November, 2007

The Department of Animal Science is pleased to provide you with this 2007 Annual Report summarizing a portion of our research, extension and teaching activities over the past year. The livestock industry continues to enjoy some excellent economic returns while also dealing with some difficult challenges including those associated with an extended period of drought. Our Department is committed to administering research and extension programs that will assist our clientele in meeting those challenges. Our research activities range from the very basic to the applied. We attempt to utilize our limited resources to help address industry problems using interdisciplinary approaches. In addition, we try to facilitate cooperation and collaboration with other universities, state and federal agencies, and private industry in addressing issues facing animal agriculture. By working together to meet these challenges we will hopefully contribute to enhanced profitability and the production of high quality, safe and wholesome consumer products.

We hope the information provided in this Annual Report will be useful to you. We welcome your comments and look forward to your continued involvement in our research, extension and teaching programs in the Department of Animal Science at the University of Wyoming.

Sincerely,

Doug L. Hixon
Head and Professor of Animal Science
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STATEMENT OF PURPOSE

The University of Wyoming encourages its faculty to engage in the discovery of knowledge and new technology when the activities are consistent with their interests, the University’s objective and the needs of the people of Wyoming.

MISSION

The dissemination of knowledge developed or acquired through the University and the provision of leadership and professional assistance in its utilization.

BIOLOGICAL VARIABILITY AND CHANCES OF ERROR

The variability among individual animals in an experiment creates problems in interpreting the results. For example, cattle on treatment X may have had a numerically larger average daily gain than those on treatment Y, but variation in individual animal weight gain within each treatment group may indicate that the difference in weight was not the result of the treatment alone. Statistical analysis attempts to “sort” the normal biological variation within groups of animals from the response attributed to the treatments imposed. From that, researchers can calculate the probability that such differences were from chance (an effect caused by normal biological variability), or produced by treatment (treatment effects).

In the research reports that follow, you will see the notation $(P<0.05)$. That means the probability of the differences resulting from chance is less than five percent. When two averages are said to be “significantly different”, the probability is less than five percent that the difference is from chance – the probability exceeds 95% that the difference results from treatment.

Some papers will report the correlation between two treatments. Correlations are a measure of the relationship between traits. The relationship may be positive (both traits tend to get bigger or smaller together) or negative (as one trait gets bigger, the other gets smaller). The perfect correlation is one (plus 1 or minus 1). If there is no relationship, the correlation is zero. Correlation does not mean cause and effect but rather gives us insight into potential relationships between traits.

In other papers, you may see an average, or mean, given as $2.50 \pm 0.10$. The first number reported is the average, or mean; 0.10 is the “standard error”. The standard error is a measure of variability, giving a range (2.40 to 2.60 in this case) where we can be 68% certain that the true average, or mean, (with limited numbers of animals) would fall.

Ways of decreasing the variability, and improving the chance of measuring differences due to treatment include: Using several animals per treatment, replicating treatments several times with pens of animals and using similar animals. The statistical analysis allows more valid, unbiased interpretation of the results regardless of the number of animals. In nearly all of the research reported here, statistical analyses are included to increase the confidence you can place in the results.
**Conversion Table**

The metric system is the unit of measurement frequently used for reporting of scientific data. To aid in interpretation of these data, conversion factors for common measurement follow:

**Length:**

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<th>=</th>
<th>.01 meter (m)</th>
<th>=</th>
<th>.3937 inches (in)</th>
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<td>=</td>
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<tr>
<td>1 m</td>
<td>=</td>
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<td>=</td>
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<td>=</td>
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**Volume:**

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<th>=</th>
<th>.034 ounces (oz)</th>
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<td>=</td>
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</tr>
<tr>
<td>3.81</td>
<td>=</td>
<td>4 qt</td>
<td>=</td>
<td>1 gallon</td>
</tr>
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</table>
In January of 2007 the Department of Animal Science was proud to learn of a Chronicle of Higher Education ranking of Departments of Animal Science which identified the University of Wyoming 7\textsuperscript{th} (tied with Michigan State University) in the U.S. based on a faculty research productivity index. This index was based on factors including federal grant dollars received, publications, citations of those publications by other researchers and awards and honors received. UW was among 166 schools ranked as large research universities with 15 or more Ph.D. programs. I believe this indicates in a highly respected, prestigious publication that we have excellent, productive scientists conducting high quality research comparable to that of any land-grant institution in the country.

We are currently in the midst of two faculty searches. We will be interviewing the final two candidates for our Food Microbiology position this week. This vacancy was created when Dr. Larry Goodridge left in August 2006. We are hopeful this second search will be successful and a new faculty member will be joining us by next fall if not by the start of the spring semester in January. In addition, we are searching for a Livestock Extension position. This position was created when Dr. Steve Paisley relocated to the new James C. Hageman Sustainable Agriculture Research and Extension Center (SAREC) at Lingle, WY. Steve is still part of our Department and will continue to work in Beef Extension while being on-site to actively administer animal research projects at SAREC. However, we received a new position in Livestock Extension to replace Steve on campus. We hope to be interviewing candidates for this position from an excellent pool by the end of the year. This individual will assist Steve with beef extension responsibilities as well as provide sheep extension support.

Faculty members of the Department of Animal Science have obtained over $1.6 M in new funding since the start of FY 07 on July 1, 2006. In addition, they have over $1.02 M in active grants obtained in previous years.

**Additional faculty recognition** received in the past year included:

- Dr. Steve Ford, Rochelle Chair, received the Legacy Award from Oregon State University (OSU) in October. Steve had received an Outstanding Alumnus Award in Ag Sciences from OSU and the Barron Lecturer Award from the University of Florida earlier this year.
- Dr. Bret Hess received an Early Career Achievement Award from American Society of Animal Science in San Antonio this past July. Bret was also elected to serve a three-year term as Western Section Director of ASAS.
- Four Animal Science faculty received “Top Prof” recognition from Mortar Board Honorary Society in October. They included Dan Rule, Kristi Cammack, Warrie Means and Doug Hixon.
- Doug Hixon was presented the “Friend of Agriculture” Award from the Albany County Cowbelles at their 2006 year-end dinner dance.

**Three ANSC staff honored.** Lance Miller, Livestock Judging Team Coach; Brandi Brewer, Accounting Associate (Laramie); and, Carrie Burke, Senior Office Assistant (Gillette), all
received “Tip of the Cap” Awards from Mortar Board Honorary Society this past spring for going “above and beyond” their assigned responsibilities to serve students.

Graduate Students Excel-

- **ANSC Students Sweep Graduate Poster Competition at Colorado Nutrition Roundtable in September.** Colt Knight, who began work on an M.S. degree in Animal Breeding with Dr. Kristi Cammack this past summer, won the top honor and received $500 from the regional American Registry of Professional Animal Scientist Chapter; Platt Price, who is finishing an M.S. with Dr. Bret Hess in nutrition and will soon be initiating a Ph.D. program, received the $250 second place award; and Francis Niemela, an M.S. student working with Dr. Steve Paisley in production and management received the $100 third place award.

- **Keith Underwood won the 2007 American Meat Science Association’s Graduate Student Research Poster Competition in Brookings, South Dakota.** Keith completed an M.S. degree with Dr. Warrie Means in Meat Science while he coached our Meat Judging Team and is now pursuing a Ph.D. under the direction of Dr. Min Du where he is working to increase marbling and enhance muscle growth in beef which could lead to higher quality grading cattle with enhanced cutability.

- **Two nutrition graduate students win Western Section, American Society of Animal Science (WSASAS) Graduate Student Competition at University of Idaho in June.** Platt Price won the $500 first place award and Rena Stohrer, an M.S. student from Buffalo, WY working with Dr. Paul Ludden, received the $200 award for third place. Evaluated on their oral presentation as well as their written manuscript, our ANSC graduate students won the $2000 Institutional Award for the fourth time in the five years the award has been presented.

Undergraduate students honored-

- **Stacia Berry, ANVS major in Business and Communications Double Option, received USA Today academic recognition and also won WyFB Young Farmer and Rancher Discussion Meet.** An outstanding senior student from Cheyenne, Stacia was named to the 2007 All-USA College Academic third team. After winning WyFB state competition, she went on to be named a Final Four contestant in the national competition in Jacksonville, FL.

- **Heather Hamilton, senior ANVS major in Communications Option from Lance Creek, WY was selected from 45 national applicants as one of 15 NCBA student intern workers for Beef Industry Convention in Nashville, TN last February.**

- **Kassi Bauman, an ANSC major in the Animal Biology Option, was selected to participate in an all-expense paid tour of Deseret Cattle & Citrus in Florida in late October sponsored by Farm Management Company.** Kassi was part of a select group of top junior students selected from approximately a dozen universities across the county to learn about internship and employment opportunities with Farm management Company’s wide range of businesses.
**Gamma Sigma Delta,** the Agricultural Honorary Society, recognizes outstanding students at their awards brunch each spring. The academic excellence of ANVS majors was recognized with the following awards:

- Outstanding Male and Female Freshmen students –
  - Travis Allen, Pre-Vet Option, Cheyenne
  - Amy Berry, Pre-Vet Option, Cheyenne

- Outstanding Junior –
  - Stacia Berry, Business and Communications Double Option, Cheyenne

- Outstanding Senior –
  - Lynn Franzkowiak, Meat Science & Food Technology, Schaller, IA

- Outstanding Ph.D. Student –
  - Qingwu Shen, Meat Science & Food Technology, Beijing, China

**Judging teams compete**-

- The Livestock Judging team started 2007 off with a strong showing in the National Western Stock Show’s (NWSS) Contests. They finished 10th out of 22 teams in the Livestock Contest, followed that with a 3rd place finish out of 22 teams in the Carload Contest the next day. They followed this with a 4th place finish at the Sioux Empire Farm Show, 4th at Iowa Beef Expo in Des Moines, 5th at San Antonio and finished the spring with a 13th place finish at the Houston Contest. Team members included Brad Mills of Stratton, CO; Lindsey Zellitti from Durango, CO; Julie Saur of Gillette, WY; Maggie Witzel of Kanorado, CO; Justin Uhrig of Alliance, NE; and Braeton Hill of Collbran, CO.

- The Meat Judging Team started the spring with a 7th place finish at the 2007 NWSS Contest. They followed that with a 6th place finish out of 16 teams in the Houston Contest and finished with a 7th place finish in the Southwestern Contest at Ft. Worth. Team members in the spring included Matt Korkow from Hanna, WY; Lander Nicodemus of Cheyenne; Lindsay Smith of Torrington, WY; Katie Kessler of Lander, WY; and Jessie Berry of Cheyenne, WY. The team was coached by Shane Thompson, Meat Lab Manager.

**Engagement**

- The Department maintains active involvement with commodity groups in the state through activities such as the Wyoming Beef Cattle Improvement Association (WBCIA) Bull Test, the WBCIA Feedlot Test and Carcass Evaluation Program, the WY Wool Growers Meat Breeds Summer Ram Test, and the WY Rambouillet Association Ram Test.

- Steve Paisley cooperates with the WY Beef Council to serve as Beef Quality Assurance (BQA) Coordinator for the State of WY. In this capacity, he has developed a Cattle for Sale website encouraging BQA practices.

- Warrie Means continues to work closely with the WY & CO Meat Processors by hosting their annual meeting and short course. He also works with the WY Department of Ag and small meat processors to develop Hazard Analysis Critical Control Point (HACCP) food safety systems.

- The Department hosted the 10th Annual Cowboy Youth Classic (CYC) on June 22 and 23rd for young people enrolled in the 4-H and FFA youth livestock (swine,
sheep and beef cattle) projects and their parents. This day and a half program includes mandatory educational workshops along with traditional livestock shows. The number of participants continues to grow with more than 118 youth enrolling in educational workshops related to their projects and approximately 200 attending the Department-sponsored meal on Friday evening. Workshops presented included animal nutrition, show lamb and show pig management, animal health and diseases, livestock anatomy and “What can UW do for you!” Judging contests and quiz bowl competitions were also administered on Friday evening. Swine (74 hd. shown), beef (49 hd.) and sheep (55 hd.) shows and their respective showmanship classes all took place on Saturday.

- North American South Devon Junior Association moved into the Hansen Livestock Teaching Arena the week after the CYC for their 2007 Annual Junior Show and associated activities. Young people from 8 states exhibited 90 head of cattle during the four-day event that also Quiz Bowl Contests, Team Marketing Competitions, an Ag Olympics and judging contest.

- The Department also continues to give leadership to traditional youth education programs such as the state 4-H Livestock Judging Contest, the State 4-H and FFA Meats Judging Contest, and the State 4-H Wool Judging Contest.

Development

- Becky Russell and her family as well as their many friends have established the Bill Russell Wheel-of-Brands Scholarship to honor her late husband and former Animal Science Professor, Dr. Bill Russell.

- We continue a partnership with the Cowboy Joe Club’s Steer-A-Year (S-A-Y) Program to enhance funding for our livestock, meat and wool judging teams. The Animal Science Judging Program receives half of the value of any newly recruited steers to this program and designated to be shared with Animal Science by the donor. 2006 S-A-Y program donors included Steve and Mary Beth Whitmire, Dahlonega, GA; Bill & Phyllis Gross, Pine Bluffs; Arlowe & Mary Hulett, Laramie; Melvin & Isla Riley, Laramie; Laura Bucholz, Saratoga; Juan & Joni Reyes, Wheatland; John & Shana Bunker, Wheatland; Doug & Marilyn Hixon, Laramie.

- The Gary and Gloria Parker Scholarship was established by family and friends of the Parkers to honor the late Gary Parker. Gary and Gloria owned and operated Shamrock Angus Ranch west of Laramie for 12 years before it was sold to Sunny Valley Farms of Yorkville, IL who now operates the ranch as SVF Shamrock LLC. They also actively participated in the establishment of this scholarship. This scholarship awarded a $2500 stipend for the 2007-08 academic year.

- The late Edna Myers Duncan bequeathed $40,000 to the Wheel-of-Brands Scholarship Program this past August. The Myers Family Ranch brand was one of the early brands put on the Wheel in 1994.

- We very much appreciate the many donors to various ANSC scholarships, endowments and foundation accounts that support our students, faculty and staff.
2007 Animal Science Contributors

ABS Global, Inc., Don Pindell, Wheatland
American Cancer Society
Brad Boner, Glenrock, WY
Burnett Enterprises, Carpenter, WY
The Butcher Block, Laramie, WY
DarLynn Cattle Company
Department of Defense Medical Research Program
Elanco Animal Health
Fort Dodge Animal Health
Julian Land and Livestock Company, Kemmerer, WY
Klein Farms, Inc.
Mountain States Lamb Cooperative
National Institutes of Health
National Science Foundation
Pfizer Animal Health
Pioneer Hi-Bred International, Inc.
Swift and Company, Inc. –Beef
Swift and Company, Inc. –Lamb
Texas Sheep and Goat Predator Management Board
USDA, APHIS National Wildlife Research Center
USDA-ARS
USDA-CREES NRICGP
University of Wyoming Agriculture Experiment Station
University of Wyoming Research Office
Western Feed Supplements
Western Region--Sustainable Agriculture Research and Education (WSARE)
Wyoming Animal Damage Management Board
Wyoming Department of Agriculture—Consumer Health Services Division
Wyoming IDeA Networks for Biomedical Research Excellence (INBRE; NIH-NCRR)
Wyoming Meat Processors Association
Wyoming Wool Growers Association
Zorko’s 7Z Livestock
Z-Tags, Inc.
The 2007 University of Wyoming Livestock Judging Team notched solid performances last spring at national events, including a top-three finish in one of the competitions at the National Western Stock Show in Denver, CO. Members of the team for the spring 2007 season included Braeton Hill, Collbran, CO; Brad Mills, Stratton, CO; Julie Saur, Gillette, WY; Maggie Witzel, Kanorado, KS; and Lindsey Zellitti, Durango, CO. In the carload competition at the NWSS, contestants had to judge a pen of 16 animals placed into groups of four. This simulates a real-life situation involving ranchers buying groups of replacement heifers or bulls. They are trying to pick groups to balance quality with uniformity. Zellitti led the team to a third-place team finish overall after placing fifth individually. Mills paced UW to a 10th-place team finish overall in the livestock judging contest by placing 11th in the individual overall competition, fifth in beef cattle, and 10th in overall reasons. His individual total of 943 is the highest score a UW contest has recorded at a 1000-point contest. The team total of 4517 is also a record for a UW team at the NWSS.

The team also competed at the San Antonio Stock Show & Rodeo in San Antonio, Texas. It was fifth out of 10 teams in the senior college division. Mills recorded the 8th highest individual score in UW history (928), while the team total of 4495 is a UW team record for that contest, as well.

UW finished fourth out of 11 teams at a contest in Sioux Falls, South Dakota, fourth out of nine at the Iowa Beef Expo in Des Moines, Iowa, and 13th out of 18 teams at the Houston Livestock Show & Rodeo. Mills was 2nd high individual overall at the contest in Sioux Falls, and Saur was 6th high individual overall at the Iowa Beef Expo in Des Moines. The spring season was capped off by a third place team finish at the Grand National Contest at the Cow Palace in San Francisco, CA.

This fall, the team has competed at the National Barrow Show in Austin, MN; the Pannell Ranch Contest in Hullet, WY; the Premier Stockman in Frankfort, IN; and the American Royal in Kansas City, MO. The team will finish their season at the North American International Livestock Exposition in Louisville, KY on November 12th.

Members of the 2007 fall team include: Daniel AlderseBaes, Clovis, NM; Jarred Bower, Worland, WY; Braeton Hill, Collbran, CO; Brad Mills, Stratton, CO; Julie Saur, Gillette, WY; and Lindsey Zellitti, Durango, CO.

The team was second at the Pannell Ranch Contest with Mills and Saur earning seventh and eighth place overall awards, respectively. Zellitti was tenth high individual overall and fourth in sheep at the Premier Stockman, while Mills was 10th in sheep and reasons, and Hill was 8th in sheep. The team set some UW records at a very competitive American Royal contest. The team set a new high score for that contest, scoring 4513 points which left them in 17th place in the contest. Individually, Mills recorded the second highest individual score (941) for a UW contestant, which was good enough for 24th high individual overall. The team is being coached by Lance Miller, who can be reached at 307-766-2159 or lrmiller@uwyo.edu.
In January 2007, a joint committee led by the Wyoming Business Council – Agribusiness Division (Scott Keith), and the University of Wyoming Animal Science Dept. (Dr. Steve Paisley) hosted the first Wyoming Winter Ag Expo. The Expo was initiated as an opportunity for Wyoming producers and agriculture-related businesses to showcase their products. The expo combined a wide variety of ag industry businesses, seedstock cattle producers, hay producers, and support industry personnel in one location, while also including educational sessions focusing on hay and livestock production. The two day expo was held at the Wyoming State Fairgrounds Pavilion building in Douglas January 22nd and 23rd.

The 2007 Winter Ag Expo hosted 34 commercial booth vendors, 22 cattle displays and 12 large equipment vendors. The expo and educational programs were free to the public, with 21 major sponsors, Converse County Tourism Promotion Board included, covering the costs of set-up, advertising, and facility lease. Exhibitor survey results and comments were positive, encouraging the committee to make the Winter Ag Expo an annual event.

**University of Wyoming / Designer Genes L.L.C.**

**Ultrasound Training Workshop July 24-26, 2007, Hansen Livestock Teaching Arena**

**Contributors:** Designer Genes Technologies, L.L.C  
Rethel and Sherry King, Harrison, AR

**Status:** Previously offered in 2002, 2003 and 2004, the course was offered July 22-26, 2007 with 8 participants.

The training is a three day workshop and certification held in cooperation with Rethel King and Designer Genes Technologies, L.L.C. Over the course of 3 days, participants learn how to interpret ultrasound images, use ALOKA ultrasound machines and BIA Software to scan cattle for backfat, ribeye area, marbling, and rump fat images. By the end of the training, participants have the opportunity to become certified using BIA software. The course is also good preparation for national UGC (Ultrasound Guidelines Council) certification at Iowa State and the University of Georgia.

**2007 Training:** 8 Participants from Wyoming, Montana, Nebraska, South Dakota, North Dakota and Minnesota.

**Cowboy Youth Classic – June 22,23 Hansen Livestock Teaching Arena**

The Cowboy Youth Classic (CYC) is an annual summer workshop and livestock show for Wyoming youth with beef, sheep and swine projects. The 2-day program (Friday - Saturday) includes mandatory educational workshops for both youth and parents, livestock fitting and showing demonstrations, and a jackpot livestock show for all three species. The CYC is supported by a large number of local sponsors, providing financial support, services, prizes, and in-kind gifts for the event. The 2007 CYC attracted over 120 participants with over 160 cattle, sheep and pigs combined, nearly double last year’s totals.

The 2007 North American South Devon Association Junior National contest was hosted by the UW Animal Science Department at the Hansen Livestock Teaching Arena at Laramie, WY. The summer contest attracted junior high and high school age youth from all over the United States, representing several states including Minnesota, Wisconsin, Ohio, Iowa, Kansas, Colorado, South Dakota, Montana, Minnesota and Wyoming.

The 2007 contest in Laramie was the largest NASDJA event to date, with 48 families attending, exhibiting over 100 head of registered South Devon cattle. In addition to the youth livestock contest, our Junior National also included additional education and public speaking opportunities including a team marketing contest, ag-related quiz bowl, and beef ambassador competition.

2007 Wyoming Beef Cattle Improvement Association

**Bull Test** – Pingetzer heifer and bull development facility, Shoshoni, WY. Approximately 270 bulls entered into the 2007 test near Riverton, WY. The annual sale is held in conjunction with the WBCIA symposium and sale, the first Saturday in April.

**Feedlot Test** - Klein Farms, Wheatland, WY. One hundred sixty four steers were consigned to the annual feedlot performance and carcass evaluation test.

**Symposium/Scholarship and Awards Banquet** – In conjunction with the WBCIA Bull Sale, the symposium committee hosts an annual educational workshop, followed by awards banquet and benefit auction, generating proceeds to provide three $1,000 to Wyoming students in ag-related college programs.

**Wyoming State Fair Carcass Contest and Live Evaluation Program** – Sponsored by several groups including the Wyoming State Fair, Wyoming Stock Grower’s Association, Wyoming Farm Bureau and UW Animal Science, the WBCIA organizes and hosts the annual beef carcass contest and live evaluation competition.

**Wyoming Supreme Cow Contest** – New to the WY State Fair this year, the WBCIA hosted a statewide supreme cow contests, with inaugural entries from Goshen, Platte and Campbell Counties.
University of Wyoming Sheep Program

Brent Larson, Livestock Manager
Robert Stobart, Associate Professor
Gary Moss, Professor

Summary

The University of Wyoming maintains approximately 120 commercial ewes, 200 purebred ewes (Rambouillet, Suffolk, Hampshire, Columbia) plus rams and replacements. These animals are used extensively to meet needs for Teaching, Research, and Extension/Service. Overriding goals of the sheep program are to: provide students and producers with the knowledge to make informed decisions regarding sheep management; conduct research that will insure long-term viability of the industry; provide superior genetics for breeders; remain cognizant of developments that affect the sheep industry and relate them to students and producers; and evaluate, test, and assist with the development of procedures and presentation of functions that enhance profitability and/or viability of the sheep industry. The Department also hosts annual white-faced and black-faced tests each year for consignor rams. Activities and functions conducted during the past year with these resources are outlined in brief below.

2006-2007 Production

In the spring of 2007, 45 Rambouillet, 43 Columbia, 69 Suffolk, 54 Hampshire, and 90 commercial ewes lambed at the University Experimental Farm. Respective weaning percentages for each of these breeds were 161, 145, 172, 170, and 165%. Also present were rams and replacement ewe lambs.

Use of Animals

Throughout the year sheep present at the experimental farm were used extensively for multiple purposes. Excess animals were shared by individuals conducting discipline oriented or multi-discipline collaborative research studies. Brief descriptions of those uses follow. Note that numbers of animals used may total to more than numbers present because some animals were used for more than one activity.

Research.

- 72 Suffolk ewes. Dr. Kristi Cammack. Effects of elevated dietary nitrate on feed intake, weight, and plasma parameters.
- 10 crossbred rams. Dr. Brenda Alexander. Role of the progesterone receptor in the development of male sexual behavior.
- Wool samples from all ewes and rams. Dr. Bob Stobart, Wool Strength and fiber diameter.
- Ram test animals. Dr. Bob Stobart. Electronic ear tags, Racewell automatic sheep handling system.
- 160 ewes. Dr. Bob Stobart. Electronic ear tags Racewell automatic sheep handling system.

Collaborative Research

- 13 ewes. Dr. Donal Skinner. Department of Zoology & Physiology.
- Wool Samples from all ewes and rams. Dr. Bob Stobart and Dr. Bruce Cameron (Dept of Family and Consumer Science), Wool Color

Academics.

- 7 lambs for fitting and showing. Little “I”.
- 10 lambs. Academic Quadrathalon
- 2 rams. Management class, vasectomy.
- 60 lambs. Processing and meats class.
- Ewes, lambs and Rams. Sheep production class and Livestock Production class.
- 10 lambs. Contest animals, State Fair.
- 10 lambs. Fitting Contest, State Fair.
- 80 lambs. University, 4-H, FFA judging workouts prior to National Western (500-600 participants).
- 150 fleeces. Wool judging workouts prior to National Western.
- 50 fleeces. Wool judging workouts for 4-H and FFA state contestants.
Producer events.

- Host 60 day blackface ram test. --- Consignor rams plus UW rams.
- Host 140 d whiteface ram test (current test is the 46th year) plus field day. --- consignor rams plus UW rams

Sales.

- 14 lambs. 4-H/FFA club lamb sale.
- 3 ram. Douglas Ram Sale
- 120 lambs. Livestock Auction
- 1 ram Tom Wilson
- 1 ram Isabella Buhr
- 1 ram CSU

Other Animals (not produced by UW).

- 50 ewes. Purchased. Dr. Steve Ford. Fetal Programming.
Food Safety

Water Pressure Effectively Reduces *Salmonella enterica* serovar Enteritidis on the Surface of Raw Almonds

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¹Department of Animal Science, University of Wyoming  
²Department of Animal Sciences, Colorado State University

**Summary and Implications**

The effects of continuous (414 MPa and 483 MPa with a holding time of 6 min) high hydrostatic water pressure (HHP) treatments on the viability of two *Salmonella enterica* serovar Enteritidis strains (PT 9c and PT 30) inoculated onto raw almonds were evaluated at 25°C. Complete inactivation of both *S. Enteritidis* isolates was achieved when the almonds were directly suspended in water, pressurized at 414 MPa and 25°C for 6 minutes, and then dried at 115°C for 25 minutes. When the almonds were pressurized at 414 MPa, followed by a dry step at 55°C for 5 minutes, a log reduction of greater than 3.58 was achieved for both isolates. Increasing the drying temperature to 65°C resulted in a log reduction of greater than 3.96 for both isolates. Increasing the water pressure to 483 MPa further decreased the *S. Enteritidis* concentration on the surface of the raw almonds. When the almonds were water pressurized at 414 MPa, the decimal reduction times were determined to be less than 35 seconds for both *Salmonella* isolates. HHP of certain dry foods appears to be feasible if the food is directly suspended in the pressurizing medium (water).

**Introduction**

Since 2001, two outbreaks of salmonellosis have been linked to consumption of raw almonds (4). Between 2000 and 2001, a *Salmonella enterica* serovar Enteritidis outbreak associated with raw almonds caused 205 cases of salmonellosis across North America. One hundred and thirty four of the cases occurred in Canada, and 71 cases were identified in the U.S. Epidemiological analysis indicated that a rare phage type of *S. Enteritidis*, phage type (PT) 30, had caused the outbreak. The 2000-2001 outbreak was the first time that salmonellosis had been associated with the consumption of raw almonds. In 2004, another outbreak of salmonellosis was associated with raw almonds (2). As with the 2000-2001 outbreak, *S. Enteritidis* was identified as the causative agent, and the CDC identified 29 cases of salmonellosis with illness onsets ranging from September 2003 to April 2004. Subtyping analysis identified another rare phage type, PT 9c, as causing the 2004 outbreak (2). Ultimately, the 2004 outbreak led to the voluntary recall of approximately 18 million pounds of raw almonds, as well as granola-type bars, muesli-type cereals and other products that contained almonds (4).

Several processes have been investigated as a way to eliminate *Salmonella* spp. From the almond surface. These treatments include propylene oxide (PPO), and various heat processes including steam (3). The FDA has recently approved the use of PPO as a method that is capable of achieving a 5 log reduction of *S. Enteritidis* on raw almonds (4).

High hydrostatic pressure (HHP) processing is considered as an emerging treatment to destroy pathogenic and spoilage microorganisms in foods, and HHP (100 -700 MPa) of foods at low or moderate temperature is being increasingly utilized as a non-thermal processing method to reduce the microbial load in foods. Previously, we reported that due to the low a,w, HHP was ineffective at reducing the *S. Enteritidis* concentration on raw almonds (5). However, when the almonds were suspended in water and then pressurized, a reduction in the *S. Enteritidis* concentration was achieved (5). The objective of this study was to further investigate the use of “water pressurization” as an effective method to reduce the *S. Enteritidis* concentration on raw almonds.

**Materials and Methods**

**Bacterial Strains**

Two *S. Enteritidis* strains were used in this study. *S. Enteritidis* PT 30 was the isolate implicated in the almond outbreak in 2000-2001, and was obtained from the National Food Laboratory in Dublin, California. *S. Enteritidis* PT 9c was identified as causing the 2004 outbreak and was obtained from the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia. Stock bacterial cultures were maintained in 30% glycerol and were frozen at -70°C.

**Isolation and preparation of Salmonella strains**

Prior to each experiment, bacterial strains were prepared according to Danyluk *et al.* (3). For growth experiments, the inocula consisted of stationary phase cells that were obtained by inoculating tryptic soy broth (TSB) with a single colony from an overnight TSA plate and
incubating the preparations overnight with shaking at 37°C. The overnight (18 h) culture was used to inoculate 150 mm x 15 mm TSA plates to produce a bacterial lawn, following incubation for 24 h at 35°C. Three plates were prepared per 400 g almond sample. Following incubation, approximately 8 to 9 ml of 0.1% peptone was added to each large plate. The bacterial lawn was loosened with a sterile spreader and cells (approximately 25 ml) were collected. Inoculum levels were determined by serial dilution in 0.1% peptone followed by plating onto TSA and XLD agar.

Inoculation procedures

Inoculation of the almonds were conducted as previously described (5). Briefly, almond samples (400 ± 1 g) were weighed into plastic polyethylene bags (30.5 cm x 30.5 cm) (Bitran, Com-Pac Int., Carbondale, IL), followed by addition of 25 ml (10^9 CFU/ml) of the pooled inoculum. This volume had been previously determined to be the least needed to completely coat the almonds with little residual liquid remaining. The bag was closed and shaken by hand through inversion for 60 s. Almonds were poured out of the bag and spread onto two sheets of 46 × 57-cm filter paper (Fisherbrand Qualitative P8, Fisher Scientific, Pittsburgh, PA) to dry for approximately 1 h.

Enumeration procedures

Fifty grams of almonds were added to an equal volume of Butterfield’s phosphate buffer. The almonds were stomached for 2 minutes in a LabBlender 400 (Seward Laboratory, London, UK) on normal speed. Serial dilutions were made in Butterfield’s phosphate buffer followed by spiral plating onto TSA and Xylose lysine deoxycholate (XLD) agar, with the use of an Autoplate 4000 spiral plate system (Spiral Biotech, Norwood, MA). After overnight incubation at 37°C, the plates were enumerated using an automated Q colony counter (Spiral Biotech), and the software associated with the instrument.

Hydrostatic Pressurization

High pressure experiments were carried out in a unit that consisted of an internal pressure chamber with dimensions of 10 cm × 24.5 cm length (Engineered Pressure Systems, Andover, MA, USA), and had the capability of pressurizing to 690 MPa at 25 to 95°C. The pressure chamber was filled with a liquid mixture of water and 5% oil (Mobil Hydrosol 78) pumped into the bottom. Prior to pressurization, the liquid was heated to the desired temperature by electric heating coils surrounding the chamber. After chamber closing, additional liquid was pumped into the instrument through a check valve to generate the desired hydrostatic pressure level. A microprocessor controlled and displayed readings of pressure level, time and temperature of pressurization. Almond samples were pressurized at different pressures, temperatures, and times, depending on the study, as described below. Pressure-come-up time was about 3 min for every 345 MPa, and come down time was within 1 min, depending on the pressure. The pressure increased in an isostatic manner (same value everywhere in the vessel and throughout the food sample). The pressure come-up and come-down times, as well as adiabatic temperature during pressurization (approximately 3°C per 100 MPa) were not used in the calculations of the pressurization time and temperature.

Water Pressurization of S. Enteritidis inoculated almonds

Twenty five gram portions of inoculated almonds were placed into 500 ml plastic bottles (Nalgene, Rochester, NY), suspended in 500 ml of sterile water pretempered to 25°C, and subjected to continuous hydrostatic pressure processes. Each experiment was conducted in triplicate. The continuous processes consisted of a treatment at 414 or 483 MPa at a temperature of 50°C for 6 minutes. Following water pressurization, the almonds were removed from the bottles, drained of excess water, and allowed to dry at four temperatures: ambient temperature (25°C), 55°C, 65°C, and 115°C. The almonds were air dried at 25°C or 115°C for 25 minutes. A handheld blow dryer was utilized to dry the almonds at 55°C and 65°C. The almonds were usually dry after 5 minutes. Following the dry step, the almonds were enumerated as described above.

Statistical Analyses

The data were compared by an analysis of variance (ANOVA) using SAS Proc GLM to determine differences (P=0.05) in the viability loss of S. Enteritidis on almonds that could be accounted to pressure, pressurization time, drying temperature without pressurization, and the interaction of pressurization with drying temperature. Pair-wise comparisons for each factor were performed using SAS lsmeans for the honestly significant difference (HSD) with a Tukey adjustment.

Results and Discussion

Results

The results of this study demonstrated the synergistic effects of water pressure and heat in reducing the S. Enteritidis concentration on the almonds. For example, water pressurizing the almonds at 414 MPa for 6 minutes, followed by drying the almonds at ambient temperature resulted in a log 4.02 decrease in the S. Enteritidis PT 9c concentration (Table 1), and a log 4.03 decrease in the S. Enteritidis PT 30 concentration (Table 2), when compared to the control (dry, unpresurized S. Enteritidis inoculated almonds). Suspending the inoculated almonds in water for 6 minutes, followed by drying at ambient temperature did not affect the S. Enteritidis concentration (Tables 1 and 2). When the almonds were suspended in water for 6 minutes and then dried at 115°C for 25 minutes, the concentration of S. Enteridits PT 9c cells on the almonds resulted in a log 4.98 decrease (Table 1), while the PT 30 concentration was reduced by log 5.10 (Table 2). When inoculated almonds that were not pressurized or suspended in water were heated at 115°C, log reductions of 2.72 (PT 9c) (Table 1) and 2.78 (PT 30) (Table 2) were observed.
The above results demonstrate the effectiveness of pressurizing the almonds directly in water, followed by a dry step. Still, drying the almonds at such a high temperature (115°C) could lead to deleterious effects on the almonds. In addition, the dry step took up to 25 minutes, and the combined HHP/drying process should be completed in as short a time as possible, making HHP processing of raw almonds on an industrial scale a realistic process. To address the concern of maintaining a raw product, we processed S. Enteritidis inoculated almonds at 414 and 483 MPa, and evaluated a rapid drying procedure at two lower temperatures (55 and 65°C). A portable hair dryer was used to simulate blowing air, and this allowed the almonds to be dried in 5 minutes, regardless of the temperature. When the almonds were pressurized at 414 MPa and dried at 55°C, a log 3.58 decrease in the S. Enteritidis PT 9c concentration was observed (Table 3). Drying the almonds at 65°C resulted in a log 3.96 decrease in the PT 9c concentration (Table 3). Pressurizing the almonds at 483 MPa followed by a dry step at 55°C decreased the concentration of S. Enteritidis on the surface of the raw almonds by log 4.41. When the almonds were dried at 65°C, a log 5.05 decrease was achieved (Table 3). The reductions for S. Enteritidis PT 30 were similar (Table 4).

Using an ANOVA procedure, the viability loss indicated significant differences (P<0.0001). Using pairwise comparisons from the HSD, pressurizing at either 414 or 483 MPa showed significantly higher (P<0.0001) viability loss values as compared to the controls. However, no significant difference (P=0.7885) was observed between 414 and 483 MPa treatments. The pressurization time also influenced the viability loss of both Salmonella isolates. The pair-wise comparisons and lsmeans showed that all pressurization times correlated with significantly higher viability loss (P<0.0001) than the control almonds. A statistically insignificant trend was observed, with 6 minutes of pressurization causing a higher viability loss value than 1.5 and 3 minutes (P=0.08). No other significant differences by pressurization time were observed (P=0.18).

With the exception of drying the almonds at 115°C (in the absence of pressurization) (P<0.001), none of the other temperature treatments showed significant differences in viability loss (P>0.15) between each other or the controls.

The effect of different pressure and temperature combinations on viability loss correlated with the data observed above. Pressure treatment combined with 115°C drying temperature showed higher viability loss values (P<0.055) as compared to all other pressure and temperature treatments except for 483 MPa/65°C, which was not significantly different (P=0.27). Additionally, all of the other pressure and temperature treatments showed significant differences (P<0.0006) from the control almonds. However, the treatments that included pressure with drying at 25°C, 55°C, or 65°C did not significantly differ (P=0.48) from one another.

Taken collectively, the results confirm our earlier observations (5) that water pressurization of raw almonds effectively reduces the S. Enteritidis concentration on the surface of raw almonds. It is also apparent that water pressure and heat act synergistically in reducing the S. Enteritidis concentration on raw almonds, and the reduction occurs irrespective of the S. Enteritidis phage type.

Discussion

Our previous work indicated that temporarily adjusting the water activity of the almonds by pressurization and a drying step would lead to a greater decrease in S. Enteritidis concentration on the surface of the raw almonds, compared to when the almonds were pressurized dry (5). In these studies, we developed a water pressurization procedure, in which the almonds were directly suspended in water prior to pressurization, pressurized, and dried at several temperatures. The rationale behind this strategy was that suspending the almonds in water would increase the a_w at the surface of the almond, allowing the pressure to impart a more destructive effect on the bacteria. In addition, the fact that the almonds would need to be dried presented an opportunity to examine the combined effects of pressure and heat (imparted through air drying) as an effective method to reduce the S. Enteritidis concentration on raw almonds.

Approximately 914 million pounds of California almonds were produced in 2005-2006 (1). Almonds are California's largest agricultural crop, with an annual value of $1.6 billion; California produces approximately 80% of the world's almonds and almost 100% of the almonds sold in the United States (1). It is apparent that any processing method, developed to reduce microbial (Salmonella) contamination on raw almonds would have to effectively reduce the microbial load on the almonds, be capable of high throughput, unalter the sensory characteristics of the almonds, and be cost effective. Water pressurization addresses most of these attributes. Furthermore, the method is rapid, easy to perform, and is amenable to high throughput with the use of industrial scale horizontal processing units, that directly employ water as the pressurizing medium. A simple air blower could be set up downstream of the pressure unit to dry the almonds prior to packaging. Due to its ability to maintain the sensory characteristics of food, HHP continues to find increased application within the food industry, although the process is still economically expensive. The use of water pressurization as a processing method for dry foods is intriguing. Several dry foods such as raw almonds, cocoa powder, cocoa beans, chocolate, dried milk, aniseed and cereal products have been implicated in outbreaks of salmonellosis (3). Depending on the physical nature of the dry food, it may be possible to suspend the food to be pressurized directly in the pressurizing medium (water). As shown in this work, such an approach would have the effect of increasing the a_w of the food, increasing the ability of HHP to reduce the concentration of vegetative bacteria on the food.

Acknowledgements

This study has been supported by a grant from the Almond Board of California.
Table 1. Viability loss of *Salmonella* Enteritidis PT 9c inoculated onto the surface of raw almonds, following water pressurization at 414 MPa, and drying at ambient temperature or 115°C.

<table>
<thead>
<tr>
<th>HPP (psi)/temperature/time</th>
<th>Log CFU/ml</th>
<th>Viability loss (log)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control1</td>
<td>6.61 (± 0.23)</td>
<td>-</td>
</tr>
<tr>
<td>Control2</td>
<td>1.63 (± 0.10)</td>
<td>4.98 (± 0.33)</td>
</tr>
<tr>
<td>Control3</td>
<td>5.67 (± 0.11)</td>
<td>0.94 (± 0.34)</td>
</tr>
<tr>
<td>Control4</td>
<td>5.89 (± 0.10)</td>
<td>0.72 (± 0.33)</td>
</tr>
<tr>
<td>Control5</td>
<td>3.89 (± 0.11)</td>
<td>2.72 (± 0.34)</td>
</tr>
<tr>
<td>60,000/50°C/6 min6</td>
<td>2.59 (± 0.007)</td>
<td>4.02 (± 0.24)</td>
</tr>
<tr>
<td>60,000/50°C/6 min7</td>
<td>6.61 (± 0.23)</td>
<td></td>
</tr>
</tbody>
</table>

1. The almonds were not subjected to any treatment.
2. The almonds were suspended in water for 6 minutes, and then dried at 115°C for 25 minutes.
3. The almonds were exposed to 115°C heat for 25 minutes.
4. Following water pressurization, the almonds were dried at ambient temperature for 25 minutes.
5. Following water pressurization, the almonds were dried at 115°C for 25 minutes.

Table 2. Viability loss of *Salmonella* Enteritidis PT 30 inoculated onto the surface of raw almonds, following water pressurization at 414 MPa, and drying at ambient temperature or 115°C.

<table>
<thead>
<tr>
<th>HPP (psi)/temperature/time</th>
<th>Log CFU/ml</th>
<th>Viability loss (log)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control1</td>
<td>6.70 (± 0.33)</td>
<td>-</td>
</tr>
<tr>
<td>Control2</td>
<td>1.60 (± 0.16)</td>
<td>5.10 (± 0.49)</td>
</tr>
<tr>
<td>Control3</td>
<td>6.11 (± 0.10)</td>
<td>0.59 (± 0.43)</td>
</tr>
<tr>
<td>Control4</td>
<td>6.10 (± 0.10)</td>
<td>0.60 (± 0.43)</td>
</tr>
<tr>
<td>Control5</td>
<td>3.92 (± 0.17)</td>
<td>2.78 (± 0.50)</td>
</tr>
<tr>
<td>60,000/50°C/6 min6</td>
<td>2.67 (± 0.009)</td>
<td>4.03 (± 0.34)</td>
</tr>
<tr>
<td>60,000/50°C/6 min7</td>
<td>6.70 (± 0.33)</td>
<td></td>
</tr>
</tbody>
</table>

1. The almonds were not subjected to any treatment.
2. The almonds were suspended in water for 6 minutes, and then dried at 55°C for 5 minutes.
3. The almonds were suspended in water for 6 minutes, and then dried at 65°C for 5 minutes.
4. Following water pressurization, the almonds were dried at ambient temperature for 25 minutes.
5. Following water pressurization, the almonds were dried at 55°C for 5 minutes.
6. Following water pressurization, the almonds were dried at 65°C for 5 minutes.

Table 3. Viability loss of *Salmonella* Enteritidis PT 9c inoculated onto the surface of raw almonds, following water pressurization at 60,000 and 483 MPa, and rapid drying at 55 or 65°C.

<table>
<thead>
<tr>
<th>HPP (psi)/temperature/time</th>
<th>Log CFU/ml</th>
<th>Viability loss (log)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control1</td>
<td>6.61 (± 0.23)</td>
<td>-</td>
</tr>
<tr>
<td>Control2</td>
<td>5.76 (± 0.006)</td>
<td>0.85 (± 0.24)</td>
</tr>
<tr>
<td>Control3</td>
<td>5.59 (± 0.10)</td>
<td>1.02 (± 0.33)</td>
</tr>
<tr>
<td>Control4</td>
<td>5.67 (± 0.11)</td>
<td>0.94 (± 0.34)</td>
</tr>
<tr>
<td>60,000/50°C/6 min5</td>
<td>2.59 (± 0.007)</td>
<td>4.02 (± 0.24)</td>
</tr>
<tr>
<td>60,000/50°C/6 min6</td>
<td>2.24 (± 0.26)</td>
<td>4.37 (± 0.49)</td>
</tr>
<tr>
<td>60,000/50°C/6 min7</td>
<td>2.44 (± 0.06)</td>
<td>4.17 (± 0.29)</td>
</tr>
<tr>
<td>70,000/50°C/6 min5</td>
<td>2.86 (± 0.29)</td>
<td>3.75 (± 0.52)</td>
</tr>
<tr>
<td>70,000/50°C/6 min6</td>
<td>2.20 (± 0.004)</td>
<td>4.41 (± 0.23)</td>
</tr>
<tr>
<td>70,000/50°C/6 min7</td>
<td>1.56 (± 0.09)</td>
<td>5.05 (± 0.32)</td>
</tr>
</tbody>
</table>

1. The almonds were not subjected to any treatment.
2. The almonds were suspended in water for 6 minutes, and then dried at 55°C for 5 minutes.
3. The almonds were suspended in water for 6 minutes, and then dried at 65°C for 5 minutes.
4. Following water pressurization, the almonds were dried at ambient temperature for 25 minutes.
5. Following water pressurization, the almonds were dried at 55°C for 5 minutes.
6. Following water pressurization, the almonds were dried at 65°C for 5 minutes.

Table 4. Viability loss of *Salmonella* Enteritidis PT 30 inoculated onto the surface of raw almonds, following water pressurization at 60,000 and 483 MPa, and rapid drying at 55 or 65°C.

<table>
<thead>
<tr>
<th>HPP (psi)/temperature/time</th>
<th>Log CFU/ml</th>
<th>Viability loss (log)</th>
</tr>
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<tbody>
<tr>
<td>Control1</td>
<td>6.70 (± 0.33)</td>
<td>-</td>
</tr>
<tr>
<td>Control2</td>
<td>6.11 (± 0.27)</td>
<td>0.59 (± 0.60)</td>
</tr>
<tr>
<td>Control3</td>
<td>5.75 (± 0.17)</td>
<td>0.95 (± 0.50)</td>
</tr>
<tr>
<td>Control4</td>
<td>6.11 (± 0.10)</td>
<td>0.59 (± 0.43)</td>
</tr>
<tr>
<td>60,000/50°C/6 min5</td>
<td>2.67 (± 0.009)</td>
<td>4.03 (± 0.34)</td>
</tr>
<tr>
<td>60,000/50°C/6 min6</td>
<td>3.12 (± 0.53)</td>
<td>3.58 (± 0.86)</td>
</tr>
<tr>
<td>60,000/50°C/6 min7</td>
<td>2.74 (± 0.15)</td>
<td>3.96 (± 0.48)</td>
</tr>
<tr>
<td>70,000/50°C/6 min5</td>
<td>2.30 (± 0.29)</td>
<td>4.40 (± 0.62)</td>
</tr>
<tr>
<td>70,000/50°C/6 min6</td>
<td>2.57 (± 0.28)</td>
<td>4.13 (± 0.61)</td>
</tr>
<tr>
<td>70,000/50°C/6 min7</td>
<td>2.09 (± 0.35)</td>
<td>4.61 (± 0.68)</td>
</tr>
</tbody>
</table>

1. The almonds were not subjected to any treatment.
2. The almonds were suspended in water for 6 minutes, and then dried at 55°C for 5 minutes.
3. The almonds were suspended in water for 6 minutes, and then dried at 65°C for 5 minutes.
4. Following water pressurization, the almonds were dried at ambient temperature for 25 minutes.
5. Following water pressurization, the almonds were dried at 55°C for 5 minutes.
6. Following water pressurization, the almonds were dried at 65°C for 5 minutes.
References


Effects of High Dietary Nitrate on Feed Intake, Weight Change, and Plasma Parameters in Sheep

Rebecca R. Cockrum1, Graduate Student  
Kathy J. Austin1, Research Scientist  
Paul A. Ludden1, Associate Professor  
Jeremy F. Taylor2, Professor  
Kristi M. Cammack1, Assistant Professor

1Department of Animal Science, University of Wyoming, Laramie, WY.  
2Division of Animal Sciences, University of Missouri, Columbia, MO

Summary and Implications

Nitrate consumption has a significant impact on livestock production worldwide due to both chronic and acute effects. During periods of severe drought relief, plants absorb high amounts of nitrate. Consumed nitrate is converted to nitrite in the rumen, whereby it is then reduced to ammonia. The initial conversion of nitrate to nitrite exceeds the reduction process when animals consume high nitrate forages. Nitrite is absorbed into the blood, binding with hemoglobin and reducing the ability of blood to carry oxygen to peripheral tissues. Impacts include decreased feed efficiency, reproductive complications, weight loss, and even death. The purpose of this study is to confirm individual variation in response to subacute levels of dietary nitrate and identify ewes more and less tolerant to elevated dietary nitrate. Purebred Suffolk sheep were administered a 300 mg NO3−/kg BW/d supplement (n = 47) or control supplement (n = 8) for 8 d. Six nitrate tolerant and six nitrate intolerant ewes were identified based on feed intake, weight change, and behavior traits, and will be used for future gene expression analyses along with six control ewes. Supplement intake of ewes was affected by the addition of excess dietary nitrate (P < 0.0001), indicating that exposure to nitrate influences feed intake. The average % NO3− intake of the tolerant and intolerant ewes was 84% and 24%, respectively. Plasma nitrite levels were not conclusive due to the binding of hemoglobin to nitrite, which would affect nitrite detection in blood plasma. Weight change was not different between nitrate treated and control ewes.

Materials and Methods

Animal Protocol

Purebred Suffolk ewes (n = 60; initial average BW = 85.7 kg) were randomly allocated to one of two project start days (contemporary groups) due to time and labor limitations. Within each contemporary group, ewes were randomly allocated to a control (n = 5) or elevated nitrate (n = 25) diet. The basal diet consisted of bromegrass hay fed at 2.5% of BW. A supplement (11.5% total dietary CP) consisting of (DM basis) 53.9% soybean meal, 28.7% beet pulp, 10.0% molasses, and 7.4% of vitamins/minerals was fed three times per day (125 g/feeding, as fed basis). After an adjustment period of 3 - 4 d, liver biopsies were performed on all ewes. Following the biopsies, ewes were randomly assigned to one of two nitrate treatments consisting of 0 (control) or 300 mg supplemental NO3−/kg BW/d. Blood was drawn for analyses at the time of biopsy, 12 h after nitrate exposure, and every 24 h for the remaining 8 d of the trial. As a follow up, blood was also taken 4 d after the cessation of treatment.

Data Analysis

Nitrate levels in the bromegrass hay and supplement were analyzed using a Standard Range Lab Nitrate Test Kit.
Plasma samples were tested for nitrite levels using the Standard Range Lab Nitrate Test Kit (L-NTK; NECi) with the omission of the nitrate reductase and NADH reagents from the assay. Plasma will also be tested for cortisol, hemoglobin, and Vitamin E in the near future. Data were analyzed using the GLM procedures of SAS (SAS Inst., Inc., Cary, NC). Mean separation of least-squares means was performed using an LSD.

RNA was isolated from liver samples with tri-reagent and further purified using RNeasy protocol. Quality and quantity of RNA were verified using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE).

Liver Biopsy Procedure

Liver biopsies were conducted according to Ferreira et al. (1996). The procedure was modified to allow for larger sample extraction. A surgical area of approximately 16 cm² was sheared and sterilized with Lugol’s iodine solution (2% iodine, 4% K+ iodide). A local anesthetic of 8cc of 2% injectable Lidocaine (VEDCO, St. Joseph, MO) was administered topically and subcutaneously around the incision area. A 1cm incision was made with a #22 scalpel blade between the intercostal space of the 10th and 11th ribs approximately 9cm below the processus spinosus. A bone marrow biopsy punch (Jorgensen Vet Supply, Loveland, CO) with a modified hose and syringe attached was inserted vertically through the intercostal space between the 10th and 11th ribs to extract a liver sample. The liver was penetrated approximately 2 - 4 times to obtain a 1 g sample. Tissue was rinsed with PBS and placed into a storage vial. All samples were snap frozen in dry ice and stored at -80°C. Incisions were sutured and cleaned with Lugol’s iodine solution, and ewes were given 3cc of penicillin for precaution against infection. An 11% death rate typically occurs in sheep when performing liver biopsies as opposed to < 0.5% in cattle (Anderson et al., 1962). A total of 121 biopsies were performed in this study, with a 7% death loss due to complications. The tissue obtained from the liver biopsies will be used to conduct microarray analyses to determine differentially expressed genes between nitrate resistant and susceptible ewes. Expression levels of differentially expressed genes will be confirmed using real-time RT-PCR.

Results and Discussion

Nitrate levels in bromegrass hay and basal supplement (without added nitrate) were < 4 ppm. Plasma nitrite levels were not significantly different between nitrate treated and control ewes, and did not differ throughout the trial (Figure 1). There was no effect \( (P > 0.10) \) of ewe age on supplement intake. Intake of supplement (Figure 2) was different \( (P < 0.001) \) between control and NO\(_3\) treated ewes and also between the two contemporary groups \( (P < 0.001) \). During treatment there was no effect \( (P > 0.10) \) of nitrate on weight change (Figure 3). There was no difference in supplement intake \( (P > 0.10) \) between control ewes and tolerant ewes (Figure 4). However, intake was lower \( (P < 0.0001) \) in intolerant ewes compared to control and tolerant ewes (Figure 4).

![Figure 1. Average nitrite plasma levels of control, tolerant, and intolerant ewes.](image1)

![Figure 2. Percent supplement intake for controls and NO\(_3\) treated ewes.](image2)

![Figure 3. Average weight change of control, tolerant, and intolerant ewes.](image3)

![Figure 4. Percent supplement intake per feeding of control, tolerant, and intolerant ewes.](image4)
Feed intake was significantly decreased by increasing dietary nitrate. Lack of differences in plasma nitrite levels between control and nitrated treated ewes may be due to the nitrite being tied up in the blood. Therefore, a test for hemoglobin may be more appropriate to verify nitrite levels.

Microarray analyses will be performed at the University of Missouri during the summer of 2008 to identify genes differentially expressed between control, tolerant, and intolerant ewes. Plasma samples will be analyzed to determine hemoglobin, cortisol, and Vitamin E levels in all ewes. Finally, elevated nitrate levels have been associated with female reproductive performance. Therefore, future studies will study the effects of nitrate toxicity on female reproduction.

Acknowledgements

This research is supported by the University of Wyoming Agriculture Experiment Station. We would also like to thank Brent Larson and Ed Van Kirk for their assistance with animal care.

References

Effects of Supplemental Cobalt on Carcass Characteristics in Feedlot Cattle

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Travis L. Smith, UW Beef Herdsman
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Department of Animal Science

Summary and Implications
Vitamin B\textsubscript{12}, a key component in the formation of glucose from propionic acid, and therefore in growth, can be synthesized from cobalt by rumen microbes (Spears, 2002). As a result of this relationship, decreased intake and growth are early signs of a Cobalt (Co) deficiency (Spears, 2002) which commonly leads to a B\textsubscript{12} deficiency. Schwarz (2000) found 0.12 parts per million (ppm) Co in dietary dry matter (DM) was optimal for maximal growth, and 0.16-0.18 ppm Co in dietary DM for maximum feed intake when feeding different levels of Co to feedlot cattle. Stangl et al. (2000) used biochemical criteria to propose 0.20 ppm Co in dietary DM as being a sufficient level for diets of growing cattle. Additional work (Tiffany, 2003) recommended 0.15 ppm Co in dietary DM as adequate for finishing steers. That additional cobalt and Vitamin B\textsubscript{12} would increase feed efficiency and overall gain in beef cattle. The objective of this project was to determine how the organic cobalt supplementation, Suppli-mix\textsuperscript{®}, affects feedlot performance and carcass characteristics in feedlot-fed beef cattle.

Materials and Methods
Seventy-one beef animals (48 steers and 23 heifers), were initially weighed and assigned to 12 feedlot pens at the University of Wyoming Livestock Center, Laramie, WY (six animals/pen). Feedlot pens received a total mixed ration fed once per day (Table 1). Top-dressed supplements were randomly assigned to 6 pens: Control supplement of 0.5 lb./day dried distillers’ grain, and Cobalt supplement of 0.5 lb./day dried distillers’ grain with Suppli-Mix\textsuperscript{®}. Animals were fed from October 19, 2006, until the kill dates, May 1, 2007, and May 20, 2007. Amount of feed fed per day was recorded, and orts were collected and measured. Animals were weighed at the beginning of the trial (October 19, 2006), and at live-animal ultrasound (April 10, 2007). Ultrasound was used to determine kill date for each animal (based on approx 0.4 in. backfat). Final weights were estimated by dividing Hot Carcass Weight (HCW) by the average dressing percentage for the kill date group.

Statistical Analysis
Proc GLM (SAS, Cary, NC) was used for statistical analysis. Animals were grouped by treatment and sex. No treatment × sex interaction was found. Proc FREQ (SAS, Cary, NC) was used to distinguish USDA Quality Grades. Experimental unit for Feedlot Performance was Pen (n = 12). Experimental unit for Carcass Characteristics was Animal (n = 71).

Results and Discussion

Table 1. Composition of trial diets.

<table>
<thead>
<tr>
<th>Ingredient, % of DM</th>
<th>Control</th>
<th>Cobalt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa-grass hay</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Corn grain</td>
<td>79.7</td>
<td>79.7</td>
</tr>
<tr>
<td>Liquid Protein</td>
<td>3.9</td>
<td>3.9</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Distillers’ grain</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Distillers’ &amp; SUPPLI-MIX\textsuperscript{®}</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Fortified TM salt</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Chemical
- DM, %: 84.7, 84.7
- CP, % of DM: 13.5, 13.5
- NDF, % of DM: 13.8, 13.8
- Co, mg/kg: 0.5, 22.0

All diets were formulated with 30 mg/kg DM Rumensin\textsuperscript{®}

Table 2. Feed performance by treatment.

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Pens\textsuperscript{a}</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Trial Start Weight (lbs.)</td>
<td>623</td>
<td>609</td>
<td>7.36</td>
</tr>
<tr>
<td>Final Weight (lbs.)</td>
<td>1187</td>
<td>1142</td>
<td>23.44</td>
</tr>
<tr>
<td>Total Gain (lbs./head)</td>
<td>534</td>
<td>503</td>
<td>21.67</td>
</tr>
<tr>
<td>Daily Gain (lbs./head)</td>
<td>2.6</td>
<td>2.5</td>
<td>0.10</td>
</tr>
<tr>
<td>Total Days on Feed</td>
<td>199</td>
<td>197</td>
<td>3.18</td>
</tr>
<tr>
<td>DFI\textsuperscript{b}</td>
<td>21.4</td>
<td>21.0</td>
<td>0.28</td>
</tr>
<tr>
<td>FE\textsuperscript{c}</td>
<td>8.0</td>
<td>8.1</td>
<td>0.37</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Calculated “deads in”
\textsuperscript{b}Daily Feed Intake (as fed, lbs./head)
\textsuperscript{c}Feed Efficiency (lbs. feed/lb. gain)
Table 3. Feed performance by sex.

<table>
<thead>
<tr>
<th>Item</th>
<th>Steers</th>
<th>Heifers</th>
<th>Sex</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Pens(^a)</td>
<td>8</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial Start Weight (lbs.)</td>
<td>638</td>
<td>594</td>
<td>8.49</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Final Weight (lbs.)</td>
<td>1195</td>
<td>1134</td>
<td>27.07</td>
<td>0.103</td>
<td></td>
</tr>
<tr>
<td>Total Gain (lbs./head)</td>
<td>557</td>
<td>480</td>
<td>25.02</td>
<td>0.037</td>
<td></td>
</tr>
<tr>
<td>Daily Gain (lbs./head)</td>
<td>2.8</td>
<td>2.3</td>
<td>0.12</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td>Total Days on Feed</td>
<td>202</td>
<td>195</td>
<td>3.67</td>
<td>0.151</td>
<td></td>
</tr>
<tr>
<td>DFI(^b)</td>
<td>21.4</td>
<td>21.0</td>
<td>0.32</td>
<td>0.330</td>
<td></td>
</tr>
<tr>
<td>FE(^c)</td>
<td>7.8</td>
<td>8.6</td>
<td>0.43</td>
<td>0.051</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Calculated “deads in”
\(^b\)Daily Feed Intake (as fed, lbs./head)
\(^c\)Feed Efficiency (lbs. feed/lb. gain)

Table 4. Carcass characteristics by treatment.

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th>Control</th>
<th>Cobalt</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Animals(^a)</td>
<td></td>
<td>36</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slaughter Group</td>
<td></td>
<td>1.53</td>
<td>1.38</td>
<td>0.09</td>
<td>0.223</td>
</tr>
<tr>
<td>Hot Carcass Wt. (lbs.)</td>
<td></td>
<td>711</td>
<td>707</td>
<td>10.67</td>
<td>0.789</td>
</tr>
<tr>
<td>Rib Eye Area (in.(^2))</td>
<td></td>
<td>12.1</td>
<td>12.3</td>
<td>0.20</td>
<td>0.625</td>
</tr>
<tr>
<td>12th Rib Backfat (in.)</td>
<td></td>
<td>0.46</td>
<td>0.46</td>
<td>0.02</td>
<td>0.944</td>
</tr>
<tr>
<td>Calculated Yield Grade</td>
<td></td>
<td>3.15</td>
<td>3.16</td>
<td>0.06</td>
<td>0.944</td>
</tr>
<tr>
<td>USDA Yield Grade</td>
<td></td>
<td>2.6</td>
<td>2.4</td>
<td>0.11</td>
<td>0.482</td>
</tr>
<tr>
<td>% Prime</td>
<td></td>
<td>1.45%</td>
<td>2.90%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Choice</td>
<td></td>
<td>88.40%</td>
<td>89.85%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Select</td>
<td></td>
<td>8.70%</td>
<td>7.25%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marbling Score</td>
<td></td>
<td>496</td>
<td>517</td>
<td>19.84</td>
<td>0.446</td>
</tr>
<tr>
<td>Carcass Value ($/cwt.)</td>
<td></td>
<td>158.68</td>
<td>160.30</td>
<td>1.33</td>
<td>0.370</td>
</tr>
<tr>
<td>Carcass Value ($/animal)</td>
<td></td>
<td>1129.76</td>
<td>1133.00</td>
<td>19.34</td>
<td>0.902</td>
</tr>
</tbody>
</table>

\(^a\)Calculated “deads in”

Table 5. Carcass characteristics by sex.

<table>
<thead>
<tr>
<th>Item</th>
<th>Sex</th>
<th>Steers</th>
<th>Heifers</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Animals(^a)</td>
<td></td>
<td>48</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slaughter group</td>
<td></td>
<td>1.36</td>
<td>1.55</td>
<td>0.10</td>
<td>0.130</td>
</tr>
<tr>
<td>Hot Carcass Wt. (lbs.)</td>
<td></td>
<td>730</td>
<td>689</td>
<td>12.80</td>
<td>0.010</td>
</tr>
<tr>
<td>Rib Eye Area (in.(^2))</td>
<td></td>
<td>12.1</td>
<td>12.3</td>
<td>0.24</td>
<td>0.548</td>
</tr>
<tr>
<td>12th Rib Backfat (in.)</td>
<td></td>
<td>0.49</td>
<td>0.44</td>
<td>0.03</td>
<td>0.108</td>
</tr>
<tr>
<td>Calculated Yield Grade</td>
<td></td>
<td>3.23</td>
<td>3.09</td>
<td>0.07</td>
<td>0.108</td>
</tr>
<tr>
<td>USDA Yield Grade</td>
<td></td>
<td>2.7</td>
<td>2.3</td>
<td>0.13</td>
<td>0.029</td>
</tr>
<tr>
<td>% Prime</td>
<td></td>
<td>2.90%</td>
<td>1.45%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Choice</td>
<td></td>
<td>86.95%</td>
<td>91.30%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Select</td>
<td></td>
<td>10.15%</td>
<td>5.80%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marbling Score</td>
<td></td>
<td>516</td>
<td>497</td>
<td>23.81</td>
<td>0.528</td>
</tr>
<tr>
<td>Carcass Value ($/cwt.)</td>
<td></td>
<td>160.05</td>
<td>158.93</td>
<td>1.59</td>
<td>0.566</td>
</tr>
<tr>
<td>Carcass Value ($/animal)</td>
<td></td>
<td>1167.95</td>
<td>1094.81</td>
<td>23.21</td>
<td>0.012</td>
</tr>
</tbody>
</table>

\(^a\)Calculated “deads in”

No differences (P>0.210) were found between control and cobalt treatments in carcass characteristics (Table 4), including slaughter group, hot carcass wt., ribeye area, 12th rib backfat, calculated yield grade, USDA yield grade, marbling score, or carcass value. Both control and cobalt treatments showed similar (88.40% vs. 89.95%) levels of animals grading Choice. Heifer calves were lighter (P=0.003; 594 lbs. vs. 638 lbs.) than steer calves at the beginning of the trial, yet final weights were similar (P=0.103; 1195 lbs. vs. 1134 lbs.). Total gain and average daily gain (ADG) over the trial were greater (P=0.037, P=0.019) for steers than for heifers (557 lbs./head vs. 480 lbs./head and 2.8 lbs./head/day vs. 2.3 lbs./head/day). No differences (P>0.151) were seen between steers and heifers in days on feed or daily feed intake. Steers demonstrated higher (P=0.051) feed efficiency (7.8 lbs. feed/lb. gain vs. 8.6 lbs. feed/lb. gain) than did heifers. In carcass characteristics, steers were shown to have a larger (P=0.01) hot carcass wt. than did heifers (730 lbs. vs. 689 lbs.). No differences (P=0.108) were found between steers and heifers in slaughter group, ribeye area, 12th rib backfat, calculated yield grade, marbling score, or carcass value/cwt. USDA yield grade for heifers (2.3) was lower (P=0.029) than that of steers (2.7). Carcass value/head was greater (P=0.012) for steers than for heifers ($1167.95 vs. $1094.81). Both sexes showed similar distributions of quality grade, with 86.95% of steers and 91.30% of heifers grading Choice. Overall feedlot performance on this study was low, potentially due to not implanting the calves, or low frequency of bunk filling. Supplementation of cobalt elicited no significant responses in feedlot performance or carcass characteristics. Heifers demonstrated lower USDA yield grade than did the steers. Steers had higher starting weights, greater daily and total gain, and larger hot carcass weights than did the heifers. Further studies are needed to consider the relationships between supplemental cobalt, ionophores, and feedlot implant regimes.

Acknowledgements

The authors would like to thank Ralco Animal Nutrition, Inc. for their support of this project.

References


Field Test of a Coyote Specific Delivery Mechanism for Reproductive Inhibitors

Marjorie MacGregor, Graduate Research Assistant
Steve Horn, Professor
Department of Animal Science

Summary and Implications

Since 1942, the sheep industry has seen an 85% decline in production. Declining economic viability coupled with coyote predation has been cited by many for this decline. Previous research at the University of Wyoming has focused on the development of a single dose oral contraceptive, RU 486, to terminate pregnancy in coyotes. In order to obtain Federal Drug Administration (FDA) approval, a species-specific delivery mechanism must be established. Our project proposed to field test the selectivity of the Coyote Lure Operative Device (CLOD) as a delivery mechanism for reproductive inhibitors. Eight research sites were selected in southeastern Wyoming. Each research site had one CLOD coated with Karo syrup and Fatty Acid Scent (FAS) from December 22nd, 2006 to April 5th, 2007. Eight additional paraffin and FAS dipped CLODs were placed in the field from February 20th to April 5th, 2007. Remote wildlife cameras with infrared flash were used to monitor CLOD activity from December 22nd, 2006 to February 8th, 2007. Weather patterns and mammalian activity were recorded for the duration of the study. A Pearson test showed a positive correlation between activations and temperature, \( r_{xy} = 0.835 \) (\( p = 0.000 \)). Preliminary scat census determined coyote density to be 1.6 coyotes/mi\(^2\). Four CLODs were activated during the study. DNA analysis confirmed three coyotes and one undetermined species that activated the CLODs. Continuing research will see modifications to the research design that include an increase in field sites with various CLOD designs that work on coyote sensory modalities.

Introduction

Coyotes (Canis latrans) have and continue to be significant predators of livestock, mainly domestic sheep (Wagner 1988). The extirpation of wolves in North America resulted in an increase in mesocarnivores. With no natural predators and a readily available food source (domestic livestock), coyote populations expanded across North America. In 1915 a Federally Funded Predator Removal program was established to decrease sheep predation by coyotes in order to increase food production during the war (US Fish and Wildlife Services 1978). Since then, the coyote has proven to be a highly successful predator and a challenge for management of the species.

At an all time high of 56.2 million animals in 1942, the sheep industry has since seen an 85% decline in production (Knowlton et al. 1999; Berger 2006). Declining economic viability of the sheep industry coupled with predation, chiefly by coyotes, is usually cited for this decline. Predator control devices and techniques including coyote getters, steel leg hold traps, denning, aerial gunning and the widespread use of predacides started coming under public scrutiny during the 1960’s. The Leopold Report of 1964 determined that predator control was indiscriminate and excessive with costs exceeding the benefits (Leopold et al. 1964). The Cain Report of 1971 found the use of Compound 1080 to be inhumane and non-selective which culminated in Executive Order 11643, banning compound 1080 for widespread predator control (Cain et al. 1971).

As we move towards awareness of the significance of carnivores in terrestrial ecosystems, there is an increasing need for publicly acceptable and humane methods of population control. A variety of non-lethal population control methods including reproductive inhibitors are currently being tested.

Research has shown that depredations are highest when coyotes have pups to provision for (Till and Knowlton 1983; Bromley and Gese 2001). Work at Hopland Research Extension Center found that if breeding pairs were removed from a territory, a new breeding pair would be established in that area within three months (Jaeger et al. 2001; Blejwas et al. 2002). The idea for reproductive control is that the absence of pups will reduce sheep depredation while leaving the breeding pair to maintain territories. Research conducted at the University of Wyoming has focused on the development of a single dose oral contraceptive, mifepristone, to terminate pregnancy (Horn et al. 2006). In order to secure Federal Drug Administration (FDA) approval, a delivery mechanism must be established.

The Coyote Lure Operative Device (CLOD) has the potential to deliver reproductive inhibitors to free-ranging coyotes. The basic CLOD design consists of a polyethylene bottle (head), filled with the compound, screwed onto an iron stake that has been pounded into the ground. Lure is applied to the head to attract coyotes and elicit a lick-bite-pull response necessary for CLOD activation. Activation consists of chewing the bottle open and ingesting the contents.

Previous CLOD research has shown an increase in activations by coyotes while decreasing non-target activations as modifications are made to the original CLOD
design (Stolzenburg 1986; Hein and Andelt 1994; Berentsen 2004; Buseck 2004). Field trials of the CLOD show an average of 5% activation success (Stolzenburg 1986; Berentsen 2007).

Lures have been the focus of previous CLOD studies to determine correlations between lures and activations. Carmines Canine Call, Government W-U Lure, and Fatty Acid Scent (FAS) have shown little difference in preference by coyotes but this does not explain the overall low rate of activations (Stolzenburg 1986; Ebbert 1988; Berentsen 2004). Despite this low activation rate, the CLOD remains the only option for delivery of oral compounds to coyotes. To date, there have been no field studies focused on what sensory modalities are used by coyotes activating or inactivating CLODs. Very little is known about the interplay between sensory perception and neophobic responses in free-ranging coyotes. Future management of the species depends on an understanding of predatory and behavioral ecology that determine the coyote’s success.

Preliminary fieldwork in Wyoming has suggested that neophobia may be affecting CLOD activations. Coyotes have shown fear avoidance response behaviors to novel stimuli in their environment (Mason and Burns 1997; Harris and Knowlton 1999). What sensory modalities are affected by novel stimuli as well as seasonal correlations needs to be established to further CLOD research.

Visitation and activations by non-target species must also be assessed to establish the efficacy of the CLOD as a coyote specific delivery mechanism. Broader implications for this field research moves towards increased success of the CLOD, culminating in the use of reproductive inhibitors as a selective and humane method of coyote control.

The primary objectives of the first year of this two-year study were to assess variables that affect visitation and activations. Current literature in this area is minimal and mainly involves captive reared species. Field data collected along with current literature helped to modify and improve the research proposal for year two, which includes assessing how sensory modalities affect CLOD activations.

**Materials and Methods**

**CLOD Design**

Our research project utilized the basic design of the CLOD with modifications made as suggested in the literature (Marsh et al. 1982; Berentsen 2004; Buseck 2004). High-density one oz. amber colored Nalgene bottles were attached with a 7/16 in. screw, bolt and rubber washer to a 12 in. iron stake. Equal portions of organic flax oil and animal lard was placed inside the Nalgene bottle. This mixture was determined to be a successful delivery medium for mifepristone in a controlled environment (Busek 2004).

**Lure**

In order to elicit a bite, chew, lick response necessary for CLOD activation, a lure was applied to the exterior of the nalgene bottle. Prior research has shown that coyotes may have a preference for Fatty Acid Scent (FAS), a commercially available product (Berentsen 2004; Buseck 2004). To cut the FAS, a 10% dilution was made with mineral oil in a ventilated hood. Dark Karo syrup was applied around the exterior of each Nalgene bottle with 0.02 oz. of 10% FAS (Figure 1).

**Figure 1.** CLOD in the field covered in dark Karo syrup with 0.02 oz. of 10% FAS.

CLODs were checked once a week at which fresh Karo syrup and FAS was applied to the exterior of the Nalgene bottle. Additional olfactory stimulation was added for a three-week trial period for dissemination of lure over a broader range. Simply Basic® cylinder loofah (surface area=47.1 in.) were dipped in 17 oz. melted paraffin and 2 oz. 10% FAS under a ventilated hood. Four dipped and undipped loofahs were randomly placed at all sites (Figure 2).

**Figure 2.** Cylinder loofah dipped in melted paraffin and 10% FAS solution.

Eight additional CLODs were placed in the field within 10 ft. of the first ones. To decrease the volatilization of the lure, two oz. of 10% FAS was added to 17 oz. of melted paraffin. Nalgene bottles were dipped weekly three times in the paraffin/FAS mixture and placed in the field (Fig.3.)
CLOD Placement

Eight research sites were established for CLOD placement, respectively called Coyote 1 - Coyote 8 in southeastern Wyoming. The goal was to select sites with recent coyote activity yet that was far enough apart to rule out activity by the same coyote. Research sites, coyote 1 – coyote 8, were determined based on visible coyote signs (scat, urine, and tracks) spatially separated by geographical borders no less than 1 mi. apart (Figure 4).

Remote Cameras

Eight Cuddeback NO Flash cameras were purchased. Each camera takes 3-megapixel daytime color images and 1.3-megapixel black/white images during darkness via infrared flash. An adjustable heat in motion sensor detects mammals up to 30 ft. away. Cameras were attached to a wood wedge with a wood screw. Two screws secured the wedge and camera to the tree 5 ft. up and at a 45° angle to the CLOD (Figure 5).

Climate Data

Temperature, wind speed, wind gusts and relative humidity were recorded daily from a National Weather Service personal weather station (Lat 41°9’2”; Lon 105°5’7”) 15 miles west of Cheyenne, Wyoming. Data was collected at 0600, 1200, 1800, and 2400 from December 22, 2006 – April 4, 2007.

Scat Census

A coyote scat deposition survey for study sites 5-8 was conducted in April to determine relative abundance within the study area. Two one mile linear transects were established along similar unimproved roads. Transects were walked on April 16th, 2007 twice with all scat removed from the area. On April 22nd, 2007 transects were walked twice with all fresh scat recorded. Number of scat recorded was divided by seven to provide a coyote scat indices of # of scat/day/mile. This information was plugged into a known regression equation, \( y = 2.66 + 11.42x \), with \( y \) = coyote scat indices and \( x \) = coyotes/square mile (Knowlton 1984).

Results and Discussion

Remote cameras recorded CLOD visitations by target and non-target species from December 22, 2006 – February 8, 2007. Visitations were recorded on compact flash cards for the eight CLOD sites for a total of 384 CLOD nights (Table 1).
Table 1. Total number of visitations by target and non-target species based on 384 CLOD nights. CLOD nights consist of total CLODs multiplied by number of field nights. Visitations recorded by Cuddeback NO-Flash digital cameras.

<table>
<thead>
<tr>
<th>Species</th>
<th>Total CLOD Nights</th>
<th>Total Visits</th>
<th>Average Visits (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canis latrans</td>
<td>384</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Leporidae family</td>
<td>384</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Odocoileus hemionus</td>
<td>384</td>
<td>23</td>
<td>6</td>
</tr>
</tbody>
</table>

Cuddeback cameras were removed February 8th, 2007 and all CLODs were moved to new locations approximately 98 ft. from previous locations. One additional CLOD dipped in paraffin and 10% FAS was placed in the field 10 ft. from the other CLODs at eight locations, bringing total CLODs in the field to 16.

Two partial activations (contents partially consumed) and two full activations (contents consumed and nalgene bottles completely shredded) were recorded during the second half of the study, February 8th – April 4th, 2007. No activations occurred with the paraffin dipped CLODs.

On numerous occasions scat and tracks were observed in the vicinity of the CLODs. On March 1st, fresh tracks were observed leading up to CLOD #6, making a complete circle around the CLOD while keeping a 5 ft. spatial distance from the CLOD. Fresh scat containing blood was found 2 ft. from CLOD #6 (Figure 6).

![Figure 6. Fresh scat (< 1 week) with blood found 2 ft. from CLOD #6 on March 1, 2007.](image)

Daily temperature, wind speed, wind gusts and relative humidity in the study area was recorded at 0600, 1200, 1800, and 2400 hours. Data was compiled into weekly averages for the duration of the study. A Pearson test showed a positive correlation between activations and temperature, \( r_{xy} = 0.835 \) (\( p = 0.000 \)).

A scat deposition survey was performed April 16th and April 22nd, 2007. All scat was removed from two one-mile transects by foot on April 16th. On April 22nd transects were walked and three scats were recorded. This gave us our coyote scat index of 3-scat/2 mi/per 7 days. To determine the number of coyotes per square mile we plugged this information in a regression equation and extrapolated for \( x \) (coyotes/sq. mi.)—\( y = 2.66 + 11.42\ x \). Coyote density estimate is 1.6 coyotes per square mile.

That coyotes will activate the Coyote Lure Operative Device (CLOD) in a captive setting in Wyoming has been established (Busek 2004). Whether or not the CLOD is successful for delivery to free-ranging coyotes in Wyoming remains to be determined. Variables must be manipulated to address factors outside of captivity, such as: the volatilization of the lure in inclement weather and the presence of novel stimuli in familiar settings. Visitations and activations by non-target species must also be assessed to establish the efficacy of the CLOD as a coyote specific delivery mechanism.

Research sites (coyote 1 – coyote 8) were established in a montane ecosystem in Southeastern Wyoming on federal, private and state lands. Species diversity, flora and fauna, and winter accessibility was the deciding factor. One Karo syrup/FAS CLOD per location was placed in the field on December 22nd, 2006. A 50/50 mixture of lard and flax oil was put inside the nalgene bottle. Not only is this the bait necessary for delivery of mifepristone but also it has shown to be highly palatable to coyotes (Busek 2004).

Cuddeback NO-Flash remote cameras were chosen based on price, ease of operation, adjustable sensitivity beam and infrared nighttime flash. The cameras through February 8th, 2007 recorded CLOD visitations. Highest visitations were by mule deer (Odocoileus hemionus) followed by lagomorphs (Leporidae family) and coyotes (C. latrans). That there were no activations by visiting non-target species is promising. Past studies have shown multiple activations by non-targets including lagomorphs and ungulates (Stolzenburg 1986; John Johnson Personal Communication 2007).

That coyotes on film showed no interest in the CLOD led us to speculate that either the lure was not affective or the coyotes were exhibiting a fear avoidance behavior of the CLOD/camera. Strong winds, high humidity and low temperatures may be affecting volatilization and dissemination of the lure. We experimented for three weeks with additional olfactory stimulation by placing a loofah sponge dipped in melted paraffin and 10% FAS solution near the CLOD.

Four controls and four treatments were randomly placed with no results. Cameras were removed and CLODs were moved to a new location within 98 feet of the original placement. This decision was based on prior research on wariness of dominant coyotes to avoid camera traps as well as avoidance behavior of novel stimuli in familiar environments (Harris and Knowlton 2001; Sequin et al. 2003; Berensten 2004).

An additional CLOD dipped in paraffin and 10% FAS was placed at each research site within 10 ft. of the other
CLOD. This was done for the remainder of the study, February 8th–April 4th 2007. During this time, two partial activations and two full activations were recorded. Partial activation indicates the CLOD was chewed on but no contents were ingested. No activations occurred with the paraffin/FAS CLOD.

Based on bite marks, tracks and scat, we theorize that coyotes are activating the CLOD. DNA analysis confirmed three coyotes and one unidentified species that activated the CLODs. That all activations occurred with Karo syrup/FAS lure supports the belief that coyotes have a preference for sweet things (Berentsen 2004; Buseck 2004).

Presence of coyote tracks paralleling and circling the CLODs lead us to believe that there is some neophobia of the device. The presence of blood marking scat on March 6th was worth noting. Females normally produce blood in their urine during estrous. We speculate a later than average estrous cycle is occurring. Whether this is indicative of coyotes in Wyoming or one individual is uncertain.

Modifications will be made to the original research design based on findings from the first field season. Emphasis will be placed on expanding upon the role of coyote sensory modalities and this relationship to the activation of the CLOD. DNA analysis in collaboration with USDA/APHIS/Wildlife Service will provide identification of species activating the CLOD. Further DNA analysis of target species will sex the individual coyote.

Acknowledgements

Special thanks to Professors Rich Olson, Steve Buskirk, and Brenda Alexander for their mentorship, Dr. Dan Rule (and Chuck) for allowing me to use the malodorous FAS in his lab, the WY Animal Damage Management Board and TX Sheep and Goat Predator Board for funding this project and Dr. John J. Johnson at USDA/APHIS/Wildlife Services for DNA analysis.

References

Horn, S.W., M. Hussain, W.He, and C. Stith. 2006. Extended release of mifepristone (RU 486) for field delivery to coyotes. In manuscript.
Summary and Implications

In areas of Montana, North Dakota, South Dakota, and Wyoming sulfur toxicity is becoming an increasing problem due to high concentrations of sulfate in stock water. High sulfate water coupled with the feeding of sulfur rich feedstuffs, including corn gluten meal and distillers dried grains (by products of the ethanol industry), lead to an increased incidence of sulfur-induced polioencephalomalacia (PEM). Alternative water sources in these areas are either unavailable or not cost-effective to cattle producers. Currently, the NRC recommends a diet of ≤ 0.3% DM sulfur for growing and adult beef cattle, with a maximum tolerable dose of 0.4% DM. However, subclinical signs of sulfur-induced PEM, including poor performance, have been associated with diets containing ≤ 0.4% DM sulfur (Gould, 2000). In this study, steers (n = 96) were assigned to one of four treatment groups for 77 d: control (CTRL; ≤ 400 ppm SO₄), high sulfate water (HS; ≥ 3000 ppm SO₄), high sulfate water plus high zeolite (HSHZ; ≥ 3000 ppm SO₄; 5% zeolite), and high sulfate water plus low zeolite (HSLZ; ≥ 3000 ppm SO₄; 2.5% zeolite). Zeolite, a hydron sink agent was used to bind hydrogen ions to prevent excess hydroden sulfide gas. The HSLZ treatment group had 5 confirmed cases of sulfur-induced PEM and 4 suspected cases, the HSHZ treatment group had 3 confirmed cases and 1 suspected case, and the HS treatment had 1 confirmed case and 3 suspected cases. No cases of PEM were observed among CTRL steers. The CTRL steers performed better overall, gaining an average of 79.9 kg during the course of the trial, compared to 68.2, 71.9, and 68.4 kg of gain for HSHZ, HSLZ and HS groups, respectively. Results from this study confirm individual variation in response to high sulfate water. Additionally, zeolite at these concentrations does not appear to be an effective ameliorator of sulfur-induced PEM.

The objective of this study was to confirm previously observed individual variation to elevated concentrations of dietary sulfur, determine if zeolite, a hydrogen sink, is an effective ameliorator of sulfur-induced PEM, and determine differences in gene expression between more and less tolerant animals.

Introduction

Dietary sulfur is required for cattle amino acid synthesis; however, negative effects on production and health have been observed when dietary sulfur exceeds 0.2% DM. (Loneragan et al., 2000). This toxicity can lead to the development of sulfur-induced PEM. Sulfide, reduced from sulfate, is either absorbed in the rumen or used by the microbes to create proteins. Normally, sulfides absorbed by the rumen are turned into sulfate in the liver and recirculated into the rumen to create essential amino acids, including methionine and cysteine. However, when excess sulfide ions are created due to increased sulfate intake H₂S gas is produced, which in excess inhibits cytochrome oxidase in the electron transport system. This inhibition causes a reduction in the production of ATP. Cortical neurons are sensitive to this change in ATP, and as a result necrosis occurs in the brain (Karamjeet, 2000).

![Sulfate Pathways](image)

**Figure 1.** Depicts brain necrosis pathways caused by excess sulfate in the rumen.
Materials and Methods

Animals and Treatments

This study was conducted at the South Dakota State University Cottonwood Range and Livestock Research Station. Steers (n = 96) were randomly assigned to one of four treatment groups: control (CTRL; ≤ 400 ppm SO_4), high sulfate water (HS; ≥ 3000 ppm SO_4), high sulfate water plus high zeolite (HSHZ; ≥ 3000 ppm SO_4; 5% zeolite), and high sulfate water plus low zeolite (HSLZ; ≥ 3000 ppm SO_4; 2.5% zeolite), with three pens of eight animals per treatment. Steers remained on trial for 77 d, and water and feed intake were recorded daily to track sulfate consumption. For this experiment, zeolite was hypothesized to bind excess hydrogen ions, and prevent the formation of hydrogen sulfide (H_2S) gas, thereby preventing sulfur-induced PEM. Steers were monitored daily for signs of sulfur-induced PEM. Liver biopsies were performed prior to and after the trial to collect tissue for future gene expression analyses. Biopsies were also performed on steers exhibiting symptoms of PEM at the time of onset. Blood samples were collected at the beginning and end of the experiment, and also on d 58 of the trial. Cattle were weighed at the start of the trial and every 28 d throughout. Upon observation of clinical symptoms of sulfur-induced PEM, steers were removed from experiment, biopsied, blood sampled, and treated promptly with thiamine, dexamethazone, and Vitamin B_12 to reduce the severity of symptoms. Surviving affected steers were monitored closely and treated with a probiotic on d 7 following onset of symptoms.

Liver Biopsies

Liver biopsies were taken from live animals with a bone marrow punch from the animal’s right side in the intercostal space between the 11th and 12th ribs. The incision for the biopsy was made on a line drawn from the point of the hip to the point of the shoulder. Using the bone marrow punch, liver samples were collected by repetitive penetrations into the liver, and extracted through a tube attached to a syringe. Samples were immediately rinsed with a 1X PBS buffer solution and snap frozen.

Statistics

Data were analyzed using the GLM procedures of SAS (SAS Inst., Inc., Cary, NC). Differences in least-squares means were tested using a LSD.

Results and Discussion

The HSLZ treatment group had the highest number of confirmed and suspected cases of sulfur-induced PEM with 5 confirmed and 4 suspected. The HSHZ treatment group had 4 cases, of which 3 were confirmed. The HS treatment group also had 4 cases, but only 1 case was confirmed. In all, 9 confirmed cases of sulfur-induced PEM were recorded along with 8 suspected across high sulfur treatment groups, with the CTRL group exhibiting no signs of sulfur-induced PEM (Table 1). Cases were confirmed by the Veterinary Diagnostic Lab at South Dakota State University by analyzing brain samples taken from deceased animals. Suspected cases exhibited clinical signs of sulfur-induced PEM, but survived the onset of symptoms. The CTRL steers had a numerically higher average weight gain (79.9 kg) than steers in the HSHZ, HSLZ, and HS treatment groups. Gains for the HSHZ, HSLZ and HS groups averaged 68.2, 71.9, and 68.4 kg, respectively (Figure 2).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Confirmed</th>
<th>Suspected</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Sulfate Low Zeolite (HSLZ)</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>High Sulfate High Zeolite (HSHZ)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>High Sulfate No Zeolite (HS)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Control Group (No Sulfate/No Zeolite)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1. Number of PEM cases across treatment groups.
Results from this study suggest that zeolite at the tested concentrations is not effective in preventing sulfur-induced PEM in roughage-fed feedlot steers. The HSLZ treatment group had a higher numerical incidence of sulfur-induced PEM, and the HSHZ treatment group had as many cases of sulfur-induced PEM as the HS group. Further testing of other hydrogen sinks is recommended to find a successful ameliorator for sulfur-induced PEM. Gene expression differences in liver samples from highly and lowly tolerant steers will be analyzed using the bovine array chip at Montana State University Functional Genomics Core Facility. A summer 2008 pasture and feedlot studies will be conducted to confirm current findings and test new feed additives.

References

Use of Mifepristone (RU 486) For Reproductive Control in Coyotes

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Summary and Implications

Coyote (Canis latrans) depredation of livestock is a problem for many agricultural producers. Research suggests that sterilization of breeding pairs in a territory will reduce the level of predation by as much as 91%. This research project investigated the use of mifepristone (RU 486) as an abortifacient for the temporary cessation of reproduction in coyotes. Five experiments were conducted during 5 breeding seasons between 1998 and 2003. In each experiment blood samples were collected to analyze serum concentration levels of RU 486. Dosage levels of RU 486 (7.5-30 mg/kg), duration (2-7 days) of treatment, and solubility of the drug were manipulated in each experiment to obtain optimal effectiveness for use in a field delivery system. Treated coyotes in 4 of 5 experiments failed to deliver pups. Due to the high degree of effectiveness and the ease of administration of oral baits, RU 486 appears to be a good candidate for controlling coyote reproduction.

Introduction

Coyote predation on domestic livestock remains a significant problem throughout North America, especially in the western United States (Knowlton et al.,1999 and Wagner and Conover, 1999). Environmentally safe, humane predator control remains a goal of livestock producers and wildlife managers. Restrictions on the use of toxic substances, limited use of trap devices, and in some cases the designation of coyotes as game animals with seasonal limits has made coyote population control difficult. The administration of an oral compound that blocks the essential functions of progesterone and terminates pregnancy in coyotes is the basis of this research.

The coyote, a seasonally monstrous, monogamous canid, breeds in late January and early February. Length of gestation is 58-60 days with an average litter size of five pups (Geir, 1968). Research done at the Mendacino County, California, Hopland Research and Extension Center (Sacks et al., 1999), as well as work done at the Deseret Ranch in northern Utah by Bromley and Gese (2001a), indicates most predation is attributed to breeding pairs of coyotes. Removal of pups from alpha pairs can reduce predation by as much as 91.6% (Bromley and Gese, 2001; Till and Knowlton, 1983).

In 1987, researchers at the pharmaceutical company Roussel Uclaf (Romanville, France) published the first papers introducing the antiprogestin mifepristone (RU 486). It has been used in a variety of species (Concannon et al., 1990) as an abortifacient with high levels of success (80%) following a single oral dose (Broden et al., 1993). When used in domestic dogs, termination of pregnancy without negative side effects was reported after 4.5 days of administration (Concannon et al., 1990 and Sankai et al., 1991) demonstrating that mifepristone was effective as an abortifacient in this species.

Materials and Methods

Study Area

Experiments I and II were conducted at the facilities of the National Wildlife Research Center (NWRC), Millville, UT. Experiments III, IV and V were conducted at the University of Wyoming Red Buttes Environmental Biology Laboratory. In both locations, coyotes were maintained without artificial heat or light.

Feeding

A constant diet of water ad libitum and 650g commercially prepared food consisting of meat, poultry, and fish with cereal (Furbreeders Agricultural Cooperative, Sandy, Utah) was used for the first two experiments. During experiments III, IV and V, dry kibble, Mazuri® Exotic Canine Diet, (PMI Nutrition International, LLC, Brentwood, MO) was fed, supplemented with meat scraps.
Breeding

Males were paired with females by placing them in adjacent kennels prior to the beginning of the project. The onset of estrus was determined by signs of receptivity by the female. Corresponding interest by the male, the presence of blood tinged vaginal discharge, and vaginal swelling were also used as visible signs of estrus (Roberts, 1971).

During estrus the male was placed with the female on alternate days. Pairings were stopped when the female no longer showed signs of receptivity toward the male. Copulatory ties were recorded for each of the breeding pairs.

Thirty days following the mid-point of the breeding period, all females were given an intramuscular dose of 0.5 ml of anesthetic consisting of a mixture of 100 mg/ml Ketaset® (ketamine HCl – Fort Dodge®), mixed with 1.0 mg/ml of PromAce® (acepromazine maleate-Fort Dodge®) tranquilizer. Palpation of embryonic vesicles (Kirk, 1977) and ultrasound examination were used to diagnose pregnancy. The ultrasound was performed using an Aloka® 500V ultrasound scanner and a 3.5 MHz convex transducer probe.

Blood was drawn from treatment and control animals at 24, 48, 72, and 96 hours after initial administration of RU 486 (treatment animals) or a placebo (control animals). High Performance Liquid Chromatography (HPLC) was used to analyse blood serum concentration levels of RU 486. The HPLC technique was a reverse-phase chromatography procedure using a C-18 separation column through which an isocratic mobile phase consisting of 55% acetonitrile (ACN), 45% water was passed. Detection of the analyte RU 486 was measured with a variable absorbance ultra-violet (UV) detector set at 305 nm wavelength. Samples were extracted using C-18 solid-phase cartridges, dried under nitrogen gas and redissolved in 100 ul of ACN, then 50 ul of this solution was injected onto the column. (Stith and Hussain, 2003).

Experiment I – 1999

Ten pregnant females (six treatment, four control) were used. Treated animals received mifepristone (RU 486, Sigma Chemical Corp., St. Louis, Missouri) weighed and packed into a gelatin capsule and administered at a dose of 7.5 mg/kg (average weight of females was 11 kg – total daily dose was 82.5 mg) given on two consecutive days. Control subjects received a capsules filled with corn starch. In both groups a single capsule was placed in the center of a 2-3 cm² ball of the coyote’s normal food, which was then placed in the kennel.

Following the two treatments of RU 486, a 200 ug Misoprostol® tablet, a synthetic prostaglandin, (GD Searle and Co., Chicago, IL) was given, once per day for 2 days in a meat bait in order to increase the effectiveness of RU 486 (Ulmann and Silvestre, 1994). Blood samples were drawn from all ten animals at 24, 48, 72, and 96 hrs. post-treatment.

Experiment II – 2000

Nine pregnant females (six treatment, three control) were used. The daily dose of RU 486 was 30 mg/kg. Each treated coyote was treated once daily for seven days. Control subjects received corresponding amounts of corn starch. Blood samples were drawn at 96 and 172 hrs. post-treatment.

Experiment III – 2001

Eight pregnant females (five treatment, three control) were used. Each treatment coyote received a single capsule containing 15mg/kg of RU 486 once daily for five consecutive days hidden in blocks of meat approximately 2 cm² and offered at the normal feeding time. Control animals received the same sized meat bait containing a capsule of cornstarch. Blood was drawn at 24, 48, and 72 hrs. post-treatment.

Experiment IV, 2002

Eight pregnant females (five treatment, three control) were used. Treatment animals received 10 mg/kg given once daily for four days. Control subjects received corresponding amounts of corn starch. Blood was drawn at 96 hrs. (four hours after the last dose of RU 486).

Experiment V, 2003

Eight pregnant females (five treatment, three control) were used. Two treatment subjects received 10 mg/kg of RU 486 once daily for 3 days. Three additional treatment subjects received the same dosage of RU 486 but with a higher solubility formulation of made by solid dispersions of RU 486 with polyethylene glycol (PEG 4000) (1:1 ratio) (Dissolution study, Hussain Laboratory, University of Wyoming, 2003). Blood for HPLC analysis of serum concentration levels of RU 486 was drawn at 72 hrs. (four hours after the last dose).

Results and Discussion

Experiment I

One subject in the treatment group (1 of 6) failed to whelp. All subjects (4 of 4) in the control groups whelped successfully.
Experiment II

All subjects in the treatment group failed to whelp. All subjects in the control group whelped successfully (3 of 3).

Experiment III

All five experimental subjects (5 of 5) failed to whelp and all controls (3 of 3) delivered live pups. In comparison to the controls, the treated animals appeared smaller abdominally and did not appear to be pregnant within a few days after the treatment period ended. The control animals had enlarged mammae and appeared pregnant at close range.

Experiment IV

All five subjects (5 of 5) in the treatment group failed to whelp. The three control subjects delivered live pups (3 of 3).

Experiment V

All five subjects (5 of 5) in the treatment group failed to whelp. The three control subjects delivered live pups (3 of 3).

The effectiveness of RU 486 in terminating pregnancy in coyotes was evaluated over five breeding seasons in pilot research experiments designed to investigate dosage efficiency. In the first experiment, a low dose of 7.5 mg/kg given for 2 consecutive days, followed by two doses of a synthetic prostaglandin induced abortion in 16.6% (1 of 6) of treated females. In the following year the dose was increased to 30 mg/kg and administration was lengthened to 7 days. This resulted in all 6 treatment coyotes aborting and all controls whelping live pups. In an effort to determine the lowest dose possible for termination of pregnancy in the coyote, experiments were then conducted using decreasing doses of RU 486 and reduced duration of treatment. In these experiments coyotes received 15 mg/kg for 5 days, 10 mg/kg for 4 days, and in the final experiment, 10 mg/kg for 3 days. In each experiment all treated females aborted and all controls whelped successfully. Further research is needed to fully define the lowest dose and shortest administration time necessary to terminate pregnancy. The use of solid dispersion formulations of RU 486 with PEG 4000 may increase the bioavailability and efficacy of RU 486. Serum concentrations of RU 486 were 868 ng/ml for coyotes that received RU 486 and PEG 4000 compared with 412 ng/ml for those that received only RU 486. Additionally, sustained release formulations may allow administration of a single treatment to induce abortion in female coyotes (He, et al., 2006).

Administration of identical amounts of RU 486 yielded very different serum levels of the drug. In Experiment I the only female that aborted had no quantifiable serum level of RU 486 in 4 of 4 blood samples analyzed. In the subsequent experiments, treated coyotes, all of which aborted, had serum concentrations of RU 486 that ranged from a high of 2976.6 ng/ml to a low of 175 ng/ml. A possible explanation for the wide range of serum concentrations may be physiological variations that exist between coyotes and their differing abilities to absorb RU 486 from the digestive tract. Diet, especially fat content, may also affect the absorbance of RU 486. The conclusion from these experiments indicates that varying serum concentration levels of RU 486, resulting from dosage regimens of as little as 10 mg/kg given for 3 days, will terminate pregnancy in coyotes.

Mifepristone (RU 486) presents an attractive alternative to conventional predator control methods. The antiprogestin RU 486 is a derivative of cholesterol as are all antiprogestins and, therefore, have many of the same properties as steroid hormones (Dence, 1980, Teutsch et al., 1995). Primary differences between native progesterone and mifepristone are the lack of a C19 methyl group and the 2-carbon side chain at C17, but most striking is the presence of a 4-(dimethylamino)phenyl group at C11 (Baulieu, 1989 and Cook et al., 1994). This substitution at the C11 position imparts binding activity to RU 486 by allowing the dimethylaminophenyl group to sit in the 11ß cleft altering the receptor-ligand conformation to an inert form (Robbins and Spitz, 1996). Association of the progesterone receptor with either native progesterone or RU 486 causes dimerization of the receptor. This association between progesterone and its receptor facilitates transcription and subsequent biological effect or, in the case of RU 486, blocks transcription and eliminates the biological effect (Wagner et al., 1996). For the RU 486-progesterone receptor complex, an inhibitory effect is exerted at the C-terminal region of the binding domain, effectively blocking transcription and, therefore, the biological effect (Robbins and Spitz, 1996). The actual mechanism of action is stabilization of the receptor-ligand complex to its corresponding heat shock protein complex. Association with the transcription factors is prevented, as is binding to the correct hormone response element of the DNA, effectively blocking transcription (Baulieu, 1990). Further evidence suggests that 16- and 17α-substituents may also play a major role in determining the agonist or antagonist activity profile of mifepristone and its analogs (Cook et al., 1994).

Current methods of managing predators are expensive. In one study, the cost of a helicopter used for aerial gunning and the required personnel to provide support was $425 per hour. The cost to keep a Wildlife Services field specialist on the job for a year (1,852 hours) was $50,000 (Wagner and Conover, 1999). A single dose (100 mg) of RU 486 is $18.00 at research catalog prices (Sigma Chemical Corp.,
St. Louis, Missouri) and would be lower in bulk. Thus, the economic advantage of placing RU 486-laced baits in coyote territories, as opposed to aerial gunning and trapping, is appealing.

Understanding the biological importance of RU 486 as it applies to reproductive physiology and its efficacy as an abortifacient in many mammalian species (Baulieu, 1985), makes the prospect of its use for coyote control invaluable. Bioadhesive nanoparticles containing RU 486 have been shown to increase oral bioavailability of mifepristone in rats (He, et al., 2006). The efficacy of solid dispersion formulations and time-release nanoparticles of RU 486 as an antiprogestin in coyotes has been established (Horn, et al., in manuscript). A field delivery mechanism for reproductive inhibitors is currently being investigated (MacGregor and Horn, 2007). With details of dose formulation and field presentation finalized, this approach may represent a new paradigm in predator management.

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References


AMP-Activated Protein Kinase is Negatively Associated with Intramuscular Fat Content in *Longissimus Dorsi* Muscle of Beef Cattle

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**Summary and Implications**

Marbling, or intramuscular fat, is an important factor in meat quality. As a key regulator of lipid metabolism, AMP activated protein kinase (AMPK) may be associated with intramuscular fat accumulation. Our objective was to evaluate the relationship among AMPK and its associated signaling mediators, with marbling and lean growth in beef cattle. Steers with high intramuscular fat content (High IMF, 5.71 ± 0.36%, n=5) and low intramuscular fat content (Low IMF, 2.09 ± 0.19%, n=5) were selected. High IMF was associated with increased tenderness (*P*<0.05) and backfat thickness (*P*<0.01). Muscle weights were higher in Low compared to High IMF (*P*<0.05). High IMF steers had a reduced AMPK activity (*P*<0.01), reduced acetyl-CoA carboxylase phosphorylation (*P*<0.05), and reduced total mTOR (*P*=0.02) content. Data provides evidence that AMPK is involved in IMF deposition in beef cattle.

**Introduction**

The quality grading of young beef carcasses is primarily based on marbling (intramuscular fat). A highly marbled beef A maturity carcass (prime grade) can be sold at a premium of $31.52 per hundred pound compared with the same maturity low marbled carcasses (standard grade) (According to USDA Market News, Dec 22, 2006). Therefore, increased marbling in young beef will garnish enormous economic returns. Mechanisms controlling the intramuscular fat accumulation, however, remain poorly defined. Such knowledge is crucial for us to develop strategies to enhance intramuscular fat accumulation.

Adenosine monophosphate-activated protein kinase (AMPK) has a central role in the control of energy metabolism (Hardie, 2005). Activation of AMPK inhibits lipogenesis while promoting lipid oxidation (Carey et al., 2006; Ravnskjaer et al., 2006; Yoon et al., 2006). Activated AMPK inhibits lipogenesis through phosphorylation and inhibition of acetyl CoA carboxylase (ACC), a key enzyme in lipogenesis. Furthermore, AMPK activity is negatively associated with lipid oxidation (Carey et al., 2006; Ravnskjaer et al., 2006; Yoon et al., 2006). Therefore, low AMPK activity is expected to promote lipogenesis and inhibit lipid oxidation, which should increase intramuscular fat accumulation. However, the role of AMPK in intramuscular fat accumulation has not been studied.

Besides its role in lipid metabolism, AMPK interacts with insulin/insulin like growth factor-1 (IGF-1) signaling (Jakobsen et al., 2001). Insulin/IGF-1 plays a major role in stimulating muscle growth. The protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway is the main pathway down-stream of IGF-1/insulin signaling (Latres et al., 2005). Alteration in AMPK activity may result in changes in mTOR signaling and affect lean growth. Our objective was to evaluate the relationship of AMPK and its associated signaling mediators, with marbling and growth of the *longissimus dorsi* muscle (LM) in beef cattle.

**Materials and Methods**

*Care and Use of Animals*

All animal procedures were approved by the University of Wyoming Animal Care and Use Committee. One hundred and sixteen multiparous Angus × Gelbvieh cows were synchronized using controlled intravaginal drug release device (CIDR, Pharmacia and Upjohn Co., Kalamazoo, MI) implants and artificial insemination to a single South Devon sire by a trained technician. Calves were vaccinated, branded, and bull calves were castrated as per the University of Wyoming herd processing procedures. Cows and calves grazed summer pasture at the University of Wyoming McGuire Ranch. Calves were weaned at 155 days of age. Steer calves were fed a three step back-ground ration for 60 days to allow for adjustment to a high energy diet. The back-ground ration began with 47% roughage and decreased stepwise to the final diet containing 13% roughage with a 1.06 Mcal/kg NEg and increasing to 1.37 Mcal/kg NEg in the final diet (Table 1). Steers were maintained on the same diet for 168 days. Steers average daily gain was 2.64 ± 0.11 lbs during the 168 day feeding period. Three weeks before slaughter, animals were stratified into harvest groups by BW and age. Forty Angus × Gelbvieh steers at 13.5 months of age with a final body weight of 1090 ± 15.9 lbs. were slaughtered at University of
Wyoming Meat Laboratory. Immediately after exsanguination and evisceration (less than 10 minutes postmortem), a sample was removed from the *longissimus dorsi* muscle (LM) muscle between the 12th and 13th rib of the right side of carcasses by inserting a knife from the body cavity. After trimming off all visible adipose and connective tissues, small pieces of muscle (1g) were collected and snap frozen in liquid nitrogen for biological analyses.

**Carcass Data**

The percentage of kidney, pelvic and heart (KPH) fat was calculated by dividing the KPH weight by the hot carcass weight + KPH and multiplying by 100. Standard carcass data measurements were collected after carcasses had cooled for 48 hours at 36-39°F. Carcasses were ribbed by bisecting the carcass between the 12th and 13th rib, perpendicular to the LM. Carcasses were allowed to bloom for 30 minutes after being ribbed to allow for LM color development and bloom of muscle. Trained, experienced technicians collected all carcass parameters. Amount of external fat thickness (FT) was measured perpendicular to the outer surface at a point three-fourths the distance from the chine bone opposite the LM using a stainless steel ruler (beef probe). The FT was adjusted to reflect fat cover of the entire carcass following standard methods (USDA, 1997). *Longissimus dorsi* muscle area was measured using a grid standardized in tenths of a square inch as designated by the Agricultural Marketing Service of USDA (USDA, 1997). Yield Grade was calculated using 2.5 + (2.50 × adjusted fat thickness, inches) + (0.20 × percent kidney, pelvic, and heart fat) + (0.0038 × HCW, pounds) – (0.32 × ribeye area, square inches). Marbling was estimated in the rib face of the bisected LM (USDA, 1997).

**Carcass Fabrication**

Carcasses were fabricated 7 days postmortem. A 0.5 in. rib steak was removed from the loin end of the left rib section using a Butcher Boy meat saw (Model B16, Lasar Manufacturing Co.; Los Angeles, CA), boned dust was removed using a hand scraper, vacuum packaged in a high barrier bag and stored at 36-39°F until day 17 postmortem, then frozen at -20°F for Warner-Bratzler shear force analysis. At 7 d postmortem, a 9-10-11 rib section was removed from the right side of each carcass according to Hankins and Howe (1946). Rib sections were packaged in a high barrier bone-guard vacuum package bag and stored at -20°F until technicians were able to dissect sections. The LM of the right side was dissected from origin to insertion during carcass fabrication. All sections were trimmed free of intermuscular fat outside the epimysium. The sections were then weighed and recorded. Weight of all sections were added together to determine LM weight. The *semitendinosus* (ST) muscle was trimmed free of all intermuscular fat external to the epimysium and weighed.

**Cooking and Warner-Bratzler-Shear Force**

Frozen steaks were thawed approximately 24 hours at 36-39°F prior to being cooked in a natural gas convection oven (Model #200, Market Forge Company, Inc.; Everett, MA) preheated to 325°F. Steaks were positioned on the rack above a pan of water used to control humidity and catch drippings. Each steak’s internal temperature was measured using a thermocouple placed in the geometric center of the LM with a calibrated Digi-Sense scanning thermometer (Cole-Parmer Instrument Company; Vernon Hill, IL) intermittently monitoring thermocouple temperature every 10 seconds. Steaks were cooked to an internal temperature of 160°F and then removed from the oven. Temperatures were taken at lateral and medial ends of the LM using calibrated dial thermometers (Koch Supplies Inc.; Kansas City, MO) immediately after removal from oven to confirm final cooked temperature. Steaks were then placed on glass plates, cooled to room temperature, and refrigerated at 36-39°C for 24 hours.

Cores were removed by a trained technician parallel to muscle fiber orientation using a sharp 0.5 in. diameter coring device. A minimum of five usable cores were removed from each steak. Cores were visually examined and discarded if excess connective tissue or holes due to thermocouple/thermometer placement were present. Shear force was measured with a Warner-Bratzler machine (G-R Electric Manufacturing Co.; Manhattan, KS) equipped with a electronic load cell (Dillion Basic Force Guage, BFG500N; EU) using a crosshead speed of 225mm/minute. Shear force of individual cores were averaged to obtain shear force of each steak.

**9-10-11 Rib Dissection**

Frozen 9-10-11 rib sections were thawed for 4 days at 36-39°F. The rib sections were removed from vacuum package bags and dissected into the lean, fat, and bone plus heavy connective tissue as an estimate of total carcass composition (Hankins and Howe, 1946). All weights were recorded and analyzed as weights and percentages.

**Proximate Analysis**

LM was used for proximate analyses according to AOAC approved methods (1990). For lipid content analyses, extraction cups with glass beads and 33 mm × 80 mm cellulose extraction thimbles (Whatman International Ltd.; Maidstone, England) were dried for 15 min at 212°F then cooled in a dessicator and weighed. After drying, 2.0 g of homogenized freeze dried LM sample were added to the dried cellulose extraction thimbles and dried at 255°F for 1 hour. Sample and thimbles were cooled in a dessicator for 15 minutes then weighed. Total lipid content was measured using a Soxtec Avanti (Model 2050, Foss Tecator; Hoganes, Sweden) fat extraction system. After the extraction process, cups and thimbles were dried for 15 minutes at 212°F. Extraction cups and thimbles were then...
Immunoblot Analysis

Electrophoresis, the proteins on the gels were transferred to nitrocellulose membranes in a tank system using a transfer buffer containing 20 mM Tris-base, 192 mM glycine, 0.1% SDS, and 20% methanol. Membranes were incubated in a blocking solution consisting of 5% nonfat dry milk in TBS/T (0.1% Tween-20, 50 mM Tris-HCl pH7.6, and 150 mM NaCl) for 1 hour. Membranes were incubated overnight at 38°F in primary antibodies with 1:1,000 dilution in TBS/T with 2% bovine serum albumin (SeraCare Diagnostics, Millford, MA). After the primary antibody incubation, membranes were washed three times for 5 minutes each with 20 ml of TBS/T. Membranes were then incubated with horseradish peroxidase-conjugated secondary at room temperature antibodies at 1:2,000 dilution for 1 hour in a solution consisting of 2% nonfat dry milk in TBS/T with gentle agitation. After three 10-minute washes, membranes were visualized using ECL (Enhanced chemiluminescent substrate) Western blotting reagents (Amersham Bioscience) and exposure to film (MR, Kodak, Rochester, NY). Density of bands was quantified by using an Imager Scanner II and ImageQuant TL software. To reduce the variation between blots, tissue lysates of both groups were run in a single gel. Band density was normalized according to the GAPDH content (Zhu et al., 2004).

Antibodies

Antibodies against ACC, phospho-ACC at Ser 79, Akt, phospho-Akt at Ser 473, mTOR, phospho-mTOR at Ser 2448, ribosomal protein S6 kinase, phospho-S6K at Thr 389, and phospho-eIF4E at Thr 37/46 were purchased from Cell Signaling (Danvers, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Ambion (Austin, TX).

LM muscle sample (0.1 g) was powdered in liquid nitrogen and homogenized in a polytron homogenizer (7-mm diameter generator) with 400 µl of ice-cold buffer containing 137 mM NaCl (Fisher Scientific, Fair Lawn, NJ), 50 mM HEPES (Sigma, St. Louis, MO), 2% SDS (w/v), Research Organics Inc., Cleveland, OH), 1% (v/v) NP-40 (Abbott Molecular Inc., Des Plaines, IL), 10% (v/v) glycerol (Sigma, St. Louis, MO), 2 mM PMSF (Fisher Scientific), 10 mM sodium pyrophosphate (EMD Chemicals Inc., Gibbstown, NJ), 10 µg/ml aprotinin (Fisher Scientific), 10 µg/ml leupeptin (Fisher Scientific), 2 mM NaF (Fisher Scientific), 100 mM NaF (JT Baker Inc., Phillipsburg, NJ), pH 7.4 (Zhu et al., 2006).

AMPK Activity Measurement

Activity was measured using a method previously described (Shen and Du, 2005a, b). Briefly, a SAMS peptide substrate was used (His-Met-Arg-Ser-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg; Invitrogen). Frozen LD muscle samples (about 0.1 g) were powdered in liquid nitrogen and homogenized in 500 µl of ice-cold homogenization buffer (0.25 M mannitol; 0.05 M Tris/HCl, pH 7.4; 1 mM EDTA; 1mM EGTA; 1 mM DTT; 50 mM NaF; 5 mM sodium pyrophosphate). The muscle homogenate obtained was centrifuged at 13,000 × g for 5 min at 4°C. The supernatant (10 µl) was removed and incubated for 10 minutes at 98°F in 40 mM HEPES, 0.2 mM SAMS peptide, 0.2 mM AMP, 80 mM NaCl, 8% (w/v) glycerol, 0.8 mM EDTA, 0.8 mM DTT, 5 mM MgCl2 and 0.2 mM ATP + 2 µCi γ-[32P]ATP, pH 7.0, in a final volume of 50 µl. An aliquot (20 µl) was removed and spotted on a 2 × 2-cm piece of Whatman P81 filter paper. The γ-[32P] ATP was removed with six washes in 1% phosphoric acid, and radioactivity was quantified after immersing the filter paper in 3 ml Scintiverse (Fisher Scientific, Hanover Park, IL). The activity was expressed as the phosphorylation of nanomolar peptide per min per gram of muscle (Zhu et al., 2004).

Statistical Analysis

Data were analyzed as a complete randomized design using GLM (General Linear Model of Statistical Analysis System, SAS, 2000). Individual animal was considered as an experimental unit. The differences in the mean values were compared by the Tukey’s multiple comparison, and mean ± standard errors were reported. Statistical significance was considered as P<0.05.
Results

Five steers with the highest intramuscular fat content (High IMF) and five steers with the low intramuscular fat content (Low IMF) were selected for further analyses based upon chemical fat analysis from 40 steers with an average fat content of 3.72 ± 0.38%. The associated carcass measurements for High IMF and Low IMF groups are listed in Table 2. The marbling score was much higher for High IMF than for Low IMF (Table 2). The average chemical fat content for High IMF LM was 5.71 ± 0.36% and for Low IMF LM was 2.09 ± 0.19% (P<0.01) (Table 2). The moisture content was higher in Low IMF LM than High IMF LM (P<0.01), which should be due to the lower fat content in Low IMF (Table 2). No difference in protein or ash content was observed (Table 2). The 9-10-11 rib separable fat content was 36.9 ± 1.2% for High IMF and 31.5 ± 1.0% for Low IMF (P<0.01). The 9-10-11 rib separable lean content was 44.9 ± 1.1% for High IMF and 50.6 ± 1.0% for Low IMF (P<0.01). No difference was observed in the content of bone & connective tissues (Table 2). The 12th fat thickness was 0.61 ± 0.05 in. and 0.39 ± 0.04 in. (P<0.01) for High IMF and Low IMF respectively (Table 2). No difference in kidney, pelvic and heart fat was observed. In addition, as indicated by shear force, the High IMF LM was more tender than Low IMF LM (Table 2). A significant muscle weight difference was observed between High IMF and Low IMF carcasses (Table 2). The weights of LM and ST muscles were significantly higher (P<0.05) in Low IMF compared to High IMF carcasses, showing enhanced muscle growth in Low IMF carcasses (Table 2).

The AMPK activity was lower (P<0.01) in High IMF muscle compared to Low IMF muscle (Figure 1). This difference in AMPK activity was not due to alteration in AMPK content. As measured by immunoblotting, no difference in AMPK content was observed (Data not shown). Seeing as AMPK directly phosphorylates acetyl-CoA carboxylase (ACC) at Ser 79, the degree of ACC phosphorylation at Ser 79 is correlated with AMPK activity. As shown in Figure 2, the phosphorylation of ACC at Ser 79 was also lower (P<0.05) in High IMF muscle compared to Low IMF muscle, consistent with AMPK activity. This phosphorylation inhibits the activity of ACC. As ACC catalyzes the formation of malonyl-CoA, a crucial substrate for lipogenesis, less phosphorylation results in higher ACC activity which promotes lipogenesis.

Protein kinase B is a key mediator down-stream of insulin/IGF-1 signaling and may be associated with the reduced lean growth in High IMF cattle. No difference in total but the phosphorylation of Akt at Ser 473 tended to be higher in High IMF than that of Low IMF LM (Figure 3). No difference in mTOR phosphorylation at Ser 2448, a site phosphorylated by Akt, was observed, though the total mTOR content was lower in High IMF LM (Figure 4). These data show that mTOR signaling was not different between High IMF and Low IMF muscle.

Discussion

AMPK is a heterotrimeric enzyme with α, β, and γ subunits (Hardie, 2004; Kim et al., 2004). AMPK is switched on by an increase in the AMP/ATP ratio, which leads to the phosphorylation of AMPK at Thr 172 by AMPK kinase (Hawley et al., 2005; Kim et al., 2004; Woods et al., 2005). Once activated, AMPK promotes glucose uptake and inhibits lipid synthesis in cells through inhibition of ACC activity, a key enzyme in lipid synthesis (Fujii et al., 2006; Hardie and Hawley, 2001). Besides controlling ACC activity and thus lipogenesis, AMPK also controls lipid oxidation (Carey et al., 2006; Ravenskjaer et al., 2006; Yoon et al., 2006). Activation of AMPK by 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) accelerates fatty acid oxidation due to a reduction in malonyl-CoA content (Merrill et al., 1997). AMPK activation also increases uncoupling protein-3 expression and mitochondrial enzyme activities which contributes to the enhanced lipid oxidation as demonstrated in rat muscle (Putman et al., 2003). Hormones and cytokines like adiponectin and interleukin-6 stimulate glucose utilization and fatty-acid oxidation by activating AMPK (Carey et al., 2006; Yamauchi et al., 2002). Therefore, a low AMPK activity in muscle is expected to promote fat accumulation, which might be associated with enhanced intramuscular fat content in beef cattle. Indeed, for the first time, our study showed that the AMPK activity was lower in High IMF compared to Low IMF LM.

Aside from the role of AMPK in fat accumulation, AMPK is also involved in adipogenesis. Enhanced adipogenesis in skeletal muscle increases the number of intramuscular adipocytes, an event contributing to enhanced marbling (Zhou et al., 2007). Activation of AMPK inhibits adipogenesis (Giri et al., 2006; Habinowski and Witters, 2001).

The role of AMPK in muscle growth remains controversial. According to current reports, AMPK may promote muscle growth through sensitizing IGF-1/insulin signaling but inhibits protein synthesis through interaction with mTOR signaling (Inoki et al., 2003). To show the possible association of mTOR signaling with AMPK in LM of beef cattle, we analyzed mTOR signaling. We observed that total mTOR concentration was lower in High IMF compared to Low IMF at the time of slaughter, however no difference in phosphorylation of mTOR at Thr 2448 was detected. These data show that mTOR signaling is not responsible for the differential muscle growth between Low IMF and High IMF cattle.

The lack of difference in mTOR signaling was unexpected because AMPK activity was lower in High IMF cattle, and AMPK inhibits mTOR signaling (Inoki et al., 2003). However, the recent report that AMPK facilitates insulin/IGF-1 signaling through phosphorylation of insulin receptor substrate-1 (IRS-1) may provide explanation for this controversy (Jakobsen et al., 2001). It is well documented that insulin/IGF-1 stimulates skeletal muscle growth (Avruch et al., 2005; Glass, 2005). Activation of IGF-1 receptor involves multi-site tyrosine auto-phosphorylation. This phosphorylation activates the catalytic activity of the receptor which phosphorylates insulin receptor substrate 1 (IRS-1). When IRS-1 is activated by phosphorylation, it serves as a docking center for recruitment for the activation of down-stream pathways,
a major pathway of which is the activation of PI3K/Akt pathway (Anthony et al., 2001; Bush et al., 2003; Vary, 2006). Indeed, phosphorylation of Akt tended to be higher (P<0.10) in High IMF compared to Low IMF muscle. One of the major targets of PI3K/Akt signaling is mTOR. Though the down-regulation of AMPK is expected to activate mTOR, the lower AMPK activity in High IMF muscle mitigates the down-stream signaling of insulin/IGF-1 including mTOR, which may neutralize the effect of AMPK on mTOR, explaining the lack of difference in mTOR signaling between High IMF and Low IMF LM.

In summary, our data showed high intramuscular fat accumulation was associated with reduced lean growth in this set of beef cattle. The AMPK activity was lower in LM with High IMF compared to Low IMF. Therefore, our study indicates that AMPK may be a molecular target to promote marbling in beef cattle. The reduced lean growth in High IMF cattle seems not to be associated with mTOR signaling and MAPK signaling. The mechanisms responsible for the negative association between intramuscular fat accumulation and lean growth, and the involvement of AMPK in this process warrant further investigation.

References


**Figure 1.** Adenosine monophosphate activated-protein kinase (AMPK) activity in *longissimus dorsi* muscle of low intramuscular (Low IMF ■) and high intramuscular fat (High IMF □) cattle. (**): P<0.01. Mean ± SE. (n = 5 per group).

**Figure 2.** Acetyl-CoA carboxylase (ACC) and its phosphorylation at Ser 79 in *longissimus dorsi* muscle of low intramuscular (■) and high intramuscular fat (□) cattle. (*): P<0.05. Mean ± SE. (n = 5 per group).

**Figure 3.** Protein kinase B (Akt) and its phosphorylation at Ser 473 in *longissimus dorsi* muscle of low intramuscular (Low IMF ■) and high intramuscular fat (High IMF □) cattle. (*): P<0.05. (#): P<0.10. Mean ± SE. (n = 5 per group).

**Figure 4.** Mammalian target of rapamycin (mTOR) and its phosphorylation at Ser2448 in *longissimus dorsi* muscle of low intramuscular (Low IMF ■) and high intramuscular fat (High IMF □) cattle. (*): P<0.05. Mean ± SE. (n = 5 per group).
### Table 1. High and low intramuscular fat steers diets throughout feeding period.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Receiving</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ration composition %, DM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn</td>
<td>43.2</td>
<td>58.1</td>
<td>71.1</td>
<td>79.2</td>
</tr>
<tr>
<td>Brome Hay</td>
<td>40.7</td>
<td>30.6</td>
<td>19.9</td>
<td>12.6</td>
</tr>
<tr>
<td>Alfalfa dehydrated pellet</td>
<td>7.0</td>
<td>2.6</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>ORE-BAC Bitter(^1,2)</td>
<td>2.4</td>
<td>2.1</td>
<td>1.9</td>
<td>1.8</td>
</tr>
<tr>
<td>ORE-BAC Sweet(^1,2)</td>
<td>2.3</td>
<td>2.0</td>
<td>1.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>2.9</td>
<td>3.3</td>
<td>2.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.5</td>
<td>0.8</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Urea</td>
<td>0.3</td>
<td>0.2</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>0.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Salt</td>
<td>0.3</td>
<td>0.3</td>
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#### Chemical and nutrient composition

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<tr>
<th></th>
<th>CP, % of DM(^3)</th>
<th>TDN, % of DM(^3)</th>
<th>DE, Mcal/kg(^3)</th>
<th>NE(_{mm}), Mcal/kg(^3)</th>
<th>NE(_{gg}), Mcal/kg(^3)</th>
<th>Calcium, % of DM(^3)</th>
<th>Phosphorus, % of DM(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13.5</td>
<td>69.8</td>
<td>3.1</td>
<td>1.9</td>
<td>1.1</td>
<td>0.6</td>
<td>0.3</td>
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</tbody>
</table>

### Table 2. Carcass measurements of high and low intramuscular fat steers.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>High intramuscular fat</th>
<th>Low intramuscular fat</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>12(^{th}) rib fat thickness, in.</td>
<td>0.61 ± 0.05</td>
<td>0.39 ± 0.04</td>
<td>0.007</td>
</tr>
<tr>
<td>Adjusted 12(^{th}) rib fat thickness, in.</td>
<td>0.60 ± 0.05</td>
<td>0.41 ± 0.04</td>
<td>0.01</td>
</tr>
<tr>
<td>Kidney, pelvic, and heart fat, % HCWT</td>
<td>5.09 ± 0.18</td>
<td>5.05 ± 0.11</td>
<td>0.87</td>
</tr>
<tr>
<td>Hot carcass weight, lbs.</td>
<td>677 ± 20.9</td>
<td>736 ± 28.0</td>
<td>0.12</td>
</tr>
<tr>
<td>USDA Yield grade</td>
<td>4.03 ± 0.14</td>
<td>3.20 ± 0.22</td>
<td>0.01</td>
</tr>
<tr>
<td>Marbling score</td>
<td>450.0 ± 5.5</td>
<td>334.0 ± 10.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Warner-Bratzler shear force, lbs.</td>
<td>8.31 ± 0.44</td>
<td>9.79 ± 0.40</td>
<td>0.04</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Longissimus muscle area, in.(^2)</td>
<td>10.9 ± 0.2</td>
<td>12.6 ± 0.5</td>
<td>0.02</td>
</tr>
<tr>
<td>Longissimus muscle, % HCWT</td>
<td>1.63 ± 0.03</td>
<td>1.80 ± 0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>Semitendinosus, % HCWT</td>
<td>0.52 ± 0.02</td>
<td>0.60 ± 0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>Proximate analysis, Longissimus muscle</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Moisture, % (^a)</td>
<td>70.78 ± 0.35</td>
<td>74.00 ± 0.44</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Protein, % (^a)</td>
<td>22.41 ± 0.32</td>
<td>22.42 ± 0.26</td>
<td>0.98</td>
</tr>
<tr>
<td>Fat, % (^a)</td>
<td>5.71 ± 0.36</td>
<td>2.09 ± 0.19</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Ash, % (^a)</td>
<td>1.05 ± 0.02</td>
<td>1.10 ± 0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>9-10-11 Rib dissection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lean, % (^b)</td>
<td>44.9 ± 1.1</td>
<td>50.6 ± 1.0</td>
<td>0.004</td>
</tr>
<tr>
<td>Fat, % (^b)</td>
<td>36.9 ± 1.2</td>
<td>31.5 ± 1.0</td>
<td>0.01</td>
</tr>
<tr>
<td>Bone &amp; Connective tissue, % (^b)</td>
<td>15.4 ± 0.74</td>
<td>15.0 ± 0.52</td>
<td>0.68</td>
</tr>
</tbody>
</table>

\(n = 5\) per group  
\(^a\) % of *Longissimus dorsi* composition.  
\(^b\) % of 9-10-11 Rib section
AMP-Activated Protein Kinase Negatively Regulates Adipogenesis in Sheep Fetal Skeletal Muscle and 3T3 cells

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Mei J. Zhu, Postdoctoral Fellow,
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Summary and Implications

Marbling, or intramuscular fat, is an important factor determining beef quality. Both adipogenesis and hypertrophy of existing adipocytes contribute to enhanced marbling. We hypothesize that the fetal stage is important for the formation of intramuscular adipocytes and AMP-activated protein kinase (AMPK) has a key role in adipogenesis during this stage. The objective of this study was to assess the role of AMPK in adipogenesis in fetal sheep muscle and 3T3 cells. Non pregnant ewes were randomly assigned to a control (Con, 100% of NRC recommendations, n=7) or over-fed (OF, 150% of NRC, n=7) diet from 60 days before to 75 days after conception when ewes were euthanized. The fetal longissimus dorsi (Ld) muscle was collected at necropsy for biochemical analyses. The activity of AMPK was significantly lower in the fetal muscle of OF sheep. The expression of PPARγ, a marker of adipogenesis, was higher in OF fetal muscle compared to Con fetal muscle. To further show the role of AMPK in adipogenesis, we used 3T3 cells. 3T3 cells were incubated in a standard adipogenic medium for 24 hr and 10 days. Activation of AMPK by 5-aminoimidazole-4-carboxamide 1-beta-d-ribonucleoside (AICAr) dramatically inhibited the expression of PPARγ and reduced the presence of adipocytes after 10 days of differentiation. Inhibition of AMPK by Compound C, enhanced the expression of PPARγ. In conclusion, these data clearly show that low AMPK activity promotes adipocyte differentiation, which should be responsible for the enhanced adipogenesis observed in OF fetal sheep muscle.

Introduction

Marbling (intramuscular fat) is a primary criterion for beef quality grading. Increased marbling in young beef will increase its quality grade and, thus, garnish enormous economic returns. Marbling is correlated with the number of adipocytes in skeletal muscle; however, mechanisms controlling adipogenesis in skeletal muscle remain poorly defined (Hausman and Poulos, 2004; Hausman et al., 2007; Hausman and Richardson, 2004). Such knowledge is crucial for us to develop strategies to enhance intramuscular fat accumulation.

Skeletal muscle cells and adipocytes are both derived from mesoderm pluripotent cells. In fetal muscle around mid-gestation, there are a large number of pluripotent cells which can differentiate into either myogenic cells or adipogenic cells. The formation of adipocytes from mesoderm begins in mid-gestation (Feve, 2005; Gnanalingham et al., 2005; Muhlhausler et al., 2006). Adipose tissue growth in later life is due to both hypertrophy and hyperplasia (Feve, 2005). However, new fat cells generated later in life are mainly located in visceral and retroperitoneal fat depot and also in subcutaneous fat depots, with few located in intramuscular fat depots (Faust et al., 1978; Miller et al., 1984; Valet et al., 2002). Thus, intramuscular adipogenesis during the fetal stage is anticipated to have a dominant effect on the number of adipocytes existing inside skeletal muscle. Adenosine monophosphate-activated protein kinase (AMPK) has a central role in the control of energy metabolism (Hardie, 2007) and we previously demonstrated that AMPK was negatively associated with marbling in beef cattle (Underwood et al., 2007). Therefore, AMPK may be a key player in adipogenesis in the muscle of fetal ruminant animals. Sheep is physiologically very similar to cattle. The objective of this study is to assess the role of AMPK in adipogenesis in fetal sheep muscle and also in 3T3 cells.

Materials and Methods

Care and use of animals

All animal procedures were approved by the University of Wyoming Animal Care and Use Committee. From 60 days before conception to day 75 of gestation (Day of mating = day 0), multiparous Rambouillet/Columbia ewes carrying singleton fetuses were fed either a concentrated diet at 100% (control, C; n=7) of National Research Council (NRC) recommendations or 150% (over-fed, OF; n=7) of NRC recommendations on a metabolic BW basis (BW^{0.75}). All ewes were weighed at weekly intervals and
rations were adjusted for weight gain, and body condition was scored at monthly intervals to evaluate changes in fatness. A body condition score of 1 (emaciated) to 9 (obese) was assigned by 2 trained observers after palpation of the transverse and vertical processes of the lumbar vertebrae (L2 through L5) and the region around the tail head (Sanson et al., 1993).

On day 75, ewes were then given an overdose of sodium pentobarbital (Abbott Laboratories, Abbott Park, IL) and exsanguinated. The longissimus dorsi (Ld) muscle from the left side of fetal sheep was dissected. After trimming off surface tissues, a small piece of muscle (1g) was sampled in the anatomical center of the muscle and snap frozen in liquid nitrogen for biological analyses.

**3T3 cell culture**

3T3 cells (ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 µg/ml of streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37°C. At 30% confluence, 3T3 cells were incubated in a standard adipogenic medium composed of 10% FBS/DMEM supplemented with insulin (1 µg/ml), dexamethasone (0.1 µg/ml), IBMX (27.8 µg/ml), and troglitazone (10 µM) (Kim and Chen, 2004; Klemm et al., 2001; Yada et al., 2006; Yamanouchi et al., 2006). In addition, 5-Aminomidazole-4-carboxamide ribonucleoside (0.1 mM and 1.0 mM AICAR, Calbiochem, San Diego, CA) and Compound C (1 µM and 10 µM, Calbiochem) were used to treat cells. Cells were collected at 24 hr for immunoblotting analyses and at 10 days (duration needed for adipocyte differentiation) for Oil-Red O staining.

**Antibodies**

Antibodies against AMPK, phospho-AMPK at Thr 172, acetyl-CoA carboxylase (ACC), phospho-ACC at Ser 79, peroxisome proliferator-activated receptor γ (PPARγ) and horseradish peroxidase linked secondary antibody were purchased from Cell Signaling (Danvers, MA). Anti-β-actin antibody was obtained from Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA 52242).

**Immunoblotting analysis**

Muscle (0.1 g) powdered in liquid nitrogen was used for immunoblotting analyses as previously described (Zhu et al., 2006; Zhu et al., 2004). Briefly, muscle sample (0.1 g) was homogenized in 400 µl of ice-cold buffer containing 137 mM NaCl, 50 mM HEPES, 2% SDS, 1% NP-40, 10% glycerol, 2 mM PMSF, 10 mM sodium pyrophosphate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 2 mM Na3VO4, 100 mM NaF, pH 7.4. Muscle homogenate was mixed with an equal volume of 2 x standard SDS sample loading buffer for SDS-PAGE separation of proteins. Proteins on the gels were transferred to nitrocellulose membranes in a transfer buffer containing 20 mM Tris-base, 192 mM glycine, 0.1% SDS, and 20% methanol. Membranes were incubated in a blocking solution consisting of 5% nonfat dry milk in TBS/T (0.1% Tween-20, 50 mM Tris- HCl pH 7.6, and 150 mM NaCl) for 1 h. Membranes were incubated overnight in primary antibodies with 1:1,000 dilution in TBS/T with 2% bovine serum albumin (SeraCare Diagnostics, Millford, MA). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies at 1:1,000 dilution for 1 h in TBS/T with gentle agitation. Membranes were visualized using ECL Western blotting reagents (Amersham Bioscience) and exposure to film (MR, Kodak, Rochester, NY). Density of bands was quantified by using an Imager II and ImageQuant TL software. Band density was normalized according to the β-actin content (Zhu et al., 2004).

**Real time quantitative PCR (RT-PCR)**

RNA was extracted from the fetal muscle using TRI reagent (Sigma, St. Louis, MO) and reverse transcribed into cDNA using a kit (Qiagen, Valencia, CA). Primer sets used were: PPARγ forward, 5'-CCGCATCTTCCAGGGGTGTC-3', and reverse, 5'-CAAGGAGGCCACATCGAAAT-3'; PPARα forward, 5'-GGCCGCTGTGATTTACGT-3', and reverse, 5'-GAAGGCGGATTGTTGTTCT-3'; PGC-1α forward, 5'-GGCCGCTGTGATTTACGT-3' and reverse, 5'-AAAACTTCAGGCGGGCTCTCAA-3'; UCPI forward, 5'-GCTAGTTTAGGAAGCGAAGTC-3', and reverse, 5'-GCCCGTGTCAGGCTCTCAA-3'; PGC-1α forward, 5'-GGCCGCTGTGATTTACGT-3' and reverse, 5'-AAAATCTCTCCAGGCGGGCTCTCAA-3'. The 18S RNA was used as a control, forward, 5'-GTAAAACGTGGTAAACCTC-3', and reverse, 5'-GCCCGCCGTGTCAGGCTCTCAA-3'. Data were expressed relative to 18S rRNA as previously described (Lomax et al., 2007).

**Oil Red O staining of intramuscular triglyceride**

After 10 days of differentiation, 3T3 cells were stained with Oil Red O working solution [2:3 mixture of 0.5% (w/v) Oil Red O in 2-propanol and distilled water] for 7 min, and rinsed with PBS to remove excessive Oil Red O dye (Kim and Chen, 2004) and, then, subjected to microscopic observation. The total area of Oil Red O staining was quantified by using Image J software (NIH, Washington DC).

**Statistical analysis**

Statistical analyses were conducted according to our previous studies in sheep (Zhu et al., 2006; Zhu et al., 2004). Briefly, each animal or a separate cell culture experiment was considered as an experimental unit. Data were analyzed as a complete randomized design using GLM (General Linear Model of Statistical Analysis System, SAS, 2000). The differences in the mean values were compared by the Tukey’s multiple comparison, and mean ± standard errors were reported. Statistical significance was considered as p < 0.05.
Skeletal muscle cells and adipocytes are both derived from mesoderm pluripotent cells. In fetal skeletal muscle around mid-gestation, there are a large number of pluripotent cells which can differentiate into either myogenic cells or adipogenic cells (Feve, 2005; Gnanalingham et al., 2005; Muhlhausler et al., 2006). Enhancing adipogenesis inside fetal muscle will provide sites for fat accumulation in the later life, increasing marbling. Adenosine monophosphate-activated protein kinase has a central role in the control of energy metabolism (Hardie, 2007). Activation of AMPK phosphorylates and inhibits the activity of ACC, a key enzyme in lipid synthesis. Activation of AMPK accelerated fatty acid oxidation due to a reduction in malonyl-CoA content (Merrill et al., 1997). We previously demonstrated that AMPK was negatively associated with marbling in beef cattle (Underwood et al., 2007). Therefore, it is highly possible that AMPK activity is associated with adipogenesis in fetal muscle. Since obesity leads to the reduction of AMPK activity in skeletal muscle (Bandyopadhyay et al., 2006; Sriwijitkamol et al., 2006), we hypothesized that maternal obesity inhibited AMPK in fetal muscle which altered adipogenesis in fetal muscle.

### Results

Down-regulation of AMPK activity in fetal muscle

The phosphorylation of AMPK at Thr 172 is correlated with its activity (Hardie, 2007). The phosphorylation of ACC at Ser 79 was exclusively catalyzed by AMPK. Phosphorylation of AMPK at Thr 172 and ACC at Ser 79 have been frequently used to measure AMPK activity (LeBrasseur et al., 2006; Takekoshi et al., 2006). AMPK phosphorylation at Thr 172 was down-regulated in the skeletal muscle of OB sheep compared to Con sheep (25.4 ± 6.6%, P<0.05) (Fig 1A). Phosphorylation of ACC was also reduced (36.2 ± 8.1%, P<0.05) in the muscle of OF sheep (Fig 1B). These data clearly show that AMPK activity was down-regulated in OF fetal muscle compared to Con muscle.

Adipogenesis in fetal muscle

Peroxisome proliferators-activated receptors are key regulators of adipogenesis. The expression of PPARγ leads to adipogenesis from pluripotent cells and it is highly expressed in adipose tissue (Spiegelman et al., 2000). To show whether there was enhanced adipogenesis, we measured the PPARγ mRNA expression. Indeed, PPARγ mRNA expression level was much higher in OF than Con fetal muscle (Fig 2A). Furthermore, we also analyzed that content of PPARγ by immunoblotting. Again, the PPARγ content was higher in OF fetal muscle (Fig 2B), indicating enhanced adipogenesis. Of course, PPARγ is also expressed in skeletal muscle, the level of expression is very low (Verma et al., 2004). Therefore, these data show that the adipogenesis was enhanced in fetal muscle.

In fetus of ruminant animals, brown adipose tissue is dominant, which rapidly transforms into white adipose tissue within the first week of life (Casteilla et al., 1987). Peroxisome proliferators-activated receptor α is preferably expressed in brown adipose tissue (Lomax et al., 2007). Its expression induces the expression of PPAR coactivator 1α (PGC-1α) which further induces the expression of uncoupling protein-1 (UCP-1, a protein conferring the thermogenic function of brown adipose tissue (Lomax et al., 2007). We data showed that the mRNA expression for both PPARα and PGC-1α were higher in OF fetal muscle than Con fetal muscle (Fig. 3AB), though no difference was observed for UCP-1 mRNA expression. These data further confirmed the enhancement of adipogenesis in OF fetal muscle. Data also indicate that at least a portion of those developing adipocytes in fetal muscle were brown adipocytes.

At mid-gestation, adipogenesis in fetal muscle just initiated and, thus, there is no mature adipocyte available in fetal muscle at this stage (Casteilla et al., 1987). Therefore, we did not conduct Oil Red O staining in fetal muscle.

Association between AMPK and adipogenesis

Above data showed that AMPK activity was inhibited and adipogenesis was enhanced in fetal muscle. However, these data do not establish the causal relationship between AMPK and adipogenesis. To answer this question, we used 3T3 cells. 3T3 cells were cultured in an adipogenic medium and treated with AICAR for 24 hr and 10 days. Cells treated for 24 hr were used for the detection of PPARγ expression and AMPK and ACC phosphorylations, while cells differentiated from 10 days were used for Oil-Red O staining of adipocytes. Ten days of differentiation is necessary for the differentiation of 3T3 cells into mature adipocytes.

At 24 hr differentiation, activation of AMPK by 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) induced a dose dependent inhibition of PPARγ, eliciting that activation of AMPK inhibited adipogenesis in 3T3 cells (Fig. 4). Compound C is a specific inhibitor of AMPK. Applying Compound C induced the increased expression of PPARγ (Fig. 4). We further analyzed the activation of AMPK in 3T3 cells due to AICAR and Compound C. The application of AICAR induced a dose-dependent activation of AMPK phosphorylation at Thr 172 and ACC phosphorylation at Ser 79, demonstrating that AMPK was activated by AICAR (Fig 5AB), except for ACC phosphorylation at 0.1 mM AICAR treatment where ACC phosphorylation was inhibited (Fig. 5B). The reason for this inhibition was unclear, but may be associated with the differential effect of low and high AMPK activation on downstream signaling, as indicated in a recent study in colon cancer cells (Park et al., 2006). On the other hand, the phosphorylation of both AMPK and ACC was inhibited by Compound C (Fig 5 AB). These data were agreement with previous reports that AMPK activation inhibited the expression of PPARγ and C/EBPs in pre-adipocytes (Giri et al., 2006). After 10 days of differentiation, activation of AMPK dramatically reduced the number of adipocytes in brown adipocytes.
3T3 cells (Fig. 6 AB). However, incubation with Compound C for 10 days induced extensive cell apoptosis and death, demonstrating the essential role of AMPK in cell survival (Data not shown). These data in 3T3 cells clearly demonstrate that AMPK has a crucial regulatory role in adipogenesis.

It is well-established that PPARγ expression induces adipogenic differentiation. This receptor functions as an obligate heterodimer with retinoid X receptor, and binds to DNA sequences called direct repeat (DR)-1 element, which induces the expression of adipocyte specific genes (Spiegelman et al., 2000). However, it remains largely unclear how AMPK regulates PPARγ expression and adipogenesis. Few reports examined the possible mechanisms associated with AMPK in adipogenesis (Giri et al., 2006; Habinowski and Witters, 2001). Activation of AMPK by AICAR induced dose-dependent apoptotic cell death and down-regulation of key adipogenic genes, and suggested that this effect was mediated by phosphorylation of eukaryotic initiation factor 2α (eIF2α) (Dagon et al., 2006). The effect of AMPK on adipogenesis in fetal muscle has not been previously evaluated.

Conclusion and implication

In conclusion, AMPK was inhibited in fetal muscle of over-fed ewes and this inhibition was correlated with enhanced adipogenesis in fetal muscle. Therefore, adipogenesis in sheep fetal muscle can be enhanced by inhibition of AMPK. Due to the physiological similarity, these data indicate that maternal nutrient supplementation can be utilized to inhibit AMPK in fetal muscle, which may be a strategy to enhance marbling in beef cattle.

References

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**Fig. 1.** Phosphorylation of AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) in fetal longissimus dorsi muscle of Con (□) and OF (■) ewes. Panel A shows representative AMPK and phospho-AMPK immunoblots and statistical data; Panel B shows representative ACC and phospho-ACC immunoblots and statistical data. (▲): Con vs. OF, P < 0.05. Mean ± SEM. (n=5 per group).

**Fig. 2.** Peroxisome proliferators-activated receptor γ (PPARγ) mRNA expression and protein content in fetal longissimus dorsi muscle of Con (□) and OF (■) sheep. Panel A shows statistical data of PPARγ expression; Panel B shows statistical data of PPARγ protein content. (▲): Con vs. OF, P < 0.05. Mean ± SEM. (n=5 per group).
Fig. 3. Peroxisome proliferators-activated receptor α (PPARα), PPAR coactivator-1α (PGC-1α) and uncoupling protein-1 (UCP-1) mRNA expression in fetal longissimus dorsi muscle of Con (□) and OF (■) sheep. Panel A shows statistical data of PPARα mRNA expression; Panel B shows PGC-1α mRNA expression; Panel C shows statistical data of UCP-1 mRNA expression. (*): Con vs. OF, P < 0.05. Mean ± SEM. (n=5 per group).

Fig. 4. Peroxisome proliferators-activated receptor γ (PPARγ) protein content in 3T3 cells at 24 hr of differentiation. AICAR: 5-Aminoimidazole-4-carboxamide ribonucleoside; Comp. C: Compound C. (*): Con vs. Treatments, P < 0.05. Mean ± SEM. (n=4 per treatment).

Fig. 5. Phosphorylation of AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) in 3T3 cells at 24 hr of differentiation. Panel A shows representative AMPK and phospho-AMPK immunoblots and statistical data; Panel B shows representative ACC and phospho-ACC immunoblots and statistical data. AICAR: 5-Aminoimidazole-4-carboxamide ribonucleoside; Comp. C: Compound C. (*): Con vs. Treatments, P < 0.05; (**:): P < 0.01. Mean ± SEM. (n=4 per treatment).
Fig. 6. Presence of adipocytes in 3T3 cells after 10 days of differentiation due to 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) treatment.


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**Caspase 3 is Not Likely Involved in the Postmortem Tenderization of Beef Muscle**

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**Summary and Implications**

Postmortem proteolysis is an important determinant in beef tenderness. Caspase 3 is a protease that functions in apoptosis and has been shown to degrade myofibrillar proteins. Our objective was to evaluate whether caspase 3 activity is related to beef tenderness and muscle growth, and whether caspase 3 is activated in postmortem beef muscle. In experiment 1, *Longissimus thoracis* (LM) and *sternomandibularis* muscle samples were obtained at 0 h, 0.25 hours, 1 hour, 3 hours, 24 hours, 72 hours, and 240 hours postmortem from 5 different steers. In experiment 2, a group of 40 beef cattle were slaughtered at the University of Wyoming Meat Lab with 10 steers of different tenderness chosen for the analysis of caspase 3 activity in the LM. In Experiment 1, no significant activation \((P = 0.70)\) of caspase 3 activity was detected, only a decreased activity at 72 hours \((P = 0.05)\) and 240 hours \((P = 0.02)\) postmortem was observed. Western blot analysis of both muscle samples showed only the pro-caspase 3 form and failed to detect the activated enzyme. In experiment 2, caspase 3 activity in the LM immediately postmortem was higher \((P = 0.05)\) for the cattle with increased Warner-Bratzler shear force values. Our results demonstrate that caspase 3 activity is not activated with its activity decreasing with time postmortem, and caspase 3 activity is not associated with Warner-Bratzler shear force at slaughter. Therefore, caspase 3 is not anticipated to be involved in postmortem tenderization of beef.

**Materials and Methods**

**Muscle sample preparation**

**Experiment 1.** Five steer were obtained from the Fort Keogh USDA Research Station and were slaughtered at University of Wyoming Meat Laboratory. *Longissimus thoracis* (LM) muscle and *sternomandibularis* muscle samples (3 g) were excised at 0 hour (immediately following exsanguination), 0.25 hour, 1 hour, 3 hours, 24 hours, 72 hours, and 240 hours after slaughter. After removing all visible fat and connective tissue, muscle samples were snap frozen in liquid nitrogen. Muscle samples were stored at -112°F until analysis was performed. Carcass data, Warner-Bratzler shear force analysis, estimates of muscle growth and carcass composition were analyzed as

**Introduction**

Postmortem storage of muscle foods is known to increase tenderness subjectively and objectively (Koohmaraie et al., 1988; Koohmaraie et al., 1991). Increased tenderness during postmortem storage has been attributed to proteolysis of myofibrillar proteins (Goll et al., 1983; Koohmaraie et al., 1988; Sentandreu et al., 2002). Researchers have attributed postmortem proteolysis to catheptic enzymes and the calpain/calpastatin system (Etherington et al., 1987; Koohmaraie et al., 1991). The calpain/calpastatin system has received much attention and is credited as a more feasible proteolytic system responsible for postmortem degradation of myofibrillar proteins (Goll et al., 2003; Koohmaraie et al., 1991; Uytterhaegen et al., 1994).

Recently other proteolytic systems have been proposed to be active in postmortem muscle and affecting myofibrillar degradation (Herrera-Mendez et al., 2006; Kemp et al., 2006a). It has been suggested the ubiquitin proteasome system and caspases could play a role in postmortem myofibrillar degradation (Herrera-Mendez et al., 2006; Kemp et al., 2006b; Sentandreu et al., 2002). This hypothesis is based on the finding that these endogenous peptidases are capable of hydrolyzing myofibrillar proteins (Condorelli et al., 2001; Lee et al., 2004). Furthermore, Sentandreu et al. (2002) and Herrera-Mendez et al. (2006) proposed a mechanism by which hypoxic conditions in postmortem muscle could cause apoptosis triggering caspase 3 activation. Up to now, the possible involvement of caspase 3 in the postmortem tenderization of beef muscle has not been evaluated. Hence, the objective of this study was to evaluate whether caspase 3 is involved in postmortem tenderization of beef muscle.
previously described by Underwood et al. (2007). Briefly, LM area were determined according to USDA (1997) at 48 hours postmortem. The whole LM and semitendinosus muscle were dissected from the left side of carcass at 7 days postmortem and weights were recorded. Carcass composition was estimated using a 9-10-11 rib dissection according to Hankins and Howe (Hankins, 1946).

Five cross-bred steers with low Warner-Bratzler shear force (8.31 ± 0.44 lb.) and five steers with high Warner-Bratzler shear force (9.78 ± 0.40 lb.) were chosen for the analyses of caspase 3 activity.

Caspase 3 Activity Assay

Caspase activity assay was performed using the EnzChek Caspase-3 assay kit#1 (Molecular Probes, Invitrogen, Carlsbad, CA). Frozen muscle samples (0.1g ) were homogenized in 500 μl extraction buffer containing 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1.0 mM EDTA, 0.0001 % Triton X-100. Samples were then frozen overnight at -4° F. Samples were thawed and centrifuged at 12,000 x g for 5 minutes. The assay was then carried according to the kit protocol. Briefly, supernatant (50 μl) of each muscle homogenate was added into an individual well of a 96 well microfluorescent plate and incubated with or without 1 μl of Ac-DEVD-CHO inhibitor for 10 minutes a room temperature. Following incubation, 50 μl of the 2x working substrate (10μ M Z-Devd-AMC) were added to each well and further incubated for 30 minutes. Fluorescence was measured at 342 nm excitation and 441 nm emission using a spectrophotometer. Caspase 3 activity was expressed as μM substrate cleaved per gram muscle per minute.

Immunoblotting Analyses

Frozen muscle samples (0.1g ) were homogenized in 500 μl extraction buffer containing 20 mM Tris-HCl (pH 7.4 at 4 °C), 2% SDS, 1% Triton X-100, 5.0 mM EDTA, 5.0 mM EGTA, 1 mM DTT, 100 mM NaF, 2 mM sodium vanadate, 0.5 mM phenylmethylsulfonyl fluoride, 10 μl/ml leupeptin, and 10 μl/ml pepstatin (Raser et al., 1995; Veiseth et al., 2001). Supernatant of each muscle homogenate was mixed with an equal amount of sample loading buffer containing 150 mM Tris-HCl (pH 6.8), 20% homogenate was mixed with an equal amount of sample loading buffer containing 150 mM Tris-HCl (pH 6.8), 2% SDS, and 20% ethanol. Membranes were incubated in a blocking solution consisting of 5% nonfat dry milk in TBS/T (0.1% Tween-20, 50 mM Tris-HCl (pH7.6), and 150 mM NaCl) for 1 hour. Then, membranes were incubated overnight in a primary antibody (rabbit-anti-caspase 3, Cell Signaling Technology, Beverly, MA) with 1 to 1,000 dilution in TBS/T with 2% nonfat dry milk. At the end of the primary antibody incubation, the membranes were washed three times for 5 minutes each with 20 ml of TBS/T. After that, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody against rabbit IgG (1:2000; Amersham Bioscience, Piscataway, NJ) for 1 hour in TBS/T with gentle agitation. After three 5-min washes, membranes were visualized using ECL Western blotting reagents (Amersham Bioscience) and exposure to film (MR, Kodak, Rochester, NY) (Zhu et al., 2006).

Statistical Analysis

Experiment 1 data were analyzed using the PROC MIXED (Mixed model) procedure of SAS (SAS Inst., Inc., Cary, NC). Animal was used as the random effect as these animals were randomly selected from a group of 15 steers slaughtered at the University of Wyoming Meat Laboratory. Time was used as the fixed effect as the sampling time points were pre-determined. Individual animal was considered the experimental unit. Experiment 2 data were analyzed as a completely randomized design using PROC GLM procedure of SAS (SAS Inst., Inc., Cary, NC). Individual animal was considered as the experimental unit. Differences in mean values were compared by the LSD comparison, and lsmean ± standard errors were reported. Statistical significance was considered as P<0.05 and trends were considered at P<0.10.

Results and Discussion

Proteolysis during the postmortem period is important for beef tenderness. Cathepsins and calpains/calpastatin system may be involved in postmortem proteolysis (Kooohmariae, 1996; Uyterhaegen et al., 1994). Current data supports the role of calpain/calpastatin system as the main participant in the postmortem tenderization (Kooohmariae, 1996; Kooohmariae et al., 1988; Uyterhaegen et al., 1994). However, calpain and calpastatin cannot explain all the variability in tenderness (Kooohmariae, 1995; Morgan et al., 1993). Kooohmariae (1995) attributed 46% of the variability in tenderness to genetic factors, and credited the remaining 54% to environmental influences. Kooohmariae (1995) goes on to explain that 30% of the variation in beef tenderness is due to calpastatin, the specific inhibitor of calpains. The difference in postmortem tenderization can not be fully explained by the variation in calpain/calpastatin system (Morgan et al., 1993). Therefore, it is possible that other proteolytic systems are active in postmortem muscle. Caspase 3 has been investigated in mouse (Condorelli et al., 2001) and pig (Kemp et al., 2006a; Kemp et al., 2006b) skeletal muscle, but has not been examined in postmortem beef muscle.

Caspases are cysteine proteinases which function in apoptosis or programmed cell death and exist as proproteins in muscle (Du et al., 2004; Fuentes-Prior and Salvesen, 2004; Herrera-Mendez et al., 2006; Suzuki et al.,
2001). These proteinases function to cleave proteins at specific aspartic acid residues after activation, which requires cleavage of the pro-domain and dimerization (Fuentes-Prior and Salvesen, 2004; Herrera-Mendez et al., 2006; Sentandreu et al., 2002) Caspases can be classified into cytokine activators that function in inflammation, apoptotic initiators, and apoptotic effectors (Fuentes-Prior and Salvesen, 2004; Herrera-Mendez et al., 2006). Apoptosis is initiated by a caspase cascade involving initiator caspases 8, 9, or 10 (Fuentes-Prior and Salvesen, 2004). Caspase 3 is an effector caspase that is activated by other caspases and is responsible to execute most protein hydrolysis tasks. It has been documented to cleave myofibrillar proteins in muscle catabolic conditions (Du et al., 2004).

In skeletal muscle, caspase-3 exists as its pro-form which is inactive. Pro-caspase 3 is activated by cleavage into a 14 kilodalon (kD) caspase 3 which is active (Turpin et al., 2006). Therefore, if caspase 3 contributes to postmortem proteolysis, it must be cleaved into 14 kD active form during postmortem. To show whether there was a 14 kD active form of caspase 3 present in beef muscle, we conducted immunoblotting analyses using a caspase 3 antibody which recognized both pro and active form of caspase 3. No active caspase 3 was detected in longissimus thoracis up to 7 days postmortem (Figure 1). Since beef longissimus muscle is mainly composed from type Ila muscle fibers (Underwood et al., 2007), to test whether a muscle mainly composed of type I fibers behaviors differently, we further analyzed caspase 3 in sternomandibularis muscle. Again, no active form of caspase 3 was detected (Figure 2). To confirm that our failure to detect 14 kD caspase 3 was not due to our technical limitation, a positive control which contains both pro and activated caspase 3 (Cell Signaling Technology, Beverly, MA) and a negative control which only contains pro-caspase 3 (Cell Signaling Technology) were loaded into gels together with muscle samples. Beef muscle samples at various postmortem stages only contain pro-caspase 3 which is also detected in the negative control (Figures 1 and 2). The active caspase 3 was detected in the positive control but absent in beef muscle samples (Figures 1 and 2). These results clearly showed that pro-caspase 3 was not cleaved into active caspase 3 at detectable quantities in postmortem muscle. However, immunoblotting is not a very sensitive method for detecting small amounts of low molecular weight proteins. Therefore, there is a chance that small amount of active caspase 3 exists in muscle samples which immunoblotting analyses fail to detect. To solve this problem, we further assessed the caspase 3 activity.

There was no significant activation of caspase 3 in longissimus thoracis muscle at postmortem (Figure 3). Caspase 3 activity at 0, 0.25, 1, 3, and 24 hours was not changed, while caspase 3 activity was decreased at 72 hours ($P = 0.05$), and 240 hours ($P = 0.02$) postmortem when compared to 0 hour (Figure 3). The lack of any significant increase in caspase 3 activity during the postmortem stage following exsanguination shows this enzyme is not activated during postmortem period, indicating that caspase 3 is unlikely to play a significant role in postmortem tenderization. This data is different from a previous report in pigs, which found that caspase 3 was activated in postmortem porcine skeletal muscle (Kemp et al., 2006b).

To further evaluate the possibility of caspase 3 in postmortem proteolysis, we analyzed the link between caspase 3 activity and beef tenderness. Since beef tenderness is affected by numerous factors, such as genetics, age, nutritional condition of cattle (Koohmaraie, 1995; Morgan et al., 1993), we used a group of cattle which had the same genetic background, age and nutritional management in order to reduce tenderness variations due to the previous factors. We selected 5 steers with a low mean Warner-Bratzler shear force of 8.31 ± 0.44 lb. and another 5 steers with a high mean shear force of 9.78 ± 0.40 lb. (Underwood et al., 2007). The low shear force group had less muscle percentage in the carcass as shown by smaller LM area size of 10.9 ± 0.2 in.$^2$ compared to 12.6 ± 0.5 in.$^2$ for high shear force group ($P<0.05$). In addition, lighter LM weights (3.6 ± 0.07 lb. versus 4.0 ± 0.11 lb., $P<0.05$) and semitendinosus muscle weights (1.15 ± 0.07 versus 1.32 ± 0.09 lb., $P<0.05$) were observed in the low shear force group. Caspase 3 assay surprisingly showed a lower ($P=0.05$) caspase 3 activity in the low shear force group compared to high shear force group (Figure 3). This suggests that caspase 3 activity at slaughter was not correlated with beef tenderness, further indicating that caspase 3 is unlikely contributing to the postmortem tenderization of beef muscle. Nevertheless, the increased caspase 3 activity showed an association with measures of muscle growth in these cross bred steers, which warrants further studies.

In summary, our results show that there is caspase 3 activity in beef muscle immediately after slaughter and during the postmortem stage, but shows no significant increase in activity at postmortem time points measured. Also, we did not detect the activation of caspase 3 in the LM and sternomandibularis muscles of beef carcasses using immunoblotting. In addition, caspase 3 activity was not correlated with Warner-Bratzler shear force of beef LM. Therefore, caspase 3 is unlikely to participate significantly in the proteolysis of postmortem beef muscle. Since caspase 3 is an executive caspase and is activated through a caspase cascade, the lack of caspase 3 activation implies that other caspases are unlikely to be activated in postmortem beef muscle either.

References


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Figure 1. Caspase 3 in beef *longissimus thoracis* muscle at 0h, 0.25h, 1h, 3h, 24h, 72h and 240h postmortem. Two representative immunoblots were shown.

Figure 2. Caspase 3 in beef *sternomandibularis* muscle at 0 h, 0.25 h, 1h, 3h, 24h, 72h and 240h postmortem. Two representative immunoblots were shown.

Figure 3. Caspase 3 activity in beef *longissimus dorsi* muscle at 0h, 0.25h, 1h, 3h, 24h, 72h and 240h postmortem. Results are presented as means ± Standard error. * Results differ from initial time point (0 h) (*P* < 0.05).

Figure 4. Panel A. Warner-Bratzler shear force of *longissimus thoracis* muscle from low and high shear force beef cattle. Panel B. Caspase 3 activity in beef *longissimus thoracis* muscle of low and high shear force beef cattle. Results are presented as means ± Standard error. * Results differ (*P* < 0.05).
The objective of this study was to examine the association of AMP-activated protein kinase (AMPK) with glycogen content in bovine muscle, and their links with intramuscular fat and muscle fiber type composition. Five steers with high intramuscular fat (High IMF, intramuscular fat content is 5.71 ± 0.36%) and five steers with low intramuscular fat (Low IMF, intramuscular fat content is 2.09 ± 0.19%) in longissimus dorsi muscle were selected for immunoblotting, glycogen and myofiber type composition analyses. The glycogen content was higher in Low IMF muscle than that of High IMF (1.07 ± 0.07 versus 0.85 ± 0.08 g/100 g muscle, P < 0.05). The phosphorylation of AMPK α subunit at Thr 172, which is correlated with its activity, was lower (P<0.05) in High IMF compared to Low IMF. In agreement with the lower AMPK phosphorylation in High IMF muscle, the phosphorylation of acetyl-CoA carboxylase (ACC) was also lower (P<0.05) in High IMF muscle than Low IMF muscle. Glycogen synthase kinase 3 (GSK3) controls glycogen synthesis through phosphorylation of glycogen synthase. The phosphorylation of GSK3 in High IMF was lower (P<0.05) than that in Low IMF, which should release its inhibition of glycogen synthase activity and but this did not enhance glycogen accumulation. Type IIB myosin isoform was absent in beef muscle. No noticeable difference in myosin isoform composition was observed between Low and High IMF muscle. In summary, data show a negative correlation between intramuscular fat and glycogen content, intramuscular fat was negatively correlated with AMPK activity, while glycogen content was positively correlated with AMPK activity. The GSK3 activity was lower in High IMF muscle compared to Low IMF muscle, which should be partially responsible for the high glycogen content in High IMF muscle. This data shows longissimus dorsi muscle lipid and glycogen metabolisms are affected by AMPK activity. This may provide a target to alter intramuscular fat and glycogen in beef cattle muscle.
which consumes oxygen and causes the formation of deoxy-myoglobin. This, in conjunction with higher water holding capacity of higher pH, alters light absorption on the muscle surface and results in a dark red color (Ashmore et al., 1973; Ashmore et al., 1971). A pH value of 5.87 in the longissimus dorsi muscle is the approximate cut-off between normal and DCB (Page et al., 2001). The high pH in postmortem muscle of DCB is due to glycogen deficiency in the muscle at slaughter (Mcveigh et al., 1982; Warriss et al., 1984). It appears that a glycolytic potential at 100 μmol/g muscle is the threshold for the occurrence of DCB (Wulf et al., 2002). Therefore, increased glycogen content in muscle at slaughter will prevent the incidence of DCB. However, up to now, mechanisms controlling glycogen accumulation in the muscle of beef cattle are poorly defined.

**Materials and Methods**

**Care and Use of Animals.**

All animal procedures were approved by the University of Wyoming Animal Care and Use Committee. Calves were vaccinated, branded, and bull calves were castrated as per the University of Wyoming herd processing procedures. Cows and calves grazed summer pasture at the University of Wyoming McGuire Ranch. Calves were weaned at 155 days of age.

Forty Angus × Gelbvieh steers at 13.5 months of age were slaughtered at University of Wyoming Meat Laboratory. Immediately after exsanguination and evisceration (less than 10 minutes postmortem), a sample was removed from the longissimus dorsi muscle (LM) muscle between the 12th and 13th rib of the right side of carcasses by inserting a knife from the body cavity. After trimming off all visible adipose and connective tissues, small pieces of muscle (1g) were cut and snap frozen in liquid nitrogen for biological analyses. Five steers with High intramuscular fat (5.71 ± 0.36%) content and five steers with Low intramuscular fat (2.09 ± 0.19%) content were selected for further analyses in this study (P<0.05) following intramuscular fat analysis.

**Intramuscular fat analyses.**

Carcasses were fabricated 7 days postmortem. A 0.5 in. rib steak was removed from the loin end of the left rib section using a Butcher Boy meat saw (Model B16, Lasar Manufacturing Co.; Las Angeles, CA), bone dust was removed by hand, and steaks were vacuum packaged in a high barrier bag and stored at -20°F until proximate analysis could be performed. All steaks were trimmed free of intermuscular fat, outside the epimysium and bones. The remaining muscles then were cut into small pieces and used for intramuscular fat (IMF) analyses by petroleum ether extraction as previously described (Underwood et al., 2007).

**Antibodies.**

Antibodies against AMPK, phospho-AMPK at Thr 172, Acetyl-CoA carboxylase (ACC), phospho-ACC at Ser 79, GSK-3, phospho-GSK3 at Ser 21/9 were purchased from Cell Signaling (Danvers, MA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was bought from Abcam Inc. (Cambridge, MA).

**Immunoblot Analysis.**

Muscle sample (0.1 g) was powdered in liquid nitrogen and homogenized in a polytron homogenizer (7-mm dia. generator) with 400 μl of ice-cold buffer containing 137 mM NaCl, 50 mM HEPES, 2% SDS, 1% NP-40, 10% glycerol, 2 mM PMSF, 10 mM sodium pyrophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM Na₃VO₄, 100 mM NaF, pH 7.4. Immunoblotting was conducted as previously described (Zhu et al., 2006).

**Glycogen content measurement.**

Glycogen was assayed using a muscle sample (0.1 g) powdered in liquid nitrogen. Glycogen, glucose, and glucose 6-phosphate were determined as previously reported by Shen et al. (Shen et al., 2005). Glycogen content was calculated using the equations of Keppler and Decker (Kepler, 1974). Glycogen is expressed as g of glycogen per 100g wet LM tissue.

**Muscle fiber typing.**

Muscle fiber typing was conducted as described previously using a method that distinguishes between fiber types based on myosin ATPase activity under various pre-incubation pH conditions (Sheehan and Hrapchak, 1987). Briefly, cryostat sections (8 µm) of the Longissimus dorsi muscles were incubated in either a pH 4.2 (0.2 M barbital acetate) solution and pH 4.5 (0.2 M barbital acetate) solution for 5 minutes, or in a pH 9.7 (33 mM sodium barbital, 60 mM CaCl₂) solution for 15 minutes. Sections were then rinsed once with water, soaked in ATP solution (25 min for the pH 4.2 and 4.5 solutions, and 15 min for the pH 9.7 solution), washed three times with 1% CaCl₂, and then incubated with 2% cobalt chloride for 10 min. After that, sections were washed with diluted 0.1 M sodium barbital and then water. Finally, sections were soaked in 2% (v/v) ammonium sulfide for 20 to 30 seconds and washed with water. The sections were observed using light microscopy (Sheehan and Hrapchak, 1987).

**Identification of myofiber isoforms.**

Longissimus muscle samples (0.1 g) were homogenized in 500 μl of buffer containing 250 mM sucrose, 100 mM KCl, 5 mM EDTA and 20 mM Tris-HCl pH 6.8. The homogenate was filtered through nylon cloth to remove debris and centrifuged at 10,000 g for 10 minutes. The pellet obtained was re-suspended in a 500 μl of washing buffer containing 200 mM KCl, 5 mM EDTA, 0.5% Triton X-100 and 20 mM Tris-HCl pH 6.8. The suspension was centrifuged at 10,000 g for 10 min. The pellet containing purified myofibrillar proteins was re-suspended in 200 μl water and 300 μl of standard 2 x sample loading buffer and then boiled for 5 minutes. After centrifugation at 12,000 g for 5 minutes, the supernatant was used for electrophoresis.
The stacking gels consisted of 4% acrylamide (acrylamide: bis = 50:1) and 5% (v/v) glycerol, 70 mM Tris-HCl pH 6.7, 0.4% (w/v) SDS, 4 mM EDTA, 0.1% (w/v) APS and 0.01% (v/v) TEMED. The separation gel contained 5% (w/v) glycerol, acrylamide: bis (50:1) at a concentration ranging from 5 to 20%, 200 mM Tris pH 8.8, 4 mM EDTA, 0.4% (w/v) SDS, 0.01% (v/v) TEMED and 0.1% (w/v) ammonium persulfate. The upper running buffer consisted of 0.1 M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 150 mM glycine, and 10 mM mercaptoethanol, and the lower running buffer consisted of 50 mM Tris-HCl pH 8.8, 0.01% (w/v) SDS and 75 mM glycine. Gels were run at 8°C in a Hoefer SE 600 (Hoefer Scientific, San Francisco, CA) unit, at constant 200 V for 24 hr (Bamman et al., 1999). After electrophoresis, gels were stained with Coomassie blue and scanned with a densitometer to determine the amount of each myosin isoform and percentage composition was reported. In addition, the identification of myosin isoforms was confirmed by immunoblotting analyses using an antibody against myosin heavy chain (DSHB, Iowa city).

**pH analysis**

A 0.5 in. rib steak was removed from the rib at 11th rib using a Butcher Boy meat saw, bone dust was removed by hand, and steaks were vacuum packaged in a high barrier bag and stored at -20°F until pH analysis could be performed. *Longissimus* muscle samples (0.1 g) were homogenized in 1000 μl of deionized water and immediately measured using a Beckman pH meter (Model PHI71, Beckman Instruments, Inc., Fullerton, CA).

**Statistical Analysis.**

Data were analyzed as a complete randomized design using GLM (General Linear Model of Statistical Analysis System, SAS, 2000). An individual animal was considered as an experimental unit. The differences in the mean values were compared by the Tukey’s multiple comparison, and mean ± standard errors were reported. Statistical significance was considered as P<0.05.

**Results and Discussion**

**AMPK, marbling and glycogen accumulation.**

Based on human and rodent studies, AMPK and GSK-3 are two kinases controlling glycogen accumulation in skeletal muscle. AMPK, a heterotrimeric enzyme with α, β, and γ subunits, is mainly recognized as a critical regulator of energy metabolism (Hardie, 2004; Kim et al., 2004). The α subunit is the catalytic unit, the γ subunit has a regulatory function, and the β unit provides anchorage sites for α and γ (Sambandam and Lopaschuk, 2003). AMPK is switched on by an increase in the AMP/ATP ratio in muscle cells, which leads to the phosphorylation of AMPK at Thr172 by AMPK kinases, including LKB-1 (a serine/threonine-protein kinase) and calmodulin-dependent protein kinase kinases (Hawley et al., 2005; Kim et al., 2004; Woods et al., 2005). Once activated, AMPK switches on pathways that generate ATP, such as glycolysis and fatty acid oxidation, while switching off ATP-consuming processes, such as the synthesis of glycogen and fatty acids (Hardie and Hawley, 2001). Since fatty acid oxidation and synthesis are expected to affect intramuscular fat content, this prompted us to hypothesize that muscle glycogen content and marbling are correlated and AMPK is involved in both processes. Data obtained in this study support our hypothesis. The average intramuscular fat content for Low IMF was 2.09 ± 0.19% and High IMF was 5.71 ± 0.36% (P<0.01) (Figure 1A). The glycogen content was also lower in Low IMF muscle than High IMF muscle (1.07 ± 0.07 versus 0.85 ± 0.08 g/100 g muscle) (Figure 1B). The pH of Low IMF (5.35 ± 0.07) and High IMF (5.34 ± 0.09) muscle was similar (P=0.42). Additionally, there were no carcasses with ultimate pH ≥ 5.87.

To explore associated mechanisms, we analyzed AMPK activity in these muscle samples. AMPK activity is correlated with the phosphorylation of α subunit at Thr 172 (Hardie, 2004). As expected, the phosphorylation was 19% ± 5.4% lower in High IMF compared to Low IMF (P<0.05) (Figure 2A). The phosphorylation of ACC at Ser 79 is catalyzed by AMPK. Therefore, the phosphorylation ratio of ACC is an indicator of AMPK phosphorylation (Ahmad et al., 2005). In agreement with the lower AMPK phosphorylation in High IMF muscle, the ratio of ACC phosphorylation was also 45% ± 10.0% lower in High IMF muscle than that of Low IMF muscle (P<0.05) (Figure 2B). These data clearly displayed that AMPK phosphorylation was decreased in High IMF muscle compared to Low IMF muscle. AMPK is a crucial kinase controlling glucose metabolism (Hardie, 2004; Sambandam and Lopaschuk, 2003). AMPK regulates glycogen synthesis and utilization through phosphorylation of phosphorylase kinase. This phosphorylation leads to its activation, which further activates glycogen phosphorylase by phosphorylation, leading to glycogenolysis (Bergeron et al., 1999; Carling and Hardie, 1989; Young et al., 1996). Therefore, the low AMPK activity in High IMF muscle should promote the accumulation of glycogen.

Acetyl-CoA carboxylase is an enzyme which catalyzes the synthesis of malonyl-CoA, a key substrate for lipogenesis. The phosphorylation of ACC by AMPK inhibits ACC activity. Hence, the reduced ACC phosphorylation in High IMF muscle promotes lipogenesis, which may provide an explanation for the high intramuscular fat content in these cattle. These data show an important role of AMPK in both glycogen and fat accumulation, suggesting that AMPK might be a target for improving beef quality.

**GSK3 and glycogen accumulation.**

GSK-3 is a serine/threonine kinase. GSK-3 negatively regulates glycogen synthase activity by phosphorylation. Since GSK-3 is constitutively active in unstimulated muscle cells, glycogen synthase primarily exists in a phosphorylated and inactive state (MacAulay et al., 2005). However, in response to the stimulation of insulin, GSK-3 is itself phosphorylated and inactivated by Akt (another name is PKB, protein kinase B), a main kinase activated by insulin receptor mediated signaling (MacAulay et al., 2005;
van der Velden et al., 2006). Due to lack of phosphorylation catalyzed by GSK3, glycogen synthase is gradually dephosphorylated by protein phosphatases and becomes active (MacAulay et al., 2005). The phosphorylation of GSK3 in High IMF was 50% \(\pm 12.6\%\) lower than that in Low IMF (\(P<0.05\)) (Figure 3), which should unleash glycogen synthase activity and enhance glycogen accumulation.

Myosin isoform composition and muscle fiber typing.

Glycogen content is known to be different in various muscle fiber types. Type II fibers have higher glycogen content compared to Type I fibers (Hambrecht et al., 2005). To make things more interesting, the expression of AMPK\(\gamma3\) subunit is muscle fiber type specific, with high levels of expression in Type IIB and Type IIA fibers, whereas its expression is very low in type I fibers (Yu et al., 2004). This prompted us to check the muscle fiber composition of Low and High IMF muscle. We used a standard myosin ATPase staining method to identify the fiber type composition. Following pre-incubation at pH 9.7, Type I were only weakly stained but Type II muscle fibers were stained, strongly resulting in a dark appearance (Figure 4A). However, we could not clearly differentiate type IIA and IIB muscle fibers despite our numerous trials using different pre-incubation pH values. This is in agreement with previous reports in sheep that the myosin ATPase staining technique only allowed the identification of type I and II muscle fibers (Suzuki, 1995; Suzuki and Cassens, 1980; Suzuki and Tamate, 1988). In addition, using immunoblotting, it was shown that the type IIB myosin isoform is absent in sheep muscle (Arguello et al., 2001). We examined the myosin isoform composition in these muscle samples. As shown in Figure 4B, there were clear bands of Type I and Type IIA isoforms, but no type IIB myosin isoform were detected. Rat extensor digitorum longus (EDL) muscle is mainly composed of Type IIB muscle fibers and soleus muscle is mainly made of Type I muscle fibers (Higaki et al., 2001). To confirm our identification of isoforms, rat EDL and soleus muscle were loaded on the same gel with cattle muscle samples. As expected, EDL muscle showed a strong Type IIB band and soleus muscle showed a strong Type I band (Figure 4B). However, the same region is blank for cattle LM, showing that cattle LM is absent of Type IIB myosin isoform. To eliminate any chance of misidentification of other bands as myosin bands, we used immunoblotting with an antibody against myosin heavy chain, which confirmed that those bands are myosin isoform bands (data not shown). As the knowledge of authors, this is the first report demonstrating that bovine muscle lacks the expression of type IIB myosin isoform. We realize this is a small sample only representing British\times continental crossbreds so can not be assumed for all breeds. We further quantified the ratio of Type I to Type II muscle fibers in cattle muscle. There was no significant difference between Low IMF and High IMF muscle (\(P=0.09\)) (Figure 4C). These data showed that fiber type composition might not be the reason for the different glycogen and fat accumulation in Low and High IMF muscle of beef cattle.

In summary, our data demonstrate a positive correlation between intramuscular fat and glycogen content, while both of them are negatively correlated with AMPK activity. The GSK3 activity was lower in High IMF muscle compared to Low IMF muscle, which may be partially responsible for the high glycogen content in High IMF muscle. No Type IIB myosin isoform was detected in the beef muscle of these British\times continental steers and the ratio of Type I to Type II fibers did not differ between High and Low IMF cattle muscle.

References


Figure 1. Intramuscular fat and glycogen content in *longissimus dorsi* muscle of low intramuscular (Low IMF ■) and high intramuscular fat (High IMF □) cattle. Panel A: intramuscular fat content; Panel B: glycogen content. (*): $P < 0.05$. Mean ± SE. (n = 5 per group).

Figure 2. AMP activated protein kinase (AMPK) and the ratio of phosphorylated Acetyl-CoA carboxylase (ACC) and total ACC in *longissimus dorsi* muscle of low (■) and high intramuscular fat (□) cattle. Panel A: AMPK and phospho-AMPK immunoblots and statistical data; Panel B: ratio of phosphorylated ACC to total ACC. (*): $P < 0.05$. Mean ± SE. (n = 5 per group).

Figure 3. Glycogen synthase kinase-3 (GSK3) and its phosphorylation at Ser 21/9 in *longissimus dorsi* muscle of low intramuscular (Low IMF ■) and high intramuscular fat (High IMF □) cattle. (*) $P < 0.05$. Mean ± SE. (n = 5 per group).

Figure 4. Myosin isoforms and muscle fiber typing of *longissimus dorsi* muscle of low intramuscular (Low IMF ■) and high intramuscular fat (High IMF □) cattle. Panel A: muscle fiber typing by pre-incubation at pH 10.5; Panel B: myosin isoform separation by electrophoresis. EDL: *extensor digitorium longus* muscle from rats. Soleus: *soleus* muscle from rats. Panel C: ratio of Type I to Type II myosin fibers. Mean ± SE. (n = 5 per group).
Nutrition

**Body Condition Score and Day of Lactation Effects on Lipogenic Enzyme Messenger RNA Abundance and Transcription Factors in Adipose Tissue of Beef Cows Fed Supplemental Fat**

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**Summary and Implications**

We hypothesized that BCS at parturition and postpartum dietary fat supplementation will alter transcript abundance of adipose tissue lipogenic enzymes during lactation in beef cows. Our objective was to determine abundance of lipogenic enzyme mRNA transcripts as well as transcription regulators in adipose tissue of three-year-old Angus × Gelbvieh beef cows nutritionally managed to achieve a BCS of 4 ± 0.07 (BW = 1056 ± 72 lb; n = 18) or 6 ± 0.07 (BW = 1276 ± 100 lb; n = 18) at parturition. Beginning 3 d postpartum, cows within each BCS were assigned to a hay diet plus low-fat control supplement or supplements with either cracked high-linoleate safflower seeds or cracked high-oleate safflower seeds until d 60 of lactation. Diets were formulated to be isonitrogenous and isocaloric, and diets with safflower seeds had 5% of DMI as fat. At d 30 and d 60 of lactation, adipose tissue biopsies were collected for RNA extraction, and quantitative RT-PCR was used to measure transcript abundance for acetyl-CoA carboxylase (ACC), hormone-sensitive lipase (HSL), and lipoprotein lipase (LPL). Western blot analysis was used to quantify signal transducer and activator of transcription-subtype 5 (STAT-5) and peroxisome-proliferator activated receptor-subtype gamma (PPAR-γ). Adipose tissue of BCS 4 cows had less mRNA for LPL (P = 0.001) and HSL (P = 0.10) compared with BCS 6 cows. Abundance of LPL mRNA was lower (P = 0.002) at d 30 postpartum compared to d 60; whereas, HSL mRNA was greater at d 30 (P < 0.001). Abundance of PPAR-γ was lower (P = 0.001) in BCS 4 cows compared to BCS 6, following the trend of LPL and HSL in adipose tissue of these cows. Both STAT-5 (P < 0.0001) and PPAR-γ (P = 0.05) were greater at d 30 compared to d 60 postpartum. We conclude that abundance of adipose tissue mRNA for HSL and LPL are influenced by cow BCS. In addition, change in mRNA abundance due to day of lactation indicates a shift in nutrient partitioning away from the mammary gland to s.c. adipose tissue. Furthermore, it is likely that STAT-5 and PPAR-γ play a role in the transcriptional regulation of these enzymes in adipose tissue during lactation in beef cows.

**Introduction**

The metabolic goal during lactation is to support mammary gland nutrient production through coordinated genetic regulation in adipose tissue (Frayn et al., 1998). Thus, repression of adipose tissue lipoprotein lipase (LPL) and acetyl-CoA carboxylase (ACC) genes (Travers et al., 1997), with simultaneous upregulation of the hormone sensitive lipase (HSL) gene (Martin-Hidalgo et al., 1994) would provide the mammary gland with endogenous energy supplies to meet the metabolic demands of lactation. Both signal transducer and activator of transcription-subtype 5 (STAT-5) and peroxisome-proliferator activated receptor-subtype gamma (PPAR-γ) are involved in transcription regulation of lipogenic enzymes such as ACC, LPL, and HSL during lactation (Schoonjans et al., 1996; Mao et al., 2002; Deng et al., 2006). Therefore, quantifying beef cow adipose tissue transcript abundance of ACC, HSL, and LPL, as well as STAT-5, and PPAR-γ proteins would provide insight into transcription regulation under the changing metabolic condition affecting these enzymes during lactation. Supplementing linoleate and oleate to beef cows postpartum enhanced biohydrogenation intermediates in adipose tissue, such as 18:1 trans-11 and 18:2 cis-9, trans-11 (Lake et al., 2007), which regulate adipose tissue lipogenesis at the transcriptional level (Griiniari et al., 1998). For the current study, our hypothesis was that BCS at parturition and biohydrogenation intermediates occurring from ruminal metabolism of either dietary high-linoleate or high-oleate lipids, fed at 5% of DMI, will alter mRNA abundance of ACC, HSL, and LPL in adipose tissue of lactating beef cows.

**Materials and Methods**

**Animals, Diets, and Tissue Collection**

The University of Wyoming Institutional Animal Care and Use Committee approved all procedures for these studies. Cows were nutritionally managed and assigned to treatments as described by Lake et al. (2005). Thirty-six three-year-old Angus × Gelbvieh beef cows were nutritionally managed to achieve either a BCS (1 = emaciated, 9 = obese; Wagner et al., 1988) of 4 (BCS 4 ±
0.07, initial BW = 1056 ± 72 lb; n= 18) or 6 (BCS 6 ± 0.07, initial BW 1276 ± 100 lb; n=18) at parturition. Briefly, cows assigned to a BCS of 4 at parturition were managed to be in an energy deficit state during the second trimester and then were fed to meet maintenance requirements throughout the third trimester of gestation to ensure that postpartum cow and calf performance was not affected. Cows assigned to a BCS of 6 at parturition were managed to meet energy requirements throughout gestation according to the NRC (1996).

At d 30 and again at d 60 of lactation, each cow was injected s.c. with approximately 400 mg of lidocain hydrochloride (Vedeo, Inc. St. Joseph, MO.) as a local anesthetic (Lake et al., 2006). Adipose tissue biopsies (5 g) were removed (Rule and Beitz, 1986), snap-frozen in liquid N, and stored at −80°C for later quantification of mRNA and protein transcription regulators.

Quantitative PCR analysis of bovine adipose tissue

Approximately 75 mg of adipose tissue was homogenized for total RNA extraction using RNeasy lipid mini kit (Qiagen Inc., Valencia, CA,) according to manufacturer’s instructions. The expression level of specific mRNAs was determined using quantitative fluorescent real-time PCR (TaqMan® methodology, Applied Biosystems, Foster City, CA). Multiplex PCR reactions were performed to quantify the target genes of interest and account for intra-assay variation.

Western blot analysis

Approximately 0.5 g of adipose tissue was homogenized in sterile, Tris buffer for Western blot of STAT-5 and PPAR-γ. Approximately 10 μg of total adipose tissue protein extract was loaded and separated using SDS-PAGE. Following electrophoresis, proteins were electrophoretically transferred onto a nitrocellulose membrane using a semi-dry transfer apparatus (BioRad, Hercules, CA). After blocking, the membrane was washed, then incubated for 1 h with anti-rabbit primary antibody against either STAT-5 or PPAR-γ (Affinity Bioreagents, Boulder, CO), then incubated for 30 min with HRP-conjugated goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA). Protein bands were visualized using chemiluminescence then digitzed for quantification (Gel Doc, BioRad, Hercules, CA).

Statistical Analyses

Cow adipose tissue mRNA data and Western blot data were analyzed as a split-plot with a 2 × 3 arrangement of treatments within a completely randomized design using the MIXED procedure of SAS (SAS Institute, Cary, N.C.). The effects of BCS at parturition, and dietary treatment were tested using individual cow as the RANDOM statement. Comparison of main effects and interactions were determined using least square means. With the exception of HSL mRNA abundance in cows fed the high-oleate diet at d 30 of lactation ($P = 0.04$), no interactions ($P = 0.19$ to 0.91) between main effects for mRNA or protein data were noted, therefore only the main effects of maternal BCS at parturition, dietary treatment, and day of lactation were presented.

Results and Discussion

Adipose tissue mRNA analysis

Effects of BCS on mRNA abundance are shown in Figure 1. Relative abundance of adipose tissue LPL mRNA was lower ($P = 0.001$) for BCS 4 cows compared to BCS 6 cows. The supply of substrates for mammary milk fat production is dependent, in part, upon the partitioning of fatty acids from adipose tissue to the mammary tissue for utilization. In the cow, initial metabolic adaptations of the adipose tissue to support lactation occur between 30 d prepartum and parturition, and are manifested by an increase in lipolysis and decreases in esterification and lipogenesis (McNamara, 1991). Circulating triacylglycerols transported by either chylomicrons or low-density lipoproteins, are hydrolyzed in adipose tissue by the catalytic activity of LPL located on the luminal surface of the capillary endothelium (Braun and Severson, 1992). The liberated free fatty acids are then made available for re-esterification and storage in the adipocyte. Lake et al. (2006) reported 20% greater LPL activity in adipose tissue of beef cows with a BCS of 4 compared to BCS 6 cows. In addition, Lake et al. (2006) reported a 50% greater in vitro palmitate esterification rates in adipose tissue of BCS 4 cows compared to BCS 6 cows, suggesting that adipose tissue was metabolically upregulated for greater storage of triacylglycerols in BCS 4 cows compared to BCS 6 cows. It is possible that the lower LPL mRNA in BCS 4 cows from the current study was a reflection of greater mRNA turnover. In addition, LPL mRNA abundance in adipose tissue might not be indicative of total tissue LPL, which was reported by Lake et al. (2006); as opposed to extracellular LPL enzyme bound to the capillary endothelium, suggesting the effect of BCS may be elicited through post-translational regulatory mechanisms. Lake et al. (2006) suggested the possibility that insulin receptors in adipose tissue of BCS 4 cows might have greater sensitivity to insulin than BCS 6 cows; thereby increasing total LPL activity. Lake et al. (2006) also suggested that, due to increased LPL activity and fatty acid esterification in adipose tissue, BCS 4 cows were more efficient in nutrient utilization and adipose tissue accretion, allowing the BCS 4 cows to gain body condition more rapidly.

Hormone sensitive lipase mRNA tended to be lower ($P = 0.10$) in adipose tissue of BCS 4 cows compared to BCS 6 cows (Figure 1). The catalytic activity of HSL involves hydrolysis of stored triacylglycerols, diacylglycerols, monoacylglycerols, and cholesterol esters in adipose cells. Although HSL has broad substrate specificity, the primary action is attributed to the hydrolysis of stored triacylglycerols. The hydrolysis of esterified fatty acids from stored adipose tissue triacylglycerols is a significant source of fatty acids for mammary tissue milk fat production during lactation. Lake et al. (2005) reported BCS 4 cows had lower circulating NEFA compared to BCS 6 cows, indicating a lower availability of NEFA to the mammary gland for milk fat production in the BCS 4 cows.
The abundance of HSL mRNA which tended \( (P = 0.10) \) to be lower in BCS 4 cows, could indicate a decrease in HSL protein, which could be responsible for the lower circulating NEFA in BCS 4 cows as reported by Lake et al. (2005).

Cow BCS had no effect on abundance of ACC mRNA \( (P = 0.34; \text{Figure 1}) \). This is supported by Lake et al. (2006), who reported no difference in ACC activity in adipose tissue of BCS 4 cows compared to BCS 6 cows. Thus, postpartum de novo fatty acid synthesis in adipose tissue was not altered by body condition of animals maintained at a suboptimal level prepartum. The lack of BCS effect on adipose tissue ACC mRNA abundance also suggests that energy utilization and storage will be predominantly provided from dietary intake of fatty acids, as indicated by increased LPL activity in adipose tissue of BCS 4 cows compared to BCS 6 during lactation (Lake et al., 2006).

The effects of day of lactation on adipose tissue mRNA abundance are shown in Figure 2. Abundance of mRNA increased both LPL \( (P = 0.05) \) and ACC \( (P = 0.001) \) from d 30 to d 60 of lactation. These results would seem contrary to Lake et al. (2006), who reported decreased LPL and ACC enzyme activities in adipose tissue from d 30 to d 60 of lactation. However, Bottger et al. (2002) reported no differences in beef cow weight change from d 60 to d 90 postpartum following significant weight loss from parturition to d 60 postpartum. Therefore, it is possible that the change in mRNA abundance for LPL and ACC at d 60 precede adipose tissue accretion and change in body condition because lipogenesis wouldn’t increase until later in lactation. Peak lactation in beef cows occurs around d 50 postpartum, and is characterized by maximal milk yield, as well as maximal energy being transported from adipose tissue to the mammary gland in support of lactation. Even though adipose tissue mRNA abundance of both LPL and ACC increased (Figure 2) at d 60 of lactation, increases in LPL and ACC protein with corresponding changes in fatty acid storage in adipose tissue may not occur until later in lactation. This suggests other post-translational regulatory mechanisms are employed in adipose tissue during lactation, such as phosphorylation (Munday, 2002). Protein Kinase A (PKA) and AMP activated Protein Kinase (AMPK) are the only two kinases known to phosphorylate Kinase A (PKA) and AMP activated Protein Kinase (AMPK) are the only two kinases known to phosphorylate 

Effects of BCS on STAT-5 and PPAR-\( \gamma \) are shown in Figure 4. Cow BCS had no effect on STAT-5 protein abundance \( (P = 0.69) \); however, PPAR-\( \gamma \) tended to be lower \( (P = 0.13) \) in BCS 4 cows compared to BCS 6 cows suggesting greater animal numbers would have been required to demonstrate a BCS effect. Hormone sensitive lipase mRNA is regulated in adipose tissue by PPAR-\( \gamma \) (Deng et al., 2006); therefore, the lower HSL mRNA in BCS 4 cows compared to BCS 6 cows could have been due to PPAR-\( \gamma \) regulation, as both followed the same trend in response to cow BCS. The lower circulating NEFA levels reported for BCS 4 cows by Lake et al. (2006), could have been associated with lower adipose tissue HSL activity. In addition, Lake et al. (2006) suggested that BCS 4 cows may have had increased insulin sensitivity in adipose tissue.

Effects of day of lactation on STAT-5 and PPAR-\( \gamma \) are shown in Figure 5. Abundance of both STAT-5 \( (P < 0.0001) \) and PPAR-\( \gamma \) \( (P = 0.05) \) decreased from d 30 to d 60 of lactation. Although STAT-5 up-regulates ACC transcription (Mao et al., 2002), ACC transcript abundance increased in adipose tissue from d 30 to d 60 of lactation (Figure 2). It is possible that the actions of PPAR-\( \gamma \) and STAT-5 could have been bidirectional, ultimately causing a decrease in mRNA abundance for ACC mRNA in adipose tissue of beef cows as lactation proceeded.

The bidirectional inhibition suggested by Shipley and Waxman, (2004) could also account for the opposite trend noted for LPL mRNA in adipose tissue, which was lower at d 30 compared to d 60 of lactation (Figure 2). Schoonjans et al., (1996) indicated PPAR-\( \gamma \) activation results in increased LPL activity and lipolysis of circulating triacylglycerols. Thus, activation of STAT-5 in adipose tissue could inhibit PPAR-\( \gamma \), causing an inverse effect on LPL mRNA abundance.

The decrease in PPAR-\( \gamma \) during lactation was consistent with the changes observed for HSL mRNA.
abundance at these two time points. Deng et al. (2006) reported nearly a 30-fold induction of HSL trancription following PPAR-γ activation in adipocytes. In addition, Lake et al. (2006) reported a decrease in circulating NEFA levels from d 30 to d 60 of lactation. Thus, PPAR-γ could be directly involved in the regulation of HSL in adipose tissue of lactating beef cows.

A concern of the producer is to provide adequate energy to the lactating cow to ensure successful, subsequent rebreeding. It has been suggested that mRNA abundance is a predictor of ultimate enzyme activity. Results of the current study suggest that lipogenic enzymes, ACC and LPL, are regulated at both transcriptional and post-translational levels during lactation; therefore, mRNA abundance alone would not be an accurate predictor of adipose tissue lipogenic activity and fatty acid partitioning in the beef cow. The energy needs of a cow are increased by lactation, and even further increased by growth in the case of a primiparous cow (Freely et al., 2006). To meet these energy demands, determining energy metabolism between d 30 and d 60 of lactation becomes paramount in order to develop and maintain an adequate feeding program. A decrease in adipose tissue HSL mRNA, around peak lactation, could indicate that the lactating cow is beginning to repartition fatty acids from the mammary gland back to adipose tissue. This information would be valuable when designing a supplementation strategy to maximize postpartum weight gain and increase in body condition to better ensure subsequent reproduction.

References


Figure 1. Messenger RNA abundance of acetyl CoA carboxylase (ACC), lipoprotein lipase (LPL), and hormone sensitive lipase (HSL) in adipose tissue of lactating beef cows due to body condition score determined by quantitative PCR. Cows were nutritionally managed to achieve a BCS of 4 or 6 at parturition (Lake et al., 2005). Data is expressed as ng mRNA of target gene/18s RNA of interest and was quantified using the TaqMan system (Applied Biosystems, Foster City, CA). Abundance of LPL ($P = 0.001; n = 18$) and HSL ($P = 0.10; n = 18$) was lower in BCS 4 cows compared to BCS 6 cows. No difference was observed in mRNA abundance for ACC ($P = 0.34; n = 18$) in adipose tissue.

Figure 2. Messenger RNA abundance of acetyl CoA carboxylase (ACC), lipoprotein lipase (LPL), and hormone sensitive lipase (HSL) in adipose tissue of lactating beef cows due to day of lactation. Data is expressed as ng mRNA of target gene of interest/18s RNA and was quantified using the TaqMan system (Applied Biosystems, Foster City, CA). Abundance for LPL ($P = 0.05; n = 36$) and ACC ($P = 0.001; n = 36$) was lower at d 30 compared to d 60 of lactation, with the opposite effect noted for HSL ($P = 0.001; n = 36$).

Figure 3. Messenger RNA abundance of hormone sensitive lipase in adipose tissue of lactating beef cows due to dietary treatment at d 30 postpartum. Data is expressed as ng mRNA of target gene of interest/18s RNA and was quantified using the TaqMan system (Applied Biosystems, Foster City, CA). Diets were hay and either a low-fat control (CON) supplement or supplements with either cracked high-linoleate (LIN) or high-oleate (OLE) safflower seeds. Lipid supplemented diets were formulated to provide 5% DMI as fat. Abundance of HSL mRNA was greatest ($P = 0.04; n=12$) for the OLE diet.

Figure 4. Abundance of signal transducer and activator of transcription-subtype 5 (STAT-5) and peroxisome-proliferator activated receptor-subtype gamma (PPAR-γ) protein in adipose tissue of lactating beef cows due to BCS. Data is expressed as optical density units (OD). Cow BCS had no effect on STAT-5 protein abundance ($P = 0.87; n = 18$). However, PPAR-γ protein abundance tended to be lower ($P = 0.13; n = 18$) in BCS 4 cows compared to BCS 6.

Figure 5. Effect of day of lactation on signal transducer and activator of transcription-subtype 5 (STAT-5) and peroxisome-proliferator activated receptor-subtype gamma (PPAR-γ) proteins in beef cow adipose tissue. Data expressed as optical density units (OD). Protein abundance for both STAT-5 ($P = 0.0001; n = 36$) and PPAR-γ ($P = 0.05; n = 36$) was greater at d 30 postpartum compared to d 60.
Nutrition

Effects of Ruminal Protein Degradability and Supplementation Frequency on Expression and Distribution of Urea Transporter- B in Lambs Fed Low Quality Forage

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Summary and Implications

Thirteen Dorset wether lambs (initial BW = 75 ± 8.8 lb) were used in a completely randomized designed experiment to examine the effects of ruminal protein degradability and supplementation frequency on the expression and distribution of urea transporter-B (UT-B) in tissues important to N recycling. Lambs were fed crested wheat grass hay (4.2% CP, 59% NDF) for ad libitum consumption plus one of four isonitrogenous supplements: 1) ruminally degradable protein (RDP) fed daily (n=3), 2) RDP fed on alternate days (n=3), 3) ruminally undegradable protein (RUP) fed on alternate days (n=3) or 4) a 50:50 mixture of RDP and RUP fed on alternate days (n=4). After 18 d, lambs were euthanized and samples (5 g) taken from the gastrointestinal tract, liver, kidney, and parotid salivary gland were snap frozen and later processed for Western blot analyses for UT-B. Immunoblotting using a rabbit polyclonal antibody to UT-B confirmed the presence of distinct 32 kDa (consistent with a non-glycosylated UT-B protein) and 47 kDa (probable N-glycosylated form of UT-B) protein bands in all nine tissues. The liver, dorsal rumen, reticulum, and ventral rumen displayed strong bands at 32 kDa and lighter bands at 47 kDa. The spiral colon, cecum, parotid salivary gland, large colon, and kidney had slight bands at 32 kDa and visible bands at 47 kDa. Although the abundance of the 47 kDa UT-B band in the ventral rumen was greater (P = 0.03) in lambs fed RDP daily, no other treatment differences (P ≥ 0.16 to 0.99) in the abundance of the 32 or 47 kDa UT-B proteins or the 32 kDa/47 kDa ratio within tissue were observed. However, the 32 kDa/47 kDa ratio was greatest (P ≤ 0.001) for the liver with no difference (P = 0.63 to 0.99) among the remaining tissues. Although protein supplementation strategy had little effect on UT-B expression in tissues other than the ventral rumen, differences in N-glycosylation among tissues may provide insight into the regulation of UT-B function.

Introduction

Decreasing the frequency of protein supplementation to ruminants consuming low quality forages has generally resulted in minimal impact on nutrient intake or digestion (Bohnert et al., 2002a), or subsequent animal performance (Bohnert et al., 2002a; Ludden et al., 2002). Hunt et al. (1989) suggested that infrequent protein supplementation may stimulate recycling of endogenous N into the rumen. Because of the positive relationship that exists between ruminally degradable protein (RDP) supplementation and forage utilization, most supplements fed infrequently include high levels of RDP. However, recent work in our laboratory (Atkinson, 2005, 2006a, 2006b) suggests that replacing a portion of the supplemental RDP with ruminally undegradable protein (RUP) may further enhance the N recycling process by altering the timing of N return to the rumen relative to the time of supplementation. The recent identification of urea transporter-B (UT-B) in the gastrointestinal tract tissues of sheep (Marini et al., 2004) and cattle (Marini and Van Amburgh, 2003; Stewart et al., 2005) provide a potential mechanism by which the N recycling process could be regulated. Despite early research, little is known about the expression, tissue distribution, and physiological role of UT-B in the N recycling process. Moreover, the effects of dietary protein on UT-B expression requires further investigation, given the importance of N recycling in ruminant livestock consuming low quality (low protein) forages. We hypothesize that UT-B is widely distributed in the gastrointestinal tract and associated tissues of ruminants, and that UT-B expression will be up-regulated in infrequently-supplemented animals, particularly when those supplements contain higher levels of RUP. Therefore, our objectives are to 1) evaluate the expression and distribution of UT-B within the gastrointestinal tract, liver, kidney, and parotid salivary gland of lambs consuming low quality forage, and 2) to assess the role of ruminal protein degradability and supplementation frequency on UT-B abundance in these tissues.
Materials and Methods

Animals and diets

Thirteen Dorset wether lambs (75 ± 8.8 lb initial BW) were randomly assigned to one of four treatments within a completely randomized design. All procedures were approved by the University of Wyoming Animal Care and Use Committee. Wethers were housed in individual metabolism crates (4.5 × 2 ft) in a temperature controlled room (68°F) under constant lighting. Details on the basal diet and the formulation of supplements fed in this experiment are described in Atkinson et al. (2005). Briefly, wethers were fed a basal diet of low-quality mature crested wheatgrass hay (4.2% CP, 59% NDF, and 42% ADF) for ad libitum consumption in two portions daily at 0630 and 1600. Wethers were supplemented at 0600 daily with one of four supplemental protein treatments: 1) a high RDP supplement (Table 1) based upon isolated soy protein fed daily (RDP-D), 2) the high RDP supplement provided on alternate days (RDP-D), 3) a high RUP supplement based upon corn gluten meal fed on an isonitrogenous basis to the RDP supplement, provided on alternate days (RUP-A), or 4) a 50:50 mixture of the RDP and RUP supplements provided on alternate days (MIX-A). The RDP and RUP supplements were fed at the daily equivalent rate of 0.236 and 0.285% of BW, respectively throughout the experiment, with alternate-day treatments fed at twice that of daily supplementation.

Table 1. Composition of supplements

<table>
<thead>
<tr>
<th>Ingredient, % of DM</th>
<th>RDP</th>
<th>RUP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated soy-protein</td>
<td>73.1</td>
<td>75.8</td>
</tr>
<tr>
<td>Corn gluten meal</td>
<td>11.7</td>
<td>11.1</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>10.2</td>
<td>8.1</td>
</tr>
<tr>
<td>Dried molasses</td>
<td>5.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Chemical

- DM, %: 95.8, 93.9
- CP, % of DM: 73.9, 54.3

RDP = ruminally degradable protein, RUP = ruminally undegradable protein

ARDEX® AF, Archer Daniels Midland Company, Decator, IL.

1Contains 3,628,739 vitamin A, 3,628,739 vitamin D3, and 18,144 vitamin E IU/kg.

Tissue Collection and Sample Analyses

After receiving their supplemented treatments for 18 days, wethers were randomly euthanized by injection of Beuthanasia-D Special (Schering-Plough Animal Health, Union, NJ) according to label directions. The lambs were immediately eviscerated, and tissue samples (5 g) collected from the dorsal and ventral rumen, reticulum, cecum, large colon, small (spiral) colon, liver, kidney, and parotid salivary gland. Samples were rinsed with PBS, and immediately snap frozen in liquid N. Samples were stored at -80°C until protein isolation and Western blot analyses were performed.

Western blot procedures used in this experiment are described in detail by Stohrer et al. (2007). Briefly, samples (500 mg tissue) were homogenized in RIPA buffer and 25 μg of protein was loaded per lane on 12% polyacrylamide SDS gels. The electrophoresed protein was transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Membranes were then probed with purified polyclonal anti-UT-B antibody (Stohrer et al. 2007) and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG. Immunoreactive protein bands were visualized by chemiluminescent substrate (Pierce, Rockford, IL). All Western blots within a tissue were processed together, with exposure time dependent upon intensity of signal within tissue type. Autoradiographs were digitized (UN-SCAN-IT™, Orem, UT) to obtain total pixel counts for the 32 kDa and 47 kDa UT-B bands as well as a 42 kDa β-actin band. Blots were normalized by dividing the total pixels for each band (32 kDa or 47 kDa) by the total pixels obtained for the corresponding β-actin of the same lane/sample. Results were expressed as arbitrary densitometry units per 32kDa and 47kDa UT-B band.

Statistical Analyses

All densitometry data were analyzed using the GLM procedures of SAS (SAS Inst. Inc., Cary, NC) using the model for a completely randomized design. Because the same tissue sample from all lambs was included in a single set of Western blots assayed simultaneously, the model used to detect treatment differences in the 32 and 47 kDa protein bands consisted of treatment only, and was conducted within a given tissue type. The model used to detect differences in the 32 kDa/47 kDa ratio also included the effects of tissue type and the treatment × tissue type interaction. No treatment × tissue type interactions (P ≥ 0.93) were detected, and thus only main effects of treatment and tissue type will be discussed. When necessary, separation of treatment means was accomplished using least squares means and Fisher’s protected LSD (P ≤ 0.05).

Results and Discussion

Immunoblotting confirmed the presence of two distinct UT-B protein bands (32 kDa and 47 kDa) in all nine tissues analyzed. Despite their presence in all tissues analyzed, the visual intensity of these UT-B bands differed among tissues. A strong 32 kDa band and a light 47 kDa band were detected in the dorsal, ventral rumen, reticulum (Figure 1), and liver. Conversely, the small (spiral) colon, cecum, large colon, and kidney displayed slight bands at 32 kDa and more visible/intense bands at 47 kDa. The parotid salivary gland displayed similar bands to the lower GI tract and the kidney (Figure 2). This is one of the first known studies (Stohrer et al., 2007) identifying UT-B within the parotid salivary gland of any species.
Other researchers have reported UT-B proteins similar in size to our 47 kDa UT-B protein in rumen papillae of dairy heifers (Marini and Van Amburgh, 2003), the duodenum, ileum, and cecum of lambs (Marini et al., 2004), and in the rumen of slaughter cattle (Stewart et al., 2005). However, we believe that the two distinct protein bands detected by our anti-UT-B antibody represent N-glycosylated (47 kDa) and non-glycosylated (32 kDa) forms of the UT-B protein. Lucien et al. (2002) predicted that the structure of human UT-B1 contained a single unique functional N-glycosylation site, which is consistent with the presence of two distinct UT-B bands as observed in the current study and that of others. Similarly, Timmer et al. (2001) and Olives et al. (1995) reported that deglycosylation of UT-B reduced the size of a larger 45-60 kDa UT-B band in human erythrocytes to 32 or 36 kDa, which is consistent with the two protein bands detected in the current experiment.

Contrary to our initial hypothesis that UT-B abundance would be greater in animals supplemented on alternate days, particularly with RUP, the only difference observed in UT-B abundance occurred in the ventral rumen. Although the abundance of the 47 kDa UT-B band in the ventral rumen was greater (P = 0.03) in lambs fed RDP daily, no other treatment differences (P ≥ 0.16) in the abundance of the 32 or 47 kDa UT-B proteins (Table 2) was observed. Similarly, Marini et al. (2004) reported no differences in UT-B abundance in the rumen in response to increasing dietary N. In contrast, Marini and Van Amburgh (2003) observed greater expression of UT-B (based upon visual evaluation) in ruminal papillae collected from the ventral sac of the rumen in dairy heifers fed a high N diet (2.97 - 3.4% N) as compared to a low N diet (1.45 – 1.89% N). The increased abundance of the 47 kDa UT-B band in the ventral rumen when lambs were fed RDP daily could be the result of high rumen ammonia concentrations associated with feeding excess RDP on the day of supplementation (Atkinson et al., 2005). Marini and Van Amburgh (2003) suggested that when a high N diet is fed and ruminal ammonia is high, urea diffuses into the gastrointestinal tract via the paracellular space and may return to the blood via UT-B. Therefore, it is possible that the level of RDP fed to RDP-D lambs was sufficient to increase UT-B abundance in the ventral rumen in the manner observed by Marini and Van Amburgh (2003). Furthermore, because it has been demonstrated that ruminal epithelial and duodenal mucosal cells are capable of undergoing ureagenesis in vitro (Oba et al., 2004), UT-B expression in these tissues may function as an excretory route for urea, rather than recycling of urea into the gastrointestinal tract.

Dietary treatment had no influence (P ≥ 0.14) on the 32 kDa/47 kDa ratio within tissues. However, the 32 kDa/47 kDa ratio differed (P = 0.06) across tissues, being greatest (P ≤ 0.001) for the liver with no difference (P ≥ 0.63) among the remaining tissues. While differences in the 32 kDa/47 kDa ratio between the liver and the remaining tissues reflect the relative differences in the degree of N-glycosylation of UT-B, the physiological importance of N-glycosylation to the function of UT-B remains largely unexplained. In other systems, N-glycosylation often determines membrane expression level and addressing of polypeptides to the membrane (Varki et al., 2002). However, site-directed mutagenesis to delete the N-glycosylation site of human UT-B expressed in Xenopus oocytes demonstrated that the lack of N-glycosylation did not affect urea uptake when compared to the wild-type UT-B (Lucien et al., 2002). Consequently, further investigation of the role of N-glycosylation on UT-B function is needed.

Both N-glycosylated and non-glycosylated forms of urea transporter-B (UT-B) are expressed in the gastrointestinal tract, kidney, liver, and parotid salivary gland of lambs fed a forage-based diet. Greater expression of the N-glycosylated form of UT-B in the ventral rumen in response to daily supplementation with ruminally degradable protein suggests that UT-B may serve an excretory function for urea, rather than recycling of urea into the gastrointestinal tract. Nonetheless, further research into the physiological significance of N-glycosylation in the regulation of UT-B function is needed.

References
Hunt, C. W., J. F. Parkinson, R. A. Roeder, and D. G. Falk. 1989. The delivery of cottonseed meal at three different time intervals to steers fed low-quality grass


Table 2. Effect of ruminal protein degradability and frequency of supplementation on the abundance of urea transporter-B protein (in arbitrary densitometry units) in the gastrointestinal tract and related tissues in lambs fed a forage-based diet.

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th></th>
<th></th>
<th></th>
<th>SEM</th>
<th>P ≤ 2</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>RDP-D</td>
<td>RDP-A</td>
<td>MIX-A</td>
<td>RUP-A</td>
<td></td>
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<tr>
<td>Reticulum</td>
<td>32 kDa</td>
<td>2.495</td>
<td>2.072</td>
<td>2.072</td>
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<tr>
<td></td>
<td>47 kDa</td>
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<td>0.348</td>
<td>0.348</td>
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<td>2.069</td>
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1Treatments consisted of RDP-D = high ruminally degradable protein (RDP) fed daily; RDP-A = RDP fed on alternate days; RUP-A = high ruminally undegradable protein (RUP) fed on alternate days; MIX-A = 50:50 mixture of the RDP and RUP supplements fed on alternate days .

2P-value of differences between treatments. abMeans with unlike superscripts within row are different (P ≤ 0.05)
Expression and Distribution of Urea Transporter-B in Lambs Fed Increasing Dietary Protein

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E. L. Belden², Professor
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²Department of Veterinary Science

Summary and Implications

Level of dietary CP may affect the expression and distribution of urea transporter-B (UT-B) in tissues important to N recycling in ruminants. Fifteen Dorset wether lambs (initial BW = 101 ± 2.9 lb) were blocked by initial BW and assigned to one of three treatments within a randomized complete block design. Lambs were fed a basal diet of mature crested wheatgrass hay (4.2% CP, 59% NDF) for ad libitum consumption plus one of three soybean meal-based supplements to achieve concentrations of 6, 9, or 12% dietary CP. Lambs were randomly euthanized within block on d 28 and samples (5 g) taken from the gastrointestinal tract, liver, kidney, and parotid salivary gland were snap frozen and later processed for Western blot analyses for UT-B. Immunoblotting using a rabbit polyclonal antibody to UT-B confirmed the presence of distinct 32 kDa (consistent with a non-glycosylated UT-B protein) and 47 kDa (probable N-glycosylated form of UT-B) protein bands in all nine tissues. A broad 32 kDa band and a slight 47 kDa band were detected in samples from the liver, reticulum, dorsal rumen, and ventral rumen. The cecum, large colon, spiral colon, and parotid salivary gland displayed a slight 32 kDa band and a visible band at 47 kDa. The kidney displayed slight bands at both 32 kDa and 47 kDa. No treatments differences in the abundance (arbitrary densitometry units) of the 32 kDa (P ≥ 0.15) or 47 kDa (P ≥ 0.51) UT-B bands or in the 32 kDa/47 kDa ratio (P ≥ 0.38) were detected within tissues. However, the 32 kDa/47 kDa ratio differed (P = 0.05) across tissues, being greatest for the ventral rumen (92.5), liver (79.7), and reticulum (27.7), intermediate for the dorsal rumen (8.0), and lowest for the kidney (1.8), large colon (0.8), spiral colon (0.8), cecum (0.6), and parotid salivary gland (0.2). Although dietary CP level had no effect on expression of either form of UT-B, differences in the 32 kDa/47 kDa ratio among tissues may suggest a possible role of N-glycosylation in the regulation of UT-B function.

Introduction

Ruminant livestock consuming low-quality (< 6% CP) forages often rely upon their ability to recycle blood urea back to the rumen to sustain microbial metabolism. Blood urea may be recycled via diffusion across the ruminal wall from the bloodstream, or it may enter the rumen via saliva (Kennedy and Milligan, 1980). Harmeyer and Martens (1980) noted that consumption of a low-protein diet increases the permeability of the gastrointestinal tract to urea, challenging the conventional wisdom that recycling occurs solely via simple diffusion (Kennedy and Milligan, 1980). The discovery of urea transporters (UT) in mammalian systems (You et al., 1993) and their role in conservation of N in animals fed low protein diets (Isozaki et al., 1994) provides a potential mechanism by which the N recycling process could be regulated. The predominant UT present in gastrointestinal tract tissues is UT-B, which has been identified in sheep (Marini et al., 2004) and cattle (Marini and Van Amburgh, 2003; Stewart et al., 2005). Despite early research, little is known about the expression, tissue distribution, and physiological role of UT-B in the N recycling process. Moreover, the effects of dietary protein on UT-B expression requires further investigation, given the importance of N recycling in ruminant livestock consuming seasonally low quality forages in the Western U.S. We hypothesize that UT-B is widely distributed in the gastrointestinal tract and associated tissues of ruminants, and that UT-B expression will be up-regulated when those animals consume low protein diets. Therefore, our objectives were to 1) evaluate the expression and distribution of UT-B within the gastrointestinal tract, liver, kidney, and parotid salivary gland of lambs consuming low quality forage, and 2) to assess the role of dietary protein supply on UT-B abundance in these tissues.

Materials and Methods

Animals and diets

Fifteen Dorset wether lambs (101 ± 2.9 lb initial BW) were blocked by initial BW and randomly assigned to one of three treatments within a randomized complete block design. Wethers were housed in individual metabolism crates (4.5 × 2 ft) in a temperature controlled room (68° F) under constant lighting. Wethers were fed a basal diet of mature crested wheatgrass hay (4.2% CP, 59% NDF) for ad

This research was supported in part by the UW Faculty Grant-in-Aid Program.
libitum consumption in two portions daily at 0630 and 1800. Forage refusals were collected and weighed daily, and amount of forage adjusted to induce a minimum of 10% refusal rate. Wethers were supplemented at 0600 and 1800 daily with one of three protein supplements to achieve dietary CP concentrations (% DM) of 6, 9, or 12% CP (Table 1). Wethers had free access to clean water and a trace mineralized salt block (Iofix T-M, Morton Salt; Chicago, IL; guaranteed analysis [% of DM] 97.1% NaCl, and ≤ 0.35% each of Zn, Mn, Fe, Cu, I, and Co). All procedures were approved by the University of Wyoming Animal Care and Use Committee.

Table 1. Composition of supplements

<table>
<thead>
<tr>
<th>Ingredient, % of DM</th>
<th>LOW¹</th>
<th>MED</th>
<th>HIGH</th>
</tr>
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<tbody>
<tr>
<td>Beet pulp</td>
<td>78.3</td>
<td>53.4</td>
<td>28.2</td>
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<tr>
<td>Molasses</td>
<td>10.0</td>
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<td>10.0</td>
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<tr>
<td>Soybean meal</td>
<td>5.5</td>
<td>27.4</td>
<td>49.2</td>
</tr>
<tr>
<td>Urea</td>
<td>0.6</td>
<td>3.1</td>
<td>5.6</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>2.3</td>
<td>3.2</td>
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</tr>
<tr>
<td>Vitamin ADE Premix²</td>
<td>2.8</td>
<td>2.8</td>
<td>2.8</td>
</tr>
</tbody>
</table>

³Supplements fed to achieve 6% (LOW), 9% (MED) or 12% total dietary CP (% of DM).
²Contains 3,628,739 IU vitamin A, 3,628,739 IU vitamin D₃ and 18,144 IU vitamin E per kg.

Tissue Collection

After receiving their supplemented treatments for 28 days, wethers were euthanized randomly within block by injection of Beuthanasia-D Special (Schering-Plough Animal Health, Union, NJ) according to label directions. The lambs were immediately eviscerated, and tissue samples (5 g) collected from the dorsal and ventral rumen, reticulum, cecum, large colon, small (spiral) colon, liver, kidney, and parotid salivary gland. Samples were rinsed with PBS, and immediately snap frozen in liquid N. Samples were stored at -80°C until protein isolation and Western blot analyses were performed.

Polyclonal antibody production

Polyclonal antibodies were prepared against a peptide (NeoMPS Inc., San Diego, CA) containing the sequence for the carboxy-terminal 19 amino acids known for human UT-B1 (EENNRYFLQAKKRMEVESPL) plus an additional cysteine residue at the amino terminus to facilitate conjugation to the antigen carrier keyhole limpet hemocyanin (KLH). Antibodies directed against the human UT-B1 peptide have been successfully used to detect UT-B in cattle (Marini and Van Amburgh, 2003) and sheep (Marini et al., 2004). Two female rabbits received multiple subcutaneous injections of the KLH-conjugated peptide for the production of polyclonal antibodies. Rabbits were bled from the central ear artery and pre-immune serum was compared to post immunization serum using ELISA to ensure that the immunized rabbits were sustaining high antibody titers to UT-B. The anti-UT-B polyclonal antibody was purified using the T-gel method (Pierce, Rockford, IL), and preabsorption with immunizing peptide was used to demonstrate polyclonal antibody specificity.

Western blot analyses

Samples (500 mg tissue) were homogenized in 3 mL RIPA buffer (phosphate buffered saline with 1%NP 40, 0.1% SDS, and 0.5% sodium deoxycholate). Protein concentration was determined using a Bradford Assay Kit (Pierce, Rockford, IL) and 25 μg of protein was loaded per lane on 12% polyacrylamide SDS gels. Gels were electrophoresed and protein was transferred to 0.2 μm polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Membranes were probed with the T-gel purified polyclonal anti-UT-B antibody diluted 1:2,500 in TBST containing 5% non-fat dry milk for 2.5 h at 20°C. Membranes were subsequently incubated in horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:10,000 (Jackson ImmunoResearch, West Grove, PA) in 5% milk/TBST solution for 1 h at 20°C. Immunoreactive protein bands were visualized by chemiluminescent substrate (Pierce, Rockford, IL). All Western blots within a tissue were processed together, with exposure time dependent upon intensity of signal within tissue type. To normalize samples within tissues, membranes were re-probed using polyclonal antibody for β-actin (Cell Signaling, Danvers, MA) diluted 1:1000 as previously described for UT-B. Autoradiographs were digitized (UNSCAN-IT™, Orem, UT) to obtain total pixel counts for the 32 kDa and 47 kDa UT-B bands as well as the 42 kDa β-actin band. Blots were normalized by dividing the total pixels for each band (32 kDa or 47 kDa) by the total pixels obtained for the corresponding β-actin of the same lane/sample. Results were expressed as arbitrary densitometry units per 32kDa and 47kDa UT-B band.

Statistical Analyses

All densitometry data were analyzed using the GLM procedures of SAS (SAS Inst. Inc., Cary, NC) using the model for a randomized complete block design. Because the same tissue sample from all lambs was included in a single set of Western blots assayed simultaneously, the model used to detect treatment differences in the 32 and 47 kDa protein bands consisted of weight block and treatment only, and was conducted within a given tissue type. The model used to detect differences in the 32 kDa/47 kDa ratio also included the effects of tissue type and the treatment × tissue type interaction. No treatment × tissue type interactions (P ≥ 0.86) were detected, and thus only main effects of treatment and tissue type will be discussed. When necessary, separation of treatment means was accomplished using least squares means and Fisher’s protected LSD (P ≤ 0.05).

Results and Discussion

Immunoblotting confirmed the presence of two distinct UT-B protein bands (32 kDa and 47 kDa) in all nine tissues analyzed. Despite their presence in all tissues analyzed, the visual intensity of these UT-B bands differed among
tissues. A broad 32 kDa band and a slight 47 kDa band were detected in the dorsal rumen, ventral rumen (Figure 1), reticulum and liver. The expression of UT-B within the liver is significant, as the bulk of recycled urea is derived from ureagenesis within the liver. Conversely, the cecum, large colon, and small (spiral) colon displayed a slight 32 kDa band, and a more intense band at 47 kDa. Interestingly, the parotid salivary gland (Figure 2) displayed an intense protein band at 47 kDa, and a much lighter band at 32 kDa. This is the first known study to have identified UT-B within the parotid salivary gland of any species. Because the parotid salivary gland plays a major role in the recycling of blood urea back to the rumen via saliva, particularly in ruminants consuming forage-based diets (Kennedy and Milligan, 1980), the identification of UT-B within the parotid salivary gland may prove to be beneficial in improving our understanding of N recycling via the salivary route. Both protein bands were apparent in the kidney at nearly the same visual intensity (Figure 3).

Marini and Van Amburgh (2003) reported a broad UT-B protein band in rumen papillae of dairy heifers, and later (Marini et al., 2004) in the duodenum, ileum, and cecum of lambs that is similar in size to our 47 kDa UT-B protein. Likewise, Stewart et al (2005) verified the presence of a 43-54 kDa UT-B protein band in the bovine rumen. However, we believe that the two distinct protein bands detected by our anti-UT-B antibody represent N-glycosylated (47 kDa) and non-glycosylated (32 kDa) forms of the UT-B protein. Lucien et al. (2002) predicted that the structure of human UT-B1 contained a single unique functional N-glycosylation site, which is consistent with the presence of two distinct UT-B bands as observed in the current study and that of others. Timmer et al. (2001) reported the presence of a broad UT-B protein band between 45 and 65 kDa in human erythrocytes, and between 37 and 51 kDa in rat erythrocytes. However, these broad bands were converted to a distinct tight band at 32 kDa following deglycosylation of those tissues. Similarly, Olives et al. (1995) confirmed the presence of a diffuse 46-60 kDa UT-B band present in human erythrocytes that, when deglycosylated, was reduced to 36 kDa.

Contrary to our initial hypothesis that UT-B abundance would be greatest for animals on the low protein diet, no treatment differences in the abundance (arbitrary densitometry units) of the 32 kDa (P ≥ 0.15) or 47 kDa (P ≥ 0.51) UT-B protein bands (Table 2) were detected. Similarly, Marini et al. (2004) reported no differences in UT-B abundance in any of the gastrointestinal tissues analyzed in response to increasing dietary N. In contrast, Marini and Van Amburgh (2003) observed greater expression of UT-B (based upon visual evaluation) in the ruminal mucosa of dairy heifers fed a high N diet (2.97 – 3.4% N) as compared to a low N diet (1.45 – 1.89 % N).

While increasing CP did not influence (P ≥ 0.38) the 32 kDa/47 kDa ratio within a given tissue, the 32 kDa/47 kDa ratio differed (P = 0.05) across tissues, being greatest for the ventral rumen (92.5), liver (79.7), and reticulum (27.7), intermediate for the dorsal rumen (8.0), and lowest for the kidney (1.77), large colon (0.83), spiral colon (0.78), cecum (0.57), and parotid salivary gland (0.20). Differences in the 32 kDa/47 kDa ratio among tissues, reflecting relative differences in the degree of N-glycosylation of UT-B, may suggest a possible role of N-glycosylation in the regulation of UT-B function in the ruminant. The importance of N-glycosylation to the function of UT-B remains largely unexplained. In other systems, N-glycosylation often determines membrane expression level and addressing of polypeptides to the membrane (Varki et al., 2002). However, site-directed mutagenesis to delete the N-glycosylation site of human UT-B expressed in Xenopus oocytes demonstrated that the lack of N-glycosylation did not affect urea uptake when compared to the wild-type UT-B (Lucien et al., 2002). The regulatory pathways affecting UT-B function in Xenopus oocytes may differ from that of ruminants, and thus further research into the physiological role of N-glycosylation on UT-B function is needed.

Implications

Both N-glycosylated and non-glycosylated forms of urea transporter-B (UT-B) are expressed in the gastrointestinal tract, kidney, liver, and parotid salivary gland of lambs fed a forage-based diet. Although dietary protein level had no effect on abundance of either form of UT-B, the relative abundance of the N-glycosylated form of UT-B differed across tissues. Further research into the physiological significance of N-glycosylation in the regulation of UT-B function is needed.

References

Kennedy, P. M., and L. P. Milligan. 1980. The degradation and utilization of endogenous urea in the

Figure 1. Western blot of samples from the ventral rumen

Figure 2. Western blot of samples from the parotid salivary gland.

Figure 3. Western blot of samples from the kidney.


<table>
<thead>
<tr>
<th>Item</th>
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<tbody>
<tr>
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<td>Med</td>
<td>High</td>
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<tr>
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<td>7.681</td>
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<td>47 kDa</td>
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<tr>
<td>47 kDa</td>
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<tr>
<td>32 kDa</td>
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<tr>
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<tr>
<td>47 kDa</td>
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<td>32 kDa</td>
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<tr>
<td>47 kDa</td>
<td>0.467</td>
<td>0.431</td>
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<td><strong>Cecum</strong></td>
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<tr>
<td>32 kDa</td>
<td>0.088</td>
<td>0.072</td>
<td>0.209</td>
</tr>
<tr>
<td>47 kDa</td>
<td>0.231</td>
<td>0.350</td>
<td>0.515</td>
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<tr>
<td><strong>Small (Spiral) Colon</strong></td>
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<td>32 kDa</td>
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<td>0.332</td>
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</tr>
<tr>
<td>47 kDa</td>
<td>0.345</td>
<td>0.316</td>
<td>0.568</td>
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1Treatments consisted of 6% (Low), 9% (Med), and 12% (High) dietary CP (% of DM).

2P-value of differences between treatments.
Summary and Implications

Four wether lambs (100.3 ± 7.5 lb. body weight) fitted with ruminal, duodenal, and ileal canulas were used in a 4 x 4 Latin square experiment to determine effects of safflower seed processing on site and extent of fatty acid digestion. Isonitrogenous diets were 33% ground (1 in.) hay, 67% concentrate (Control), with safflower lipid replacing enough of the concentrate to provide 3% added fat from either high-linoleate whole or cracked safflower seeds or oil extracted from the seeds. Orthogonal contrasts included Control vs. fat-supplemented diets, and linear and quadratic effects of degree of safflower seed processing on site and extent of fatty acid digestion. Isonitrogenous diets were 33% ground (1 in.) safflower seed processed to different degrees. Our hypothesis was that whole safflower seeds will provide partial protection from ruminal biohydrogenation of fatty acids, but may also reduce digestibility in the small intestine. Our objective was to determine site and extent of digestion of safflower fatty acids fed as whole or cracked seeds, or top-dressed oil. We conclude that duodenal flow of 18:2 of 16:0 (P = 0.08) was the lowest of all sites. Quadratic flow of 18:0 (P = 0.02) was greatest in Control vs. fat-supplemented diets, and flow of biohydrogenation intermediates 18:1 (Control, P = 0.07), 18:1 (Control, P = 0.03), and 18:1 (Control, P = 0.11) were least for Control lambs. Duodenal flow of 18:0 and 18:2 was greatest (P = 0.03) for Control, but flow of biohydrogenation intermediates 18:1 (P = 0.08), 18:1 (P = 0.07), 18:1 (P = 0.02), 18:1 (P = 0.02), 18:1 (P = 0.03), and 18:1 (P = 0.11) were least for Control lambs. Duodenal flow of 18:0 in Control lambs was nearly half (P < 0.001) that of lambs supplemented fat. Quadratic responses for duodenal flow of 18:2 (P = 0.01), 18:2 (P = 0.07) and 18:2 (P = 0.09) were due to greater flow of those fatty acids in lambs fed oil. Apparent small intestinal disappearance (g/d entering the duodenum) of 16:0 (P = 0.02), 18:0 (P = 0.002), 18:2 (P = 0.01), and 18:2 (P = 0.09) were greater for fat-supplemented lambs than Control. Percentage of 16:0 (P = 0.05) and 18:0 (P = 0.01) disappearing from the small intestine demonstrated a quadratic response because lambs fed oil had the lowest disappearance values for those fatty acids. Percentage of 18:2 digested in the small intestine, however, was greater (quadratic, P = 0.08) in lambs fed oil. We conclude that duodenal flow of biohydrogenation intermediates increased when lambs were fed fatty acids from safflower seeds. Supplementation fatty acids in the form of extracted oil seems to be the most effective strategy to increase status of linoleic acid and biohydrogenation intermediates in lambs fed diets containing 3% added fat.

Materials and Methods

General

All procedures for the following experiment were approved by the University of Wyoming Animal Care and Use Committee. Four wether lambs (100.3 ± 7.5 lb. body weight) were fitted with ruminal, duodenal, and ileal canulas. Lambs were placed in metabolism crates (4.6 × 2 ft.) in a climate controlled room under continuous lighting where they had free access to water.
Following the design of a 4 x 4 Latin square experiment, lambs were assigned to 1 of 4 dietary treatments with 13-d collection periods. The Control diet contained no supplemental fat and consisted of 33% ground (1.0 in.) hay and 67% concentrate (as-fed basis; Table 1). Safflower fatty acids from either high-linoleate whole (76.2% 18:2c9c12) or cracked (76.8% 18:2c9c12) safflower seeds or oil (76.8% 18:2c9c12) extracted from the seeds were supplemented to the Control diet at the expense of the safflower seed meal so that fatty acid-supplemented diets contained 3% added fat. All dietary treatments were formulated to be isonitrogenous (actual dietary Crude Protein (CP) = 13.3% of Dry Matter (DM)) and to meet the requirements of a 100 lb. growing lamb gaining 0.66 lb/day (NRC, 1985). Lambs were offered their daily ration at 0530 and 1730. As an external marker of digesta flow, 2.5 g (NRC, 1985). Lambs were offered their daily ration at 0530 and 1730. As an external marker of digesta flow, 2.5 g of TiO2 was dosed intraruminally immediately before each feeding (Myers et al., 2006). Each 10-d adaptation period was followed by 3 days of duodenal and ileal sampling. Sampling began at 0500 on day 11 of each period with the collection of 150 mL of duodenal and ileal digesta repeated every 6 hours. Collection times were advanced by 2 hours on both day 12 and 13 so that digesta was collected every other hour of a theoretical 24-hour clock.

### Laboratory Analysis

Beginning 2 day before and continuing through the 3-day collection of digesta, samples of all feedstuffs were taken on a daily basis for laboratory analysis. Feed refusals were collected 2 day before and throughout the sampling period. Refusals were weighed back and collected for laboratory analysis. Feed and refusal samples were analyzed for DM (AOAC, 1990) and fatty acid content (Whitney et al., 1999 as modified by Kucuk et al., 2001) to determine dry matter intake (DMI) and fatty acid intake (Table 2). Duodenal and ileal samples were frozen at -20° C, lyophilized (Genesis 25 freeze dryer, The VirTis Co., Gardiner, NY), and composited within lamb for each collection period for analysis of TiO2 (Myers et al., 2004) and fatty acids (Kucuk et al., 2001).

### Calculations and Statistical Analysis

### Duodenal Flow of Fatty Acids

Unlike previous experiments published by our laboratory in which heifers were fed a high-forage diet plus cracked high-linoleate safflower seeds (Scholljegerdes et al., 2004) or lambs were fed a high-concentrate diet plus safflower oil (Atkinson et al., 2006), Control lambs in the present experiment had greater (P = 0.03) duodenal flow of 18:1c9 (Table 2). Because intake of 18:1c9 was least for Control lambs, greater flow of 18:1c9 may be attributable to less ruminal biohydrogenation in lambs fed the Control diet. Scholljegerdes et al. (2004) noted that ruminal biohydrogenation of C18 unsaturated fatty acids in heifers fed a no added fat control supplement was 87% of that for heifers fed high-linoleate safflower seeds formulated to provide 3% added dietary fat. Support for greater biohydrogenation in lambs fed safflower lipids is provided by greater (P < 0.001) duodenal flow of 18:0, as this fatty acid is the product of complete biohydrogenation (Jenkins, 1993). Consistent with increased duodenal supply of ruminal biohydrogenation intermediates in heifers (Scholljegerdes et al., 2004) or lambs (Atkinson et al., 2006) fed high-linoleate safflower lipids to provide 3% added dietary fat, we observed greater flow of the biohydrogenation intermediates 18:1c9 (P = 0.08), 18:1r11 (P = 0.07), 18:1r12 (P = 0.02), 18:1r13 (P = 0.02), 18:1c11 (P = 0.03), and 18:1c12 (P = 0.11) in lambs fed fat.

Among the fat-supplemented lambs, duodenal flow of 18:1c9 increased linearly (P = 0.04) as safflower seeds were processed more extensively. Quadratic responses were also noted for 18:2c9r11 (P = 0.01), 18:2r10c12 (P = 0.07), and 18:2c9c12 (P = 0.009) flowing to the duodenum because of greater flow of these fatty acids in lambs fed oil. Greater flow of biohydrogenation intermediates in lambs fed oil may be due to accumulation of lipid in the rumen, which would reduce the extent of biohydrogenation (O’Kelly and Spiers, 1991). Triacylglycerol (TAG) from oil would presumably be more available than TAG from the other two sources. Lipolysis of TAG in the rumen of lambs fed oil should occur within 2 hours (Batmen and Jenkins, 1998), whereas lipolysis of TAG in the seed would be delayed because the TAG would not be as readily accessible for ruminal enzyme attack as the TAG in oil. Subsequently,
free 18:2c9c12 would be liberated more rapidly in the rumen of lambs fed oil. Accumulation of free 18:2c9c12 inhibits complete biohydrogenation (Noble et al., 1974) because of inhibition of bacterial growth (Harfoot et al., 1973) and inhibition of bacterial hydrogenases (Grinari and Bauman, 1999).

**Intestinal Disappearance**

Apparent small intestinal disappearance of 16:0 ($P = 0.02$), 18:0 ($P = 0.002$), 18:1n9 ($P = 0.07$), 18:1n11 ($P = 0.07$), 18:2c9c12 ($P = 0.008$), and 18:2r10c12 ($P = 0.09$) were greater for the diets supplemented with safflower fatty acids (Table 3). An increase in absorption of these fatty acids is expected to increase concentration in bodily tissues (Bolte et al., 2002) and milk (Lake et al., 2007) of ruminant animals fed these lipid sources.

Among the fat supplemented lambs there was a linear increase for 18:1r10 ($P = 0.03$) and 18:1n9 ($P = 0.05$), and a quadratic response for apparent small intestinal disappearance of 18:2c9c11 ($P = 0.005$) and 18:2r10c12 ($P = 0.06$) primarily because of an increase in oil-fed lambs.

Expressed as a percentage of fatty acids entering the duodenum, a quadratic response was noted for 16:0 ($P = 0.05$) because of an increase for lambs fed cracked seeds (Table 3). A quadratic response for intestinal disappearance was noted for 18:0 ($P = 0.01$) because the oil treatment had the lowest digestibility coefficient for this fatty acid. A linear increase ($P = 0.08$), however, was noted for percentage of 18:2c9c12 digested in the small intestine because of greater digestion of this fatty acid by lambs fed oil. Our observations were consistent with Kucuk et al. (2004) who reported that intestinal digestibility of 18:2c9c12 was greater than intestinal digestibility of 18:0 in lambs that had an increase in 18-carbon poly-unsaturated fatty acids (PUFA) entering the small intestine.

Contrary to the original hypothesis, feeding lambs whole seeds did not protect safflower fatty acids from ruminal biohydrogenation. Furthermore, amount of total fatty acids disappearing from the small intestine was not affected ($P \geq 0.48$) by form of supplemental safflower lipid (Table 3). Supplementing fatty acids in the form of oil extracted from high-linoleate safflower seeds enhanced intestinal digestibility of 18:2c9c12, but decreased intestinal digestibility of 18:0. Supplementing fatty acids in the form of oil seems to be the most effective strategy to increase status of 18:1c9 and 18-carbon PUFA in lambs fed diets containing 3% added fat.

Total energy available from fatty acids digested in the small intestine would be comparable regardless of how safflower seeds were processed. Nevertheless, producers are more likely to change composition of food products derived from ruminants fed oil vs. whole or cracked seeds when diets are supplemented to provide 3% added fat.

**References**


Table 2. Dry matter intake (DMI) (grams/day (g/d)), fatty acid intake (g/d), and duodenal flow of fatty acid (g/d) in lambs fed a basal diet (Con) or diets with safflower fat in the form of whole (Wh) or cracked (Cr) seeds or oil extracted from the seeds

<table>
<thead>
<tr>
<th>Item</th>
<th>Dietary treatment</th>
<th>Con vs. Fat-suppl</th>
<th>Con</th>
<th>Wh</th>
<th>Cr</th>
<th>Oil</th>
<th>SEM</th>
<th>L</th>
<th>Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMI, g/d</td>
<td></td>
<td></td>
<td>1163.7</td>
<td>1083.2</td>
<td>1134.4</td>
<td>1162.6</td>
<td>50.4</td>
<td>0.55</td>
<td>0.50</td>
</tr>
<tr>
<td>16:0 fatty acid intake, g/d</td>
<td></td>
<td></td>
<td>3.80</td>
<td>5.39</td>
<td>4.80</td>
<td>5.02</td>
<td>0.36</td>
<td>0.02</td>
<td>0.48</td>
</tr>
<tr>
<td>18:1n9 fatty acid intake, g/d</td>
<td></td>
<td></td>
<td>0.13</td>
<td>0.24</td>
<td>0.10</td>
<td>0.29</td>
<td>0.04</td>
<td>0.07</td>
<td>0.48</td>
</tr>
<tr>
<td>18:1n10 fatty acid intake, g/d</td>
<td></td>
<td></td>
<td>0.08</td>
<td>0.12</td>
<td>0.10</td>
<td>0.91</td>
<td>0.21</td>
<td>0.26</td>
<td>0.03</td>
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<tr>
<td>18:1n11 fatty acid intake, g/d</td>
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<td>0.11</td>
<td>0.26</td>
<td>0.25</td>
<td>0.28</td>
<td>0.04</td>
<td>0.02</td>
<td>0.99</td>
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<tr>
<td>18:1n12 fatty acid intake, g/d</td>
<td></td>
<td></td>
<td>0.26</td>
<td>0.66</td>
<td>0.59</td>
<td>0.59</td>
<td>0.10</td>
<td>0.02</td>
<td>0.62</td>
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<tr>
<td>18:1n13 fatty acid intake, g/d</td>
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<td>1.98</td>
<td>1.31</td>
<td>1.29</td>
<td>1.98</td>
<td>0.19</td>
<td>0.08</td>
<td>0.05</td>
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<tr>
<td>18:1n14 fatty acid intake, g/d</td>
<td></td>
<td></td>
<td>0.21</td>
<td>0.35</td>
<td>0.37</td>
<td>0.36</td>
<td>0.05</td>
<td>0.03</td>
<td>0.91</td>
</tr>
<tr>
<td>18:1n15 fatty acid intake, g/d</td>
<td></td>
<td></td>
<td>0.05</td>
<td>0.33</td>
<td>0.18</td>
<td>0.11</td>
<td>0.08</td>
<td>0.13</td>
<td>0.09</td>
</tr>
<tr>
<td>18:2n9 fatty acid intake, g/d</td>
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<td></td>
<td>0.01</td>
<td>0.02</td>
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<td>0.04</td>
<td>0.002</td>
<td>0.008</td>
<td>0.002</td>
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<td>18:2n10 fatty acid intake, g/d</td>
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<tr>
<td>18:2n11 fatty acid intake, g/d</td>
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<td>2.25</td>
<td>1.91</td>
<td>2.93</td>
<td>0.29</td>
<td>0.02</td>
<td>0.15</td>
</tr>
<tr>
<td>18:2n12 fatty acid intake, g/d</td>
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<td></td>
<td>0.19</td>
<td>0.14</td>
<td>0.08</td>
<td>0.18</td>
<td>0.02</td>
<td>0.39</td>
<td>0.18</td>
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<tr>
<td>18:2n13 fatty acid intake, g/d</td>
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<td></td>
<td>28.36</td>
<td>47.32</td>
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<td>47.32</td>
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<tr>
<td>18:2n14 fatty acid intake, g/d</td>
<td></td>
<td></td>
<td>84.98</td>
<td>76.62</td>
<td>83.26</td>
<td>72.95</td>
<td>1.95</td>
<td>0.02</td>
<td>0.23</td>
</tr>
</tbody>
</table>

1Orthogonal contrasts included Control (Con) vs. Fat-supplemented (Fat-suppl) diets and linear (L) and quadratic (Q) effects of degree of safflower seed processing (whole, cracked, and oil).

2n = 4.

Table 3. Apparent disappearance from the small intestine (g/d) and digestibility coefficients (% entering the duodenum) in lambs fed a basal diet (Con) or diets with safflower fat in the form of whole (Wh) or cracked (Cr) seeds or oil extracted from the seeds

<table>
<thead>
<tr>
<th>Item</th>
<th>Dietary treatment</th>
<th>Con vs. Fat-suppl</th>
<th>Con</th>
<th>Wh</th>
<th>Cr</th>
<th>Oil</th>
<th>SEM</th>
<th>L</th>
<th>Q</th>
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</thead>
<tbody>
<tr>
<td>Intestinal disappearance, g/d</td>
<td></td>
<td></td>
<td>3.80</td>
<td>5.39</td>
<td>4.80</td>
<td>5.02</td>
<td>0.36</td>
<td>0.02</td>
<td>0.48</td>
</tr>
<tr>
<td>Intestinal digestibility coefficient, %</td>
<td></td>
<td></td>
<td>85.68</td>
<td>82.79</td>
<td>86.01</td>
<td>81.38</td>
<td>1.34</td>
<td>0.19</td>
<td>0.48</td>
</tr>
<tr>
<td>16:0</td>
<td></td>
<td></td>
<td>88.20</td>
<td>74.64</td>
<td>83.32</td>
<td>68.72</td>
<td>2.67</td>
<td>0.06</td>
<td>0.17</td>
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<tr>
<td>18:1n9</td>
<td></td>
<td></td>
<td>95.87</td>
<td>97.49</td>
<td>98.80</td>
<td>92.40</td>
<td>2.43</td>
<td>0.90</td>
<td>0.19</td>
</tr>
<tr>
<td>18:1n10</td>
<td></td>
<td></td>
<td>92.93</td>
<td>96.85</td>
<td>97.49</td>
<td>82.42</td>
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<td>91.52</td>
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<tr>
<td>18:1n12</td>
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<td></td>
<td>90.66</td>
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<tr>
<td>18:1n13</td>
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<td></td>
<td>94.69</td>
<td>88.43</td>
<td>92.00</td>
<td>86.97</td>
<td>1.52</td>
<td>0.02</td>
<td>0.52</td>
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<tr>
<td>18:1n14</td>
<td></td>
<td></td>
<td>73.17</td>
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<td>0.91</td>
<td>0.34</td>
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<td>86.09</td>
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<td>91.72</td>
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<td>0.81</td>
<td>0.001</td>
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<td></td>
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<td>100.00</td>
<td>99.44</td>
<td>100.00</td>
<td>73.85</td>
<td>7.92</td>
<td>0.37</td>
<td>0.06</td>
</tr>
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<td>57.78</td>
<td>67.01</td>
<td>63.00</td>
<td>80.64</td>
<td>5.13</td>
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<td>0.11</td>
</tr>
<tr>
<td>18:2n11</td>
<td></td>
<td></td>
<td>74.30</td>
<td>74.70</td>
<td>74.53</td>
<td>84.43</td>
<td>3.26</td>
<td>0.38</td>
<td>0.08</td>
</tr>
<tr>
<td>18:3n12</td>
<td></td>
<td></td>
<td>74.99</td>
<td>68.73</td>
<td>77.22</td>
<td>76.72</td>
<td>3.55</td>
<td>0.86</td>
<td>0.16</td>
</tr>
<tr>
<td>18:3n13</td>
<td></td>
<td></td>
<td>84.98</td>
<td>76.62</td>
<td>83.26</td>
<td>72.95</td>
<td>1.95</td>
<td>0.02</td>
<td>0.23</td>
</tr>
</tbody>
</table>

1Orthogonal contrasts included Control (Con) vs. Fat-supplemented (Fat-suppl) diets and linear (L) and quadratic (Q) effects of degree of safflower seed processing (whole, cracked, and oil).

2n = 4.

1Insufficient observations for statistical analysis.
Ruminant Nutrition

Supplementary Ruminally Undegradable Protein Containing Fishmeal for Cattle Fed Limited Amounts of Forage: Effects on Long-Chain Fatty Acid Flow to the Duodenum

Bret W. Hess, Associate Professor
Eric J. Scholljegerdes, Former Graduate Student
Charles M. Murrieta, Research Scientist
Daniel C. Rule, Professor
Department of Animal Science

Summary and Implications

Twelve Angus crossbred cattle (8 heifers and 4 steers; average initial BW = 1310 lbs) fitted with ruminal and duodenal cannulas and fed restricted amounts of forage plus a ruminally undegradable protein (RUP) supplement were used in a triplicated 4 × 4 Latin square design experiment to determine intestinal supply of long-chain fatty acids. Cattle were fed 4 different levels of chopped (1 in.) bromegrass hay (11.4% CP, 57% NDF; OM basis): 30, 55, 80, or 105% of the forage intake required for maintenance. Cattle fed below maintenance were given specified quantities of a RUP supplement (6.8% porcine blood meal, 24.5% hydrolyzed feather meal, and 68.7% menhaden fishmeal; DM basis) designed to provide duodenal essential AA flow equal to that of cattle fed forage at 105% of maintenance. Experimental periods lasted 19 d (17 d of adaptation and 2 d of sampling). Although fatty acid intake from hay increased linearly (P < 0.001) as cattle consumed more forage, total fatty acid intake increased (cubic, P < 0.001) as total OM intake decreased because fatty acid consumption increased (cubic, P < 0.001) as cattle consumed more supplement. As a result, total fatty acid flow to the duodenum increased linearly (P < 0.001) as intake of supplement increased. Duodenal flow of 14:1, 15:0, 15:1, and 18:0 increased linearly (P < 0.05) with increased forage consumption. A quadratic response (P < 0.05) was noted for duodenal flow of myristic, oleic, linoleic, and linolenic acids largely because duodenal flow of these fatty acids was least for cattle consuming forage at 105% of maintenance. The biohydrogenation intermediates 16:1c-t11, 18:1r11, 18:1r10, 18:1r12, and 18:1r13 responded to dietary treatment in a quadratic fashion (P < 0.09), but duodenal flow of CLA was not affected (P ≥ 0.149) by dietary treatment. We conclude that a supplement consisting of 6.8% porcine blood meal, 24.5% hydrolyzed feather meal, and 68.7% menhaden fishmeal (DM basis) can be fed to maintain or improve intestinal supply of fatty acids in cattle consuming limited amounts of forage.

Introduction

Studies evaluating nutritional effects on reproduction in ruminants often use restricted feed intake to limit the supply of dietary energy (Schillo, 1992). As an initial step toward separating physiological effects of protein from energy in ruminants, Scholljegerdes et al. (2005a) used an RUP supplement consisting of 6.8% porcine blood meal, 24.5% hydrolyzed feather meal, and 68.7% menhaden fishmeal (DM basis) to balance intestinal supply of essential AA in cattle consuming restricted quantities of forage. Menhaden fishmeal contains 10.5% crude fat (NRC, 1982) and 8% of DM as oil with relatively high concentrations of ≥ 20C PUFA (Mattos et al., 2002). Ashes et al. (1992) reported that in vitro ruminal biohydrogenation was reduced in the presence of PUFA from fish oil. Biohydrogenation rates determined in vitro, however, may be much less than the actual in vivo rates (Moate et al., 2004). Because ruminal disappearance of long-chain fatty acids is minimal (Jenkins, 1993), we hypothesized that the RUP supplement used by Scholljegerdes et al. (2005a) would increase intestinal supply of total fatty acids in cattle fed forage at levels below NRC (2000) recommendations for maintenance. Our objective was to determine long-chain fatty acid flow to the duodenum of beef cattle fed limited amounts of forage plus supplementary RUP from a combination of porcine blood meal, hydrolyzed feather meal, and menhaden fishmeal.

Materials and Methods

General

Twelve ruminally and duodenally cannulated Angus cross cattle were used in a triplicated 4 × 4 Latin square experiment (8 heifers in a duplicate 4 × 4 Latin square and 4 steers in a single 4 × 4 Latin square conducted simultaneously; average initial BW = 1310 lbs) in accordance with an approved University of Wyoming Animal Care and Use Committee protocol. Cattle were fed chopped (1 in.) bromegrass hay (9.4% ash, 11.4% CP, and 57% NDF on an OM basis) at 30, 55, 80, and 105% of the forage intake required for maintenance (NRC, 2000). Cattle fed below maintenance were given specified quantities of an RUP supplement (6.8% porcine blood meal, 24.5% hydrolyzed feather meal, and 68.7% menhaden fishmeal; DM basis) designed to provide duodenal essential AA flow equal to that of cattle fed forage at 105% of maintenance (Scholljegerdes et al., 2005a). Basal flows of total essential fatty acids in cattle consuming forage at 105% of maintenance equal to that of cattle fed forage at 105% of maintenance. Experimental periods lasted 19 d (17 d of adaptation and 2 d of sampling). Although fatty acid intake from hay increased linearly (P < 0.001) as cattle consumed more forage, total fatty acid intake increased (cubic, P < 0.001) as total OM intake decreased because fatty acid consumption increased (cubic, P < 0.001) as cattle consumed more supplement. As a result, total fatty acid flow to the duodenum increased linearly (P < 0.001) as intake of supplement increased. Duodenal flow of 14:1, 15:0, 15:1, and 18:0 increased linearly (P < 0.05) with increased forage consumption. A quadratic response (P < 0.05) was noted for duodenal flow of myristic, oleic, linoleic, and linolenic acids largely because duodenal flow of these fatty acids was least for cattle consuming forage at 105% of maintenance. The biohydrogenation intermediates 16:1c-t11, 18:1r11, 18:1r10, 18:1r12, and 18:1r13 responded to dietary treatment in a quadratic fashion (P < 0.09), but duodenal flow of CLA was not affected (P ≥ 0.149) by dietary treatment. We conclude that a supplement consisting of 6.8% porcine blood meal, 24.5% hydrolyzed feather meal, and 68.7% menhaden fishmeal (DM basis) can be fed to maintain or improve intestinal supply of fatty acids in cattle consuming limited amounts of forage.

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AA were predicted for each animal at each experimental forage intake level using the equation reported by Scholljegerdes et al. (2004b; total essential AA flow to the small intestine, g/d = [0.055 × g of OM intake] + 1.546). The quantity of RUP supplement delivered was adjusted for anticipated RUP values at 30, 55, and 80% intake as reported by Scholljegerdes et al. (2005b). Amount of feed delivered was provided in equal portions at 0600 and 1800 daily. Each period of the Latin square lasted 19 d, with 17 d for diet adaptation to allow for adjustment of the digestive system to forage intake level and RUP supplement. Cattle had ad libitum access to water and trace-mineral salt (Champions Choice; Akzo Nobel Salt Inc., Clarks Summit, PA; guaranteed analysis [% of DM]: NaCl, 95 to 99; Co, Cu, I, Mn, Zn, and Fe, <1%) until d 14 of each sampling period. On d 14 of each sampling period, to avoid any confounding effects of salt intake on water intake and fluid passage rate, trace mineral salt was no longer provided. Feed refusals were not observed from d 10 through 19 of the experimental periods.

Sampling

As a marker for digesta flow, boluses of 0.18 oz of Cr₂O₃ were dosed intraruminally at each feeding (total = 0.36 oz of Cr₂O₃/d) from d 8 to 19 of each sampling period. Duodenal samples were collected for 2 d after the adaptation period. To account for feed composition throughout the trial, feeds were sampled every day and composited within each period. Beginning at 0400 on d 18 of each sampling period, duodenal (7 oz) samples were taken every 4 h. On d 19, collection times were advanced 2 h, so that samples were collected to represent every 2-h segment of a 24-h period. Duodenal digesta samples were frozen immediately before being lyophilized (Genesis SQ 25 Super ES Freeze Dryer; The VirTis Co., Gardiner, NY), ground to pass a 1-mm screen, composited within animal, and then stored for subsequent analyses.

Laboratory Analyses

Feed and duodenal samples were analyzed for DM and ash (AOAC, 1990). Nitrogen content of feed was determined using a LECO FP-528 N analyzer (LECO Corp., Henderson, NV), and NDF content of feed was determined using an ANKOM 200 fiber analyzer (ANKOM Technology, Fairport, NY). Chromium concentration of duodenal digesta was determined (Hill and Anderson, 1958) by atomic absorption spectrophotometry (Model 210 VGP AA Spectrophotometer; Buck Scientific, Norwalk, CT) with an air-plus-acetylene flame. Feed (Table 1) and duodenal digesta samples were subjected to direct transesterification and analyzed for fatty acids as described by Scholljegerdes et al. (2004a) except H₂ replaced He as the carrier gas.

Calculations and Statistical Analyses

Flow of digesta was calculated by dividing the amount of Cr dosed by the concentration of Cr in the respective duodenal sample. Duodenal flow of fatty acids was calculated by multiplying the fatty acid concentration in duodenal digesta by duodenal digesta flow. Data were analyzed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) for a Latin square in a randomized complete block design experiment. The effect of gender was included in the model as a block, and animal was used as the random effect. Orthogonal polynomial contrasts were used to compare linear, quadratic, and cubic responses to level of forage intake (Steel and Torrie, 1980).

Results and Discussion

Total Fatty Acid Intake

As anticipated, total fatty acid intake from hay increased linearly (P < 0.001) as cattle consumed more forage (Table 2). Total fatty acid intake, however, increased (cubic, P < 0.001) as total OM intake decreased because fatty acid consumption increased (cubic, P < 0.001) as cattle were offered more supplement.

Fatty Acid Flow

Total fatty acid flow to the duodenum increased linearly (P < 0.001) as intake of supplement increased (Table 3). In a review of the literature on factors affecting lipid balance in the rumen, Jenkins (1993) concluded that flow of fatty acids to the duodenum is generally closely related to intake of dietary lipids. In the same review, the author determined that 8% of dietary lipids disappeared from the rumen and microbial lipid synthesis was 15 g/kg of lipid-free OM digested in the rumen. Using those estimates in addition to values for OM truly digested in the rumen and microbial lipid synthesis, Jenkins (1993; Moate et al., 2004). Lipid disappearance from the rumen also was more common for diets with added fat than for control diets in Jenkins (1993). The difference may be partially explained by reduced de novo synthesis as a result of enhanced uptake of exogenous lipid by microbial cells (Jenkins, 1993; Moate et al., 2004). Lipid disappearance from the rumen also was more common for diets with added fat than for control diets in the 15 studies reviewed by Jenkins (1993).

Duodenal flow of fatty acids was calculated by dividing the amount of fatty acids flowing to the duodenum by duodenal digesta flow. Data were analyzed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) for a Latin square in a randomized complete block design experiment. The effect of gender was included in the model as a block, and animal was used as the random effect. Orthogonal polynomial contrasts were used to compare linear, quadratic, and cubic responses to level of forage intake (Steel and Torrie, 1980).
flow of 14:0 as forage intake decreased was also expected. In agreement with our results, Klusmeyer and Clark (1991) observed less flow of 18:0 to the duodenum in dairy cows fed fishmeal. Those authors also reported that ruminal biohydrogenation of C18 unsaturated fatty acids was reduced in cows fed fishmeal. Stearic acid is the final product of ruminal biohydrogenation of C18 unsaturated fatty acids (Jenkins, 1993). Therefore, the decrease in duodenal flow of 18:0 for cattle consuming supplement in the present study may be attributable to less extensive ruminal biohydrogenation of C18 unsaturated fatty acids.

Further support for less extensive biohydrogenation of C16 and C18 unsaturated fatty acids in cattle fed supplement is provided by the quadratic response \( (P < 0.05) \) for duodenal flow of 16:1c9, 18:1c9, 18:2n-6, and 18:3n-3 (Table 3). Intake of 18:3n-3 was slightly greater whereas intake of 18:2n-6 was nearly twice as great for cattle fed forage at 105 vs. 30% of maintenance. Intake of 16:1c9 and 18:1c9 was greater in cattle fed supplement. Biohydrogenation of C16 and C18 MUFA occurs at a much slower rate than biohydrogenation of C18 PUFA (Moate et al., 2004). Thus, greater flow of 16:1c9 and 18:1c9 for cattle fed supplement was to be expected. Biohydrogenation of dietary 16:1c9 may have also contributed to the previously mentioned increase in duodenal flow of 16:0 in cattle fed supplement; however, this process was not going to completion as evidenced by the increase in duodenal flow of 16:1c9 \( (linear, \ P = 0.045) \) and 16:1c11 \( (quadratic, \ P = 0.01) \). Quadratic \( (P < 0.085) \) responses to dietary treatment for duodenal flow of the biohydrogenation intermediates 18:1r11, 18:1r10, 18:1r12, and 18:1r13 again supports the notion that ruminal biohydrogenation of C18 unsaturated fatty acids was less complete in cattle fed supplement. Duodenal flow of 18:2c9r11 CLA, however, was not affected \( (P \geq 0.149) \) by dietary treatment. It has been proposed that the longer chain PUFA from fish oil inhibit complete ruminal biohydrogenation of 18:2n-6 by inhibiting growth of bacteria responsible for hydrogenating 18:1r11 or through inhibition of their hydrogenases (Griinari and Bauman, 1999). In their review of the literature, Khanal and Olson (2004) noted that fish oil supplementation increases concentration of milk fat CLA. Those authors also concluded that the highest concentration of milk fat CLA with fish oil supplementation was achieved when it was included at 2% of the diet DM. Fatty acids from supplement in the current experiment were included at 1.7% of OM for the treatment in which hay was fed at 55% of maintenance. The quadratic response \( (P = 0.068) \) for flow of 18:1r11 occurred because of the increase observed in cattle fed forage at 55% of maintenance. Our results are consistent with the suggestion that, because intestinal supply of 18:1r11 far exceeds that of 18:2c9r11 CLA, 18:1r11 serves as a precursor for conversion to 18:2c9r11 CLA via \( \Delta-9 \) desaturase in mammary tissue (Bauman et al., 2003). Our laboratory documented that milk of beef cows increased from 0.3 to 0.97 g of 18:2c9r11 CLA/100 g of freeze-dried milk (Lake et al., 2007) by feeding cracked high-linoleate safflower seeds. In a companion study in which beef cattle were fed the same diets as those fed by Lake et al. (2007), Scholljegerdes et al. (2004a) reported that duodenal flow of 18:2c9r11 CLA was only increased from 0.2 to 0.3 g/d whereas duodenal flow of 18:1r11 increased from 12.7 to 72.4 g/d in cattle fed cracked high-linoleate safflower seeds. Fievez et al. (2003) illustrated that altered milk fat 18:2c9r11 CLA content was mainly dependent on supply of 18:1r11 and to a lesser extent on the activity of \( \Delta-9 \) desaturase.

As opposed to evidence for less extensive biohydrogenation of C18 unsaturated fatty acids, biohydrogenation of \( \geq 20-C \) PUFA seemed quite extensive for cattle fed supplement. Slightly greater flow of 20:5n-3 and 22:5n-3 to the duodenum in cattle fed forage at 80% of maintenance requirements coupled with a steady decline for the 55 and 30% of maintenance treatments resulted in detection of quadratic responses \( (P = 0.079 \) and 0.102, respectively) to dietary treatment (Table 3). These responses occurred despite increased intake of \( \geq 20-C \) PUFA as forage intake decreased and supplement intake increased. Our results were not consistent with observations from in vitro experiments conducted by Ashes et al. (1992) and Gulati et al. (1999) in which \( \geq 20-C \) PUFA from fish oil were not hydrogenated to any significant extent. Literature reports on in vivo experiments indicate that ruminal biohydrogenation of 20:5n-3 ranged from 76 (Wachira et al., 2000) to 92.6% (Chikunya et al., 2004) in sheep and 89.8 to 92.4% (Scollan et al., 2001) in steers fed fish oil. Ruminal biohydrogenation of 22:6n-3 ranged from 72.2 (Wachira et al., 2000) to 91.5% (Chikunya et al., 2004) in sheep and 86.6 to 90.4% (Scollan et al., 2000) in steers fed fish oil. Duodenal flow of 20:5n-3 was approximately 11% of 20:5n-3 intake in cattle fed the greatest quantity of supplement in the present experiment. The inability to detect 22:6n-3 in duodenal contents suggests that this fatty acid was completely hydrogenated in the rumen. This latter response may explain why Burns et al. (2003) did not detect an increase in endometrial content of 22:6n-3 in beef cows fed fishmeal.

**Implications**

Supplementation with a combination of 6.8% porcine blood meal, 24.5% hydrolyzed feather meal, and 68.7% menhaden fishmeal (DM basis) should improve fatty acid status when beef cattle consume limited amounts of forage.

**Literature Cited**


Table 1. Concentration of predominant long-chain fatty acids in feed ingredients offered to beef cattle consuming restricted amounts forage plus supplemental ruminally undegradable protein

<table>
<thead>
<tr>
<th>Fatty acid, mg/g</th>
<th>Bromegrass hay</th>
<th>Menhaden fishmeal</th>
<th>Hydrolyzed feather meal</th>
<th>Porcine blood meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0</td>
<td>2.27</td>
<td>20.38</td>
<td>9.74</td>
<td>1.94</td>
</tr>
<tr>
<td>C18:0</td>
<td>0.35</td>
<td>4.28</td>
<td>4.67</td>
<td>0.93</td>
</tr>
<tr>
<td>C18:1n-9</td>
<td>0.80</td>
<td>5.69</td>
<td>11.13</td>
<td>2.02</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>1.55</td>
<td>1.12</td>
<td>5.29</td>
<td>1.78</td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>0.43</td>
<td>1.08</td>
<td>0.23</td>
<td>-</td>
</tr>
<tr>
<td>C20:5n-3</td>
<td>-</td>
<td>9.43</td>
<td>0.36</td>
<td>-</td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>-</td>
<td>9.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>6.01</td>
<td>84.00</td>
<td>37.64</td>
<td>6.26</td>
</tr>
</tbody>
</table>

Table 2. Intake by beef cattle consuming restricted amounts of bromegrass hay plus supplemental ruminally undegradable protein

<table>
<thead>
<tr>
<th>Intake</th>
<th>Forage intake level, % of maintenance</th>
<th>SEM(^3)</th>
<th>Contrast P-value(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>55</td>
<td>80</td>
</tr>
<tr>
<td>OM, lb/d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forage</td>
<td>7.0</td>
<td>12.8</td>
<td>18.7</td>
</tr>
<tr>
<td>Supplement</td>
<td>5.6</td>
<td>3.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Total</td>
<td>12.6</td>
<td>16.6</td>
<td>20.4</td>
</tr>
<tr>
<td>Fatty acids, lb/d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forage</td>
<td>0.05</td>
<td>0.08</td>
<td>0.12</td>
</tr>
<tr>
<td>Supplement</td>
<td>0.46</td>
<td>0.27</td>
<td>0.14</td>
</tr>
<tr>
<td>Total</td>
<td>0.51</td>
<td>0.35</td>
<td>0.26</td>
</tr>
</tbody>
</table>

\(^1\)Cattle were fed 30, 55, 80, or 105% of the forage intake required for maintenance (NRC, 2000) plus a rumen undegradable protein supplement comprised of 6.8% porcine blood meal, 24.5% hydrolyzed feather meal, and 68.7% menhaden fishmeal (DM basis).

\(^2\)Orthogonal contrasts included linear (L), quadratic (Q), and cubic (C) effects among forage intake levels.

\(^3\)n = 12.
Table 3. Long-chain fatty acid flow to the duodenum of beef cattle consuming restricted amounts of bromegrass hay and supplemental ruminally undegradable protein containing fishmeal\(^1\)

<table>
<thead>
<tr>
<th>Fatty acid, g/d</th>
<th>Forage intake level, % of maintenance</th>
<th>SEM(^3)</th>
<th>Contrast P-value(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>55</td>
<td>80</td>
</tr>
<tr>
<td>C14:0</td>
<td>13.7</td>
<td>7.8</td>
<td>5.9</td>
</tr>
<tr>
<td>C14:1</td>
<td>1.6</td>
<td>2.6</td>
<td>2.8</td>
</tr>
<tr>
<td>C15:0</td>
<td>3.5</td>
<td>4.5</td>
<td>5.6</td>
</tr>
<tr>
<td>C15:1</td>
<td>0.6</td>
<td>1.2</td>
<td>1.9</td>
</tr>
<tr>
<td>C16:0</td>
<td>78.7</td>
<td>59.8</td>
<td>50.0</td>
</tr>
<tr>
<td>C16:1t(_9)</td>
<td>1.8</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td>C16:1t(_9)</td>
<td>5.4</td>
<td>5.9</td>
<td>4.2</td>
</tr>
<tr>
<td>C16:1c(_9)</td>
<td>2.1</td>
<td>2.3</td>
<td>2.6</td>
</tr>
<tr>
<td>C16:0</td>
<td>43.8</td>
<td>46.7</td>
<td>58.9</td>
</tr>
<tr>
<td>C18:1t(_9)</td>
<td>1.2</td>
<td>1.6</td>
<td>2.2</td>
</tr>
<tr>
<td>C18:1t(_9)</td>
<td>1.1</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>C18:1t(_11)</td>
<td>7.9</td>
<td>10.1</td>
<td>8.8</td>
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<td>C18:1t(_12)</td>
<td>1.6</td>
<td>2.0</td>
<td>1.5</td>
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<tr>
<td>C18:1t(_13)</td>
<td>3.6</td>
<td>4.5</td>
<td>2.4</td>
</tr>
<tr>
<td>C18:1c(_9)</td>
<td>7.6</td>
<td>8.0</td>
<td>7.4</td>
</tr>
<tr>
<td>C18:1c(_11)</td>
<td>2.0</td>
<td>2.2</td>
<td>1.9</td>
</tr>
<tr>
<td>C18:1c(_13)</td>
<td>0.5</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>C18:2c(_9)</td>
<td>1.6</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>5.2</td>
<td>6.4</td>
<td>6.0</td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>3.9</td>
<td>2.8</td>
<td>1.5</td>
</tr>
<tr>
<td>C20:5n-3</td>
<td>2.7</td>
<td>3.1</td>
<td>3.4</td>
</tr>
<tr>
<td>C22:5n-3</td>
<td>1.0</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Unidentified</td>
<td>30.4</td>
<td>22.0</td>
<td>20.1</td>
</tr>
<tr>
<td>Total</td>
<td>221.3</td>
<td>200.0</td>
<td>193.3</td>
</tr>
</tbody>
</table>

\(^1\)Cattle were fed either 30, 55, 80, or 105% of the forage intake required for maintenance (NRC, 2000) plus 2,556, 1,491, 762, or 0 g/d, respectively, of a high ruminally undegradable protein supplement that contained 6.8% porcine blood meal, 24.5% hydrolyzed feather meal, and 68.7% menhaden fish meal (DM basis).

\(^2\)Orthogonal contrasts included linear (L), quadratic (Q), and cubic (C) effects among forage intake levels.
Ruminant Nutrition

**Supplementary Ruminally Undegradable Protein for Beef Cows Fed Limited Amounts of Forage From Early Through Mid- Gestation: Effects on Beef Cow Performance**

Price L. Platt, Graduate Research Assistant  
Min Du, Assistant Professor  
Steve I. Paisley, Assistant Professor  
Venerand Nayigihugu, Research Scientist, Assistant  
Jennifer D. Hess, Lab Technician  
Bret W. Hess, Associate Professor  
Department of Animal Science

**Summary and Implications**

Twelve triparous and 24 diparous cows (1102 ± 16.8 lb. initial body weight) were individually fed native grass hay plus 1 of 3 supplements from day 45 through day 185 of gestation to evaluate effects of gestational dietary treatment on body weight (BW), body condition score (BCS), and ultrasonographic longissimus muscle (LM) area, fat within the LM, and fat depth over the 12th rib. Dietary treatments were native grass hay plus a soybean meal-based supplement formulated to achieve 1.12 lb/day of BW gain (C), 70% of net energy for maintenance (NE\text{m}) provided by C (NR), and 70% of NE\text{m} provided by C plus a ruminally undegradable protein (RUP) supplement (6.8% porcine blood meal, 24.5% hydrolyzed feather meal, and 68.7% menhaden fishmeal; dry matter (DM) basis) designed to provide duodenal essential amino acid (AA) flow equal to that of cattle fed C (NRP). Data were analyzed as a split-plot in a randomized complete block (parity) design. Dietary treatment x day of gestation interactions were noted (P < 0.05) for all variables except for fat depth over the 12th rib. Cows fed C had significantly greater BW, BCS, and LM area than NR from day 73 through 185 (final BW = 1287 vs. 1149 lb). Body weight and LM area of NRP cows were intermediate until day 115; NRP cows had significantly greater BW and LM area than NR cows thereafter, but BW and LM area of NRP cows did not differ from C cows throughout the experiment. Body condition score of NRP cows was similar to both C and NR throughout the experiment. Fat within the LM was greatest for C on day 157; fat within the LM was similar among dietary treatments at all other collection dates. Average dry matter intake (DMI) by NR was 6.8 lb/day less than C cows, and NRP cows consumed 4.4 lb/day less DM than cows fed C. Differences in beef cow performance during early to mid-gestation were attributable to differences in plane of nutrition; however, cows fed a RUP supplement designed to balance intestinal supply of essential AA were able to withstand this period of nutrient restriction. Provision of supplemental protein balanced for intestinal supply of essential AA may be an effective nutritional management strategy to increase production efficiency of pregnant beef cows consuming limited amounts of forage.

**Introduction**

Drought is a recurring meteorological phenomenon in Wyoming and surrounding areas (NWS, 1988-1989; USGS, 2004), which results in arid and semi-arid conditions, causing a significant reduction in rangeland forage production (Derner et al., 2007). Furthermore, occurrence of precipitation early in the growing season followed by dry conditions can cause a rapid decline in forage quality by advancing phenological maturity of rangeland forage plants (Ganskopp and Bohnert, 2001). Spring-calving beef cows grazing rangelands affected by those conditions are likely to experience nutrient restriction during early to mid-gestation. Feed intake restriction is often practiced in experimental settings to emulate nutrient restriction that may occur in production settings (Dunn and Moss, 1992). As an initial step toward separating physiological effects of protein from energy in ruminants, Scholljegerdes et al. (2005a) used a ruminally undegradable protein (RUP) supplement consisting of 6.8% porcine blood meal, 24.5% hydrolyzed feather meal, and 68.7% menhaden fishmeal (DM basis) to balance intestinal supply of essential AA in cattle consuming restricted quantities of forage. However, production responses by pregnant beef cows fed restricted amounts of forage plus the aforementioned RUP supplement have not been determined. Our objectives were to evaluate effects of gestational plane of nutrition and RUP supplementation on BW, BCS, and ultrasonographic measurements of LM area, intramuscular fat within the LM, and fat depth over the 12\textsuperscript{th} rib.

**Materials and Methods**

**General**

The University of Wyoming Animal Care and Use Committee approved all procedures for the following study. All 3- and 4-yr old, lactating Angus x Gelbvieh cows from the University of Wyoming beef herd were estrous synchronized to be bred via AI on June 2, 2006. Cow-calf pairs were then allowed to graze a 4450 acre pasture on the University of Wyoming’s McGuire Ranch located...
approximately 35 miles northeast of Laramie at an elevation of 6,637 ft. Dominant native forage species within the pasture (Weston et al., 2005) include Sandberg’s bluegrass (Poa secunda), western wheatgrass (Pascopyrum smithii), and prairie junegrass (Koeleria pyramidata). On day 33 of gestation, cows were separated from their calves, then cows were evaluated for pregnancy via palpation per rectum by a licensed veterinarian using ultrasound technology. Calves were permanently separated from 50 of the cows (18 triparous and 32 diparous) that were diagnosed pregnant. Forty-two (18 triparous and 24 diparous) of the most uniform cows were then transported to the University of Wyoming Livestock Center located in Laramie, WY. Cows were pen-fed (6 cows/pen) native grass hay (6.2% crude protein (CP), DM basis) plus supplemental protein to provide a diet with 10% CP until initiation of experimental diets. Cows were reconfirmed pregnant by a licensed veterinarian on day 40 of gestation. On day 45 of gestation, 36 of most uniform cows (12 triparous and 24 diparous) were then selected to be individually fed native grass hay plus 1 of 3 supplements from day 45 through 185 of gestation.

**Dietary Treatments**

The control (C) diet consisted of native grass hay plus a soybean meal-based supplement formulated for pregnant replacement heifers (1,300 lb. mature BW) to achieve 0.95 lb/day of BW gain (NRC, 2000), which we estimated would be comparable to daily gain of 1.12 lb/day of BW gain for non-lactating cows pregnant with their second and third calf. The other dietary treatments were 70% of NE\textsubscript{m} provided by C (NR), and 70% of NE\textsubscript{m} provided by C plus a RUP supplement (6.8% porcine blood meal, 24.5% hydrolyzed feather meal, and 68.7% menhaden fish meal; DM basis; Scholljegerdes et al., 2005a) designed to provide duodenal essential AA flow equal to that of cattle fed C (NRP). Basal flows of total essential AA were predicted for each treatment using the equation reported by Scholljegerdes et al. (2004; total essential AA flow to the small intestine, g/d = [0.055 × g of OM intake] + 1.546). The quantity of RUP supplement delivered was adjusted for more extensive ruminal degradation of RUP supplement in cattle fed restricted amounts of forage (Scholljegerdes et al., 2005b). Using actual mineral content of each dietary ingredient, the C supplement was fortified to ensure that cows fed C would consume the same amount of mineral per unit BW as cows fed the RUP supplement.

The feeding protocol followed procedures outlined by Whitney et al. (2000) as modified by Lake et al. (2005). Briefly, supplement was offered daily at 0600 and 1600 in equal allotments. Supplement was always consumed within 20 min, after which one half of the daily hay allotment was offered. Cows were allowed to consume hay for the remainder of each 2-hour feeding period. Refusal of hay was seldom more than 2.2 lb. by a single cow at any given feeding time. Feed offered daily was adjusted for biweekly changes in BW and increased NE\textsubscript{m} requirements as gestation proceeded.

**Sampling and Laboratory Analyses**

Cow BW was the average of 2 consecutive-d BW recorded every 14 d from d 45 to 185 of gestation. Cow BCS was recorded every 28 d as the average of 3 trained technicians. Area of the LM, percentage i.m. fat within the LM, and fat covering the 12th rib were collected on d 45, 101, 157, and 185 of gestation via ultrasound using the “New” Aloka SSD-500 with a 17.2-cm transducer (Aloka Co., Ltd, Wallingford, CT). Images were collected on Beef Image Analysis (BIA) software (Designer Genes Technologies, L.L.C.).

Grades samples of supplement and hay were collected every 2 wk throughout the experiment. Feed samples were ground through a Wiley Mill (Thomas Hill and Sons, Philadelphia, PA) to pass a 1-mm screen. Ground samples were analyzed for DM (AOAC, 1990), N (LECO model FP-528 Nitrogen Determinator, LECO, St. Joseph, MO), NDF (ANKOM 200 fiber analyzer, ANKOM Technology Fairport, NY), and IVDMD (ANKOM Daisy\textsuperscript{11} Incubator, ANKOM Technology Fairport, NY). Diet composition is reported in Table 1.

<table>
<thead>
<tr>
<th>Table 1. Ingredient and diet composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient, % as fed</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>Native grass hay</td>
</tr>
<tr>
<td>Soybean meal</td>
</tr>
<tr>
<td>Molasses</td>
</tr>
<tr>
<td>DiCal</td>
</tr>
<tr>
<td>Limestone</td>
</tr>
<tr>
<td>Premix\textsuperscript{3}</td>
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<td>Fishmeal</td>
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<td>Feather meal</td>
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<table>
<thead>
<tr>
<th>Diet composition</th>
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<tbody>
<tr>
<td>DM, %</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>92.3</td>
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<td>CP, % of DM</td>
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<td>62.7</td>
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<tr>
<td>IVDMD, %</td>
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<td>47.2</td>
</tr>
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</table>

\textsuperscript{1}Dietary treatments consisted of native grass hay plus soybean meal-based supplement formulated to achieve 0.51 kg/d of BW gain (C), 70% of NE\textsubscript{m} provided by C (NR), and 70% of NE\textsubscript{m} provided by C plus a RUP supplement (6.8% porcine blood meal, 24.5% hydrolyzed feather meal, and 68.7% menhaden fish meal; DM basis). \textsuperscript{2}227g of 110,000 IU of vitamin A/kg, 27,500 IU of vitamin D/kg, and 660 IU of vitamin E/kg was added to 907 kg (as fed) of each protein supplement mixture. \textsuperscript{3}68.3% KCL, 27.6% FeSO\textsubscript{4}, 3.1 ZnO, 0.6% MnO, 0.4% CuSO\textsubscript{4}

\textsuperscript{1}Actual DMI from d 48 through 163 of gestation

**Statistical Analyses**
Data were analyzed as a split-plot in a randomized complete block (parity) design using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC). The MODEL statement included fixed effects of dietary treatment, parity, sampling period, in addition to all possible interactions. The random effect of cow within dietary treatment × block (specified in the RANDOM statement) accounted for the correlations among repeated observations on the same cow. Means for dietary treatment, sampling period, and the dietary treatment × sampling period interaction were separated using the LSMEANS option.

**Results and Discussion**

**Intake**

Dietary treatment x day of gestation interactions were noted ($P < 0.05$) for all variables except for fat depth over the 12\textsuperscript{th} rib (Table 2). Average DMI was 15.2, 17.6 and 21.2 lb/day during the first trimester (day 45 through 91) then was increased to 15.7, 18.1, and 22.9 lb/day from day 92 to 163 of gestation for NR, NRP, and C, respectively. Compared with C cows, DMI was 6.8 lb/day less for NR and 4.4 lb/day less for NRP from day 45 through 163 (Table 1). Greater DMI by NRP vs. NR cows was largely attributable to greater intake of RUP by NRP cows. Hay intake by NRP cows also was 3.9% greater than NR. Total feed offered was adjusted for bi-weekly BW change, and BW of NRP cows averaged 6.1% more than BW of NR over the course of the feeding period.

**Body Weight**

Cows fed C had significantly greater BW than cows fed NR from day 73 through 185. Body weight of cows fed NRP was intermediate until day 115 of gestation; NRP cows had significantly greater BW than NR cows thereafter, but BW of NRP cows did not differ from C cows throughout the experiment. Total BW change from day 45 to 101 of gestation by C and NR cows in the present experiment was comparable to values previously reported by our laboratory in which Miller et al. (2004) conducted a similar experiment with multiparous cows. Those authors reported that C cows gained 48.9 lb. from day 45 to 101 of gestation whereas NR cows lost 28.2 lb. over the same period. Although the magnitude of change in BW from day 45 to 101 of NR in the present experiment was 10.1 lb. more than that reported by Miller et al. (2004), due to much greater variance, change in BW for NR cows in the present experiment was not statistically significant indicating that the NR cows maintained BW. In summarizing 27 yr of data collected at the High Plains Grassland Research Station located in Cheyenne, WY, Garrelts (2006) noted that average BW gain by 3- and 4-yr old March-calving cows nursing calves while grazing rangelands from mid-June through mid-October was approximately 66.1 lb. during dry years. Some of the 3- and 4-yr old cows experienced minimal gain, and it was not uncommon for mature cows in the data set of Garrelts (2006) to experience BW loss during dry years. Cows that have not reached mature BW should be expected to support growth of their bodies in addition to growth and development of their fetuses (NRC, 2000). Despite NR cows in the present experiment not consuming adequate nutrient intake to accomplish growth, pregnancy was maintained because maternal and placental systems compensate for maternal undernourishment to provide the fetus with adequate nutrients (Bassett, 1986; 1991). Cows fed NRP on the other hand, began to gain BW between day 129 and 143 of gestation. The ability of NRP cows to achieve BW similar to C cows may be related, in part, to intake of digestible energy. Using in vitro dry matter digestibility (IVDMD) plus DMI values in Table 1, intake of digestible DM would be 7.3, 9.0, and 10.4 lb/day for NR, NRP, and C cows, respectively. Differences in dietary IVDMD are corroborated by in vivo estimates of total tract OM digestibility reported by Scholljegerdes et al. (2004; 2005a), although the magnitude of increased IVDMD for the NRP treatment was nearly twice that of differences in total tract organic matter digestibility (Scholljegerdes et al., 2005a). Adjusting dietary IVDMD of NRP to be of the same magnitude reported by Scholljegerdes et al. (2005a), however, would only decrease digestible dry matter intake by 0.2 lb/day. Hence, differences in beef cow performance during early to mid-gestation were attributable to differences in plane of nutrition. It is also possible that cows fed NRP were utilizing AA from the RUP to support or maintain protein deposition because cows fed NRP had similar BW as C cows throughout the experiment.

**Body Condition Score**

Similar to BW, cows fed C had significantly greater BCS than NR cows from day 73 through 185. Body condition score of NRP cows was similar to both C and NR throughout the experiment. Maintenance of BCS for NR was consistent with maintenance of BW throughout the experiment. Alternatively, cows fed C and NRP were expected to gain BCS according to relationships published in the NRC (2000). Changes in BW over the course of the experimental period included the weight of the gravid uterus (Ferrell et al., 1976; Prior and Laster, 1979). Thus, we concur with Miller et al. (2004) who suggested that apparent associations between changes in BW and BCS in pregnant are likely confounded by weight of the gravid uterus.

**Ultrasonic Measurements**

Cows fed C had significantly greater LM area than NR from day 101 through 185; LM area of cows fed NRP were intermediate on day 101 of gestation. Cows fed NRP had significantly greater LM area than NR cows on day 157 and 185 of gestation, but BW and LM area of NRP cows did not differ from C cows throughout the experiment. Differences in LM area among dietary treatments are consistent with differences in BW, which was in agreement with results of Miller et al. (2004). The ability of NRP cows to finish the experimental feeding period with an LM area similar to C cows provides further evidence for the suggestion that cows fed NRP were utilizing AA from the RUP to support protein deposition.
Intramuscular fat within the LM was greatest for C on day 157 of gestation; intramuscular fat within the LM was similar among dietary treatments at all other collection dates. Cows fed C began and finished the feeding period with greater fat thickness covering the 12th rib than NR cows. Similar to BCS, fat thickness over the 12th rib of NRP cows did not differ from either C or NR at d 185 of gestation. Maintenance of LM area and fat cover over the 12th rib for NR cows was consistent with maintenance of BW and BCS over the 140-day feeding period. Likewise, differences in fat cover over the 12th rib among cows with differing BCS in the present study were comparable to our previous reports (Miller et al., 2004; Lake et al., 2005).

Provision of supplemental protein balanced for intestinal supply of essential AA may be an effective nutritional management strategy to increase production efficiency of pregnant beef cows consuming limited amounts of forage.

References

Table 2. Effects of early to mid-gestation nutrient restriction and dietary supplementation with ruminally undegradable protein on body weight and composition of beef cows

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Day of gestation</th>
<th>SEM²</th>
<th>Diet</th>
<th>Day</th>
<th>Diet × Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, lb.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1,092&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1,092&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1,122&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>1,140&lt;sup&gt;dI&lt;/sup&gt;</td>
<td>1,152&lt;sup&gt;ae&lt;/sup&gt;</td>
</tr>
<tr>
<td>NR</td>
<td>1,109&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>1,068&lt;sup&gt;dD&lt;/sup&gt;</td>
<td>1,073&lt;sup&gt;eD&lt;/sup&gt;</td>
<td>1,073&lt;sup&gt;eD&lt;/sup&gt;</td>
<td>1,087&lt;sup&gt;SC&lt;/sup&gt;</td>
</tr>
<tr>
<td>NRP</td>
<td>1,123&lt;sup&gt;E&lt;/sup&gt;</td>
<td>1,082&lt;sup&gt;eF&lt;/sup&gt;</td>
<td>1,101&lt;sup&gt;fE&lt;/sup&gt;</td>
<td>1,115&lt;sup&gt;deE&lt;/sup&gt;</td>
<td>1,120&lt;sup&gt;eF&lt;/sup&gt;</td>
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<tr>
<td>BCS, 1-9 scale</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>5.34&lt;sup&gt;aB&lt;/sup&gt;</td>
<td>5.73&lt;sup&gt;dA&lt;/sup&gt;</td>
<td>5.82&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>5.89&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>5.96&lt;sup&gt;aA&lt;/sup&gt;</td>
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<td>5.05&lt;sup&gt;haA&lt;/sup&gt;</td>
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<td>5.50&lt;sup&gt;abA&lt;/sup&gt;</td>
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<td></td>
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<tr>
<td>C</td>
<td>9.77&lt;sup&gt;aB&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>NR</td>
<td>9.39&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>-</td>
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<tr>
<td>NRP</td>
<td>9.29&lt;sup&gt;aB&lt;/sup&gt;</td>
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<tr>
<td>i.m. fat, %</td>
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</tr>
<tr>
<td>C</td>
<td>3.93&lt;sup&gt;aC&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>NRP</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>12th rib fat, in.</td>
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<tr>
<td>C</td>
<td>0.22&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>-</td>
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<tr>
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<td>0.13&lt;sup&gt;bA&lt;/sup&gt;</td>
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<tr>
<td>NRP</td>
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</tr>
</tbody>
</table>

1Diets consisted of native grass hay plus a soybean meal-based supplement formulated to achieve 1.12 lb/day of BW gain (C), 70% of NE<sub>in</sub> provided by C (NR), and 70% of NE<sub>in</sub> provided by C plus a RUP supplement (NRP).

2<sup>n</sup> = 36 cows per dietary treatment.

a,b,c,d,e,f,g,h,i,j,k,l,m,n Means within a column lacking a common superscript differ (P < 0.05).

a,b,c,d,e,f,g,h,i,j,k,l,m,n,m Means within a row lacking a common superscript differ (P < 0.10).

a,b,c,d,e,f,g,h,i,j,k,l,m,n,m,n,m Means within a row lacking a common superscript differ (P < 0.05).
Curcumin Enhances Cytotoxic Effects of Cisplatin in Human Ovarian Cancer Cells

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Summary and Implications

Curcumin, the active ingredient of turmeric, has anti-proliferative and chemo-sensitizing properties in cancer cells. Curcumin, through the actions of the tumor suppressor protein TP53, can arrest the cell-cycle in the G2/M phase leading to the release of cytochrome c from mitochondria causing apoptosis, or by down-regulation of cyclin D1 preventing apoptosis. The objective of this experiment was to determine the effects of curcumin on cell proliferation and apoptosis in human epithelial ovarian cancer SKOV-3 cells. It was hypothesized that the anti-proliferative properties of curcumin would enhance the TP53-mediated apoptotic effect of cisplatin, a traditional chemotherapeutic. Cultured SKOV-3 cells were treated with curcumin (0, 10, or 20 µM) for 4, 8, 16 and 24 h at 37°C and expression of TP53 and cyclin D1 was determined by Western blot analysis. Viability of similarly treated cells was determined by trypan blue staining. Incidence of apoptosis was detected by TUNEL assay. Effects of curcumin and cisplatin (0.25 µg/mL) on numbers of metabolically active cells were determined by MTT assay.

Curcumin at 10 and 20 µM decreased the numbers of viable cells and decreased the expression of cyclin D1 while TP53 was only increased with 20µM curcumin. Although curcumin decreased the numbers of viable cells, numbers of cells with positively-stained apoptotic nuclei were < 10%. Cisplatin or curcumin alone decreased the numbers of metabolically active cells which were further decreased when cells were treated with 20 µM curcumin in combination with cisplatin. The reduced number of metabolically active cells following treatment with curcumin may be due to the arrested cell cycle mediated through increased TP53 and decreased cyclin D1 expression. Lack of a robust apoptotic effect suggests that curcumin, while possibly lytic at high doses, at low doses may primarily arrest the cell cycle facilitating an increased cytotoxic effect of cisplatin.

Introduction

The majority of cancers of the ovary are thought to originate by clonal expansion of a surface epithelial cell perturbed by genotoxic reactive oxidants generated during the mechanics of ovulatory ovarian rupture. Oxidative damages to DNA are normally reconciled by TP53-mediated tumor suppressor (cell-cycle arrest and base-excision repair) mechanisms; it is a unifocal 'escape' that could be problematic (i.e., a genetically-altered progenitor cell, with un repaired DNA, but not committed to death, giving rise to a transformed phenotype; Murdoch and McDonnel, 2002). There are four basic stages of advancement in common epithelial ovarian cancer. Stage I is defined by the formation of an epithelial inclusion cyst that invades the ovarian cortex. Cancerous cells are extruded into and seed the abdominal cavity when an inclusion cyst ruptures. Pelvic spread and generation of ascites fluid are the hallmarks of Stage II disease. Stage III is characterized by tumor implants involving the small intestine, mesentery, and superficial liver. Distant disseminated metastasis (e.g. to the parenchymal liver) occur in Stage IV. Traditional chemotherapy treatments are somewhat problematic in that patients often become refractory to treatment and relapse (Ozols, 2002). Treatments that intervene during the early stages of disease development or attenuate disease recurrence following cytoreductive surgery would improve survival rates among women diagnosed with this insidious disease. Curcumin ( diferuloyl methane), a major component of the spice tumeric (Curcuma longa) and the traditional herbal medicine Zedoriae rhizoma, possesses antiproliferative and apoptotic effects (Shi et al., 2005). It was hypothesized that curcumin would attenuate cell proliferation, cause apoptosis, and by blocking the cell cycle, enhance the cytotoxic effects of the traditional chemotherapeutic agent cisplatin.

Materials and Methods

Cell Culture

Human ovarian cancer cells SKOV-3 were maintained in RPMI 1640 media supplemented with 10% FBS. Cells were counted using trypan blue and seeded into 96 well plates for the MTT assay or 6 well plates for viability staining or poly-D-Lysine chamber slides for TUNEL Assay. Cells grown in 6 well plates were treated with 0, 10, 20 or 40 µM curcumin for viability counts. Similarly
treated cells were collected in Laemmli buffer and used for Western blot analysis. Cells in 96 well plates were treated with curcumin alone (10 or 20 μM) or in combination with cisplatin (0.25 mg/mL).

Western Blot Analysis

Cell lysates (25 μg) were loaded onto 12 % PAGE and transferred to 0.2 μm nitrocellulose in Towbin buffer. Membranes were blocked in TBST containing 5% nonfat dry milk and probed with rabbit polyclonal antibodies directed towards TP53 or Cyclin D1 (1:1000; Santa Cruz Biotech) followed by HRP conjugated secondary antibody (Jackson Labs). After washing with TBST, membranes were incubated for 5 min in Pierce Super Signal chemiluminescence reagent and exposed to X-ray film.

TUNEL Assay

To detect apoptotic nuclei, cells were stained using the Promega TUNEL assay. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% TX-100, and incubated with TdT reaction mixture (1 μL TdT, 1 μL biotinylated nucleotide, 98 μL equilibration buffer) for 1 hour at 37°C. Free nucleotide was washed away and endogenous peroxidase was blocked by incubating in 0.3% H2O2. Slides were incubated with Streptavidin-HRP solution (1:500) for 30 min. After washing, positive staining was observed with DAB.

MTT Assay

SKOV-3 cells in 96 well plates were treated with 0, 10 or 20 μM curcumin alone or in combination with 0.25 μg/ml cisplatin for 24 hours at 37oC/5% CO2. MTT reagent (0.01mL) was added to each well of the microtiter plate and the plate was incubated for 2-4 hours. Detergent (0.1mL) was added and incubated for 2-4 hours. Absorbance was read in a microplate reader at 570 nm. Average values were determined from triplicate readings with average value of the blank subtracted.

Statistics

Data were analyzed by analysis of variance using GLM procedures of SAS.

Results and Discussion

Curcumin alone decreased numbers of viable SKOV-3 cells (Figure 1). Curcumin has demonstrated cytotoxic and antiproliferative effects in many cancer cells lines with curcumin causing cell arrest in the G2/M phase of the cell cycle (Sharma et al., 2005). In the present study SKOV-3 ovarian cancer cells exposed to 20 μM of curcumin had decreased cyclin D1 and increased TP53 expression (Figure 2). Cell division can be induced by cyclin D1, but is generally tightly regulated to prevent proliferation in unfavorable environments. However in transformed (i.e. cancer) cells, the cell cycle is generally deregulated with cancer cells commonly having increased expression of cyclin D1 (Choudhuri et al., 2005). The decrease in cyclin D1 expression noted in the present experiment may indicate a pause in cell proliferation. Curcumin may also induce cell death (apoptosis) via a TP53-dependent pathway.

TP53 is a tumor suppressor protein, and mutation of the TP53 gene is common in tumor tissue. Expression of high-levels of TP53 can lead to either cell cycle arrest or apoptosis. Curcumin treated cells had an increased expression of TP53 (Figure 2). It is unclear, however, if expression of TP53 lead to apoptosis or cell cycle arrest in the present experiment. Although numbers of viable cells were decreased with curcumin treatment (Figure 1), numbers of positively stained apoptotic nuclei were small (data not shown). It is possible that apoptosis had occurred in cells which had lost adhesion to the cell plate and were not quantified. Alternatively, the lack of a robust apoptotic effect may suggest that curcumin, while lytic at high doses, at low doses primarily arrests the cell cycle facilitating an increased cytotoxic effect of cisplatin.

Since chemoresistance is common in patients suffering from ovarian cancer, treatments which increase sensitivity to traditional chemotherapeutics would be clinically beneficial. Curcumin at 10 and 20 μM decreased the number of metabolically active cells; however, curcumin at 20 μM in combination with cisplatin further decreased numbers of metabolically active cells when compared to cisplatin or curcumin alone (Figure 3). Reduced numbers of metabolically active cells following treatments with curcumin may be due to an arrested cell cycle mediated through increased TP53 and decreased cyclin D1 expressions. In conclusion, treatment of SKOV-3
human ovarian epithelial cancer cells with curcumin causes cell death while sensitizing cells to the toxic effect of cisplatin.

Supported by INBRE P20RR016474.

![Figure 3](image)

**Figure 3.** Metabolically active cells as measured by MTT assay. Cells were treated with 10 or 20 μM curcumin (Cur10 and Cur20) alone or in combination with 0.25 μg cisplatin (Cis). All treatments decreased \( P < 0.001 \) the number of metabolically active cells. Columns with differing subscripts differ \( P < 0.001 \).

**References**


Down-Regulation of Growth Signaling in Cotyledonary Arteries of Overnourished, Obese Pregnant Sheep

Mei J. Zhu, Postdoctoral Fellow, Min Du, Assistant Professor, Peter W. Nathanielsz, Professor, Bret W. Hess, Associate Professor, Gary E. Moss, Professor, Stephen P. Ford, Professor, Department of Animal Science, University of Wyoming

Summary and Implications

Maternal obesity and over-nutrition reduce arteriolar density in cotyledonary (COT) tissues and decrease protein kinase B (Akt) and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling, but the mechanism(s) for this down-regulation is unknown. Both Akt and ERK1/2 are downstream components of insulin/insulin like growth factor-1 (IGF-1) signaling, and AMP-activated protein kinase (AMPK) is known to sensitize insulin/IGF-1 signaling through phosphorylation of insulin receptor substrate-1 (IRS-1). The objective of this study was to assess the activity of AMPK and its role in the observed down-regulation of insulin/IGF-1 signaling in COT arteries of the ewe placenta. Non pregnant ewes were randomly assigned to a control (CF, 100% of NRC recommendations, n=10) or obeseogenic (OB, 150% of NRC, n=10) diet from 60 days before to 75 days after conception. At necropsy, the smallest terminal arteries that entered the COT tissues (0.5–1.0 mm in diameter) were immediately collected. Fetal plasma concentrations of glucose, insulin and IGF-1 were higher (P<0.05) in the blood of fetuses from OB than CF ewes (43.63 ± 5.58 mg/dl, 7.29 ± 1.31 IU/ml, and 53.30 ± 1.76 ng/ml versus 25.35 ± 2.10 mg/dl, 1.14 ± 0.35 IU/ml and 44.62 ± 0.90 ng/ml, respectively). Total AMPK and phosphorylated AMPK at Thr 172 (the active form) was reduced (p<0.05) by 19.7± 8.4 % and 25.9 ± 7.7 %, respectively in the COT arterial tissues of OB ewes. Total acetyl-CoA carboxylase (ACC), a down-stream target of AMPK, and its phosphorylated form was also reduced (p<0.05) by 32.9 ± 9.2% and 45.4 ± 14.6%, respectively in OB compared to CF COT arteries. The phosphorylation of IRS-1 at Ser789, a site phosphorylated by AMPK, was 24.5% ± 9.0% lower (p<0.05) in COT arteries of OB ewes. The down-stream insulin signaling was down-regulated in COT arteries of OB ewes, showing insulin/IGF-1 resistance. The possible reason for this resistance might be due to the down-regulation of AMPK, which reduced phosphorylation of IRS-1 at Ser789, reducing the PI3K activation mediated by IRS-1 and mitigating down-stream insulin/IGF-1 signaling in arteries of OB ewes despite increased plasma glucose, insulin and IGF-1 concentrations.

Introduction

In the USA, pre-pregnancy obesity increased from 13.0% in 1993 to 1994 to 22.0% in 2002 to 2003, with a net increase of 69.3% (Kim et al., 2007). More importantly, a shift towards higher gestational weight gain appears evident, resulting from excessive nutrient uptake during pregnancy (Siega-Riz et al., 2006). Adequate placental vascularity is essential for the delivery of nutrients to the rapidly growing fetuses. Up to now, the effect of maternal over-nutrition and obesity on placental vascular development and underlying mechanisms are unclear.

The sheep is one of the most frequently used animal models for human pregnancy. In the ruminant, 70 to 120 placentomes compose the individual units of fetal: maternal exchange (Ford, 2000) and each placentome is composed of fetal (cotyledon; COT) and maternal (caruncular, CAR) components. Cotyledons are tufts of chorionic villi which develop adjacent to uterine CAR on the uterine wall, and interdigitate with corresponding crypts of the uterine caruncle to form the placentomal units (Ford, 2000). Previously, we reported that the size of placentome decreased while the vascularity of the placentome increase due to maternal under-nutrition (Zhu et al., 2007a; Zhu et al., 2007b), which impacts the ability of the fetus to acquire optimal delivery of nutrient and oxygen (Reynolds & Redmer, 2001). We further demonstrated that the mitogen-activated protein kinase (MAPK/ERK1/2) pathway and the phosphoinositide-3 kinase/protein kinase B (PIP3-K/Akt) pathway were up-regulated in placentome arteries under maternal nutrient restriction, in association with increased placentomal vascularity (Zhu et al., 2007a; Zhu et al., 2007b). However, the impact of over-nutrition and obesity on MAPK and Akt signaling has not been examined.

Insulin exerts its biological effects mainly through activation of the PI3K/Akt signaling pathway (Bush et al., 2003; Latres et al., 2005; Park et al., 2005; Song et al., 2005; Subramaniam et al., 2005; Vary, 2006). The binding of insulin to its receptor leads to phosphorylation of insulin receptor substrate-1 (IRS-1), a key mediator of insulin signaling (Anthony et al., 2001; Bush et al., 2003; Vary, 2006). Phosphorylated IRS-1 recruits and activates PI3K/Akt signaling, which further activates mammalian
target of rapamycin (mTOR) signaling (Chiang & Abraham, 2005). In addition, phosphorylation of IRS-1 also leads to the activation of MAPK (Choi & Sung, 2004; Hwang & Hur, 2005). Therefore, it is quite possible that insulin/IGF-1 signaling was involved in the overall regulation of PI3K/Akt and MAPK signaling in response to nutrient availability. AMP-activated protein kinase (AMPK) has a central role in the control of energy metabolism, which sensitizes insulin signaling through phosphorylation of IRS-1 at Ser 789 (Jakobsen et al., 2001). Thus, AMPK may mediate the insulin/IGF-1 signaling in sheep placentomes due to the availability of nutrients. The objective in the current study is to assess the alteration in insulin/IGF-1 signaling and AMPK, and their roles in placental vascular density changes in response to maternal over-nutrition and obesity in the ewes.

Materials and Methods

Care and use of animals

All animal procedures were approved by the University of Wyoming Animal Care and Use Committee. From 60 days before conception to day 75 of gestation, multiparous Rambouillet/Columbia ewes were placed in individual pens and fed either a highly palatable diet at 100% (control, C; n=10) of National Research Council (NRC) recommendations or 150% (obesogenic, OB; n=10) of NRC recommendations on a metabolic BW basis (BW0.75). Ewes were weighed at weekly intervals so that individual diets could be recalculated, with rations adjusted for weight gain, and body condition scored at monthly intervals to evaluate changes in fatness as previously described (Ford et al., 2007).

Immediately prior to necropsy, on day 75, pregnant ewes were weighed and a sample of fetal blood was collected via jugular venipuncture into a chilled nonheparinized vacutainer tube (no additives, Sigma, St. Louis, MO). Serum obtained was frozen at -80°C until assayed for insulin. Blood was also collected in a separate chilled tube (heparin plus sodium fluoride; 2.5 mg/ml; Sigma, St. Louis, MO), and plasma was frozen at -80°C until assayed for glucose.

Tissue collection

On the day of necropsy, ewes were sedated with Ketamine, anesthetized by isofluorane inhalation and exanguinated while remained under general anesthesia. All placentomes present in the uteri of both CF and OB ewes were classified as type A using the criteria described previously (Vonnahme et al., 2006). On each necropsy day, the smallest terminal arteries that entered the COT tissues (0.5–1.0 mm in diameter) were immediately collected from five similar size placentomes and frozen in liquid nitrogen for protein extraction and western blotting. An additional two placentomes of similar sizes were randomly selected from each ewe and utilized for vascular area density analysis as described below. The fetus was removed and weighed, and all remaining placentomes were dissected from the uterus.

Vascular area density measurement

The arteriolar vascular density was determined by procedures previously published from our laboratory (Vonnahme et al., 2003). Briefly, two placentomes were dissected from the surrounding tissue and weighed. A cross section of each placentome containing CAR and COT tissue was placed in a tissue cassette (Tissue Tek, Miles Labs, Elkhart, IN) and fixed with 4% (w/v) paraformaldehyde in a phosphate buffer (0.12 M; pH 7.4) and paraffin embedded. Twelve 5-μm sections evenly spaced over a 450-μm area of each placentome were evaluated for resistance arteriolar vascular area density (i.e., CAR blood vessel area/ CAR area; COT blood vessel area/COT area) via image analysis (Optimus Image Analysis Software, Bothell, WA). Briefly, for each placentome, maternal and fetal blood vessels were counted and traced within four fields for each of the twelve 5-μm section at points where the CAR and adjacent COT tissue of the placentome could be visualized, and values were averaged.

Antibodies

Antibodies against AMPK (Cat # 2532), phospho-AMPK at Thr 172 (Cat # 2535), ACC (Cat # 3662), phospho-ACC at Ser 79 (Cat # 3661), IRS-1 (Cat # 2382), phospho-IRS-1 at Ser 789 (Cat # 2389), Akt (Cat # 9272), phospho-Akt at Ser 473 (Cat # 9271), mTOR (Cat # 2972), phospho-mTOR at Ser 2448 (Cat # 2971), ERK (Extracellular regulated kinase)1/2 (Cat # 9102), phosphorylated ERK1/2 (Thr202/Tyr204, Cat#9101), Insulin receptor beta (Cat # 3025), IGF-1 receptor (Cat # 3027) and phosphor-IGF-I Receptor (Tyr1131 / Insulin Receptor (Tyr1146) (Cat # 3021) were purchased from Cell Signaling (Danvers, MA). Anti-β-actin (Cat # JAL20) antibody was obtained from Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA 52242).

Immunoblot analysis

Cotyledonary arteries which were pooled for each ewe were powdered in liquid nitrogen, then 0.1g powdered artery was used for immunoblotting analyses as previously described (Zhu et al., 2007a).

Glucose, Insulin and IGF-1 analyses

Glucose was analyzed using the Infinity™ (ThermoTrace Ltd, Cat. # TR15498; Melbourne, Australia) colorimetric assay modified in the following manner; plasma was diluted 1:5 in dH2O, and 10μL of diluted plasma was added to 300μL reagent mix. All samples were run in triplicate. The intra-assay and inter-assay CVs are 3% and 5%, respectively.

Insulin was measured by RIA in accordance with manufacturer recommendations (Coat-A-Count Diagnostic Products Corp., Los Angeles, CA) and completed in a single array. When using ovine serum, the intra-assay CV is < 3 % and sensitivity was 0.05ng/ml. IGF-1 was run on fetal serum
samples using Immulite test kits on an Immulite 1000 (Diagnostic Products Corp., Los Angeles, CA) in a single array, the intra-assay CV is < 5 %and sensitivity was 20 ng/ml

Statistics

Data were analyzed as a complete randomized design using GLM (General Linear Model of Statistical Analysis System)(SAS, 2000). Means separation was performed using LSMEANS. Means ± SEM were considered different when P < 0.05.

Results and Discussion

OB ewes increased their weight by ~50% from diet initiation to necropsy (65 ± 2 to 96 ± 2 kg), while CF ewes increased their body weight only ~7% over the same time period (64 ± 2 to 69 ± 2 kg). The body weight and empty carcass weight of OB fetuses were higher than that of CF fetuses (P<0.05, Table 1). However, no differences were observed in total placentome weight or the total weight of CAR or COT tissues across dietary groups (Table 1). Further, neither placentome number nor average placentome weight of OB or CF ewes differed (Table 1). At necropsy, plasma concentrations of glucose, insulin and IGF-1 were higher (P<0.05) in the blood of fetuses from OB than CF ewes (43.63 ± 5.58 mg/dl, 7.29 ± 1.31 IU/ml, and 53.30 ± 1.76 ng/ml versus 25.35 ± 2.10 mg/dl, 1.14 ± 0.35 IU/ml and 44.62 ± 0.90 ng/ml, respectively). Vascular density was lower (P<0.05) in COT of OB sheep compared to CF sheep (Figure 1).

Western blotting analysis indicated that no alteration in the content of total insulin receptor, total IGF-1 receptor and their phosphorylated forms were observed (Figure 2), but there was a reduction of the down-stream insulin signaling, Akt, mTOR and ERK1/2 and their phosphorylated forms, in COT arterial tissues of OB ewes (P<0.05; Figure 3). Further, total AMPK and phosphorylated AMPK at Thr 172 (the active form) was down-regulated by 19.7± 8.4 % (p<0.05) and 25.9 ± 7.7 % (p < 0.05) respectively in the COT arterial tissues of OB ewes (Figure 4). Total acetyl-CoA carboxylase (ACC), a down-stream target of AMPK, and its phosphorylated form was also reduced by 32.9 ± 9.2% (p<0.05) and 45.4 ± 14.6% (p<0.05) respectively in OB compared to CF COT arteries (Figure 4). The phosphorylation of IRS-1 at Ser 789, a site phosphorylated by AMPK, was 24.5% ± 9.0% (p<0.05) lower in COT arteries of OB ewes.

These data demonstrate that overnourishment of sheep during early to mid-gestation reduced the vascular density of COT tissue. This deduction in COT vascular density was not due to increased matrix tissue, since there was no difference in average placentome weight. The reduction in vascular density might be due to the decreased branching angiogenesis, since CAR vascular beds grow primarily by increased capillary size with little increase in capillary number while COT capillary beds grow primarily by branching angiogenesis over the last two-thirds of gestation in the sheep (Reynolds et al., 2005a). The decreased vascularity within the COT tissue of OB ewes should abate nutrient transfer from mothers to fetuses and made the overall nutrient transport relatively constant. Indeed, there were huge difference in maternal plasma glucose, insulin and IGF-1 concentrations (Data not shown). However, their differences in fetal plasma were much milder. This observation was reminiscent of our previous report showing that vascularity was increased in placentomes of under-nourished ewes, which increases placentomal efficiency (Zhu et al., 2006, 2007a).

To investigate the underlying mechanism(s) associated with this reduced vascularity under our over-nutrient regimen, two key pathways associated with cell growth, proliferation and angiogenesis were evaluated in COT arteries of ewe placentomes. The phosphorylation of both Akt and ERK1/2, key intermediates in the PI3K/Akt and MAPK pathways respectively, were decreased in day 75 COT arteries of OB ewes. The data are consistent with our previous report that the PI3K/Akt and MAPK (Erk) were up-regulated in placentomal arteries under nutrient deficiency (Zhu et al., 2007a). The PI3-K/Akt pathway promotes endothelial cell survival and protein synthesis (Gerber et al., 1998; Fujio & Walsh, 1999), migration and capillary-like structure formation (Shiojima & Walsh, 2002) and its down-regulation in COT arteries are expected to reduce angiogenesis. The PI3-K/Akt pathway has also been previously reported to be important for placental growth. The placenta of Akt knockout mice display significant hypotrophy and decreased vascularization (Yang et al., 2003). The MAPK pathway is mainly involved in cell mitogenesis and very recently, MAPK/ERK1/2 has been shown to mediate the mammalian target of the rapamycin (mTOR) signaling pathway and thus links to the control of protein synthesis (Ma et al., 2005). ERK1/2 was also reported to be crucial for endothelial cell proliferation in sheep and human placental artery during NO mediated angiogenesis (Zheng et al., 2006). Similar to Akt, we found that ERK1/2 and its phosphorylated form were down-regulated in COT arteries of OB ewes compared to CF ewes (Figure 3).

Since both Akt and ERK1/2 signaling are down-stream events of insulin/IGF-1 signaling, we further investigated the upstream mediators of insulin/IGF-1 signaling in the COT arteries. Insulin and IGF-1 share a common pathway in promoting cell growth and proliferation (Avruch et al., 2005). However, beyond the presence of insulin receptors, little is known of the mechanisms underlying the biological effects of insulin in the placenta. Insulin stimulation of placentonal cells enhanced the phosphorylation of ERK1/2 and Akt by 286 ± 23% and 393 ± 17%, hinting the important role of insulin in placental development and, very possibly, angiogenesis (Boileau et al., 2001).

Insulin exerts its biological effects mainly through activation of the phosphoinositide-3 kinase/protein kinase B (PI3K/Akt) signaling pathway (Bush et al., 2003; Latres et al., 2005; Park et al., 2005; Song et al., 2005; Subramaniam et al., 2005; Vary, 2006). IRS-1 is a key site mediating insulin signaling (Shao et al., 2002).

AMPK is a heterotrimeric enzyme with α, β, and γ subunits (Hardie, 2004; Kim et al., 2004). AMPK is activated by phosphorylation at Thr 172. Once activated, AMPK promotes glucose uptake and inhibits lipid synthesis.
in cells (Hardie & Hawley, 2001; Fujii et al., 2006). Activated AMPK phosphorylates IRS-1 at Ser 789 which enhances insulin signaling (Jakobsen et al., 2001). We observed a reduction in the phosphorylation of AMPK and its down-stream effector, ACC at Ser 79. Since ACC phosphorylation at Ser 79 was exclusively catalyzed by AMPK, phosphorylation of AMPK at Thr 172 coupling with ACC phosphorylation at Ser 79 were commonly used to measure AMPK activity. These data showed that AMPK activity was reduced in the COT arteries of OB ewes. This reduction was accompanied by a reduction in IRS-1 phosphorylation at Ser 789, a site phosphorylated by AMPK. To be a phosphorylation site beneficial to IRS-1 mediated activity was reduced in the COT arteries of OB ewes. This decrease in IRS-1 phosphorylation mitigates down-stream insulin/IGF-1 signaling, including Akt/mTOR and MAPK (ERK1/2) signaling. This notion is consistent with several very recent reports showing that AMPK inhibition was associated with insulin resistance in skeletal muscle (Lee et al., 2006; Steinberg et al., 2006; Watt et al., 2006).

Most previous studies regarding to angiogenesis in placentomes were focused on growth factors (Reynolds et al., 2005b; Pfarrer et al., 2006; Babischkin et al., 2007). In this study, despite the augmented insulin and IGF-1 levels in the fetal blood stream of OB mother, the Akt and ERK 1/2 signaling were down-regulated in OB COT arteries. These data suggest that in addition to growth factors intracellular signaling events may be equally important in mediating angiogenesis in arteries. We hope our studies can ignite the association between intracellular signaling events and angiogenesis in placentomes.

These data show that the phosphorylation of AMPK and its down-stream target ACC were reduced in COT arterial tissue of OB mothers. This reduction in AMPK activity decreased the phosphorylation of IRS-1 at Ser789, which is expected to reduce the PI3K activation mediated by IRS-1, mitigating down-stream insulin/IGF-1 signaling in OB ewes despite increased plasma glucose, insulin and IGF-1 concentrations. The mitigation of insulin/IGF-1 downstream signaling in COT arteries should be associated with the reduced vascular density in COT tissue.

References


Table 1. Fetal and placental measurements from CF and OB d78 sheep

<table>
<thead>
<tr>
<th>TRT</th>
<th>Fetal wt, g</th>
<th>Em. Carcass wt, g</th>
<th>Total CAR wt, g</th>
<th>Total COT wt, g</th>
<th>Plac wt, g</th>
<th>Plac No</th>
<th>Avg. Plac wt, g</th>
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<tbody>
<tr>
<td>CF(N=10)</td>
<td>185.72 ± 6.89</td>
<td>147.64 ± 4.88</td>
<td>115.09 ± 9.76</td>
<td>541.85 ± 30.91</td>
<td>656.94 ± 36.27</td>
<td>44.3 ± 2.7</td>
<td>15.04 ± 0.83</td>
</tr>
<tr>
<td>OB(N=10)</td>
<td>234.36 ± 6.61</td>
<td>166.34 ± 2.97</td>
<td>108.93 ± 7.45</td>
<td>507.63 ± 33.82</td>
<td>616.55 ± 36.57</td>
<td>46.7 ± 2.7</td>
<td>13.54 ± 1.01</td>
</tr>
<tr>
<td>P-value</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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</tr>
</tbody>
</table>

*Plac. = Placentomes; Em. = empty; *: P<0.05

Figure 1. Phosphorylation of IGF-1/insulin receptors in COT arteries of placentomes from day75 CF and OB sheep. Panel A shows representative immunoblots; Panel B shows statistical data. (n=10 per group).

Figure 2. Total and phosphorylated Akt, mTOR, and ERK1/2 in COT arteries of placentomes from day75 CF and OB sheep. Panel A shows representative Akt and phospho-Akt immunoblots; Panel B shows representative mTOR and phospho-mTOR immunoblots; Panel C shows representative ERK1/2 and phospho-ERK1/2 immunoblots; Panel D shows statistical data. (*): P < 0.05. (n=10 per group).
Figure 3. Total and phosphorylated AMPK and ACC in COT arteries of placentomes from day75 CF and OB sheep. Panel A shows representative AMPK, ACC, phospho-AMPK and phospho-ACC immunoblots; Panel B shows statistical data. (*): P < 0.05. (n=10 per group).
Reproductive Physiology

Effects of Progesterone on Ram Reproductive Behavior

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Summary and Implications

Progesterone is necessary in males for spermiogenesis and testosterone biosynthesis. The current study tested the hypothesis that progesterone is also a crucial modulator of sexual behavior in rams. Intact Columbia rams (n = 6) and Columbia rams gonadectomized (GNX; n = 5) at 6 – 7 mo of age were exposed to ewes in estrus at 10 – 11 mo of age. Expressed reproductive behaviors were recorded and categorized as investigatory (investigatory sniffs, flehmen, foreleg kicks, nudge, vocalization) or consummatory (mount attempts, mounts, ejaculations) behaviors. Following determination of baseline behaviors, intact rams were treated with the specific progesterone receptor antagonist, mifepristone (RU486; 25 mg), twice daily, and re-tested. Gonadectomized rams were implanted subcutaneously with 4 doses of Synovex-h (200 mg testosterone and 20 mg estradiol per dose). Concentrations of serum testosterone did not differ among intact and GNX males treated with Synovex-h. Expression of reproductive behavior was determined one month following insertion of Synovex-h implants. Pursuant to behavior testing, GNX rams were treated with 5 mg of progesterone twice daily and behavior was monitored. Two intact rams were removed from the study due to a lack of sexual behavior. Mifepristone treatment did not affect the expression of investigatory behavior in intact rams, but tended to decrease the expression of consummatory behavior. Testosterone and estradiol (Synovex-h) alone tended to increase the number of investigatory behaviors in GNX males, but consummatory behavior was not observed. Investigatory behaviors tended to be further increased following treatment of GNX males with progesterone. Mounts and mount attempts were observed in two of the five GNX males following progesterone treatment, but consummatory behavior was not increased overall. Progesterone appears to facilitate the expression of ram reproductive behavior and may be especially important for the expression of consummatory behavior.

Introduction

Typical breeding practices for food-animal species utilize limited numbers of males to inseminate large numbers of females. Therefore, it is critical that libido (sexual interest or motivation), mating competence (ability to inseminate females), and fertility (sperm quality) of males is adequate to ensure reproductive success. Libido in rams is highly variable and is influenced by developmental(Roselli et al., 2003) and environmental (Price, 1987) factors.

Progesterone is named for its progestational role in maintaining pregnancy in mammals, and is traditionally regarded as a “female hormone”. The facilitory and inhibitory effects progesterone exerts on female reproductive behavior is well documented (Blaustein and Erskine, 2002). Progesterone is a precursor for both androgen and estrogen synthesis. In the male, androgens are necessary for the development of secondary sex characteristics and testosterone is considered the primary male sex hormone. However the role of testosterone in the expression of male-typical behavior has been overstated since there is little correlation between plasma testosterone concentrations and male behavior (reviewed in: Andersen and Tufik, 2006). Testosterone is aromatized to estradiol 17β in specific hypothalamic nuclei and is considered the centrally active hormone in the male (reviewed in Resko et al., 1999). Progesterone receptors are also present in behaviorally relevant nuclei of the male brain, and progesterone receptor knock-out male mice exhibit sexual-behavior deficits (Phelps et al., 1998).

The physiological significance of progesterone, outside of its role as a precursor for androgen production, is not well understood. Traditionally progesterone was thought to have little or no function in the control of male sexual behavior. Early studies, utilizing supraphysiologic doses of progesterone, were shown to inhibit male sexual behavior (reviewed in Wagner, 2006). However, treatment of rodents with physiological concentrations of progesterone facilitated mounting and copulatory behavior (Andersen and Tufik, 2006). Therefore the objective of this experiment was to determine the role of progesterone in the expression of ram reproductive behavior.

Materials and Methods

Sexually naive Columbia rams (n = 6) and rams gonadectomized (GNX; n = 5) at 6 – 7 mo of age were tested for sexual behavior at 10 – 11 mo of age. Rams were housed together, fed alfalfa hay, and provided water ad...
libitum. Estrus was induced in ovariectomized ewes by intravaginal progesterone treatment (CIDR) for 10 d followed by 50 μg daily injections (i.m.) of estradiol-17β. Ewes exhibited estrus by 48 h following CIDR removal and estradiol treatment. Ewes remained in estrus for 96 h when treated daily with 50 μg estradiol (i.m.). Sexual behavior was evaluated in rams tested individually for three minutes in a 2.0 x 2.0 m pen with three estrous ewes. The testing environment was a partition of their home pen with rams tested in sight of pen mates. Sexual behaviors exhibited by the rams were recorded and classified as either investigatory (investigatory sniff, flehmen, nudge, foreleg kick, and vocalizations) or consummatory (mount attempts, mounts, and ejaculations) behavior.

Baseline behaviors were established in all rams by the average of expressed sexual behavior in rams on three consecutive days. Following baseline testing, GNX rams were implanted with four Synovex-h implants (200 mg testosterone propionate and 20 mg estradiol benzoate each; Syntex Laboratories, Inc. Palo Alto, CA). Approximately 30 d following insertion of Synovex-h implants, GNX rams were exposed to ewes in estrus and observed for the expression of sexual behavior on three consecutive days. Effect of progesterone on the expression of sexual behavior was determined in Synovex-h treated rams following the administration of exogenous progesterone (5 mg s.c. in 95% ethanol) twice per day. Progesterone treatment was initiated 2 d prior to testing and continued throughout the 3 d testing period.

Following the establishment of baseline behavior, intact rams were treated with 25 mg mifepristone (RU486; 20% ethanol 80% Tween 80) twice per day and exposed to estrus ewes. Mifepristone treatment was initiated 2 d prior to behavior testing.

Blood samples for analysis of serum concentrations of testosterone were collected from GNX rams prior to castration and from both GNX and intact rams three times during the testing period. Serum was separated by centrifugation and stored at -20 °C until testosterone analysis.

Concentrations of testosterone were determined in a single RIA using a solid phase DPC (Diagnostic Products Corporation, Los Angeles, CA) kit with 6.1% intra-assay variation. Standards were diluted in charcoal-stripped wether serum and were parallel to kit standards.

Statistical Methods.

Effects of mifepristone (RU486) and Synovex-h (testosterone and estradiol) on sexual behavior were determined by a paired T Test (SAS 9.1, Cary, NC). Additive effects of progesterone in GNX rams were determined by paired T Test utilizing behavior expressed pre-Synovex-h or following Synovex-h as the baseline. Differences in concentration of serum testosterone were determined by GLM methods of SAS (Ver. 9.1, Cary, NC).

Results and Discussion

Differences in mating behavior exist among individuals of all species studied (Meisel and Sach, 1994). Mating performance of rams is important for the profitability of the sheep industry. Stellflug et al. (2006) indicated twice as many poor-performing rams were needed to obtain breeding results equal to a single high-sexually performing ram. With nearly 30% (Fitzgerald and Perkins, 1991) of rams classified as non-performers, the importance of ram sexual behavior is well recognized.

Two intact rams in the current study were removed from the study due to a lack of sexual behavior. One of these rams had low serum concentrations of progesterone and may have been sexually immature. The other ram had serum concentrations of testosterone comparable to his sexually-active cohorts.

All GNX rams had detectable quantities (4.4 ± 0.9 ng/mL) of testosterone prior to castration, and had likely reached sexual maturation prior to castration. Following castration and treatment with Synovex-h, serum concentrations of testosterone were not different (P = 0.62) from intact rams (7.1 ± 2.9 ng/mL). Serum concentrations of testosterone in GNX rams did not differ (P = 0.43) over time, and although progesterone is a precursor hormone for testosterone, serum concentrations of testosterone was not increased (P > 0.21) during progesterone treatment.

The testes and the adrenal cortex are the sites of synthesis for progesterone in the male. Circulating progesterone in the male is most likely of adrenal origin (Wagner, 2006). Treatment of intact rams with the progesterone receptor antagonist, RU486, did not affect (P = 0.70) investigatory behavior, but tended (P = 0.09) to decrease consummatory behaviors (Figure 1). Progesterone appears to affect male behavior by acting at its own receptor, independent of its role as a precursor hormone.

Figure 1. Investigatory and consummatory behavior expressed by intact rams prior to (Baseline) and following mifepristone treatment. * P = 0.09 paired T Test change from Baseline.

Although Synovex-h implants alone tended (P = 0.06) to increase investigatory behavior. Synovex-h implants in combination with exogenous progesterone further increased (P = 0.02) the expression of investigatory behavior compared to pretreatment baseline (Figure 2).
Mounting behavior was not observed in any of the GNX rams prior to progesterone treatment. Following progesterone treatment mounting behavior was observed in two of the five GNX rams although consummatory behavior did not increase overall ($P = 0.25$).

Figure 2. Investigatory behavior expressed in gonadectomized rams prior to treatment (Baseline), following treatment with Synovex-h implants, and Synovex-h implants in combination with progesterone. **$P = 0.02$ *$P = 0.07$, Paired T Test change from Baseline.

Similar to rodents (Wagner, 2006), progesterone appears to be more important for the expression of mounting behavior than for sexual interest in the ram. Intact rams treated with the progesterone receptor antagonist, mifepristone, had decreased numbers of mounts while investigatory behavior was unaffected. Mounting behavior was only observed in GNX rams after treatment with progesterone, and was not observed when rams were treated with testosterone and estradiol (Synovex-h) alone. Clearly GNX rams displayed increased sexual interest, as indicated by the increased expression of investigatory behaviors; however, this increase may reflect learning (possibly independent of a hormonal affect) in these sexually naïve rams. Although results depict only a trend, numbers were limited and more robust results would be expected with a larger experimental population.

In conclusion progesterone, acting at its receptor, appears to facilitate the expression of ram sexual behavior and may be especially important for the expression of mounting behavior.

References


Reproductive Physiology

**Fetal Steroid Changes Associated With Maternal Obesity in the Sheep**

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**Summary and Implications**

Steroid hormones are associated with long-term organizational effects during development. Prenatal glucocorticoid (cortisol) overexposure appears to program adverse effects on blood pressure, metabolism and the hypothalamic-pituitary-adrenal (HPA) axis, while elevated prenatal testosterone exposure is linked to reproductive dysfunction. Utilizing our newly developed sheep model of maternal obesity, we evaluated its impacts on fetal cortisol and testosterone concentrations at midgestation. Twin bearing ewes were assigned to a control (C, 100% of NRC recommendations, n=6) or obesogenic (OB, 150% of NRC, n=7) diet from 60 days before to 75 days after conception when ewes were anesthetized and maternal jugular and umbilical venous blood samples collected. Serum was stored at -80ºC until assayed for cortisol and testosterone by RIA (Diagnostic Products, Los Angeles, CA). OB and C ewes increased their body weights by 50% and 7%, respectively, from diet initiation until sample collection. Fetuses from OB ewes were heavier than those from C ewes (374 ± 10 vs. 268 ± 12g; P<0.05). Maternal blood concentration of cortisol were greater for OB vs. C ewes (31.8 ± 5.9 vs. 3.6 ± 0.8 ng/ml; P<0.05), and was greater in the blood of their fetuses (7.7 ± 2.1 vs. 3.8 ± 0.8 ng/ml; P<0.05), regardless of fetal sex. Male fetuses exhibited greater blood concentration of testosterone than female fetuses regardless of dietary treatment (0.47 ± 0.11 vs. 0.04 ± 0.01 ng/ml; P<0.05). While blood concentrations of testosterone were similar for female fetuses from OB and C ewes (0.05 ± 0.01 and 0.04 ± 0.02 ng/ml), testosterone concentrations of male fetuses from OB ewes were greater than those from C ewes (0.83 ± 0.17 vs. 0.26 ± 0.13 ng/ml; P<0.05). These data demonstrate that maternal obesity elevates fetal blood cortisol, which may result from increased uptake of elevated maternal cortisol or de novo synthesis. Further, testosterone concentrations were elevated in the blood of male fetuses from OB vs. C ewes and may impact their reproductive function in later life.

**Introduction**

Most studies to date have utilized maternal nutrient restriction models to study in utero programming of placental, fetal and postnatal development. Of the hormones known to regulate fetal development, it is the glucocorticoids that are the most likely to have widespread programming effects in utero. They affect the growth and development of all tissues and organ systems that are at increased risk of adult pathophysiology when fetal growth is impaired (1). It has been determined that if nutrient restriction occurs at the time of fetal organogenesis (early to mid-gestation), the changes may be severe and lead to a permanent developmental deficit. For example, there are changes in the relative proportions of cell types in the pancreatic islets, liver, kidneys and skeletal muscles after IUGR that are associated with adult insulin resistance, glucose intolerance, and hypertension (2,3,4,5). Sexual development of the CNS depends in large part on the steroid hormone milieu from day 50-80 of gestation (ovine gestation length = 147days). Evidence suggests that aromatization of testosterone to estradiol in the hypothalamus and amygdala at that time is necessary for masculinization of the sheep brain (6,7). Male fetuses have markedly higher circulating levels of testosterone from day 35 to day 80 of gestation than do female fetuses(8).

The prevalence of obesity increased in the United States at a rate of 50% per decade through the 1980s and 1990s (9). Further, as compared to women with normal body mass index, overweight, obese and morbidly obese women had an increased risk of preeclampsia, gestational hypertension, gestational diabetes, preterm birth, cesarean section and macrosomia(10,11). Further, children exposed to maternal obesity are at increased risk of developing metabolic syndrome(12,13), which suggests that obese mothers even in the absence of gestational diabetes may still have metabolic factors that affect fetal growth and postnatal outcomes. The objective of this study was to evaluate the impacts of maternal obesity and high energy intake in the ewe from 60 days before conception to mid-gestation on fetal size, as well the blood concentrations of cortisol and testosterone.
Materials and Methods

Animals

From 60 days before conception (first day of mating = day 0) to day 75 of gestation multiparous ewes carrying twin fetuses were fed either a highly palatable diet at 100% (control, C; n=6) of National Research Council (NRC) recommendations or 150% (obesogenic, OB; n=7) of NRC recommendations on a metabolic BW basis (BW0.75). Immediately prior to necropsy, on day 75, each ewe was weighed and a sample of blood was collected via jugular venipuncture and serum frozen at -80°C until assayed for cortisol and testosterone. Under anesthesia, fetal blood was collected from the umbilical vein of each fetus, and serum was collected and stored as described for maternal blood. Ewes were then euthanized and the gravid uterus quickly removed. Fetal weights, crown rump lengths and sex were determined as well as weights of selected fetal tissues. Five female and 7 male fetuses were recovered from the C ewes, and 8 female and 6 male fetuses were recovered from the OB ewes.

Cortisol and Testosterone Analyses

Cortisol was quantified utilizing a commercial antibody coated tube RIA kit (Diagnostic Products Corp., Los Angeles, CA). Briefly, standards are prepared as doubling dilutions from 12.8 to 0.2 ng of cortisol (Sigma, St. Louis, MO) in 0.1 ml of 0.01 M PBS containing 2.5% BSA. Samples are analyzed in duplicate with 0.1 ml of serum, 0.1 ml of 0.01 M PBS containing 0.1% (wt/vol) gelatin, and 1 ml of tracer solution. Tubes were incubated at room temperature for 4 h, decanted, and counted for 1 min in a gamma counter (Cobra II, Packard Instrument Corp. Meriden, CT). The intra-assay and inter-assay CVs was 2.1% and 4.3%, respectively. Testosterone was quantified using a commercial antibody coated tube RIA kit (Diagnostic Products Corp., Los Angeles, CA) according to manufacturer’s directions. Briefly, samples were analyzed in duplicate with 0.05 ml of plasma and 1 ml of 125I testosterone tracer, incubated for 3 hours at 37 C, decanted, and counted for 1 min in a gamma counter (Cobra II, Packard Instrument Corp. Meriden, CT). All samples were run in a single assay with a sensitivity of 0.04 ng/ml.

Statistics

Data were analyzed as a complete randomized design using GLM (General Linear Model of Statistical Analysis System) (Institute, 2000). Means separation was performed using LSMEANS. Means ± SEM were considered different when P<0.05 unless otherwise stated.

Results and Discussion

Body weight of ewes on the OB diet increased (P<0.05) ~30% from diet initiation to mating, and increased (P<0.05) an additional 13% from mating to necropsy, while ewes fed the C diet maintained similar weights from diet initiation to necropsy. Fetal weight and crown rump length of ewes on the OB diet were greater than that of fetuses from ewes on the C diet on day 75 of gestation (Table 1). There was no effect of sex on size or wt of fetuses at necropsy. Concentrations of cortisol were greater (P<0.05) in the blood of ewes and their fetuses on the OB diet than those of ewes and their fetuses on the C diet, regardless of fetal sex (Figure 1). Concentrations of testosterone in the blood of female fetuses of ewes on the OB and C diets were similar (Figure 2). Blood concentrations of testosterone in male fetuses were higher (P<0.05) than that of female fetuses across both dietary groups, but male fetuses of ewes in the OB group had markedly higher (P<0.05) testosterone concentrations than male fetuses of ewes in the C dietary group (Figure 2).

These data demonstrate that the maintenance of maternal obesity and high energy consumption from conception through midgestation results in a marked increase in fetal size and weight. The elevated concentrations of cortisol in the blood of fetuses from OB ewes at midgestation may lead to health problems in postnatal life, as fetal glucocorticoids have been implicated in early-life programming of adult disease. Glucocorticoid receptors are expressed on most fetal tissues from the early embryonic stages, and overexposure may alter the rate of maturation of fetal organs, leading to alterations in structure and function in postnatal life. Further, the elevated circulating levels of testosterone in the blood of male fetuses from OB versus C ewes may alter the development of sexually behaviors in later life through alterations in the growth, maturation and remodeling of the developing CNS.

References

Table 1: Data collected for fetuses on day 78 of gestation in ewes fed 100% or 150% of NRC recommendations

<table>
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<tr>
<th>Treatment</th>
<th>Fetal Wt (g)</th>
<th>Empty Carcass Wt (g)</th>
<th>Crown rump length (cm)</th>
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<td>198 ± 9^a</td>
<td>22.1 ± 0.2^a</td>
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<tr>
<td>OB</td>
<td>374 ± 10^b</td>
<td>283 ± 7^b</td>
<td>24.6 ± 0.2^b</td>
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^a,bMeans ± SEM within a column with different superscripts differ (P<0.05).

Figure 1. Concentrations of cortisol in the blood for male and female fetuses on day 75 of gestation.
Figure 2. Concentrations in the testosterone in the blood of female and male fetuses on day 75 of gestation.
Reproductive Physiology

Impact of Maternal Obesity on Growth and Pancreatic Function in the Fetal Sheep

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Summary and Implications

Maternal obesity is increasing at an alarming rate in the United States, and is thought to be associated with the progressive increase in obesity and type 2 diabetes observed in children and young adults. The sheep is often used as a model to study human pregnancy problems. Ewes were assigned to a control (C, 100% of NRC recommendations, n=5) or obesogenic (OB, 150% of NRC, n=6) diet from 60 days before conception to 75 days of gestation when animals were euthanized and the gravid uterus recovered. Paraffin-embedded fetal pancreatic tissue sections were incubated with guinea pig anti-porcine insulin or mouse anti-glucagon antibodies at 4° C overnight, then with fluorescent labeled 2° antibodies Rhodamine labeled goat anti-guinea pig or AlexaFluor 488 labeled goat anti-mouse for 60 min at 22° C. Insulin and glucagon positive cell number was determined/unit area of pancreatic tissue. OB ewes increased (P<0.05) their body weight by ~50%, while C ewes increased their body weight only ~7% from diet initiation until necropsy. Fetuses from OB ewes were heavier than those from C ewes (374 ± 10 vs. 268 ± 12 g, P<0.05). Although all organs were heavier (P<0.05) in fetuses from OB vs. C ewes, only pancreatic weight was increased relative to fetal body weight (0.05 ± 0.01 vs. 0.12 ± 0.01 %, P<0.05). Numbers of insulin positive cells/unit pancreatic area were 50% greater (P<0.06) in fetuses from OB vs. C ewes, while numbers of glucagon positive cells were similar. Further, both the concentrations of glucose and insulin were elevated (P<0.05) in the blood of OB vs. C ewes (65.5 ± 6.6 vs. 52.1 ± 3.5 mg/dL and 25.0 ± 9.0 vs. 4.8 ± 1.6 uIU/ml, respectively) and fetuses (40.7 ± 4.2 vs. 26.2 ± 2.3 mg/dL and 5.8 ± 0.8 vs. 1.6 ± 0.1 uIU/ml, respectively) on day 75. The increase in blood insulin concentrations of fetuses gestated by OB vs. C ewes may result from increased maternal glucose delivery into the fetal compartment, or may reflect developing insulin sensitivity. The observed acceleration of pancreatic development in OB vs. C fetuses may alter pancreatic function in later life, resulting in alterations in growth efficiency and carcass quality.

Introduction

Recent data from the 1999-2000 National Health and Nutrition Examination Survey (NHANES) (www.cdc.gov/nchs/products/pubs/pubd/hestats/obese/obse99.htm) show that almost 65% of the adult population in the United States is overweight, defined as having a body mass index (BMI) greater than 25 kg/m2, compared to 56% seen in NHANES III, conducted between 1988 and 1994. The prevalence of obesity, defined as BMI greater than 30 kg/m2, has also increased dramatically from 23 to 31% over the same time period. The World Health Organization (WHO) has declared obesity to be one of the top ten adverse health risk conditions in the world and one of the top five in developed nations (www.who.int/nut/obs.htm). Obesity among the women of reproductive age ranges from 20.2 to 34% and children exposed to maternal obesity are at increased risk of developing metabolic syndrome, which suggests that obese mothers even in the absence of gestational diabetes may still have metabolic factors that affect fetal growth and postnatal outcomes. Maternal obesity has been associated with either intrauterine growth restriction (IUGR) or large for gestational age fetuses. Both conditions are connected to offspring exhibiting altered insulin secretion and adiposity. Studies on Pima Indian indicate that the intrauterine environment plays an important role in diabetes development in the offspring. The interplay of factors involved in maternal health and nutrient restriction (NR) both in the pre-conceptional period and during pregnancy is currently under extensive investigation in rodents and sheep. The objective of this study was to evaluate the impact of maternal obesity on fetal growth and pancreatic function at midgestation.

Materials and Methods

Animals

From 60 days before conception (Day 1 of mating = day 0) to day 75 of gestation, multiparous ewes were fed either a highly palatable diet at 100% (control, C; n=5) of
National Research Council (NRC) recommendations or 150% (obeseogenic, OB; n=6) of NRC recommendations on a metabolic BW basis (BW0.75). Immediately prior to necropsy, on day 75, each ewe was weighed and a sample of blood was collected via jugular venipuncture and plasma and serum frozen at -80°C until assayed for glucose and insulin, respectively. Under anesthesia, fetal blood was collected from the umbilical vein, and serum and plasma were collected and stored as described for maternal blood. Ewes were then euthanized and exanguinated, and the gravid uterus quickly removed. Fetal weights, crown rump lengths and sex were determined as well as weights of selected fetal tissues. The weight of the fetal pancreas was recorded and a portion immediately collected and frozen in liquid nitrogen for protein extraction and immunoblotting. The remaining pancreatic tissue was placed in a tissue cassette (Tissue Tek, Miles Labs, Elkhart, IN), fixed with 4% (w/v) paraformaldehyde in a phosphate buffer (0.12 M; pH 7.4) and paraffin embedded.

Immunohistochemistry

Six 5 µm sections were obtained from paraffin-embedded blocks of C and OB fetal pancreatic tissue maintaining at least 50 µm between sections. Paraffin embedded sections were then deparaffinized and hydrated by routine methods before the antigen retrieval procedure. Nonspecific antigenic sites were blocked by a 5-min incubation in 1.5% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS with 0.1% Triton X-100 (Union Carbide Corp, Somerset, NJ) and 0.05% Tween 20 (Bio-Rad Laboratories Inc., Hercules, CA) then for 30 minutes with FX signal enhancer (Invitrogen, Carlsbad, CA) before the sections were incubated with guinea pig anti-porcine insulin primary antibody (Dako, Carpinteria CA, 1:500) and mouse anti-glucagon (Sigma-Aldrich, St. Louis, MO, 1:500) antibodies at 4° C overnight, then with fluorescent labeled 2° antibodies Rhodamine labeled goat anti-guinea pig (Millipore, Billerica, MA 1:500) and AlexaFluor 488 labeled goat anti-mouse (Invitrogen, Carlsbad, CA, 1:500) for 60 min at 22° C. Images were visualized using an Olympus BX50 microscope and captured digitally using a Retiga EXiFast camera. Pictures at 400 X magnification were taken using QED Imaging software (Media Cybernetics, Silver Spring, MD) for 20 fields of view for each section. Insulin and glucagon positive cells were counted for each field of view by two experienced individuals and the counts averaged.

Dot blotting assay

Triplicate 2 µl samples from each extracted SDS-PAGE protein sample were spotted onto a nitrocellulose membrane (0.45µm; Bio-Rad Laboratories Ltd) and air dried under a hood. Then membranes were blocked with 5% nonfat milk powder in TBST (50 mM Tris-HCl, pH7.6, 150 mM NaCl, 0.05% Tween-20,) for 2 h. Blocked membranes were then incubated overnight at 4°C with guinea pig anti-porcine insulin primary antibody (Dako, Carpinteria CA, 1:1000, Cat# A0564). At the end of the primary antibody incubation, the membranes were washed with TBST three times, for 10 min each. After that, membranes were incubated with horseradish peroxidase-conjugated anti-guinea pig secondary antibody (Chemicon, Temecula, CA, 1:5000, Cat # AP193P) for 1 h at RT. After three 15 min washes, membranes were visualized using ECLTM Western blotting detection reagents (Amersham Bioscience) and exposure to film (MR, Kodak, Rochester, NY). The density of each dot was quantified using an Imager Scanner II (Amersham Bioscience) and ImageQuant TL software (Amersham Bioscience).

Glucose and Hormone Analyses

Glucose was analyzed using the InfinityTM (ThermoTrace Ltd, Cat. # TR15498; Melbourne, Australia) colorimetric assay modified in the following manner; plasma was diluted 1:5 in dH2O, and 10µL of diluted plasma was added to 300µL reagent mix. All samples were run in triplicate. The intra-assay and inter-assay CVs are 3% and 5%, respectively. Insulin was measured by RIA in accordance with manufacturer recommendations (Coat-A-Count®, Diagnostic Products Corporation, Los Angeles, CA). When using ovine serum, the intra-assay CV is < 3 %, while the inter-assay CV is < 5%.

Statistics

Data were analyzed as a complete randomized design using GLM (General Linear Model of Statistical Analysis System) (Institute, 2000). Means separation was performed using LSMEANS. Means ± SEM were considered different when P<0.05 unless otherwise stated. Data are presented as means ± SEM and are considered different when P<0.05. Throughout the experiment, n = 5 for C animals and 6 for OB animals unless otherwise stated.

Results and Discussion

OB ewes increased their body 30% from diet initiation to conception and an additional 13% from mating to necropsy, while C ewes maintained similar body weights throughout the experimental period. Fetal body weight and Crown Rump Length were greater (P<0.05) in OB than C ewes on day 75 (374 ± 10g and 24.6 ± 0.2cm vs. 286 ±12g and 22.1±0.2cm, respectively). Most fetal tissue weights from OB ewes were greater (P<0.05) than those from C ewes, but only pancreatic weight was increased (P<0.05) as a percentage of fetal weight. (Table 1). Glucose and insulin concentrations were greater (P<0.05) in both fetal and maternal blood of OB ewes when compared to C ewes (Figures 1 and 2). Numbers of insulin positive cells were ~50% greater (P<0.05) per field in the fetal pancreas of OB versus C ewes (Immunohistochemistry). Insulin concentrations in fetal pancreatic tissue of OB ewes was also greater (P<0.05) than that of C ewes (Figure 3).
Conclusions

In conclusion, we have demonstrated that maternal obesity and access to an increased amount of a highly palatable diet results in maternal and fetal hyperglycemia and hyperinsulinemia by mid gestation in the sheep model, in association with an asymmetric increase in fetal weight. Pancreatic weight increased markedly in fetuses gestated by obese mothers, in part due to an increased mitotic rate of β cells, resulting in increased β cell numbers and insulin content by mid gestation. Increases in β cell numbers and insulin availability during fetal development may be stimulated, in part, by increases in maternal glucose concentrations which are known to diffuse readily into the fetal compartment. Over stimulation of pancreatic endocrine function may define a mechanism whereby the fetus adapts its somatic cell growth to respond to excess nutrient availability. An acceleration of fetal pancreatic growth and cell number, as observed in OB ewes in this study could be expected to alter cellular composition and function of the pancreas in later life. Failure of the pancreas to return to a normal cellular composition and function postnatally could lead to obesity, altered insulin secretion and diabetes in offspring. More specifically, in ruminant species such as sheep and cattle, these changes in pancreatic structure and function could lead to decreased growth efficiency and growth rate of lambs and calves, reducing producer profit.

Table 1. D78 fetal tissue and organ weights (g) from control ewes or ewes

<table>
<thead>
<tr>
<th>Tissues and Organs</th>
<th>Control ewe wt (g)</th>
<th>Fat ewe wt (g)</th>
<th>C ewe wt/unit fetal wt (%)</th>
<th>OB ewe wt/unit fetal wt (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>7.15 ± 0.23</td>
<td>10.12 ± 0.35*</td>
<td>2.69 ± 0.11</td>
<td>2.70 ± 0.069</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.37 ± 0.09</td>
<td>2.09 ± 0.09*</td>
<td>0.51 ± 0.35</td>
<td>0.56 ± 0.012</td>
</tr>
<tr>
<td>Adrenal</td>
<td>0.055 ± 0.004</td>
<td>0.075 ± 0.017</td>
<td>0.021 ± 0.002</td>
<td>0.020 ± 0.005</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.14 ± 0.02</td>
<td>0.47 ± 0.03*</td>
<td>0.05 ± 0.01</td>
<td>0.12 ± 0.01*</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.32 ± 0.04</td>
<td>0.44 ± 0.03*</td>
<td>0.12 ± 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>Lung</td>
<td>6.84 ± 0.36</td>
<td>9.81 ± 0.32*</td>
<td>2.55 ± 0.08</td>
<td>2.63 ± 0.11</td>
</tr>
<tr>
<td>Liver</td>
<td>15.92 ± 0.99</td>
<td>24.26 ± 0.97*</td>
<td>5.93 ± 0.22</td>
<td>6.46 ± 0.15</td>
</tr>
<tr>
<td>Testis</td>
<td>0.069 ± 0.015</td>
<td>0.077 ± 0.005</td>
<td>0.024 ± 0.005</td>
<td>0.021 ± 0.002</td>
</tr>
<tr>
<td>Ovary</td>
<td>0.019 ± 0.003</td>
<td>0.026 ± 0.004</td>
<td>0.008 ± 0.001</td>
<td>0.007 ± 0.001</td>
</tr>
<tr>
<td>St muscle</td>
<td>0.49 ± 0.01</td>
<td>0.65 ± 0.05*</td>
<td>0.20 ± 0.01</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>Ld muscle</td>
<td>2.40 ± 0.16</td>
<td>3.46 ± 0.20*</td>
<td>0.97 ± 0.02</td>
<td>0.95 ± 0.04</td>
</tr>
<tr>
<td>IHeart</td>
<td>2.56 ± 0.14</td>
<td>3.27 ± 0.18*</td>
<td>0.96 ± 0.05</td>
<td>0.87 ± 0.04</td>
</tr>
</tbody>
</table>

Figure 1. Glucose content in both maternal and fetal serum
Figure 3. Insulin content in both maternal and fetal serum.

Figure 3. Insulin dot blot of pancreas tissues. Top shows representative insulin dot blot of pancreas tissues on day 78 gestation; Bottom shows group means ± SEM (n=5 per group*: P<0.05).
Maternal Nutrition Influences Expression of Fetal Steroids and their Receptors

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Summary and Implications

Sexual differentiation of the brain is thought to occur between d 30 and d 70 in the fetal lamb. The uterine environment may affect fetal expression of steroids and their receptors altering adult animal endocrine systems. To determine how maternal over-nutrition affects synthesis of fetal steroids and their receptors, fetuses were collected from ewes fed at 100\% (Control; \(n = 5\)) or 150\% (Obese; \(n = 6\)) of NRC requirements from 60 d prior to breeding until collection at 75 d of gestation. Fetal serum concentrations of testosterone were determined in umbilical blood. Neural tissues from fetuses twinned male/female were utilized in this study. Hypothalamic and amygdala RNA was isolated and message for progesterone, estrogen, and androgen receptors were quantified. Concentration of testosterone was greater in male fetuses compared to female fetuses. Male fetuses from Obese ewes had greater serum concentrations of testosterone than male fetuses from Control ewes, but differences in testicular steroidogenic enzymes mRNA were not detected. Quantity of Hypothalamic estrogen receptor (ER) \(\beta\), but not ER-\(\alpha\), RNA tended to differ by a sex by treatment interaction. Estrogen receptor \(\beta\) message was greater in female fetuses compared to male fetuses from Control-fed ewes. Although there were no differences in ER-\(\beta\) RNA among male fetuses, message tended to be decreased in female fetuses from obese ewes compared to those from Control ewes, and did not differ from male fetuses. Expression of RNA for hypothalamic progesterone receptor tended to be greater in male fetuses than female fetuses at d 75 of gestation, but did not differ by dam nutritional treatment or any sex by treatment interaction. Hypothalamic RNA for the androgen receptor did not differ by sex, dam nutritional treatment, or their interaction. Amygdala RNA for the estrogen or androgen receptor did not differ by sex, treatment, or their interaction. Over nutrition of the dam appears to alter the expression of the estrogen receptor in the female fetuses while increasing serum concentrations of testosterone in male fetuses which may affect the adult response to stress, expression of sexual behavior and/or the pattern of gonadotropin release in response to gonadal steroids.

Introduction

Brain development of the fetal sheep is sensitive to exposure to fetal gonadal steroids. Exposure of sheep fetuses to steroids during critical periods of neural development causes permanent changes in the brain (Ford and D’Occhio, 1989). The fetal testis, through the actions of testosterone or its metabolite (estradiol), actively masculinizes and defeminizes the male brain resulting in sex-typical behavior and a differential response of the gonadotropin hormones to steroidal feedback (Roselli, 2007). Steroids have permanent effects on the brain through specific binding of their receptors. During this critical period of development, changes in the steroid receptors or exposure to altered steroid concentrations have the potential to permanently affect the expression of adult sexual behavior, fertility (through effects on gonadotropin release; Roselli, 2007), and the response to stress (Weiser et al., 2007). It was hypothesized that maternal over-nutrition could affect neural development through changes in the expression of steroid receptors.

Materials and Methods

Animal care and use was approved by the University of Wyoming Animal Care and Use board.

Animals

Sixty days prior to mating, multiparous, whitefaced crossbred ewes were weighed and individually fed a diet supplying either 100\% (Control; \(n = 5\)) or 150\% (Obese; \(n = 6\)) of National Research Council (NRC, 1990) recommended dietary requirements. At d 75 of pregnancy ewes were weighed and anesthetized. Under anesthesia fetal blood was collected from the umbilical vein. Following blood collection, ewes were killed by heart puncture and fetuses were collected and weighed. Empty carcass weights for ewes and lambs were collected following evisceration.

Tissue collection
Brains were removed from the fetal cranium, mid-sagittally sectioned and dissected using surface landmarks. The hypothalamus (including the preoptic area) consisted of tissue caudal to the optic chiasm to the posterior border of the mammillary bodies and dorsally to the roof of the third ventricle. The amygdale consisted of a block of tissue from the ventromedial temporal lobe with approximately the same rostral-caudal dimension of the hypothalamus containing entorhinal cortex as well as the major cortical, medial and basal amygdaloid nuclei. Brain tissue was snap frozen and stored at -80°C until RNA extraction. Only brain tissue from male/female twinned fetuses were utilized in this study. Twelve male/female twinned fetuses, balanced by dam dietary treatment, were utilized in this study.

**RNA Isolation and cDNA synthesis**

Total Cellular RNA was isolated using TRI Reagent (Sigma Chemical; St. Louis, MO). Briefly, 100mg brain tissue (hypothalamus and amygdala) was homogenized in 1 mL of Tri Reagent and allowed to sit at room temperature for 5 minutes before adding 0.2 mL chloroform. After 10 minutes, the homogenate was centrifuged for 15 minutes at 4°C at 12,000 rpm. The aqueous layer was transferred to a new tube and the RNA was precipitated with 0.5 mL of isopropanol by centrifuging for 10 minutes at 12,000 rpm. The RNA pellet was washed once with 70% ethanol and suspended in 100 μL RNAse free water. The concentration was determined using a NanoDrop spectrophotometer and 10 μg was further purified using RNEASY (Qiagen Inc; Santa Clara, CA) with on-column DNase digestion. Approximately 2.0 μg of RNA was mixed with 4 μL reverse transcription buffer (5X) and 1 μL of iScript reverse transcriptase (Bio-Rad Laboratories, Richmond, CA). The mixture was placed in a thermocycler for 5 minutes at 25°C, 30 min at 42°C, 5 min at 85°C and held at 4°C. The cDNA was diluted with 100 μL nuclease free water and stored at -20°C until quantitative PCR was performed.

**Semi-Quantitative Real Time PCR**

Diluted cDNA (10 μl) was used as a template for semi-quantitative Real Time PCR amplification in 25 μL reactions consisting of 12.5 μL SYBR Green Supermix (Bio-Rad Laboratories), 0.5 μL H2O and 1μL each forward and reverse primer. Ovine GAPDH, androgen receptor (AR), estrogen receptor (ER) beta, ER-α, and progesterone receptor (PR) primers were designed using Primer 3 software to generate ~ 100bp amplicons. Semi-quantitative RT-PCR was performed using 40 cycles of 95°C for 30 sec and 62°C for 30 sec. Following amplification, cDNAs were melted (melting curve analysis) to ensure the quality of amplification by incubating RT-PCR products for 10 sec at each step with increase in temperature by 0.5°C from 55°C to 95°C in each cycle. AR, ER-β, ER-α and PR mRNA expression levels were quantified and reported relative to GAPDH.

**Hormone analysis**

Fetal blood was allowed to clot overnight at 4°C. Serum was separated by centrifugation at 1500 g for 20 min and was stored at -20°C until analysis for concentrations of serum testosterone. Hormones were quantified in duplicate according to manufacturer’s directions using a commercially available solid phase RIA kit (Diagnostic Products Corp., Los Angeles, CA). Testosterone was quantified in a single assay with a sensitivity of 0.04 ng/ml.

**Statistical analysis**

Data were analyzed as a complete randomized design utilizing GLM methods of SAS (Ver. 9.1, Cary, NC). Preplanned means comparisons, when appropriate, were analyzed utilizing PDIFF option of GLM; LSMEANS ± SEM are reported.

**Results and Discussion**

Ewes fed 150% of NRC increased (≥ 30%; P < 0.05) body weight from diet initiation until breeding and increased an additional 13% from mating until tissue collection. Ewes on the control diet maintained weight from diet initiation until tissue collection. Weights and crown rump length were greater in fetuses from obese ewes than control fed ewes (See Ford et al., this volume).

Serum concentrations of testosterone were greater (P < 0.05) in male than in female fetuses, and differed (P < 0.05) by sex by ewe-diet interaction. Although serum concentrations of testosterone did not differ by ewe-diet in female fetuses, concentrations of testosterone was increased (P < 0.05) in male fetuses from obese ewes compared to fetuses from control-fed ewes. Since it was not known whether differences were due to changes in steroid synthesis or steroid clearance, mRNA for testis steroidogenic enzymes were determined. Message for the first and rate limiting enzyme for the biosynthesis of all steroids, CPY11A necessary for the conversion of cholesterol to pregnenolone (Payne and Hales, 2004), did not differ among fetal sex or maternal dietary regimen. Message for other steroidal enzymes (3βHSD – conversion of pregnenolone to progesterone through the delta four steroidogenic pathway, CYP17A- conversion of pregnenolone through the delta five pathway, or 17βHSD-conversion of testosterone to estradiol) did not differ among male fetuses from control or obese ewes. Differences in fetal serum testosterone were, therefore, not due to changes in synthesis but may be attributed to alterations in steroid clearance. Lipophilic testosterone would likely be stored to a greater extent in the fatter fetuses from obese ewes. Although expression of AR in the hypothalamus or amygdale did not differ by sex, ewe dietary treatment or their interaction (P ≥ 0.25; data not reported), the increased exposure of the developing brain to testosterone could affect neural development by increased activation of the expressed receptors.
Fetal expression of RNA for hypothalamic ER-β did not differ by ewe diet (P = 0.28) or sex (P = 0.17) but tended (P = 0.1) to differ by a sex by treatment interaction (Figure 1). Ewe diet did not (P = 0.7) affect expression of ER-β RNA in the male fetuses, but female fetuses from obese ewes tended (P = 0.06) to have decreased expression of ER-β compared to fetuses from control-fed ewes (Figure 1) and expression did not differ from male fetuses. Fetal expression of amygdala RNA for ER-β did not differ by sex or ewe diet or any sex by diet interaction (P ≥ 0.6). ER-β is expressed in discrete nuclei of the hypothalamus, but does not appear to be necessary for normal reproductive function. ER-β may, however, be involved in the control of anxiety behaviors and the response to stress (Weisner et al., 2007). Changes in the expression of ER-β during critical periods of neural development may alter adult behavior and the response to stress through the hypothalamic-pituitary-adrenal axis. ER-α is necessary for normal reproductive function (Weisner et al., 2007). Similar to Roselli et al. (2006) differences in the expression of ER-α were not detected.

Expression of hypothalamic PR RNA tended (P = 0.06) to be increased in male more than in female fetuses (Figure 2). In addition there was a one fold increase (P = 0.06) in fetal expression of PR RNA in fetuses from control compared to obese ewes (Figure 2). There was no ewe-diet by fetal-sex interaction (P = 0.6). Roselli et al. (2006) also noted an increase in the expression of hypothalamic progesterone receptor in 65 d fetuses. Differences in the expression of the PR at this developmentally relevant time period suggests that progesterone acting at its receptor may facilitate sexual differentiation of the brain.

In conclusion, the maternal environment affects expression of hypothalamic ER-β, and fetal serum concentrations of testosterone which may affect adult sexual behavior and the response to stress.

References


Anticancer efficacies of cisplatin-releasing pH-responsive nanoparticles.

P Xu, EA Van Kirk, Y Zhan, DD Isaak, M Radosz, Y Shen, and WJ Murdoch. The objective of these investigations was to test the hypothesis that a rapid cytoplasmic release profile from nanoparticles would potentiate the anticancer activity of cisplatin. Cisplatin-loaded nanoparticles with pH-responsive poly[2-(N,N-dieethylamino)ethyl methacrylate] (PDEA) cores were synthesized from PDEA-block-poly(ethylene glycol)(PDEA-PEG) copolymer using a solvent-displacement(acetone-water) method. Nanoparticles with pH-nonresponsive poly(epsilon-caprolactone)(PCL) cores made from PCL-block-PEG (PCL-PEG) were used for comparison. Nanoparticle sizes, zeta potentials, drug-loading capacities, and pH responsiveness were characterized. Cellular uptake and localization in lysosomes were visualized using confocal fluorescence microscopy. Cytostatic effects of free and encapsulated cis-diamminedichloroplatinum(II) dichloride (cisplatin) toward human SKOV-3 epithelial ovarian cancer cells were estimated by using the MTT metabolic activity assay. Intraperitoneal tumor responses to cisplatin and cisplatin/PDEA-PEG were evaluated in athymic mice at 4-6 weeks postinoculation of SKOV-3 cells. PDEA-PEG nanoparticles dissolved at pH < 6 and rapidly internalized and transferred to lysosomes; it therefore was predicted that the PDEA nanoparticles would release cisplatin upon integration into acidic lysosomes and thereby overwhelm the chemoresistant properties of SKOV-3 cells. Indeed, relative proportions of viable cells were diminished to a greater extent by exposure in vitro to fast-releasing compared to slow-releasing nanoparticles or an equivalent dose of free cisplatin. Incidences of cellular pyknosis (a morphological indicator of apoptosis) were most evident within intestinal/mesentery tumors of mice treated with cisplatin/PDEA-PEG; tumor burdens were correspondingly reduced.

Economics of feeding cull cows using different management strategies.

Steve Paisley, Extension Beef Cattle Specialist, Frances Niemela, Graduate Research Assistant

Chris Loehr, Graduate Research Assistant UW Animal Science, SAREC. In many parts of the Four-State region, corn is not the most cost-effective source of energy for beef cattle diets. However, Many ranchers in the Four-State region do not have feeding facilities or appropriate mixing equipment on their operations. This project is designed to investigate the effect of three different feeding systems (using different feed ingredients and equipment) on the performance and carcass characteristics of cull cows and the economics associated with each of the systems. The cull cow project will include the feeding of cull cows at three locations, the Cottonwood Range and Livestock Research Station (Cottonwood), Philip, SD, the Hettinger Research and Extension Center (HREC), Hettinger, ND; and the Sustainable Agriculture Research and Extension Center (SAREC), Lingle, WY. Cattle were purchased in November, 2007 from local auction markets within 100 miles of each station during November of 2007. The initial purchase serves as a control in the economic analysis. Cows purchased for the UW study at the James C. Hageman SAREC near Lingle, WY averaged 1129 lbs, and ranged in age from 3 to 10+ years of age. All cows were vaccinated, dewormed, and assigned to one of three treatments:

1) CON – corn-based control diet fed using traditional feeding methods.
   UW-SAREC diet: alfalfa/grass hay and barley straw, rolled corn and feedlot supplement containing Rumensin®.

2) LOCAL – diet based on locally-grown feeds, fed using traditional feeding methods
   UW-SAREC diet: corn silage and rolled corn, with commercial feedlot supplement containing Rumensin®.

3) LIMIT – diet based on corn and a commercial supplement formulated to control intake. This diet will be fed using self-feeders and utilize little or no hay.
   UW-SAREC diet: Purina accuration and impact self-fed rations.

All diets are formulated to contain approximately 60 Mcal NEg/cwt and 11.5% crude protein. All cows were implanted with Finaplix-H® (200 mg trenbolone acetate) upon arrival. At the conclusion of the 100 day feeding period, cows will be shipped from each location to Cimpl Meats in Yankton, SD, where carcass data (hot carcass weight, backfat, longissimus muscle area, marbling, lean color, fat color) will be collected.


Ram selection is fundamental to the profitability of a flock and is based on desired physical and performance traits. Selection processes, however, rarely include an evaluation of sexual behavior even though the ability and desire to mate with ewes in estrus is required for the incorporation of superior genetics into a flock. Differences in mating behavior exist among individuals of all species studied. If evaluated, as many rams could be culled for poor mating behaviors as are culled for physical limitations or poor semen quality. Stellflug and co-workers (2006) confirmed that twice as many poor-performing rams were needed to obtain breeding results equal to a single high-sexually performing ram. Low mating behavior results in
the need for additional rams, extends the lambing season, and decreases the number of lambs born per ewe lambing (SID, 1996; Carr et al., 2001). Based on serving-capacity tests, 29.6% of rams at the USDA-ARS Sheep Experiment Station (USSES) were identified as non-performers. Although sexual interest, or libido, of rams is necessary for the incorporation of superior genetics into a flock, ram mating behavior is rarely evaluated due to constraints of time, labor, and physical facilities necessary for such tests. Because the economic benefit of behavior testing rams is high, alternative means of identifying rams with undesirable mating behavior is warranted.

Ninety-eight percent of the 68,280 sheep producers in the U.S. have small and medium sized operations with less than 500 breeding animals (NASS 2005) generating less than $500,000 per year. This proposed research seeks to enhance economic opportunities for agriculture producers by identifying and eliminating rams with poor reproductive performance. The elimination of those rams would save sheep producers $13.5 million dollars in ram costs, decrease the number of rams required in breeding flocks, and decrease grazing pressure on natural resources. The overall objective of this project is to increase the profitability of sheep production by eliminating non- and low- performing rams from the flock.

**Insulin and glucose dynamics in mature ewes born from nutrient restricted dams.**

*Lindsey George, PhD student and Stephen Ford, Professor and Rochelle Chair.* Both under- and overnourishment during pregnancy have been linked to a predisposition for the development of a variety of health problems (e.g. cardiovascular disease, altered growth, dyslipidemia, and metabolic abnormalities) in the resulting offspring. Ewes born from nutrient restricted (NR) dams at the Center for the Study for Fetal Programming represent a valuable model for the study of the effects of maternal NR on metabolism and reproductive efficiency in mature offspring. It has been observed in our lab that female offspring of maternal NR dams exhibit altered progesterone concentrations throughout the estrous cycle compared to those born from control (C) dams. Other research also suggests changes in insulin sensitivity occurring throughout the menstrual/estrous cycle and throughout pregnancy, possibly linked to sex steroid concentrations. We intend to confirm our previous observation of differences in progesterone concentrations throughout the estrous cycle and simultaneously evaluate glucose and insulin dynamics in mature ewes born from NR versus C dams. Additionally, we hypothesize that ewes born from NR dams will exhibit different insulin sensitivity and glucose dynamics through pregnancy compared to C offspring. From d 28 to 78 of gestation, dams were fed either a NR (50% NRC requirements) or C (100% NRC requirements) diet. Two to four year old ewes born from the NR (n=15) and C (n=14) dams will be monitored daily for estrus and blood sampled d 0, 3, 6, 9, and 12 of their cycle, as well as at the time of the next estrus. A glucose tolerance test will be conducted in a subset of these ewes around d 10 of their estrous cycle and again, following breeding, at approximately d 45, 90, and 135 of pregnancy. Basal glucose, insulin, insulin:glucose ratio, and indices of insulin sensitivity based on basal glucose and insulin concentrations will be used to evaluate glucose and insulin dynamics throughout the estrous cycle, and glucose tolerance tests will be appropriately assessed for estimation of insulin sensitivity and glucose dynamics prior to and throughout pregnancy. A better understanding of the links between maternal nutrition, fetal development and energy metabolism will help producers optimize nutrition in reproducing animals, potentially enhancing feed efficiency, improving animal health, and optimizing growth and fat deposition. Also, understanding how energy metabolism in mature animals is influenced by poor nutritional conditions in utero will further our knowledge of factors contributing to metabolic diseases and associated morbidities in the human population (e.g. obesity, diabetes, cardiovascular disease).