Contrasting Effects of a Dietary Copper Deficiency in Male and Female Mice (43697)

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Abstract. **Female rats are protected from the lethal effects of a dietary copper (Cu) deficiency, but female mice fed a Cu-deficient diet develop atrial thromboses and die. To further investigate the effect of sex on Cu status in mice (n = 16), male and female adult Swiss-Webster mice were fed Cu-supplemented (8.4 mg Cu/kg) or Cu-deficient (0.3 mg Cu/kg) diets with deionized water for 43–49 days. Six female mice, but only one male mouse, fed the Cu-deficient diet died during the experiment. Both male and female mice fed the Cu-deficient diet exhibited typical features of deficiency. The severity of anemia and the values observed for several indicators of Cu status (plasma ceruloplasmin [EC 1.16.3.1.] and erythrocyte copper-zinc superoxide dismutase [EC 1.15.1.1.] activities, cardiac Cu) were similar in both male and female Cu-deficient mice. However, cardiac enlargement (0.97 vs 0.73 g/100 g body wt, $P < 0.05$), cardiac edema (79.9% vs 78.2% cardiac water, $P < 0.05$) and depletion of renal Cu (10.4 vs 12.5 μg/g dry weight, $P < 0.05$) were more severe in female compared with male, Cu-deficient mice. Furthermore, although hepatic Cu was significantly ($P < 0.05$) lower in female Cu-deficient compared with Cu-supplemented mice, it was not significantly decreased by deficiency in male mice. These data indicate that the female mice experienced a more extreme form of Cu deficiency than the males.**

[P.S.E.B.M. 1994, Vol 205]

There is considerable evidence, from experiments using rats, that the sex of an animal mediates the effects of copper (Cu) deficiency (1–8). Most experiments have shown that female rats are protected from the deleterious effects of a dietary Cu deficiency (1–3, 5–7). However, two studies (4, 8) have shown that under certain conditions female rats fed a Cu-deficient diet may develop typical signs of deficiency. Furthermore, a previous study in this laboratory (9) found that female mice fed a Cu-deficient diet died with massive occlusive atrial thromboses. However, male mice were not included in the experiment. In the present investigation, male and female mice were fed Cu-supplemented or Cu-deficient diets and several indices of Cu status were measured in an attempt to further elucidate the mediating role of sex on the effects of Cu deficiency.

Materials and Methods

Groups (n = 16) of 6-week-old male and female Taconic Swiss-Webster mice (Taconic Farms, Germantown, NY)[2] were fed Cu-supplemented (8.4 mg Cu/kg diet) or Cu-deficient (0.3 mg Cu/kg diet) diets (Table I) *ad libitum* with deionized water for 43–49 days. Dietary Cu was determined by flame atomic absorption following $H_2SO_4/HNO_3/H_2O_2$ digestion (10) with National Institute of Standards and Technology (NIST) citrus leaves (1572) as a standard. At the end of the experiment mice were anesthetized with sodium pentobarbital and exsanguinated from the inferior vena cava. Blood (0.5 ml) was collected in microcentrifuge tubes containing 3.8% (w/v) trisodium citrate (0.026 ml) as anticoagulant (11). The blood:anticoagulant ratio was altered from that usually used (9:1) as plasma from these mice would be used for coagulation studies (12, 13). Platelet-poor plasma (PPP) was prepared by centrifugation of whole blood at 2000g for 20 min in a refrigerated (4°C) centrifuge and stored at

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Received September 20, 1993. [P.S.E.B.M. 1994, Vol 205]
Accepted October 27, 1993.

0037-9727/94/2052-0190$10.50/0

Table I. Diet Composition[a]

Diet component	g/kg Diet
Sucrose[b]	544.5
Salt mix[c]	70.5
l-Cysteine[d]	5.0
Vitamin-free casein[d]	80.0
Vitamin mixture[e]	20.0
Lard[f]	280.0

[a] Diets, salt and vitamin mixes were prepared in our diet kitchen using the sources given below or, unless otherwise indicated, reagent grade chemicals.
[b] American Crystal Sugar Co., Moorhead, MN.
[c] Modified Salt Mixture No 2 (USP XIII). Contained (g/kg diet): $Ca(H_2PO_4)_2 \cdot H_2O$, 5.85; $CaCO_3$, 3.37; $Fe(C_6H_5O_7) \cdot 5H_2O$, 1.19; $MgSO_4$, 5.48; K_2HPO_4, 9.59; $NaH_2PO_4 \cdot H_2O$, 3.09; NaCl, 1.74; $MnSO_4$, 30.76 (mg/kg); KI, 0.66 (mg/kg); $Zn(C_2H_3O_2)_2 \cdot 2H_2O$, 167.82 (mg/kg); $CuSO_4 \cdot 5H_2O$, 39.28 (mg/kg); sucrose, 39.96. Copper-deficient diets contained an identical salt mix but with $CuSO_4 \cdot 5H_2O$ omitted and sucrose, 40.00 g/kg diet.
[d] ICN Nutritional Biochemicals, Cleveland, OH.
[e] Contained (mg/kg diet): *p*-aminobenzoic acid, 110; ascorbic acid, 1,000; *i*-inositol, 110; nicotinamide, 100; *alpha*-tocopherol acetate (powder, 250 IU/g), 110; retinol concentrate (500,000 IU/g), 100; biotin, 0.44; Ca pantothenate, 2; cyanocobalamin, 0.03; menadione, 0.05; pyridoxine HCl, 0.02; riboflavin, 0.02; thiamin HCl, 0.02; ergocalciferol concentrate (500,000 IU/g), 0.005; sucrose, 18.47 (g/kg).
[f] Armour & Co., Phoenix, AZ.

−70°C. Erythrocyte pellets were also stored at −70°C.

Plasma total cholesterol (TC) was determined by an enzymatic colorimetric method (Sigma Procedure No. 352; Sigma Diagnostics, St. Louis, MO). Plasma ceruloplasmin (EC 1.16.3.1.; Cp) and erythrocyte copper-zinc superoxide dismutase (EC 1.15.1.1.; CuZnSOD) activities were measured by standard methods (14, 15). Protein estimations were performed on erythrocyte lysates assayed for CuZnSOD activity by Pierce BCA Protein Assay Reagent (Pierce Catalog No. 23225 B; Rockford, IL). Organ metals were determined by flame atomic absorption spectrophotometry following lyophilization and $H_2SO_4/HNO_3/H_2O_2$ digestion (10) with NIST bovine liver (1577a) as a standard.

Data were analyzed (16) by two-way analysis of variance (ANOVA). Scheffé contrast analyses were performed on data from any ANOVA with a significant ($P < 0.05$) Cu × sex interaction. Mortality data were evaluated by the Mantel-Haenszel method (17).

Results

Mice were allocated initially to treatment groups so that male and female groups fed the Cu-supplemented or Cu-deficient diet were weight-matched to within 0.5 g (Table II). Female mice were significantly ($P < 0.001$) smaller than male mice at the start of the study. However, at the end of the study, female mice fed the Cu-supplemented diet had increased their body weight to approximately equal that of the male mice (both Cu-supplemented and Cu-deficient) while female mice fed the Cu-deficient diet failed to grow and exhibited a slight decrease in body weight (Table II). The failure of the Cu-deficient female mice to gain weight was a direct result of decreased food intake in this group (Lynch & Klevay, personal observation). A significant ($P < 0.01$) Cu × sex interaction was observed for liver size and Scheffé contrast analysis revealed that Cu-adequate males had significantly ($P < 0.05$) larger livers than any other group. The reason for increased liver size in this group compared with all other groups is unknown.

Six (of 16) female mice fed the Cu-deficient diet died (Days 40–48) during the experiment. In contrast, only one (of 16) male mouse fed the Cu-deficient diet died (Day 43). The difference in mortality was significant ($P < 0.05$) and estimates of common relative risk indicated that female, compared with male, mice had a 6-fold higher risk for mortality when fed the Cu-deficient diet. Postmortem examination revealed that both male and female Cu-deficient mice had enlarged hearts but only female Cu-deficient mice exhibited atrial thromboses similar to those reported previously (9).

Table II. Initial and Final Body Weights and Relative Liver Size for Male (M) and Female (F) Mice Fed Copper-Supplemented (S) or Copper-Deficient (D) Diets[a]

	M-S	F-S	M-D	F-D	Cu[b]	Sex[b]	Cu × Sex[b]
Initial body weight (g)	31.5 (0.4)	25.9 (0.3)	30.9 (0.5)	26.0 (0.2)	NS[c]	0.001	NS
Final body weight (g)	33.8[e] (0.7)	32.5[e] (0.8)	31.7[e] (0.5)	25.3[f] (0.6)	0.001	0.001	0.001
Liver (g/100 g)[d]	6.7[e] (0.3)	5.2[f] (0.2)	5.2[f] (0.3)	5.0[f] (0.1)	0.001	0.001	0.01

[a] Results given as mean (standard error).
[b] $P <$ values from 2-way ANOVA.
[c] NS, Not significant.
[d] Reported as g/100 g final body weight.
[e,f] Means in the same row with different superscripts are significantly ($P < 0.05$) different (Scheffé contrast analysis).

A number of cardiovascular parameters are reported in Table III. Cardiac wet and dry weights were significantly ($P < 0.001$) increased in both male and female Cu-deficient mice, and female mice had significantly ($P < 0.05$) lower cardiac dry weights than males. A significant ($P < 0.01$) Cu × sex interaction was noted for cardiac wet weight. Significant ($P < 0.001$) Cu × sex interactions were also observed for both cardiac water and cardiac size. Cu deficiency significantly ($P < 0.001$) increased cardiac water and cardiac weight and female Cu-deficient mice had significantly ($P < 0.05$; Scheffé contrast) more cardiac water and heavier hearts than any other experimental group. Cu deficiency significantly lowered hematocrit ($P < 0.001$) in both male and female mice, and a significant ($P < 0.001$) Cu × sex interaction was observed for this parameter.

A statistically significant ($P < 0.01$) Cu × sex effect was observed for plasma Cp activity (Table IV) and Scheffé contrast analysis showed that Cu deficiency significantly ($P < 0.05$) lowered Cp activity only in male mice. Although female Cu-deficient, compared with Cu-supplemented, mice had lower Cp activity, this effect was not statistically significant ($P = 0.11$; Scheffé contrast). The Cp activity was significantly ($P < 0.05$; Scheffé contrast) lower in Cu-supplemented female, compared with male, mice. Significantly lower ($P < 0.001$) erythrocyte CuZnSOD activity was observed in Cu-deficient, compared with Cu-supplemented, mice (Table IV). Plasma TC (Table IV) was uninfluenced by Cu status but was significantly ($P < 0.05$) lower in female, compared with male, mice.

Results from the organ metal analyses are given in Table V. ANOVA showed that Cu deficiency significantly ($P < 0.01$) decreased hepatic Cu in Cu-deficient female mice. A nonsignificant decrease was observed in the corresponding male groups. Significant Cu × sex interactions were found for hepatic ($P < 0.05$), cardiac ($P < 0.01$) and renal ($P < 0.001$) Cu. Cu deficiency significantly ($P < 0.001$) decreased both cardiac and renal Cu and this effect was exacerbated in female, compared with male, mice. Statistically significant ($P < 0.001$) and independent effects of Cu deficiency and sex on hepatic iron (Fe) were observed. Cu-deficient mice had higher hepatic Fe than their Cu-supplemented counterparts and females exhibited higher hepatic Fe than males. No statistically significant effects of either Cu status or sex were observed for hepatic zinc (Zn).

Discussion

Conditions of Cu deficiency have been defined under which male rats are more likely to die than females (2, 3, 7). In the current investigation, however, female mice had a 6-fold higher risk for mortality than males when fed the Cu-deficient diet. Although Cu-deficient male mice had a lower mortality risk than females in this experiment, these animals exhibited a number of the typical features of Cu deficiency. Cu-deficient, compared with Cu-supplemented, male mice had lower hematocrits, enlarged hearts, decreased Cp and CuZnSOD activities, increased hepatic Fe and decreased cardiac and renal Cu (Tables III–V). However, Cu-deficient male mice did not have significantly lower hepatic Cu than Cu-supplemented males (Table V). Female Cu-deficient mice exhibited features similar to those observed in male Cu-deficient mice (Tables III–V). In contrast, however, Cu-deficient female mice did not have significantly lower Cp activity but did have significantly lower hepatic Cu than female Cu-supplemented mice (Tables IV and V). In a separate experiment, we have found similar Cp activities in Cu-supplemented and Cu-deficient female mice which

Table III. Cardiovascular Parameters in Male (M) and Female (F) Mice Fed Copper-Supplemented (S) or Copper-Deficient (D) Diets[a]

	M-S	F-S	M-D	F-D	Cu[b]	Sex[b]	Cu × Sex[b]
Cardiac wet weight (g)	0.171[e] (0.004)	0.145[e] (0.004)	0.231[f] (0.010)	0.245[f] (0.010)	0.001	NS[c]	0.01
Cardiac dry weight (g)	0.040 (0.001)	0.035 (0.001)	0.050 (0.002)	0.049 (0.002)	0.001	0.05	NS
Cardiac water (% wet weight)	76.7[e,f] (0.2)	76.0[e] (0.4)	78.2[f] (0.4)	79.9[g] (0.4)	0.001	NS	0.001
Cardiac weight (g/100 g)[d]	0.51[e] (0.01)	0.45[e] (0.01)	0.73[f] (0.04)	0.97[g] (0.04)	0.001	0.001	0.001
Hematocrit (% PCV)	33.4[e] (2.2)	40.0[e] (1.1)	10.4[f] (1.5)	5.5[f] (1.0)	0.001	NS	0.001

[a] Results given as mean (standard error).
[b] $P <$ values from 2-way ANOVA.
[c] NS, Not significant.
[d] Reported as g/100 g body weight.
[e,f,g] Means in the same row with different superscripts are significantly ($P < 0.05$) different (Scheffé contrast analysis).

Table IV. Plasma Ceruloplasmin (Cp) Activity, Erythrocyte Copper-Zinc Superoxide Dismutase (CuZnSOD) Activity and Plasma Total Cholesterol (TC) in Male (M) and Female (F) Mice Fed Copper-Supplemented (S) or Copper-Deficient (D) Diets[a]

	M-S	F-S	M-D	F-D	Cu[b]	Sex[b]	Cu × Sex[b]
Cp	30.0[e]	15.6[f]	4.3[f]	4.8[f]	0.001	0.01	0.01
(Units/l)	(3.4)	(1.8)	(0.7)	(0.3)			
CuZnSOD	1.07	0.98	0.31	0.18	0.001	NS[c]	NS
(Units/mg)[d]	(0.11)	(0.04)	(0.06)	(0.04)			
TC	108	96	109	78	NS	0.05	NS
(mg/dl)	(10.0)	(12.4)	(7.6)	(4.4)			

[a] Results given as mean (standard error).
[b] $P <$ values from 2-way ANOVA.
[c] NS, Not significant.
[d] Reported as Units/mg erythrocyte protein.
[e,f] Means in the same row with different superscripts are significantly ($P < 0.05$) different (Scheffé contrast analysis).

were significantly different (Lynch & Klevay; unpublished data). The altered Cp activity reported here in Cu-deficient female mice is, therefore, thought to be physiologically relevant even though it was not found to be statistically significant. It is of interest to note that cardiac enlargement, cardiac edema and depletion of hepatic and renal Cu were more extreme in female, compared with male, Cu-deficient mice (Tables II–IV). Cu deficiency is usually associated with hypercholesterolemia (18). However, although both male and female mice fed the Cu-deficient diet in this experiment exhibited a number of features consonant with Cu deficiency (anemia, heart enlargement, decreased CuZnSOD activity, decreased organ Cu, and increased hepatic Fe, 18), hypercholesterolemia was absent (Table IV). This observation is in agreement with a previous experiment (9) performed in this laboratory using the same diet fed to female Taconic Swiss-Webster mice. The reason for the lack of effect of Cu deficiency on plasma total cholesterol under these experimental conditions is presently unknown.

Although it has been suggested that the cardiac enlargement associated with Cu deficiency (19–21) is due to decreased coronary resistance and hemodynamic overload related to anemia (22, 23), anemia cannot explain all of the enlargement (19). Recent evidence indicates that edema may play a role in the cardiac enlargement of Cu deficiency (24, 25). In the current investigation, anemia was severe in both male and female Cu-deficient mice but cardiac enlargement was greater in Cu-deficient female mice (Table III). Furthermore, although cardiac water followed the same pattern as enlargement (Table III) with edema greatest in the female Cu-deficient mice, the relative change in cardiac size associated with Cu-deficiency was much greater than the corresponding change in cardiac water in both male and female mice. These results tend to indicate that although edema, rather than anemia, may be a more important determinant of cardiac enlargement in Cu-deficient mice, other factors, such as mitochondrial swelling (26, 27) and increased mitochondrial myofibrillar volume density (28), may also be relevant to the pathogenesis of this phenomenon.

Increased hepatic Fe is a sensitive indicator of Cu deficiency (29). Both male and female Cu-deficient

Table V. Hepatic, Cardiac, and Renal Trace Mineral Analyses from Male (M) and Female (F) Mice Fed Copper-Supplemented (S) or Copper-Deficient (D) Diets[a]

	M-S	F-S	M-D	F-D	Cu[b]	Sex[b]	Cu × Sex[b]
Hepatic Cu	8.0[e]	10.8[f]	7.2[e]	6.6[e]	0.001	NS[c]	0.05
(μg/g)[d]	(0.9)	(0.6)	(0.8)	(0.2)			
Hepatic Fe	0.49	1.36	1.88	2.67	0.001	0.001	NS
(mg/g)	(0.05)	(0.16)	(0.22)	(0.17)			
Hepatic Zn	65.0	60.7	70.5	68.9	NS	NS	NS
(μg/g)	(11.5)	(3.8)	(8.6)	(2.5)			
Cardiac Cu	27.6[e]	28.5[e]	19.7[f]	16.8[f]	0.001	NS	0.01
(μg/g)	(0.6)	(0.5)	(0.9)	(0.3)			
Renal Cu	16.6[e]	16.7[e]	12.5[f]	10.4[g]	0.001	0.01	0.001
(μg/g)	(0.4)	(0.2)	(0.4)	(0.2)			

[a] Results given as mean (standard error).
[b] $P <$ values from 2-way ANOVA.
[c] NS, Not significant.
[d] Organ metals reported on dry weight basis.
[e,f,g] Means in the same row with different superscripts are significantly ($P < 0.05$) different (Scheffé contrast analysis).

mice had significantly increased hepatic Fe and female mice were also found to have significantly more hepatic Fe than males. A number of other studies (1, 5, 6, 30, 31) have also demonstrated that female rats have higher hepatic Fe than males. Although female rats have been shown to experience a less severe anemia than male rats exposed to a dietary Cu deficiency (1, 4, 5), in the present study both male and female mice experienced a severe anemia. Furthermore, the less severe anemia associated with Cu deficiency in female, compared with male, rats may be prevented by supplementation with dietary Fe, indicating that some of the changes associated with Cu deficiency in female rats may be reversed by changes in Fe metabolism (5). In the present study, the statistically significant effects of both Cu status and sex on hepatic Fe were independent of one another. Thus, altered Fe metabolism may not be responsible for the sex-specific effects of a dietary Cu deficiency in these mice.

Although both cardiac and renal Cu were significantly decreased in both male and female Cu-deficient mice, hepatic Cu was significantly decreased only in female Cu-deficient animals. Cu deficiency is usually associated with lowered hepatic Cu (29), but a previous study (6) has found that some biochemical changes indicative of a Cu-deficient state may occur in animals fed a Cu-deficient diet in the absence of an effect on hepatic Cu. That both cardiac and renal Cu were lower in Cu-deficient female mice than any other experimental group confirms that this group experienced a more severe form of Cu deficiency than the male mice.

This investigation clearly demonstrates that female mice experienced a more extreme form of Cu deficiency than males. This observation is in contrast to numerous studies with rats which have shown that the male rat is more susceptible to the effects of a dietary Cu deficiency (1–8). The reason for this species difference in the effect of sex on Cu metabolism is presently unknown. Limited data are available regarding the effect of sex on Cu status in humans, but it has been shown that supplementation of healthy young men and women with 150 mg Zn/day (>10 times the current RDA [12 mg/day]; see 32) for six weeks significantly decreased serum Cp and erythrocyte CuZnSOD activities only in women (33). Plasma Cu and hematocrit were unaffected by Zn supplementation. Zn is a well known antagonist of Cu status (34–39) and supplementation with 150 mg Zn/day may, therefore, have resulted in a mild form of Cu deficiency in the women. The reported changes in Cp and CuZnSOD activities (33) tend to suggest that human females, like the female mice in this experiment, may be more susceptible to some of the deleterious effects of a dietary Cu deficiency.

The diet used here was developed three decades ago by Ball *et al.* (40) who emphasized its improved nutritional qualities over other thrombogenic diets. In a series of papers, they showed that consumption of the diet produced numerous anatomical lesions similar to those found in people with ischemic heart disease. The earlier work was reviewed, some further improvements in the diet were made, and experiments revealed that the major defect of this diet in the production of cardiovascular lesions was a deficiency of copper (9). We then followed our earlier procedure (9) and that of the originators of the diet in using adult animals so that growth was nearly complete at the beginning of the experiment.

The Taconic Swiss-Webster mice used in the present experiment are not uniquely susceptible to the thrombogenic effects of this diet. Ball *et al.* (41) compared three strains of mice and found 64%, 48% and 10% of mice had atrial thrombosis between 6 and 40 weeks. Swiss mice were most susceptible and C mice were least susceptible.

Although this diet may not be ideal, mice consuming it do rather well. Without a Cu supplement (41, 42), they will grow and survive at least 66 to 68 weeks. With Cu supplementation, substantial numbers will survive more than 500 days even with frequent handling and anesthesia for the recording of electrocardiograms, *etc.* (9).

The diet is lower in protein than is usually recommended. "The traditional approach to recommendation of a protein content for mouse diets has been to start with the minimal requirement of the most demanding strains and add several percent to allow for variations in protein quality" (43). Several examples of diets containing far less than the usual 20% have been found adequate for mice (43). In our experience, mice fed this diet will grow (Table II) (9) and bear young (9). Thus, the amount of protein probably is adequate for adult mice.

The amount of Fe in the diet is higher (198 mg Fe/kg) than that usually recommended. However, diets adequate for mice sometimes contain Fe in the 255 to 299 mg/kg range (43).

The amount of fat in the diet is greater than that usually recommended; however, the caloric density requirements for mice have not been determined (43). The thrombogenic effect of the diet probably is not mediated via the fat because increasing the amount of lard from 28% to 40% did not increase the incidence of thrombosis of C mice (41).

Our experience with this diet enhances its utility in the study of the thrombotic process (44–46) in atherogenesis and acute myocardial infarction. Diets used to produce atherosclerosis or thrombosis in animals in the study of related human illness generally are not perfect and generally are improved nutritionally over several decades without impairing their usefulness in producing pathology. As risk of ischemic heart disease

is generally considered to be related to dietary imperfections, experimenters have some latitude in dietary characteristics. The imperfect human diet that produces the atherosclerosis that leads to heart disease also produces small stature (47–49), which can be interpreted as another indication of dietary imperfection. Some people even consume diets as high in fat as in the present experiment (50, 51).

It has been suggested (52, 53) that Cu deficiency may be an important factor in the etiology and pathophysiology of coronary heart disease (CHD). The observations of Fields *et al.* (2, 3) that Cu-deficient male rats are more susceptible than females to death have provided partial support to this hypothesis since it has been shown that male sex is one of the best documented risk factors for CHD (54). Comparison of the present study with the results of Fields is not as contradictory as it may seem at first glance. When people die with acute myocardial infarction, men are more likely to experience sudden death while women die with thromboembolism (55). Cu-deficient male rats of Fields *et al.* (2, 3) died suddenly. Here female mice died with thrombosis. Although there are differences between these two experimental models, the combined results replicate an otherwise puzzling observation in heart disease epidemiology.

In summary, female mice experienced a more extreme form of Cu deficiency than males. All but one of the tabulated variables (hepatic Zn) were affected by either dietary Cu or the sex of the animals; more than half demonstrated an interaction between Cu and sex. Comparison of these results with earlier studies of rats were reconciled by consideration of the causes of death. Male rats die suddenly, female mice die with thrombosis when deficient in copper. This difference resembles that found in people with acute myocardial infarction. The experimental conditions of these experiments are useful in the study of the thrombotic aspects of atherosclerosis and ischemic heart disease. Although these conditions may seem unique, the experiment clearly reveals dietary Cu and sex to be the major factors that influenced the results.

The authors acknowledge the assistance of Denice Schafer and staff with diet preparation and animal care, Terry Wolf and Randy Perila for technical assistance, LuAnn Johnson and Shiela Bichler for statistical analyses, and Marie Swenson for manuscript preparation.

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