Precalving and early lactation factors that predict milk casein and fertility in the transition dairy cow

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ABSTRACT

Multiparous Holstein cows ($n = 82$) of either high or low genetic merit (GM) (for milk fat + protein yield) were allocated to 1 of 2 diets in a 2×2 factorial design. Diets differed in the ratio of rumen-undegradable protein (RUP) to rumen-degradable protein (37% RUP vs. 15% RUP) and were fed from 21 d precalving to 150 days in milk. This study evaluated the effects of these diets and GM on concentrations of milk casein (CN) variants and aimed to identify precalving and early lactation variables that predict milk CN and protein yield and composition and fertility of dairy cows. It explored the hypothesis that low milk protein content is associated with lower fertility and extended this hypothesis to also evaluate the association of CN contents with fertility. Yields (kg/d) for CN variants were 0.49 and 0.45 of α -CN, 0.38 and 0.34 of β -CN, 0.07 and 0.06 for κ -CN, and 0.10 and 0.09 of γ -CN for high- and low-RUP diets, respectively. Increased RUP increased milk, CN, and milk protein yields. Increased GM increased milk protein and γ -CN yields and tended to increase milk CN yield. The effects of indicator variables on CN variant yields and concentrations were largely consistent, with higher body weight and α -amino nitrogen resulting in higher yields, but lower concentrations. An increase in cholesterol was associated with decreased CN variant concentrations, and disease lowered CN variant yield. A diet high in RUP increased proportion of first services that resulted in pregnancy from 41 to 58%. Increased precalving metabolizable protein (MP) balance decreased the proportion of first services that resulted in pregnancy when evaluated in a model containing CN percentage, milk protein yield, diet, and

GM. This finding suggests that the positive effects of increasing dietary RUP on fertility may be curvilinear because cows with a very positive MP balance before calving were less fertile than those with a lower, but positive, MP balance. Prepartum MP balance was important to production and reproductive outcomes, but surprisingly, metabolizable energy balance was not. The hazard of pregnancy in the first 150 d of lactation was 28% lower in cows producing milk with the lowest quartile of protein percentage compared with cows with milk in the upper 3 quartiles. Milk CN percentage was positively associated with improved pregnancy at first service. This study demonstrates the importance of protein metabolism to reproductive performance of the dairy cow.

Key words: fertility, protein degradability, casein

INTRODUCTION

Good nutritional management of dairy cattle during the transition period can improve their responses to the metabolic challenges posed by late pregnancy and early lactation (Bell, 1995; Lean et al., 2014). Hence, metabolic status and dietary interventions before calving are valuable to study as predictors of future productivity and reproductive performance (Bell, 1995). Much of the research in this area has focused on nutritional interventions after calving or around the time of conception (Patton et al., 2007), but these metabolic processes can be influenced before calving and in early lactation because the body tissue reserves the cow has available affect subsequent productivity and health (LeBlanc, 2010; Lean et al., 2013; Bradford et al., 2015). Identifying the key indicators associated with better productive and reproductive outcomes may allow cattle to be better fed and managed to achieve maximum productivity. Evidence exists that higher producing cows have lower fertility (Spalding et al.,

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1975; Lean et al., 1989) and that genetic selection for increased production can reduce fertility (Hageman et al., 1991; Buckley et al., 2000; Horan et al., 2005; Pollott and Coffey, 2008). These observations suggest the need to investigate differences in productive and reproductive performance and metabolism in cows of differing genetic merit (**GM**).

Bovine milk contains 2.5 to 3.5% true protein, approximately 80% of which is CN (Coulon et al., 1998). Casein has 4 main variants (α_{S1} -CN, α_{S2} -CN, β -CN, and κ -CN) and an additional class, γ -CN, which is a product of postsecretion proteolytic breakdown of β -CN (Fox and Mulvihill, 1982). Each CN variant may have distinct properties, but it is not known if different CN variants are associated with better production or reproductive outcomes, or what factors affect production of specific variants. It is well recognized that increased dietary RUP and metabolizable AA flux can increase milk yield when fed during early lactation (Carroll et al., 1994; Cunningham et al., 1996; McCormick et al., 1999; Doepel et al., 2004), but knowledge of the effects of protein nutrition during the precalving period is more limited, and 2 meta-analyses failed to identify optimal estimated MP (Lean et al., 2003) or dietary CP concentrations (Lean et al., 2012). The role of specific AA nutrition precalving on production and reproductive performance remains to be determined, but nutritional principles dictate that AA nutrition must be important.

Cattle can use nutrients from the diet and from endogenous body reserves, hence diet is important both before calving, when body reserves are being accreted, and during lactation and the breeding period. Dietary protein intake is of particular interest because improved protein nutrition in transition and early lactation can improve production, fertility, and health in the dairy cow (van Saun et al., 1993). Milk yield was unchanged when RUP concentration in the diet was increased before calving (van Saun et al., 1993), but milk protein percentage increased from 2.96 to 3.18%. Increasing the amount or degradability of dietary protein lowers the proportion of cows pregnant to service (Butler, 2000; Lean et al., 2012); however, decreasing the degradability of the dietary protein can improve reproductive outcomes (Folman et al., 1981; Carroll et al., 1994). Further, large observational studies suggest that reproductive performance is improved in cows with high milk protein concentration (Moss, 2001; Buckley et al., 2003; Morton, 2004; Madouasse et al., 2010). Given that CN represents 80% of milk proteins, it can be hypothesized that evaluating the precalving factors that influence CN variants and milk protein concentration may provide insight into the metabolic pathways that are associated with both lower milk protein and lower

fertility. Further, the effects of GM on CN, milk, milk protein production and content, and fertility should be examined in the context of these studies. Given that very few detailed studies of milk CN variants exist and none of these provide reproductive data, this study provides a unique opportunity to examine relationships between these factors and diet, GM, and metabolic status as reflected by precalving indicators and immediate postcalving measures, albeit with limited numbers of cattle.

The indicator variables that most reliably predict future reproductive and productive performance are yet to be defined. Body weight and BCS are often used as easily measured proxies for energy or, more critically, nutrient reserves (Edmondson et al., 1989), and excessive loss or low levels of these nutrients are associated with lower fertility (Garnsworthy and Topps, 1982; Buckley et al., 2003; López-Gatius et al., 2003). Similarly, changes in or absolute values for blood metabolites, including glucose, free fatty acids, BHB, insulin, and IGF-I, have been associated with changes in reproductive performance (Leroy et al., 2005, 2008; LeBlanc, 2010; Ospina et al., 2010; Chapinal et al., 2012); however, these indicators are not uniformly associated with adverse reproductive outcomes (Chapinal et al., 2012). The importance of metabolic factors during lactation that influence fertility was examined by Westwood (1998) and Westwood et al. (2000, 2002), but metabolite concentrations during the period before calving have not been as comprehensively evaluated as indicators of future performance. As such, one goal of this study was to screen factors during the prepartum period as potential indicators of future productive and reproductive performance.

In the current study, we reanalyzed data from an older study (Garvin, 1999; Westwood et al., 2000, 2002) and modeled some new data to (1) describe the effects of protein nutrition and GM on CN variants; (2) to explore precalving and early lactation factors that may predict production, especially protein and CN production, and reproductive performance; (3) to evaluate an *a priori* hypothesis that low milk protein content is associated with poor fertility and to understand the factors, including CN variant yield and composition, that contribute to this situation.

MATERIALS AND METHODS

Cows and Feeding

This study is a re-examination and extension of data obtained from studies by Garvin (1999) and Westwood et al. (2000, 2002). Multiparous Holstein-Friesian cows

($n = 82$) were blocked by GM (high or low Australian Breeding Value [ABV] of milk fat + milk protein yield) and maintained on 1 of 2 diets differing in protein degradability from 21 d precalving to 150 d postpartum in a 2×2 factorial arrangement. Within GM groups, cows were paired according to calving date, age, and lactation number before random allocation to dietary group. Diets were isonitrogenous (dry cow 10.5% CP; lactating cow 19.3% CP) and isoenergetic (dry cow 10.0 MJ/kg of ME; lactating cow 11.3 MJ/kg of ME) and differed in the ratio of RUP to RDP protein (37% RUP:63% RDP vs. 15% RUP:85% RDP). Nineteen cows of high GM and 21 cows of low GM received the diet high in RUP, and 21 cows in each of the high and low genetic groups received the diet low in RUP. Cows were housed in an outdoor, shaded corral with a dirt floor, and feed was individually delivered, removed, and measured in a TMR via a Calan feeding system (American Calan Inc., Northwood, NH) located under shade and on a concrete base that was scraped daily. Before entry into the experiment cows were trained in use of this system. Total mixed rations consisted of chaffed alfalfa and oaten hays and a concentrate pellet. Full details of feed composition and analysis are available in Westwood et al. (2000) and in Supplementary Table S1 (<http://dx.doi.org/10.3168/jds.2015-10275>).

Data Collection

Daily DMI was calculated individually for each cow and averaged weekly. Weekly BW and BCS (1–5 scale;

Edmondson et al., 1989) were recorded every 7 ± 3.5 d from entry into trial until week 10 of lactation.

Sample Collection and Analysis

Coccygeal blood samples were collected weekly, commencing 3 wk before parturition until week 10 of lactation ($n = 14/\text{cow}$). Samples were analyzed for plasma BHB, cholesterol, glucose, urea, α -amino nitrogen (AAN), and serum free fatty acid concentrations as described in Westwood et al. (2000).

Cows were milked twice daily, with milk yields recorded manually at each milking and an average daily production calculated weekly for the first 10 wk of lactation. Milk fat and protein concentrations were determined weekly for the first 10 wk of lactation using infrared spectrometry (Milkoscan 605 analyzer, Foss Electric, Hillerød, Denmark). Skim milk was retained for CN precipitation and analysis at week 1 to 10 of lactation for casein yield and percentage, and weeks 2, 6, and 10 of lactation for CN variant yields and percentages. This was multiplied by the amount of skim milk as a percentage of whole milk to obtain the CN concentration in whole milk (mg/mL). Individual CN variant yields were determined by fast protein liquid chromatography, using SDS-PAGE. The method used was described by Davies and Law (1987) with minor modifications as described by Garvin (1999). α -Casein concentration was a combination of both α_{S1} -CN and α_{S2} -CN. For this study, average concentrations of blood metabolites and estimated ME and MP balance taken

Table 1. Description of variables included in models

Variable ¹	Description ²	Unit
AAN	Plasma AAN, average for wk –3 to –1	mmol/L
Genetic merit	Australian Breeding Value (ABV) for milk fat + protein yield	
BW	Body live weight, average of wk –3 to –1	kg
BCS	BCS, average for wk –3 to –1	1–5 scale, assessed to quarter points
Calcium	Plasma calcium, average for wk –3 to –1	mmol/L
Cholesterol	Plasma cholesterol, average for wk –3 to –1	mmol/L
BHB	Plasma BHB, average for wk –3 to –1	mmol/L
Glucose	Plasma glucose, average for wk –3 to –1	mmol/L
ME balance	ME balance, average for wk –3 to –1	MJ of ME/d
MP balance	MP balance, average for wk –3 to –1	g/d
Milk yield	Unless otherwise stated, average for milk yield for wk 1–3 of lactation	kg/d
Milk protein yield	Milk protein yield, average for wk 1–3 of lactation	kg/d
Milk protein percentage	Milk protein percentage, average for wk 1–3 of lactation	% whole milk
Milk CN yield	Milk CN yield, average for wk 1–3 of lactation	kg/d
Milk CN percentage	Milk CN percentage, average for wk 1–3 of lactation	% whole milk
CN variant yield	Milk α -, β -, γ -, or κ -CN yield, average for wk 2, 7, and 10 of lactation	kg/d
CN variant percentage	Milk α -, β -, γ -, or κ -CN percentage, average for wk 2, 7, and 10 of lactation	% whole milk
Free fatty acids	Serum free fatty acids, average for wk –3 to –1	$\mu\text{mol/L}$
Urea	Plasma urea, average for wk –3 to –1	mmol/L

¹AAN = α -amino nitrogen.

²Week in relation to parturition.

in the 3 wk before calving and milk yield and composition in the first 3 wk after calving were evaluated as indicator variables for inclusion in statistical models evaluating production and reproduction (Table 1).

Reproduction

Commencing 21 ± 3.5 d after calving, all cows were monitored by rectal palpation of the reproductive tract to identify any condition that might compromise reproductive performance. The monitoring was repeated every 3 wk for cows that had not been inseminated during the previous 42 d. A voluntary waiting period of 45 d was observed after which cows were bred 6 to 18 h after the observation of primary or secondary heat signs. Estrus detection, starting immediately after calving, was aided by visual observation for at least 14 h/d and by placing KaMaR Heatmount detectors (Steamboat Springs, CO) on the tail-head of each cow. Cows were paired (one from each dietary group) and artificially inseminated with semen from the same ejaculate. All cows were inseminated by the same technician. Successful pregnancy was identified by a positive pregnancy test via palpation 42 d after a mating had occurred. Palpation was repeated 7 d later to confirm pregnancy, and once a pregnancy was confirmed, routine palpation was not continued. The interval from calving to pregnancy was defined as the number of days between calving and mating that resulted in a positive pregnancy test by palpation 42 d after mating. Full details of reproductive management and measurement are available in Westwood et al. (2000).

Disease

Treatment records were used to identify incidence of disease. Cows were classified as either being affected or not affected by "severe disease" between 6 wk precalving to 150 DIM. Severe disease was classified as one or more of the following conditions being present: acute mastitis, retained fetal membrane or retained placenta, metritis or peritonitis, hypocalcaemia, ketosis, or displaced abomasum. The workers coding the diseases were not aware of the cows' production or reproductive outcomes.

Nitrogen and Energy Balance Modeling

Weekly MP balance was estimated for each cow from 3 wk prepartum until wk 10 of lactation using CPM dairy (version 3.08; Cornell-Penn-Miner, <https://cahpwww.vet.upenn.edu/doku.php/software:cpm:purchase>). Calculations were based on dry cow rations in wk -3 to 0 (weeks numbered in relation to parturition at wk

0), early lactation rations in estimates for wk 1, and lactation ration for wk 2 to 10 inclusive. Calculations included individual weekly DMI, BW, BCS, milk yield, milk fat percentage, milk protein percentage, parity, lactation status, age, and days pregnant. Dietary evaluations were based on the feed analysis results for the individual components of the diet. Feed evaluations were conducted on change of pellet or forage. Weekly ME balances were calculated based on formulas described by Agricultural and Food Research Council (AFRC, 1993) as detailed by Westwood et al. (2000).

Statistical Analysis

All data analysis was performed using Stata (Intercooled Stata v. 13, StataCorp, College Station, TX).

Production Variables. Descriptive analysis of variables was undertaken, and the effects of diet and GM for each variable are provided in Table 2. Diet and GM were included in all models as categorical variables. Mixed models (XTMIXED, Stata version 13.1) were used to explore the significance of the precalving indicator variables on milk yield and milk protein and CN yield and composition. Precalving indicator variables refer to serum free fatty acids, plasma AAN, calcium, cholesterol, BHB, glucose, and urea, ME balance, MP balance, BW, BCS, and the severe disease variable (Table 1). Effects of season had been evaluated previously and were found to be insignificant (Westwood et al., 2002). For all factors other than disease, an average value for the 3 wk before calving was used. The following linear mixed model was used:

$$Y_{ijkl} = \mu + \beta_i + \gamma_j + \theta_k + (\beta\gamma)_{ij} + (\beta\gamma\theta)_{ijk} + X_l + \varepsilon_{ijkl}$$

where Y_{ijkl} = response to diet i (i = high RUP or low RUP) and GM j (j = high or low ABV for milk fat + protein) at the k th time (k = week 1 to 10 of lactation) for cow number l (l = 1 to 82); μ = overall mean; β_i = fixed effect of diet; γ_j = fixed effect of GM; θ_k = fixed effect of week (time); $(\beta\gamma)_{ij}$ = effect of diet by GM interaction; $(\beta\gamma\theta)_{ijk}$ = effect of diet by GM by week interaction; X_l = random effect of animal; ε_{ijkl} = random residual error within animal l , on diet i , with GM j at week k . Indicator variables were included in models containing diet, GM, and week of lactation as a priori fixed effects. An autoregressive (AR1) covariance structure was used based on superior fit to other covariance structures. Variables that had a univariable P -value of less than 0.2 were included for evaluation in multivariable models using stepwise modeling with backward elimination. Once removed, variables were not eligible for re-entry into the model. Final models were

Table 2. Descriptive analysis of BW, BCS, and predictor variables [mean (SD) averaged for the 3 wk before calving]¹

Variable	Diet		Genetic merit	
	High RUP	Low RUP	Low ABV	High ABV
BW (kg)	665.73 (74.06)	655.41 (65.73)	650.65 (68.65)	669.77 (70.21)
BCS (1–5)	3.39 (0.32)	3.44 (0.23)	3.42 (0.30)	3.41 (0.25)
MP balance (g/d)	511.38 (183.34)	356.18 (128.14)	444.83 (168.89)	426.95 (183.58)
ME balance (MJ of ME/d)	27.44 (33.34)	57.14 (30.85)	44.99 (23.77)	39.00 (43.52)
Free fatty acids (μmol/L)	264.57 (149.63)	298.95 (243.40)	258.90 (145.37)	305.33 (246.76)
Urea (mmol/L)	5.66 (1.34)	5.87 (1.20)	5.72 (1.22)	5.81 (1.33)
α-Amino nitrogen (mg/100 mL)	3.73 (0.47)	3.69 (0.47)	3.83 (0.50)	3.59 (0.41)
Glucose (mmol/L)	3.63 (0.52)	3.62 (0.40)	3.71 (0.43)	3.55 (0.49)
Calcium (mmol/L)	2.29 (0.27)	2.26 (0.25)	2.30 (0.26)	2.24 (0.25)
Cholesterol (mmol/L)	2.58 (0.81)	2.50 (0.58)	2.58 (0.67)	2.50 (0.73)
BHB (mmol/L)	1.82 (1.51)	1.34 (0.36)	1.36 (0.31)	1.30 (0.31)

¹ABV = Australian Breeding Value for milk fat + protein yield.

assessed based on Wald tests and Akaike's information criterion (AIC) to evaluate model fit and confounding was assessed by evaluating changes in coefficients as new terms were added into the models. A similar mixed model with an independent covariance structure (XTMIXED, Stata version 13.1) was used to explore the significance of the precalving indicator variables on 8 factors of milk CN variant composition and yield (yield and percentage for each of α-, β-, γ-, and κ-CN). Multivariable models were determined in the same way as for milk yield and protein variables.

Reproductive Outcomes. The proportion of first services that resulted in pregnancy was assessed using logistic regression models (LOGISTIC, Stata version 13.1) with diet and GM included as a priori fixed effects. Indicator variables and early lactation production factors were included as individual covariables. Early lactation production factors included milk yield and protein and CN yield and percentage, and an average of the first 3 wk of lactation was used. Those with *P*-values <0.2 were evaluated in multivariable backward stepwise modeling. A logistic normal nonparametric accelerated time failure survival analysis (STREG, Stata version 13.1) was used to further explore the effect of these precalving indicator variables and early lactation production factors on the risk of pregnancy up to 150 DIM. Cows that were not pregnant at 150 DIM were considered nonpregnant, even if they became pregnant after 150 DIM, resulting in right censoring of the data. Cows were then separated into quartiles based on their milk protein percentage (average of first 3 wk after calving) using the lowest quartile as reference group. A further analysis was then performed to assess the following time-varying covariables using a Cox's proportional hazards regression model (STCOX, Stata version 13.1) for calving to pregnancy interval: ME balance, MP balance, milk yield, milk protein per-

centage, milk protein yield, CN percentage, CN yield, serum free fatty acids, and plasma urea, AAN, glucose, calcium, cholesterol, and BHB.

Milk Protein Percentage. Cows were again separated into quartiles based on their milk protein percentage, and cows with the lowest milk protein percentage were compared with those of the upper 3 quartiles using canonical linear discriminant analysis (CANDISC, Stata version 13.1). Significant differences that existed between the 2 groups in the precalving indicator variables and early lactation production traits were identified. A backward stepwise logistic regression (LOGISTIC, Stata version 13.1) was also performed to assess the results of the discriminant analysis and validated that these variables did differ (data not shown) between the groups with the lowest and higher milk protein content.

RESULTS

Effect of Dietary Protein Degradability and GM on Indicator Variables

Descriptive analyses of the average blood concentrations of indicator variables in the 3 wk prepartum are included in Table 2. Cows receiving more RUP in the diet or those of higher GM had numerically higher BW and lower BCS. An increased proportion of RUP in the diet increased prepartum MP balance (Table 3 and Figure 1), and cows of high GM tended to have a lower prepartum MP balance (Table 3). Figure 1 shows that this pattern continued into early lactation.

Production Responses

Univariable and multivariable relationships between indicator and production variables, in which diet, GM,

Table 3. Effects of diet and genetic merit (GM) on MP balance; model includes effects of diet, GM, and diet $\times$ GM interaction¹

Evaluation	Predicted mean (SE) (g/d)	95% CI	Significance (P-value)
Diet			0.001
Low RUP	87.59 (24.08)	40.40–134.78	
High RUP	405.77 (23.50)	359.72–451.82	
Genetic merit			0.096
High ABV ²	223.15 (23.50)	177.10–269.20	
Low ABV	279.34 (24.08)	232.15–326.53	
Diet $\times$ GM ³			0.794

¹Week $P = 0.0001$; diet $\times$ GM $\times$ week $P = 0.6295$.²ABV = Australian Breeding Value for milk fat + protein yield.³Predicted means for each of the 4 diet $\times$ GM interactions did not differ significantly and are not shown.

and week were included as a priori factors, are provided in Supplementary Table S2 (<http://dx.doi.org/10.3168/jds.2015-10275>) and Table 4, respectively.

Milk Protein Yield and Percentage. The increase in milk protein production with the higher RUP diet was marked (0.13 kg/cow per day) (Table 4). Milk protein yield increased by approximately 0.08 kg/cow per day in the group with higher GM for milk solids production (Table 4). Protein yield was associated with higher precalving plasma AAN concentrations, BW, and MP balance (Table 4, Supplementary Table S2; <http://dx.doi.org/10.3168/jds.2015-10275>). Milk protein percentage was unaffected by diet or GM (Table 4). Increased prepartum blood cholesterol, BW, and plasma AAN were associated with lower milk protein percentage (Table 4, Supplementary Table S2).

Milk Casein Yield and Percentage. Decreasing the degradability of the protein in the diet increased milk CN yield by 0.09 kg/d (Table 4, Figure 2). Body weight, MP balance, glucose, and disease all had significant associations with CN yield (Supplementary Table S2; <http://dx.doi.org/10.3168/jds.2015-10275>). Higher

CN yield was observed in cows with higher precalving BW, consistent with the positive influence of BW on total milk protein yield. Incidence of severe disease lowered CN yield, when it was included in the model with BW. Increased prepartum plasma glucose concentrations were associated with lower CN concentrations, as was disease and plasma calcium and cholesterol when explored individually (Supplementary Table S2), but these latter 3 factors were confounded by glucose concentrations and became nonsignificant in multivariable models containing plasma glucose (Table 4). Milk CN concentration was consequently lower with higher glucose, BW, and AAN (Table 4). Diet and GM did not influence milk CN percentage (Table 4, Figure 3).

Casein Variant Yields and Percentages. Yields (kg/d) for CN variants were 0.49 and 0.45 of α -CN, 0.38 and 0.34 of β -CN, and 0.07 and 0.06 of κ -CN, for high- and low-RUP diets, respectively. γ -Casein had yields of 0.10 and 0.09 for high- and low-RUP diets, respectively. Reducing the degradability of protein in the diet increased yields of α - and β -CN, but it did not significantly influence concentrations of any CN variants (Table 4). Largely consistent associations were found in the models evaluating the effects of individual indicator variables on CN variant yields and concentrations, with higher BW and AAN resulting in higher yield, but lower concentrations; decreased protein yield was associated with disease, and an increase in cholesterol was associated with decreased CN concentration. α -Casein and β -CN yields were affected by the same variables as CN yield; increased by BW and decreased by disease (Table 4). Increased BW lowered α - and β -CN percentage but increased κ -CN yield. Increased BCS increased only γ -CN yield. Increased cholesterol concentrations were associated with lowered α -, γ -, and κ -CN percentages, and higher precalving plasma calcium concentrations lowered κ -CN yield. Increased AAN concentrations precalving increased κ -CN yield and decreased α - and β -CN percentages.

Figure 1. Effect of diet and genetic merit on estimated MP balance. ABV = Australian Breeding Value for milk fat + protein yield.

Table 4. Multivariable models for milk yield and composition including indicator variables, and diet and genetic merit as a priori factors

Dependent variable	Covariable	Regression coefficient (SE)	Significance	Diet [Mean (SE)]		Genetic merit [Mean (SE)]		P-value	P-value
				High RUP	Low RUP	High ABV ¹	Low ABV		
Milk yield (kg/d)	BW	0.043 (0.008)	0.001	39.66 (0.85)	36.30 (0.82)	39.07 (0.84)	36.74 (0.85)	0.004	0.053
	AAN ²	4.042 (1.270)	0.001						
Milk protein yield (kg/d)	BW	0.001 (0.001)	0.001	1.26 (0.02)	1.13 (0.02)	1.23 (0.02)	1.15 (0.02)	0.001	0.014
	MP balance	0.001 (0.001)	0.002						
Milk protein %	AAN	0.101 (0.019)	0.001						
	Cholesterol	-0.079 (0.029)	0.007	3.07 (0.03)	3.09 (0.03)	3.07 (0.03)	3.09 (0.03)	0.664	0.689
	AAN	-0.096 (0.046)	0.035						
	BW	-0.001 (0.001)	0.038						
Milk CN yield (kg/d)	BW	0.001 (0.001)	0.001	1.04 (0.02)	0.95 (0.02)	1.01 (0.02)	0.97 (0.02)	0.002	0.097
	Disease	-0.077 (0.029)	0.009						
Milk CN %	BW	-0.001 (0.001)	0.001	2.54 (0.03)	2.58 (0.03)	2.54 (0.03)	2.58 (0.03)	0.353	0.408
	Glucose	-0.134 (0.047)	0.005						
	AAN	-0.214 (0.048)	0.001						
	BW	0.001 (0.001)	0.001	0.49 (0.01)	0.45 (0.01)	0.48 (0.01)	0.46 (0.01)	0.002	0.248
Milk α-CN yield (kg/d)	Disease	-0.033 (0.016)	0.034						
	BW	-0.001 (0.001)	0.005	1.19 (0.02)	1.22 (0.02)	1.19 (0.02)	1.22 (0.02)	0.039	0.126
	AAN	-0.076 (0.028)	0.007						
	Cholesterol	-0.045 (0.019)	0.015						
Milk β-CN yield (kg/d)	Disease	-0.036 (0.013)	0.006	0.38 (0.01)	0.34 (0.01)	0.36 (0.01)	0.36 (0.01)	0.001	0.555
	BW	0.001 (0.001)	0.003						
Milk β-CN %	BW	-0.001 (0.001)	0.001	0.93 (0.02)	0.92 (0.02)	0.91 (0.02)	0.95 (0.02)	0.685	0.119
	AAN	-0.078 (0.025)	0.002						
Milk γ-CN yield (kg/d)	BCS	0.026 (0.010)	0.009	0.10 (<0.01)	0.09 (0.004)	0.10 (<0.01)	0.09 (<0.01)	0.149	0.015
	Cholesterol	-0.029 (0.012)	0.011	0.24 (0.011)	0.25 (0.011)	0.25 (0.011)	0.24 (0.011)	0.539	0.341
Milk κ-CN yield (kg/d)	BW	0.001 (0.001)	0.001	0.07 (<0.01)	0.06 (<0.01)	0.07 (<0.01)	0.07 (<0.01)	0.442	0.944
	AAN	0.014 (0.003)	0.001						
Milk κ-CN %	Ca	-0.014 (0.006)	0.020						
	Cholesterol	-0.019 (0.006)	0.001	0.16 (0.01)	0.18 (0.01)	0.16 (0.01)	0.18 (0.01)	0.063	0.174
	AAN	0.019 (0.009)	0.028						

¹ABV = Australian Breeding Value for milk fat + protein yield.

²AAN = α-amino nitrogen.

Table 5. Summary of reproductive variables; mean (no of cows) for pregnancy to first service, mean (SD) for interval from calving to pregnancy

Reproductive variable ¹	Diet		Genetic merit ²	
	High RUP	Low RUP	Low ABV	High ABV
Pregnancy to first service (%)	58 (23/40)	41 (17/41)	46 (18/39)	52 (22/42)
Interval from calving to pregnancy (d)	94.75 (32.47)	104.05 (36.28)	102.90 (5.33)	96.26 (33.92)

¹Cows that were not pregnant at 150 DIM were considered nonpregnant, even if they became pregnant after 150 DIM, resulting in right censoring of the data.

²ABV = Australian Breeding Value for milk fat + protein yield.

Reproductive Responses

Increasing the percentage of RUP in the diet increased the percentage of pregnancies to first service from 41 to 58% and tended to decrease the calving to pregnancy interval by 9 d (Table 5), although diet and GM were not significant when assessed in the multivariable model (Table 6). In the multivariable model, higher early lactation CN percentage and milk protein yield (kg/d) and decreased prepartum MP balance (g/d) improved the proportion of first services that resulted in pregnancy when diet, GM, and their interaction were included in the statistical model (Table 6). Individually, improved CN yield tended to be associated with increased pregnancy to first service (Supplementary Table S3; <http://dx.doi.org/10.3168/jds.2015-10275>), a result consistent with the positive effect of the higher RUP diet on CN yield and pregnancy to first service. None of the other factors tested univariably or as time-varying covariables influenced calving to pregnancy interval (data not shown).

Low Milk Protein and CN

To explore the hypothesis that low milk protein can predict poor fertility, cows were divided into quartiles and the group producing the lowest milk protein percentage was compared with the cows in the upper 3 quartiles. The hazard of pregnancy was 28% lower in cows producing the lowest quartile of milk protein percentage during the first 150 DIM compared with the

upper 3 quartiles (Table 7, Figure 4). Differences in the precalving indicator variables between the 2 groups were explored using discriminant analysis and logistic regression. Both methods identified the same factors associated with lowest milk protein percentage (Table 8). Cows producing a lower milk protein percentage produced 3.87 L/cow per day more milk on average than cows in the other 3 quartiles (Table 9). They also had higher prepartum plasma calcium and glucose and estimated MP balance compared with the group producing a higher milk protein percentage (Tables 7 and 8). The group with low milk protein had 0.17 mmol/L less urea in the blood prepartum on average than the group producing a higher milk protein percentage.

DISCUSSION

This study re-examines and builds on the original work of Garvin (1999), Westwood (1998), and Westwood et al. (2000, 2002), using the milk composition data set of the first study and the reproductive data sets of the latter two. The detail and high quality of these data provided the opportunity to explore new hypotheses and improve metabolic understanding of milk protein and CN variants. This study had 3 main aims: (1) to describe the effects of protein nutrition and GM on CN variants; (2) to explore precalving and early lactation factors that may predict production, especially protein and CN production, and reproductive performance, and (3) to evaluate an a priori hypothesis that low milk protein content is associated with poor fertility and to

Table 6. Logistic regression model of factors affecting the proportion of first services that resulted in pregnancy

Variable	Odds ratio (SE)	95% CI	Significance (P-value)
Diet ¹	0.86 (0.622)	0.210–3.545	0.839
Genetic merit (GM) ²	0.86 (0.603)	0.217–3.401	0.828
Diet × genetic merit	1.10 (1.090)	0.156–7.697	0.926
CN %	9.86 (10.261)	1.283–75.782	0.028
Milk protein yield (kg/d)	20.09 (27.721)	1.344–300.264	0.030
MP balance (g/d)	1.00 (0.002)	0.993–0.999	0.044

¹The diet higher in RUP was used as the reference group. The effect of diet is at the reference category of GM.

²The higher ABV was used at the reference group; the effect of GM is at the reference category of diet.

Figure 2. Average milk casein yield by week for diet and genetic merit groups. ABV = Australian Breeding Value for milk fat + protein yield.

Figure 3. Average milk casein percentage by week for diet and genetic merit groups. ABV = Australian Breeding Value for milk fat + protein yield.

Figure 4. Kaplan-Meier survival plot of the time to pregnancy within 150 d, separated for cows producing the lowest quartile of milk protein percentage and the upper 3 quartiles of milk protein percentage.

Table 7. Multivariable model of factors predicting time to pregnancy within 150 d of calving using a logistic normal nonparametric accelerated time failure survival analysis, with milk protein percentage as a binary variable (lowest quartile as reference group)

Variable	Hazard ratio (SE)	95% CI for hazard ratio	Significance (<i>P</i> -value)
Diet ¹	1.05 (0.115)	0.850–1.303	0.640
Genetic merit ²	1.01 (0.110)	0.812–1.245	0.962
Milk protein %	0.72 (0.096)	0.556–0.936	0.014

¹The diet higher in RUP was used as the reference group.

²Genetic merit is defined as Australian Breeding Value (ABV) for milk fat + protein yield. The higher ABV was used as the reference group.

Table 8. Multivariable model for factors affecting low milk protein percentage, created using backward stepwise logistic regression

Variable	Odds ratio	95% CI	Significance (<i>P</i> -value)
Average milk yield (kg/d)	0.91 (0.041)	0.831–0.993	0.034
Average urea (mmol/L)	1.97 (0.624)	1.063–3.666	0.031
Average glucose (mmol/L)	0.24 (0.170)	0.058–0.971	0.045
Average calcium (mmol/L)	0.04 (0.055)	0.002–0.653	0.024
Average MP balance (g/d)	1.00 (0.002)	0.992–1.000	0.029

understand the factors, including CN variant yield and composition, that contribute to this situation. Factors after calving and around breeding that can be used as predictors of fertility and production were well explored by Westwood et al. (2000, 2002). This study explores factors before calving and in very early lactation that influence fertility and production. Because of the large amount of analyses, we have not sought to address all of these factors in the Discussion but have focused on the most important results, which are summarized in the conclusion, and on consistently identified metabolic influences on production and reproduction in the study.

Individual CN composition may affect milk-processing outcomes; however, little information exists on yields of CN variants (Barry and Donnelly, 1980; Kroeker et al., 1985; Davies and Law, 1987) or nutritional and metabolic factors that may determine individual CN yields or percentages. Average CN content varied considerably between individual cows (range 1.3–4.5% total milk, average 2.6%) (Garvin, 1999). In this study, α -, β -, κ -, and γ -CN represented approximately 47, 37, 7, and 9% of total CN, respectively (Garvin, 1999). α -Casein concentrations were similar to those presented by Davies and Law (1980), Barry and Donnelly (1980), and Kroeker et al. (1985) (48.3, 48.7, and 59.9%, respectively). The β -CN percentage was similar to that of Davies and Law (1980) and Barry and Donnelly (1980) (35.7 and 33.8%, respectively), although κ -CN was lower and γ -CN was higher than previously reported (12.8 and 3.2% for κ - and γ -CN, respectively) (Davies and Law, 1980). Different milk protein polymorphisms may also be associated with changes in milk, milk fat, and milk protein yields (Ng-Kwai-Hang et al., 1984), but these were not explored in this study. It is unsurprising that

the factors affecting α - and β -CN yields were the same those identified for total CN yield because these collectively accounted for more than 80% of total CN yield.

Milk yield and milk protein responses to changes in dietary protein and AA supply are variable (Doeppel et al., 2004). In this study, increasing the ratio of RUP:RDP in the diet by using a high-quality protein source increased early lactation milk, milk protein, and milk CN yield. The milk yield response is similar to linear increases in production in response to increased RUP supplementation (Wright et al., 1998). However, in a review of 127 trials that replaced soybean meal with sources higher in RUP, only 17% of comparisons identified significantly higher milk yield in groups fed higher RUP (Santos et al., 1998). This effect varied, based on the type of RUP source used, possibly because of changes in profile of absorbed AA. Sannes et al. (2002) did not find an effect on milk yield of feeding increased RDP, nor did Reynal and Broderick (2005). These varying responses reflect the complexity of assessing and improving MP status and a limitation in study design of nutritional studies, in which the substitution of one feed for another creates a complex series of changes that may not simply increase the MP supply of the most rate-limiting AA nor improve the overall nutrient balance. Importantly, in the current study, lower protein degradability significantly increased estimated MP balance (Table 3) and increased milk, protein, and CN yields, all indicating a positive effect of the diet higher in RUP (Tables 2 and 4). Further, milk α -, β -, and γ -CN yields were significantly increased by feeding the diet higher in RUP (Tables 2 and 4), with only the percentage of α -CN declining significantly with diet or GM. In general, increased yield of milk proteins is as-

Table 9. Group means (SD) of indicator variables differing between cows of lowest quartile for milk protein percentage or in the 3 higher quartiles identified using canonical linear discriminant analysis

Variable	Low milk protein	Average-high milk protein
Average milk yield (L/d)	40.70 (6.901)	36.83 (6.844)
Average urea (mmol/L)	5.64 (1.247)	5.81 (1.302)
Average glucose (mmol/L)	3.77 (0.380)	3.57 (0.486)
Average calcium (mmol/L)	2.37 (0.295)	2.24 (0.235)
Average MP balance (g/d)	497.38 (174.013)	414.43 (175.375)

sociated with lower concentrations, often as a result of increased milk production.

The tendency for increased early lactation milk yield for cows with higher ABV (Table 4) has been identified in other studies (Flux et al., 1984). Increased prepartum MP balance increased milk protein yield (Table 4). Cows in both dietary groups had excellent weight gain before calving, suggesting that neither group was limited by ME or MP availability prepartum (Table 2). The cows with higher ABV tended to have lower prepartum estimated ME and MP balances (Westwood et al., 2000; Tables 2 and 4). Abundant literature examines the detrimental effects of a negative energy balance on fertility (Wathes et al., 2007). However, in this study, ME balance before calving did not contribute to any production or fertility model (Table 4). Milk production and DMI are key determinants of ME and MP balances, and based on the current results, the importance of MP balance should be given further consideration in fertility studies.

Further insight into the factors present before calving that influence production traits, is provided in Table 4 and Supplementary Table S2 (<http://dx.doi.org/10.3168/jds.2015-10275>). Prepartum BW and AAN increased production for most production variables studied (Table 4, Supplementary Table S2). Body weight reflects the endogenous nutrient reserves available to the cow, and it is unsurprising that this factor increased milk, protein, CN, α -CN, β -CN, and κ -CN yields because it reflects an improved energy and nutrient status of cows. However, percentages of CN and β -CN were lower with higher BW precalving. Free circulating AA, as indicated by a higher AAN concentration, are the main precursors for milk protein and perhaps reflect labile protein stores and were associated with higher milk, protein, and κ -CN yields. Again lower milk CN concentration, including α - and β -CN, and consequently lowered milk protein percentage were associated with higher prepartum AAN (Table 4). The lower percentages of proteins may possibly have resulted from the positive effect of BW and AAN on milk yield. A negative effect of cholesterol on milk protein and α -, γ -, and κ -CN percentages (Table 4) was unexpected, and a causal link is not immediately obvious. However, increased cholesterol reflects a more positive energy balance (Lean et al., 1992) and may indicate a more anabolic state in which milk production is increased, decreasing milk protein and CN content. Therefore, the association of cholesterol itself with milk protein and CN content may not be causal but reflect this relationship with yield. Higher blood glucose, calcium, and cholesterol concentrations were associated with lower CN concentration, but glucose statistically confounded the other variables, which became nonsignificant in

multivariable models. Prepartum plasma calcium was a significant factor in several models (Supplementary Table S2; <http://dx.doi.org/10.3168/jds.2015-10275>), but it only increased κ -CN yield in the multivariable model. This finding may reflect the metabolic interactions between calcium and other indicator variables. For example, bone calcium and glucose are causally linked through osteocalcin and insulin metabolism, as was shown in murine studies (Lee et al., 2007), and reviewed in a bovine context (Lean et al., 2014). Elegant intervention studies by Martinez et al. (2014) demonstrated the central role of hypocalcaemia in influencing insulin and glucose concentrations. Further work is required to confirm the importance of these observations, but they suggest that relationships among bone and energy metabolism may be reflected in production and reproductive outcomes.

Westwood et al. (2002) identified a positive effect of increased RUP intake on fertility. However, in a multivariable model containing diet, GM, diet $\times$ GM interaction, CN percentage, and milk protein yield, higher prepartum MP balance lowered the risk of pregnancy to first service (Table 6). Other factors in this model, namely, CN percentage and milk protein yield, increased the proportion pregnant to first service, suggesting that cows receiving a diet higher in RUP that resulted in higher milk CN percentage were less successful reproductively if they retained more MP precalving or were less efficient with MP utilization. On average, cows in both dietary groups had substantial positive MP balances before calving. These findings, in conjunction with the results in Table 4 and Supplementary Table S2 (<http://dx.doi.org/10.3168/jds.2015-10275>), suggest the possibility that the reproductive response to anabolic stimuli precalving, such as BW, MP balance, and AAN that produced more milk, protein, and CN yields, may be curvilinear. Diets after calving may not be able to provide the protein, energy, fats, and other nutrients required to produce the extra proteins, fat, lactose, and other components of increased milk production stimulated by improved protein status prepartum.

Support for this concept comes from the observation that cows in the lowest quartile of milk protein percentage had slower times to pregnancy within 150 d of calving compared with cows in the other 3 quartiles. Further, a numerically lower proportion of the cows in this group were pregnant by 150 DIM (Table 7, Figure 4). Cows with higher milk CN percentage had greater odds of pregnancy to first service (Table 6). The multivariable model that described the linear milk protein percentage response (Table 4) differs from the factors that were associated with the lowest milk protein percentage quartile group (Table 9); however, considerable consistency is present because both models indicate

that factors that reflect an increased stimulus to milk reduced milk protein percentage. Further, the cows in these groups differed between the analyses because the latter compared 1 quartile of cows with the other 3. Cows in the lowest protein group had higher milk yield on average than those producing milk of a higher protein percentage. Plasma calcium and glucose and estimated MP balance were all higher before calving in the group producing low milk protein percentage. The latter measures reflect anabolic processes and support the observation that these cows had increased early lactation milk yield. The lowest milk protein group would therefore have had a greater irreversible loss of nutrients in milk, particularly thorough increased lactose production, and possibly an increase in protein and glucose used for mammaryogenesis in the early lactation period. Bickerstaffe et al. (1974) estimated that 69 to 98% of circulating glucose was used by the mammary gland. Most of this glucose is used as a precursor for lactose synthesis, but it is also essential as the main source of energy for the mammary gland (Wood et al., 1965) and ovary (Rabiee et al., 1997). Consequently, low milk protein and CN content may reflect disordered metabolic control in cows stimulated to produce more lactose and protein. Indicator variables before calving and a diet higher in RUP that had positive effects on reproduction and production reflected anabolic processes; however, it appears that not all cows are capable of integrating the nutrient demands for lactation with those required for reproductive outcomes. This population may be identified through a low milk protein or CN concentration.

CONCLUSIONS

The objectives of this study were (1) to describe the effects of protein nutrition and GM on CN variants; (2) to explore precalving and early lactation factors that may predict production, especially protein and CN production, and reproductive performance; and (3) to evaluate an a priori hypothesis that low milk protein content is associated with poor fertility and to understand the factors, including CN variant yield and composition, that contribute to this situation. We were able to conclude that decreasing protein degradability in the diet increased milk, milk protein, and CN yields, and increased GM increased milk protein and γ -CN yields and tended to increase milk CN yield. We also found that the effects of indicator variables that may predict productive and reproductive outcomes were largely consistent, confirming the importance of some well-determined causal factors such as BW and disease and identifying some less well-explored indicators, including cholesterol and AAN that are worthy of further exploration. A diet high in RUP increased the

proportion of first services that resulted in pregnancy, suggesting a positive effect of increased MP on fertility. However, when combined in a model containing CN yield, diet, GM, and diet $\times$ GM interaction, higher prepartum MP balance lowered the risk of pregnancy to first service. This finding suggests that the effects of increasing dietary RUP on fertility may be curvilinear. Anabolic factors measured prepartum, including BW, AAN, cholesterol, and glucose, increase production of milk, protein, CN, and CN variants, but they tended to reduce the percentage of these components in milk. Finally, we found low milk protein percentage in early lactation was associated with a lower risk of pregnancy within 150 DIM and a low milk CN percentage was associated with a lower proportion of cows pregnant at first service. Early lactation milk yield and prepartum MP balance and glucose and calcium concentrations were higher in the cows producing lower milk protein percentage, and prepartum urea was higher in this group. Metabolizable protein balance was significant and important to both production and reproduction, but surprisingly, ME balance was not. This study demonstrates the importance of protein metabolism to productive and reproductive performance.

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