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ORIGINAL ARTICLE

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Liver mitochondrial oxygen consumption and efficiency of milk production in lactating Holstein cows supplemented with copper, manganese and zinc

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Summary

The objective of this study was to examine the relationship between mitochondrial proton leak and feed efficiency with supplementation of different levels of Cu, Mn and Zn (Bioplex, Alltech) at levels above Nutrient Requirements of Dairy Cattle (NRC, 2001). Milk yield and composition, mineral excretion in milk and faeces, feed efficiency and liver mitochondrial proton leak were measured in 60 Holstein dairy cows at approximately 70 days in milk on a commercial dairy. Treatments reflect total Cu, Mn and Zn intake/day and are as follows: (i) High: 444 mg/day Cu, 3492 mg/day Mn, 2234 mg/day Zn; (ii) Med: 436 mg/day Cu, 3002 mg/day Mn, 2047 mg/day Zn; (iii) Low: 420 mg/day Cu, 2764 mg/day Mn, 2186 mg/day Zn; (iv) LowMn: 391 mg/day Cu, 2617 mg/day Mn, 1849 mg/day Zn; and (v) Control: 264 mg/day Cu, 2850 mg/day Mn, 1593 mg/day Zn. Proton leak-dependent respiration was lowest in Control ($p < .10$). However, measures of efficiency were greatest in Med and least in High ($p < .10$). Therefore, measures of efficiency did not reflect efficiency due to low proton leak and there appears to be an upper limit to beneficial supplementation of Cu, Mn and Zn.

KEYWORDS
copper, manganese, mitochondria, proton leak, zinc

1 | INTRODUCTION

Many dairies supplement lactating dairy cows with levels of Cu, Mn and Zn that are greater than levels recommended by Nutrient Requirements of Dairy cows (National Research Council, 2001). Levels are high because sources of Cu, Mn and Zn that are endogenous to forages and grains are highly variable (Swecker, 2014) and are ignored in ration formulation. Recommended requirements are met by adding exogenous mineral in both inorganic and chelated forms (National Research Council, 2001) regardless of mineral levels in feeds. There is some evidence that relatively high levels of Cu, Mn and Zn need to be maintained in order to support an immune response to a challenge before the challenge is evident. However, little data to support supplementation at high levels are available and its effects on production are unknown.

Cu, Mn and Zn are important to many metabolic functions including immune responses and reproduction. All three minerals function as antioxidants in their roles as cofactors in SOD[1] and prevention of formation of ROS (Halliwell & Gutteridge, 1989; Macmillan-Crow & Cruthirds, 2001). Mitochondria, because of their role in oxidative metabolism, are particularly susceptible to ROS damage, which can induce proton leak (Brookes, 2005). Therefore, SOD is an important enzyme to maintain mitochondrial membranes and protect mitochondrial electron transport and ATP formation. The Cu, Zn-SOD is associated with the outer mitochondrial membrane where superoxide anions are formed and Mn-SOD is associated with the mitochondrial matrix and clears ROS formed as a result of electron transport. Because Cu, Mn and Zn are cofactors of SOD, supplementation of those trace minerals is associated with an increase in SOD activity to maintain mitochondrial efficiency and integrity (Halliwell & Gutteridge, 1989).

Inefficiency in this process results in increased proton leak across the inner mitochondrial membrane, decreased yield of ATP, has been correlated with a lower feed efficiency (Kolath, Kerley, Golden, & Keisler, 2006) and impacts feed efficiency because mitochondrial inefficiency

TABLE 1 Total mixed ration (TMR) ingredients and nutrient composition

Ingredients	% DM	SD (n = 11)
Corn silage	18.4	
Rolled corn	16.0	
Wheat silage	13.5	
Canola meal	10.0	
Haylage	6.27	
Alfalfa hay	5.27	
Whole cottonseed	5.27	
Dried distiller grain	4.79	
Mill run	3.75	
Whey	2.73	
Almond hulls	2.55	
Soy Best[a]	2.36	
Molasses	2.10	
Soy hulls	1.31	
Blood meal	1.23	
Calcium carbonate	1.06	
Wheat straw	0.91	
Palmit 80[b]	0.82	
Sodium bicarbonate	0.77	
Salt	0.27	
Diamond V XP[c]	0.26	
Premix[d]	0.19	
Magnesium oxide	0.12	
Alimet 88%[e]	0.071	
Smartamine[f]	0.052	
Rumensin 90[g]	0.0,066	
Nutrients[h]		
CP	17.0	0.38
ADF	20.1	0.93
NDF	31.0	1.0
Starch	20.3	0.70
Fat	4.06	0.20
Lignin	3.41	0.25
Ash	9.46	0.37
Ca	0.786	0.069
P	0.496	0.071
Mg	0.290	0.019
S	0.273	0.017
K	1.53	0.080
Na	0.414	0.047
Fe (ppm)	418	43

(Continues)

TABLE 1 (Continued)

Ingredients	% DM	SD (n = 11)
Co (ppm)	755	136
Se (ppm)	633	55

[a]Soy Best, West Point, NE.
[b]Natu'oil Services, Port Coquitlam, BC Canada.
[c]Diamond V, Cedar Rapids, IA.
[d]Premix was composed of ground limestone, distillers dried grains, magnesium oxide, di-alpha-tocopherol acetate, vegetable oil, sodium selenite, ferrous sulphate, selenium yeast, vitamin A acetate, vitamin D3 supplement, cobalt carbonate, ethylenediamine dihydriodide, saccharomyces cerevisiae fermentation solubles, brewers dried yeast, d-Biotin.
[e]Novus International, Saint Charles, MO.
[f]Adisseo USA, Alpharetta, GA.
[g]Elanco, Greenfield, IN.
[h]Cu, Zn and Mn contents of the TMR are presented in Table 2.

may account for up to 30% of resting energy requirements (Brand & Divakaruni, 2011).

The relationship between antioxidant nutrients such as Cu, Mn and Zn and proton leak is not known. The objectives of this study are to determine whether supplementing lactating dairy cows with high levels of Cu, Mn and Zn influences mitochondrial proton leak, milk yield, milk components, feed or energetic efficiency and thus to determine whether Cu, Mn and Zn could impact feed efficiency through changes in mitochondrial efficiency. Understanding the role of mitochondrial efficiency in feed efficiency will aid in feeding decisions to improve production efficiency.

2 | MATERIALS AND METHODS

All procedures involving animals were approved by the University of California, Davis Animal Care and Use Committee. All cows were housed at a commercial dairy. Therefore, treatments, procedures and measurements were designed to not interfere with feed management and daily routine of the dairy. Cows included in the study were a subset of a freestall pen with a capacity of 210 cows and all cows had equal access to the same diet (Table 1). Cows were individually dosed with their respective supplements (Table 2) daily at morning feeding and all procedures, including dosing and sample collection, occurred during the regular lock-up times for the pen.

2.1 | Cows and treatments

Sixty lactating Holstein dairy cows approximately 70 DIM were randomly assigned to five treatment groups: (i) High with high Cu, Mn and Zn intakes: 444 mg/day Cu, 3,492 mg/day Mn, 2,234 mg/day Zn; (ii) Med with medium Cu, Mn and Zn intakes: 436 mg/day Cu, 3,002 mg/day Mn, 2047 mg/day Zn; (iii) Low with low Cu, Mn and Zn intakes: 420 mg/day Cu, 2,764 mg/day Mn, 2,186 mg/day Zn; (iv) LowMn with low Mn intakes: 391 mg/day Cu, 2,617 mg/day Mn, 1849 mg/day Zn; and (v) Control: 264 mg/day Cu, 2,850 mg/day Mn, 1,593 mg/day Zn. Treatments were designed to supply approximately half the

requirements (National Research Council, 2001) in inorganic (sulphate) or organic (chelated) forms (Bioplex, Alltech) by daily dosing an oral solution of minerals and 100 ml of distilled water ("Supplemented exogenously," Table 2). This was true for Cu levels, but Mn and Zn contents of the TMR were well above requirements due to high levels in the diet. Therefore, treatment names are associated with "Total Mineral Intake" in Table 2.

Experimental periods were 28 days in length with 14 days of oral supplementation followed by 14 days of oral supplementation and chromium oxide (Cr_2O_3) to determine faecal output. During the last 4 days of oral supplementation, milk, feed, residual feed and faecal samples were collected. Liver biopsies were performed on days 28. Cows that were biopsied were parity 2 or greater and had glucose and ketone body levels of 60-80 mg/dl and < 12 mg/dl respectively. Because liver samples had to be fresh for analyses of mitochondrial respiration, only five cows could be biopsied and mitochondrial respiration measured per day. Therefore, six cows per treatment were enrolled in each cohort each week, and then treatments were replicated

with a second cohort composed of another six cows per treatment per week for a total of 10 cows per treatment and trial length of 13 weeks. Six cows were enrolled each week, but only five were biopsied so that cows could be excluded for metabolic problems (assessed by blood glucose and blood betahydroxy butyric acid levels), lameness, mastitis or behaviour.

2.2 | Nutrient analyses

Faecal, feed and refusal samples were dried at 60°C for 48 hr in a forced-air oven and ground through a 1-mm screen (Wiley mill, Arthur Gill Thomas, Swedesboro, NJ). Samples were analysed for DM, ADF, NDF, CP, fat, ash and lignin using wet chemistry analyses (American Association for Analytical Chemists reference methods 935.29, 973.18, 2002.04, 990.03, 920.39, 942.05, 973.18, respectively), starch using near-infrared reflectance spectrophotometry (based on predictive equations developed at Analab, Agri-King) and mineral analyses using inductively coupled plasma–mass spectrophotometry for Ca, P,

TABLE 2 Effect of Cu, Mn and Zn supplementation levels on mineral intake and mineral excretion in milk and faeces

Mineral intake and excretion	Treatments[1]					SEM	NRC[2]
	High	Med	Low	LowMn	Control		
Supplemented exogenously (mg/day)	inorganic	organic	organic	organic			
Mn	246	308	185	246	0		
Cu	155	194	116	155	0		
Zn	443	554	332	443	0		
Total mineral intake[3] (mg/day)							
Mn	3,492[a]	3,002[ab]	2,764[ab]	2,617[b]	2,850[ab]	380	490
Cu	444[a]	436[a]	420[a]	391[a]	264[b]	35	370
Zn	2,234[a]	2,047[a]	2,186[a]	1,849[ab]	1,593[b]	220	1,300
Milk mineral excretion (mg/day)							
Mn	0.500[b]	0.778[a]	0.642[a]	0.651[a]	0.658[a]	0.098	
Cu	1.93[ab]	2.64[a]	2.04[ab]	1.79[b]	1.68[b]	0.36	
Zn	145	148	126	131	147	14	
Faecal mineral excretion (mg/day)							
Mn	2,905[a]	2,787[a]	2,885[a]	2,554[b]	3,041[a]	220	
Cu	361[c]	456[a]	396[b]	395[b]	291[d]	16	
Zn	1,720[b]	2,058[a]	1,925[a]	1,737[b]	1,656[b]	92	
Retained minerals[4] (mg/day)							
Mn	559[a]	223[ab]	-74.9[ab]	-2.65[ab]	-441[b]	340	
Cu	82.0[a]	-22.4[bc]	21.5[b]	-5.45[bc]	-51.8[c]	29	
Zn	360[a]	-157[bc]	140[ab]	-19.4[bc]	-358[c]	190	

Means within a row not followed by the same superscript letter are significantly different ($p < .05$).

[1]High treatment contains highest levels of Cu, Zn and Mn all well above National Research Council (2001) requirements. Med treatment contains intermediate levels of Cu, Zn and Mn above National Research Council (2001) requirements. Low treatment contains lower levels of Cu, Zn and Mn but still above National Research Council, 2001 requirements. LowMn treatment contains the lowest levels of Mn (and lower levels of Cu and Zn) but still above National Research Council (2001) requirements and Control treatment contains the lowest levels of Cu and Zn, which are close to National Research Council (2001) requirements.

[2]Mineral requirements based on National Research Council (2001) assuming 24 kg/day DMI and milk yield of 50 kg/day.

[3]Total mineral intakes were calculated based on DMI determined using iNDF (Table 5).

[4]Assuming no urinary excretion.

Mg, K, S, Na, Cl, Fe, Co, Cu, Mn and Zn (American Association for Analytical Chemists reference methods 985.01 for Ca, P, Mg, K, Na, Fe, Co, Cr, Cu, Mn and Zn, 923.01 for S and 915.01 for Cl; Table 1.).

2.3 | Milk analyses

Milk production was measured once per day during the morning milking using the Milk Check device (Dickinson & Tomaszewski, 1968). Samples were collected for analysis of components by the Tulare Dairy Herd Improvement Association (Tulare, CA) and mineral analysis by UC Davis Analytical Lab using inductively coupled plasma–mass spectrometry (Cope, Mackenzie, Wilde, & Sinclair, 2009). One day prior to biopsy, blood samples were drawn from the coccygeal vein with vacuum tubes containing sodium fluoride and potassium oxalate (Becton-Dickinson Vacutainer®) to evaluate blood glucose and ketone body levels to assess cow health. Samples were centrifuged at 648 g for 10 min, and a drop of plasma was transferred to the sensor of the test strip (Precision Xtra β-ketone and Blood Glucose, Abbott; Iversen, Falkenberg, Voigtsberger, Forderung, & Heuwieser, 2009).

2.4 | Estimation of DMI

Amounts of Cu, Mn and Zn supplied endogenously in the diet were estimated using a double marker system to estimate DMI based on faecal output and apparent dry matter digestibility. Then, DMI was multiplied by Cu, Mn and Zn concentration in the diet to determine total mineral intake (Table 2). To determine faecal output, Cr_2O_3 (10 $g^{-1}d^{-1}$) was used as an indigestible faecal marker administered once daily with the cows' supplement at 07:30 hr for 14 days. Faecal grab samples were collected at 08:00 and 20:00 hr over the last 4 days of the 28-days dosing period and combined to estimate 24 hr Cr_2O_3 excretion. DMI was calculated based on the assumption that Cr intake had equilibrated with Cr excretion after 14 days. In the same way, apparent dry matter digestibility was estimated using iNDF, iADF or iLignin concentration in the diet and faeces using the equations in Huhtanen, Kaustell, and Jaakkola (1994) and Bargo, Muller, Delahoy, and Cassidy (2002). iADF, iNDF and iLignin were determined using samples of oven-dried (60°C forced-air oven) faecal and feed material composited by day, weighed into 5 × 15 cm dacron bags and ruminally incubated in three rumen-fistulated dairy cows for 28 days. After incubation, bags were removed from the rumen, rinsed with cold tap water for 30 min and dried in a forced-air oven at 60°C for 24 hr. Dry bags and residues were weighed, and DM disappearance was corrected for bag losses during digestion by subtracting weights of digested, blank bags. The incubated residue was analysed for NDF, ADF and lignin to estimate iNDF, iADF and iLignin (Huhtanen et al., 1994). Faecal output and digestibility were then used to estimate individual cow feed intake according to equations described in Bargo et al. (2002).

Faecal, diet and diet refusal samples used in the determination of DMI were collected over the last 4 days of the 28-days dosing period. Diet samples were collected once daily and subsampled from three tubs set along the feed bunk during feed delivery (Rossow & Aly, 2013). Refusal samples were collected once daily before the a.m.

feeding, and average diet sample compositions were corrected with average residual compositions.

2.5 | Liver sampling and mitochondrial isolation

Liver biopsies were performed by a veterinarian, and approximately 1 g of liver tissue was obtained using the Sontech® liver biopsy tool. The sample was placed in a Corning® vial with mitochondrial isolation media (220 mM mannitol, 70 mM sucrose, 20 mM Tris, 1 mM EDTA and 0.1% (w/v) bovine serum albumin, pH 7.4 at 4°C) in ice, and mitochondria were isolated immediately (Cawthon, Beers, & Bottje, 2001; Cawthon, McNew, Beers, & Bottje, 1999). Minced tissue was homogenized in a Potter-Elvehjem vessel with a Teflon pestle of 0.16 mm clearance incubated in ice. The homogenate was centrifuged at 500 × g for 10 min and the resulting supernatant centrifuged at 10,000 × g for 10 min to obtain the mitochondrial pellet. Fatty acid-free BSA was used in the isolation of mitochondria to sequester free fatty acids and to act as a free radical scavenger. The resulting pellet was resuspended and washed in 10 ml of isolation solution with BSA at 8,100 × g for 10 min and then was resuspended again and washed in 10 ml of isolation solution without BSA at 8,100 × g for 10 min. The resulting mitochondrial pellet was suspended in 200 μl of isolation medium and placed in ice until used for oxygen consumption and proton leak kinetics assays. Protein concentration was determined using the Bradford protein assay with BSA as the standard.

2.6 | Measurement of mitochondrial oxygen consumption

Mitochondrial oxygen consumption was measured using a Hansatech Clark-type oxygen electrode (Norfolk, UK). Mitochondria (0.35 mg protein/ml final concentration) were incubated in 1 ml of oxygen consumption medium (120 mM KCl, 5 mM KH_2PO_4, 5 mM $MgCl_2$, 5 mM Hepes and 1 mM EGTA) in a magnetically stirred incubation chamber maintained at 30°C. Rotenone (5 μM) was used to block electron transport chain at Complex I, and State 4 respiration (non-phosphorylating respiration) was determined in mitochondria following the addition of 5 mM succinate. State 3 respiration was measured in mitochondria incubated in the presence of 5 mM succinate and 100 μM ADP. RCR was determined by dividing State 3 respiration by State 4 respiration.

2.7 | Measurement of mitochondrial membrane potential

Mitochondrial proton motive force was assessed using a TPMP+-sensitive electrode. All measurements were completed in duplicate to determine mitochondrial oxygen consumption and membrane potential simultaneously. Rotenone (5 μM) and oligomycin (8 μg/mg protein) were used to block electron transport chain at Complex I and ATP synthase respectively. Nigericin (0.4 μg/ml) was added to convert the pH component of Δp to membrane potential units (mV) allowing Δp to be measured in mV units (Ramsey et al., 2004). Data from the two electrodes (oxygen and TPMP+) were collected by data acquisition software

(Hansatech Oxygraph System, Norfolk, UK) allowing real-time simultaneous measurements of mitochondrial oxygen consumption and Δp. MMP in mV was calculated based on the Nernst equation as:

$$MMP = 61.5 \log([TPMP] \text{ added} - \text{external } [TPMP]) \times$$
$$TPMP \text{ binding correction}/(0.001 \times mg \text{ of protein}/ml \times [TPMP])$$

A TPMP binding correction of 0.4 (µl/mg of mitochondrial protein)$^{-1}$ was used (Rolfe, Hulbert, & Brand, 1994).

2.8 | Mitochondrial proton leak kinetics

The kinetic responses of proton leak to membrane potential were determined by titrating the electron transport chain with malonate (0.1 to 2.5 mм), an inhibitor of succinate dehydrogenase, in the presence of oligomycin (8 µg/mg mitochondrial protein). Proton leak kinetics were visualized by plotting mitochondrial membrane potential against oxygen consumption. Proton leak was compared among groups by calculating oxygen consumption at a common membrane potential (165 mV).

2.9 | Statistical analysis

Individual cow was the experimental unit of interest because all measurements were made on individual cows including DMI estimated using markers for digestibility and faecal output. All statistical analyses were performed using SAS (SAS Institute, version 9.4). Standard deviations of nutrient composition (Table 1) were calculated using the univariate procedure. Differences among dry matter intake estimation, and total intake, milk excretion, faecal excretion and retained Cu, Mn or Zn (Table 2) and milk yield and components (Table 3) least-square means by treatment were estimated using general linear models with replication and parity as covariates. Least-square means of RCR, State 3 respiration and State 4 respiration (Table 4) and DM digestibility and efficiency (Table 5) were analysed by treatment using the mixed model procedure with replication and parity as fixed effects, and DIM as a random effect. Overall comparisons of digestibility and efficiency with RCR and State 3 and State 4 respiration regardless of treatment (Table 6) were analysed using the general linear model procedure.

3 | RESULTS AND DISCUSSION

Nutrient composition of the diet was fairly constant for the 11 weeks of the research trial (Table 1). The highest standard deviations were for the mineral contents of the diet. As mineral concentrations in the diet are small, it is expected that small deviations can result in high variability relative to other nutrients in the diet.

TABLE 3 Differences in milk yield and milk components among treatments

	Treatments[1]					
	High	**Med**	**Low**	**LowMn**	**Control**	**SEM**
Milk, kg	47.4[ab]	50.9[a]	46.0[ab]	43.6[b]	49.7[a]	2.9
FCM[2], kg	46.1	49.0	45.4	44.8	48.0	2.8
ECM[3], kg	45.4	48.1	45.0	43.4	47.3	2.7
MilkE[4], mcal/kg	0.652[ab]	0.647[b]	0.677[ab]	0.684[a]	0.653[ab]	0.018
Fat, kg	1.58	1.66	1.58	1.60	1.64	0.11
Fat, %	3.31[b]	3.28[b]	3.50[ab]	3.70[a]	3.29[b]	0.18
Protein, kg	1.38[ab]	1.44[a]	1.40[ab]	1.23[b]	1.43[a]	0.090
Protein, %	2.91	2.85	3.03	2.83	2.90	0.11
Lactose, kg	2.23[ab]	2.40[a]	2.18[ab]	2.05[b]	2.37[a]	0.15
Lactose, %	4.70	4.72	4.69	4.70	4.77	0.098
Total solids, kg	4.04[ab]	4.30[a]	3.93[ab]	3.67[b]	4.25[a]	0.25
Total solids, %	8.50	8.45	8.49	8.41	8.58	0.15

Means within a row not followed by the same superscript letter are significantly different ($p < .05$).
[1]High treatment contains highest levels of Cu, Zn and Mn all well above National Research Council (2001) requirements, Med treatment contains intermediate levels of Cu, Zn and Mn above National Research Council (2001) requirements, Low treatment contains lower levels of Cu, Zn and Mn but still above National Research Council (2001) requirements, LowMn treatment contains the lowest levels of Mn (and lower levels of Cu and Zn) but still above National Research Council (2001) requirements, and Control treatment contains the lowest levels of Cu and Zn, which are close to National Research Council (2001) requirements.
[2]Fat-corrected milk calculated as (milk yield, kg/day*0.432) + (fat yield, kg/day*16.216).
[3]Energy-corrected milk calculated as (0.323*milk yield, kg/day) + (12.82*fat yield, kg/day) + (7.13*protein yield, kg/day).
[4]Milk energy content, mcal, calculated as (milk fat content, kg/kg * 9.29 + milk protein content, kg/kg * 5.47 + milk lactose content, kg/kg * 3.95) * milk yield, kg (Phuong et al., 2013).

nmol O mg^{-1} protein min^{-1}	Treatments[1]					SEM
	High	Med	Low	LowMn	Control	
State 3[2]	75.8	64.4	78.2	73.0	64.1	13
State 4[3]	26.2[ab]	22.6[ab]	25.9[ab]	27.1[a]	22.0[b]	3.0
RCR[4]	2.89	2.76	2.98	2.65	2.83	0.27

TABLE 4 Effect of Cu, Mn and Zn supplementation on liver mitochondrial oxygen consumption from dairy cows at 70 DIM—mixed model

Means within a row not followed by the same superscript letter are significantly different ($p < .10$).

[1]High treatment contains highest levels of Cu, Zn and Mn all well above National Research Council (2001) requirements, Med treatment contains intermediate levels of Cu, Zn and Mn above National Research Council (2001) requirements, Low treatment contains lower levels of Cu, Zn and Mn but still above National Research Council (2001) requirements, LowMn treatment contains the lowest levels of Mn (and lower levels of Cu and Zn) but still above National Research Council (2001) requirements, and Control treatment contains the lowest levels of Cu and Zn, which are close to National Research Council (2001) requirements.

[2]Oxygen consumption during State 3 respiration (ATP-dependent respiration).

[3]Oxygen consumption during State 4 respiration (proton leak-dependent respiration).

[4]Respiratory control ratio (RCR) is State 3 Oxygen consumption/State 4 Oxygen consumption.

Total mineral intake was different among treatments and did not reflect levels of minerals that were supplemented exogenously (Table 2). The statement by Weiss (2005) that "variation in feed composition and DMI can be high making precise and accurate measurements of trace minerals difficult" is reflective of the treatment groupings based on total mineral intake. Treatment names reflect both exogenous supply of Cu, Mn and Zn and DMI. For example, exogenously supplemented mineral levels were the same for the High and LowMn groups, but the High supplement contained all inorganic forms of Cu, Mn and Zn and the LowMn supplement contained all organic forms of Cu, Mn and Zn. The LowMn group had much lower total Mn intake. Therefore, the low Mn content in the LowMn group is due to low DMI. Level of DMI determined total Cu, Mn and Zn intake for High, LowMn and Control groups. The Med and Low groups were given the highest and lowest levels of exogenous supplemented minerals, but had the lowest and highest levels of DMI respectively. Therefore, total mineral intake for these groups represents an interaction between exogenously supplemented mineral and DMI. Other research on Cu, Mn and Zn supplementation has ignored mineral content supplied by the feed in analyses or treatment results (DeFrain, Socha, Tomlinson, & Kluth, 2009; Formigoni et al., 2011; Nemec et al., 2012; Nocek, Socha, & Tomlinson, 2006; Rabiee, Lean, Stevenson, & Socha, 2010). Therefore, this study is unique in that it attempts to quantify both dietary and exogenously supplied Cu, Zn and Mn effects on milk production, mitochondrial respiration and efficiency.

As the iNDF marker-determined DMI had the lowest SEM, iNDF-based estimates of DMI were used for all estimates of efficiency. Other comparisons of DMI estimates based on iNDF, iADF and iLignin have also found that iNDF is the most consistent marker to determine apparent digestibility, which is then used to estimate DMI (Bargo et al., 2002; Maulfair, Fustini, & Heinrichs, 2011). The use of iNDF as a more consistent indicator may be due to higher levels of NDF in diets compared with iADF and iLignin.

Retained minerals were highest in the High group reflecting the highest total mineral intake. Milk mineral excretion and faecal mineral excretion were also lowest in the High group. The Control group was lowest in mineral retention reflecting the lowest total Cu and Zn intake and low Mn intake with high milk and faecal Mn excretion. Retained minerals were not different between the Med, Low and LowMn groups. Results from Olson et al. (1999) were similar in that decreasing supplementation reduced Cu, Mn and Zn faecal excretion. They also fed Cu, Mn and Zn at levels higher than recommendations in 2-year-old beef cows over a 2-year period and measured faecal Cu, Mn and Zn output and liver concentrations. Faecal Cu, Mn and Zn decreased with a decrease in supplementation level over time (3 days) and control heifers had significantly lower faecal mineral excretion than supplemented cows. Cu liver levels also increased with supplementation, but Zn and Mn liver levels did not change, indicating that other organs may be better indicators of Zn and Mn status. Although liver levels were not measured in this study, retention was higher for Cu, Mn and Zn in the High treatment and lowest in the Control treatment, indicating that estimating retention may be a better indicator of Mn and Zn status. Estimates of retention in this study did not include urine excretion. However, milk and urinary excretion of Mn and Zn has been shown to be negligible and not responsive to level of supplementation (Nemec et al., 2012; Weiss & Socha, 2005).

Milk, total solids and milk protein yields were highest in Med and Control and lowest in LowMn (Table 3). Low milk and milk component yields in LowMn could be due to low total Mn intake, or the lowest DMI. DMI has been shown to be an important determinant of milk yield and also plays a role in milk component yields. However, Med also had low DMI but was highest in milk and milk protein yields. Med and Control, which were highest in milk, solids and milk protein yield, were not affected by total Cu and Zn intake as they were highest and lowest in Cu and Zn intake respectively. Therefore, it appears that supplementation of Mn at high levels could benefit milk production and oversupplementation of Cu and Zn does not affect milk and milk component yields.

In a meta-analysis by Rabiee et al. (2010), they found that organic forms of Cu, Mn and Zn supplementation increased milk, ECM, solids,

TABLE 5 Effect of Cu, Mn and Zn supplementation on energy and N efficiencies in dairy cows at 70 DIM

	Treatments[19]					
	High	**Med**	**Low**	**LowMn**	**Control**	**SEM**
Faecal output Cr[1] (kg DM/day)	9.20[ab]	8.88[b]	8.89[b]	7.68[c]	10.1[a]	0.68
DM digestibility (%)						
iNDF[2]	62.3[a]	61.0[a]	61.7[a]	56.9[b]	54.4[b]	1.8
iADF[2]	72.8[a]	72.8[a]	71.5[a]	71.0[a]	68.2[b]	1.8
iLignin[2]	70.5[a]	64.9[b]	68.0[a]	68.7[a]	64.7[b]	2.1
DM intake (kg/day)						
iNDF	28.8[a]	22.3[c]	27.0[ab]	20.8[c]	24.2[bc]	2.8
iADF	35.3[a]	27.2[b]	37.8[a]	31.1[ab]	32.3[ab]	4.7
iLignin	34.9[a]	24.0[c]	33.9[ab]	28.1[bc]	29.8[abc]	3.7
Efficiency						
FCE[3]	1.77[c]	2.44[a]	1.86[bc]	2.15[ab]	2.21[a]	0.18
NUE[4]	32.6[c]	41.4[a]	35.0[bc]	36.5[abc]	38.4[ab]	3.1
FEL[5]	1.31[c]	1.71[a]	1.4[bc]	1.58[ab]	1.55[ab]	0.12
FCR[6]	0.637[a]	0.443[c]	0.593[ab]	0.471[c]	0.503[bc]	0.064
GEE[7]	0.322[c]	0.420[a]	0.344[bc]	0.388[ab]	0.380[ab]	0.030
GECP[8]	0.332[c]	0.422[a]	0.357[bc]	0.372[abc]	0.392[ab]	0.031
CPB[9]	3.46[a]	2.41[b]	3.16[a]	2.30[b]	2.73[ab]	0.45
FE[10]	0.128[c]	0.165[a]	0.138[bc]	0.153[ab]	0.149[abc]	0.012
FEECM[11]	1.89[c]	2.48[a]	2.03[bc]	2.29[ab]	2.24[ab]	0.18
MEMEI[12]	0.525[c]	0.683[a]	0.560[bc]	0.631[ab]	0.619[ab]	0.050
EEF[13]	0.437[c]	0.569[a]	0.467[bc]	0.526[ab]	0.516[ab]	0.041
NEFF[14]	0.435[b]	0.561[a]	0.475[b]	0.494[ab]	0.551[a]	0.044
NeffEeff[15]	0.946[b]	0.964[b]	0.971[ab]	0.915[b]	1.04[a]	0.045
MilkE[16]	0.686[abc]	0.679[c]	0.708[ab]	0.710[a]	0.680[bc]	0.018
ECE[17]	0.181[c]	0.238[a]	0.194[bc]	0.219[ab]	0.214[ab]	0.017
MilkEGE[18]	0.322[c]	0.420[a]	0.344[bc]	0.388[ab]	0.380[ab]	0.030

Means within a row not followed by the same superscript letter are significantly different ($p < .10$).

[1]Faecal output determined using Cr as an external marker (Bargo et al., 2002).

[2]Apparent total tract digestibilities using indigestible markers iNDF, iADF or iLignin based on methods from Bargo et al. (2002), Huhtanen et al. (1994) and Krizsan and Huhtanen (2013).

[3]FCE is feed conversion efficiency calculated as milk kg/DMI kg (Arndt et al., 2015).

[4]NUE is N-use efficiency calculated as milk N g/100 g N intake (Arndt et al., 2015).

[5]FEL is feed efficiency for lactation calculated as MilkE mcal/DMI kg (Arndt et al., 2015).

[6]FCR is feed conversion ratio calculated as DMI kg/milk kg (Zamani, 2012).

[7]GEE is gross energy efficiency calculated as MilkE mcal/energy intake mcal (Zamani, 2012).

[8]GECP is gross efficiency of CP calculated as CP in milk kg/CP intake kg (Zamani, 2012).

[9]CPB is crude protein balance calculated as CP intake kg - CP milk kg (Zamani, 2012).

[10]FE is feed efficiency calculated as milk fat and protein yield kg/DMI kg (Xue et al., 2011).

[11]FEECM is feed efficiency based on ECM calculated as ECM kg/DMI kg (Xue et al., 2011).

[12]MEMEI is ME efficiency calculated as MilkE mcal/ME intake mcal (Xue et al., 2011).

[13]EEF is energy efficiency calculated as MilkE mcal/DE intake mcal (Phuong et al., 2013).

[14]NEFF is N efficiency calculated as N in milk kg/digestible N intake kg (Phuong et al., 2013).

[15]NeffEeff is ratio of N efficiency to energy efficiency calculated as NEFF/EEF (Phuong et al., 2013).

[16]MilkE is milk energy content mcal calculated as (milk fat content kg/kg * 9.29 + milk protein content kg/kg * 5.47 + milk lactose content kg/kg * 3.95) * milk yield kg (Phuong et al., 2013).

[17]ECE is energy conversion efficiency calculated as ECM kg/ME intake mcal (Mantysaari et al., 2012).

[18]MilkEGE is gross energy efficiency calculated as MilkE mcal/GE intake mcal (Vandehaar, 1998).

[19]High treatment contains highest levels of Cu, Zn and Mn all well above National Research Council (2001) requirements, Med treatment contains intermediate levels of Cu, Zn and Mn above National Research Council (2001) requirements, Low treatment contains lower levels of Cu, Zn and Mn but still above National Research Council (2001) requirements, LowMn treatment contains the lowest levels of Mn (and lower levels of Cu and Zn) but still above National Research Council (2001) requirements, and Control treatment contains the lowest levels of Cu and Zn, which are close to National Research Council (2001) requirements.

TABLE 6 Relationship between measures of efficiency and liver mitochondrial oxygen consumption for dairy cows at 70 DIM

		State 3			State 4			RCR		
	Mean	I[18]	Slope	R^2	I	Slope	R^2	I	Slope	R^2
DM digestibility (%)										
iNDF[1]	60.1	52	0.23	0.19	50	0.85	0.13	45	5.2	0.15
iADF[1]	73.9	69	0.13	0.13	68	0.48	0.092	66	2.8	0.094
iLignin[1]	68.4	63	0.15	0.14	61	0.58	0.10	60	3.1	0.093
Efficiency										
FCE[2]	2.03	2.6	−0.015	0.18	2.7	−0.55	0.12	3.0	−0.36	0.18
NUE[3]	33.5	42	−0.25	0.16	46	−1.0	0.13	48	−5.2	0.11
FEL[4]	1.34	1.7	−0.010	0.18	1.8	−0.039	0.14	2.0	−0.23	0.17
FCR[5]	0.552	0.43	0.0036	0.090	0.44	0.0094	0.034	0.30	0.089	0.10
GEE[6]	0.326	0.41	−0.0023	0.15	0.43	−0.0084	0.10	0.47	−0.052	0.12
GECP[7]	0.342	0.43	−0.0026	0.16	0.47	−0.010	0.13	0.49	−0.053	0.11
CPB[8]	2.99	1.9	0.031	0.14	1.8	0.099	0.070	0.74	0.80	0.14
FE[9]	0.127	0.16	−0.00092	0.15	0.17	−0.0035	0.11	0.18	−0.021	0.12
FEECM[10]	1.94	2.4	−0.014	0.15	2.6	−0.051	0.10	2.9	−0.33	0.13
MEMEI[11]	0.531	0.66	−0.0037	0.15	0.70	−0.014	0.10	0.77	−0.085	0.12
EEF[12]	0.443	0.55	−0.0031	0.15	0.58	−0.011	0.10	0.64	−0.071	0.12
NEFF[13]	0.429	0.57	−0.0039	0.17	0.63	−0.017	0.15	0.64	−0.075	0.10
NeffEeff[14]	0.956	1.0	−0.0023	0.086	1.1	−0.011	0.10	1.0	−0.031	0.024
MilkE[15]	0.660	0.65	0.00017	0.0035	0.66	−0.00036	0.00085	0.63	0.012	0.028
ECE[16]	0.186	0.23	−0.0013	0.15	0.25	−0.0048	0.10	0.27	−0.031	0.13
MilkEGE[17]	0.326	0.41	−0.0023	0.15	0.43	−0.0084	0.10	0.47	−0.052	0.12

[1] Apparent total tract digestibilities using indigestible markers iNDF, iADF or iLignin based on methods from Bargo et al. (2002), Huhtanen et al. (1994) and Krizsan and Huhtanen (2013).

[2] FCE is feed conversion efficiency calculated as milk kg/DMI kg (Arndt et al., 2015).

[3] NUE is N-use efficiency calculated as milk N g/100 g N intake (Arndt et al., 2015).

[4] FEL is feed efficiency for lactation calculated as MilkE mcal/DMI kg (Arndt et al., 2015).

[5] FCR is feed conversion ratio calculated as DMI kg/milk kg (Zamani, 2012).

[6] GEE is gross energy efficiency calculated as MilkE mcal/energy intake mcal IE (Zamani, 2012).

[7] GECP is gross efficiency of CP calculated as CP in milk kg/CP intake kg (Zamani, 2012).

[8] CPB is crude protein balance calculated as CP intake kg - CP milk kg (Zamani, 2012).

[9] FE is feed efficiency calculated as milk fat and protein yield kg/DMI kg (Xue et al., 2011).

[10] FEECM is feed efficiency based on ECM calculated as ECM kg/DMI kg (Xue et al., 2011).

[11] MEMEI is ME efficiency calculated as MilkE mcal/ME intake mcal (Xue et al., 2011).

[12] EEF is energy efficiency calculated as MilkE mcal/DE intake mcal (Phuong et al., 2013).

[13] NEFF is N efficiency calculated as N in milk kg/digestible N intake kg (Phuong et al., 2013).

[14] NeffEeff is ratio of N efficiency to energy efficiency calculated as NEFF/EEF (Phuong et al., 2013).

[15] MilkE is milk energy content mcal calculated as (milk fat content kg/kg * 9.29 + milk protein content kg/kg * 5.47 + milk lactose content kg/kg * 3.95) * milk yield kg (Phuong et al., 2013).

[16] ECE is energy conversion efficiency calculated as ECM kg/ME intake mcal (Mantysaari et al., 2012).

[17] MilkEGE is gross energy efficiency calculated as MilkE mcal/GE intake mcal (Vandehaar, 1998).

[18] I is intercept and R^2 is coefficient of determination from regression of efficiency on mitochondrial respiration parameter.

fat and protein yield but not milk fat or protein per cent. In most of these studies, Cu, Mn and Zn were fed at recommended levels. In this study, milk, solids and protein yields were highest with the highest level of supplementation of organic forms (Med) and the lowest level of supplementation (Control). Therefore, amount and form of exogenous mineral did not affect milk, solids or protein yields. However, as Med treatment represents high levels of exogenously supplied organic Cu, Mn and Zn at levels above recommendations, Cu and Zn may have less of a negative impact on yields if they are in organic forms.

State 4, maximum proton leak-dependent respiration, was affected by total intake of Cu, Mn and Zn ($p < .10$) but not by the form of minerals. State 3 respiration (maximum ATP stimulated respiration) and respiratory control ratio (RCR = State 3/State 4) did not differ among treatments (Table 4). However, State 4 respiration was highest in LowMn and lowest in Control, indicating that Mn plays an important role in minimizing proton leak-dependent respiration and high levels of Cu and Zn, whether they are in inorganic or organic forms, increase State 4 respiration. Mn through Mn-SOD is known to reduce ROS in the mitochondrial matrix

and reduce proton leak (Halliwell & Gutteridge, 1989). Proton leak is an important component of basal energy requirements. Therefore, reducing State 4 respiration through Mn supplementation and/or not feeding high levels of Cu and Zn could improve efficiency.

Very few studies exist examining mitochondrial function in dairy cattle and none have examined the relationship between feeding minerals that support antioxidant function (Cu, Mn and Zn), mitochondrial respiration and efficiency. Other studies have shown feed efficiency decreases with increased proton leak or State 4 respiration (Bottje et al., 2002; Kolath et al., 2006; Lancaster et al., 2014), but these measurements have not been reported in dairy cows. Because mitochondrial proton leak is a major factor contributing to resting energy expenditure, it is expected that animals with higher efficiencies may also have lower mitochondrial proton leak. To examine the impact of Cu, Mn and Zn supplementation on mitochondrial oxygen consumption and efficiency, Table 5 compares results from 16 different methods for measuring efficiency with Cu, Mn and Zn treatments, and Table 6 compares the same feed efficiency methods to measurements of mitochondrial oxygen consumption in dairy cow livers. In Tables 5 and 6 (footnotes), eight methods to estimate efficiency are based on weights of input vs. weight of output: FCE (Arndt, Powell, Aguerre, Crump, & Wattiaux, 2015), NUE (Arndt et al., 2015), FCR (Zamani, 2012), GECP (Zamani, 2012), CPB (Zamani, 2012), FE (Xue, Yan, Ferris, & Mayne, 2011), FEECM (Xue et al., 2011) and NEFF (Phuong, Friggens, de Boer, & Schmidely, 2013). The other eight methods estimate efficiency based on energy input or output or both: FEL (Arndt et al., 2015), GEE (Zamani, 2012), MEMEI (Xue et al., 2011), EEF (Phuong et al., 2013), NeffEeff (Phuong et al., 2013), MilkE (Phuong et al., 2013), ECE (Mantysaari, Liinamo, & Mantysaari, 2012) and MilkEGE (Vandehaar, 1998). Even though each efficiency measure is based on different inputs, outputs and units of measure, comparison of results is the same for both levels of supplementation (Table 5) and liver mitochondrial oxygen consumption (Table 6). This implies that differences in methods to estimate efficiency may be inconsequential as long as a relationship of outputs/inputs is represented. While FCR and CPB may appear to be exceptions, they represent inverses of other efficiency methods. Therefore, they also represent greatest efficiency in Med and smallest in High. However, NEFF and NeffEeff, which represent N in milk relative to digestible N intake and NEFF relative to energy efficiency (based on digestible energy intake), respectively, are highest in Control. Control cows had the lowest digestibility and both NEFF and NeffEeff are based on digestibility. Therefore, these methods reflected differences due to digestibility rather than differences due to Cu, Mn and Zn supplementation.

Efficiencies of milk production, N or CP use were consistently highest in Med and lowest in High. Nayeri et al. (2014) found that FCE increased linearly with increased supplemental Zn in an amino acid complex. Similarly in this study, Med had the highest level of Zn supplemented in organic forms and the highest FCE. In addition to Med being highest in efficiency, Med was also lowest in DMI and highest in milk, solids and milk protein yields and High was highest in DMI, but lower in milk and milk protein yields. These results imply that there is a decrease in efficiency when supplementation levels of Cu, Mn and Zn are too high. Med also supplied the highest levels of exogenous

organic forms of Cu, Mn and Zn while High supplied high levels of exogenous inorganic forms of these minerals supporting the idea that high levels of supplementation of Cu and Zn may have a more positive or less negative impact if they are in organic forms.

Low levels of Mn may be associated with low DMI (Table 2) and low milk, solids and protein yields (Table 3), but low levels of Mn could be related to mitochondrial dysfunction because of increased State 4 respiration (Table 4). MilkE was highest in LowMn (Table 5), but this is due to LowMn having the highest milk fat % and lowest milk yield, lowest lactose and lowest protein (Table 3). Proportions of milk fat, lactose and protein, kg/kg, are the main contributors to calculating MilkE. Because LowMn had high State 4 respiration, relatively low DMI, low milk yield and therefore moderate efficiencies, it appears mitochondrial proton leak was not associated with energy, N or CP utilization or feed efficiency. However, because most efficiencies are expressed as ratios, low DMI and low milk yield could translate into improved efficiency without considering that low milk yield is an undesirable condition.

To examine the relationship between efficiency and mitochondrial oxygen consumption, Table 6 lists slopes, intercepts and coefficients of determination (R^2) for regressing measurements of efficiency on State 3, State 4 or RCR regardless of Cu, Mn and Zn supplementation. Overall R^2 were low but highest R^2 were obtained for State 3 respiration and RCR with efficiency methods FCE and FEL indicating that these methods may be better predictors of mitochondrial contributions to efficiency. Both FCE and FEL also include DMI, which has been shown to be linked to mitochondrial oxygen consumption through residual feed intake (RFI). RFI was not measured in this study and typically highlights differences in feed intake relative to gain. Kolath et al. (2006) and Lancaster et al. (2014) found that the rate of liver mitochondrial respiration (and RCR) was increased in low RFI steers, but there was no difference in proton leak. In the current study, State 4 respiration also had lowest R^2 for all methods of measuring feed efficiency, indicating that mitochondrial oxygen consumption due to proton leak was not captured well in any of the methods used to measure efficiency. Results from Kolath et al. (2006) and Lancaster et al. (2014) were also relative to gain and not lactation and so may not reflect differences in methods used to measure efficiency relative to a different physiological state, lactation. Brown, DeNise, and McDaniel study (1988) is the only other study that has measured milk production and mitochondrial oxygen consumption. The authors found a trend that mitochondrial oxygen consumption was correlated with higher milk yields, but did not measure intake or efficiency. Therefore, FCE and FEL may be the best measures of efficiency in lactating dairy cattle, especially relative to mitochondrial oxygen consumption, but none of these methods appear to capture State 4 respiration, which may be the biggest contributor to metabolic efficiency.

4 | CONCLUSION

Current Nutrient Requirements of Dairy Cattle (National Research Council, 2001) recommendations are to ignore Cu, Mn and Zn endogenous levels in forages and grains and to add Cu, Mn and Zn

exogenously to meet requirements. These recommendations may lead to excessive levels in diets if supplied in inorganic forms and may decrease efficiency and decrease milk, solids and protein yields. LowMn supplementation increases mitochondrial oxygen consumption due to proton leak and may decrease milk, solids and protein yields.

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