A revised representation of urea and ammonia nitrogen recycling and use in the Molly cow model

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ABSTRACT

Accurately predicting nitrogen (N) digestion, absorption, and metabolism will allow formulation of diets that more closely match true animal needs from a broad range of feeds, thereby allowing efficiency of N utilization and profit to be maximized. The objectives of this study were to advance representations of N recycling between blood and the gut and urinary N excretion in the Molly cow model. The current work includes enhancements (1) representing ammonia passage to the small intestine; (2) deriving parameters defining urea synthesis and ruminal urea entry rates; (3) adding representations of intestinal urea entry, microbial protein synthesis in the hindgut, and fecal urea-N excretion; and (4) altering existing urinary N excretion equations to scale with body weight and adding purine derivatives as a component of urinary N excretion. After the modifications, prediction errors for ruminal outflows of total N, microbial N, and nonammonia, nonmicrobial N were 29.8, 32.3, and 26.2% of the respective observed mean values. Prediction errors of each were approximately 7 percentage units lower than the corresponding values before model modifications and fitting due primarily to decreased slope bias. The revised model predicted ruminal ammonia and blood urea concentrations with substantially decreased overall error and reductions in slope and mean bias. Prediction errors for gut urea-N entry were decreased from 70.5 to 26.7%, which was also a substantial improvement. Adding purine derivatives to urinary N predictions improved the accuracy of predictions of urinary N output. However, urinary urea-N excretion remains poorly predicted with 69.0% prediction errors, due mostly to overestimated urea-N entry rates. Adding representations of undigested microbial nucleic acids, microbial protein synthesized in the hindgut, and urea-N excretion in feces decreased prediction

errors for fecal N excretion from 21.1 to 17.1%. The revised model predicts that urea-N entry into blood accounts for approximately 64% of dietary N intake, of which 64% is recycled to the gut lumen. Between 48 and 67% of the urea recycled to the gut flows into the rumen largely depending on diet, which accounts for 29 to 54% of total ruminal ammonia production, and 65 to 76% of this ammonia-N is captured in microbial protein, which represents 17% of N intake. Based on model simulations, feeding a diet with moderately low crude protein and high rumen-undegradable protein could increase apparent ruminal N efficiency by 20%.

Key words: utilization efficiency, model, nitrogen, Molly cow model

INTRODUCTION

The ability to recycle urea-N to the rumen represents an evolutionary advantage of ruminants, allowing them to better utilize N, especially during periods of dietary protein deficiency. Many current models are empirical and based on data fits, so the level of aggregation of these models generally does not allow detailed analyses of specific metabolic reactions. Baldwin and coworkers included concepts and essential features of N metabolism in a mathematical model of the dairy cow named Molly (Baldwin et al., 1987a,b,c). Molly is a dynamic and mechanistic model that has potentially greater flexibility and functionality when evaluating different management strategies and feeding practices being considered for implementation. However, the added complexity also increases the difficulty of model parameterization. Based on the original model structure, Hanigan et al. (2013) reported biased predictions of ruminal ammonia concentrations and microbial N outflow, and Li et al. (2018) observed high proportions of ruminal ammonia and blood urea prediction errors due to slope bias. The latter work identified improper representations of urinary urea-N excretion and urea-N cycling between blood and the rumen as the primary drivers of the ammonia and blood urea prediction errors.

Received November 5, 2018.

Accepted January 27, 2019.

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To address such existing model problems, measurements of gut urea-N entry and urinary urea-N excretion rates are necessary to uniquely define N recycling between the rumen and blood and to define urea balance. Lobley et al. (2000) described a double-labeled urea approach to measure urea kinetics in ruminants, and developed a steady-state, mass balance model to estimate flux rates. The application of this approach has resulted in the generation of a significant number of flux observations in cattle in the last 20 yr, which can be used to examine model assumptions and address potential errors in the model.

The objectives of this study were to update the representations of N cycling across the gut wall, fecal N excretion, and urinary N excretion in the Molly model to more closely reflect the underlying biology, and to evaluate model prediction accuracy with respect to N and urea kinetics. The work included (1) representing ammonia passage to the small intestine; (2) deriving parameters defining urea synthesis and ruminal urea entry rates; (3) adding representations of intestinal urea entry rate, microbial protein synthesis in the hindgut, and fecal ammonia excretion derived from blood urea; and (4) altering the existing urinary N excretion equations to scale with BW and adding purine derivatives as

a component of urinary N excretion. We hypothesized that these updates would result in robust improvements in predictions of ruminal N cycling, microbial outflow from the rumen, and urinary N excretion.

MATERIALS AND METHODS

Data Collection

Observations of total urea entry, gut urea entry, and urinary urea elimination rates were collected from 21 published papers (Archibeque et al., 2001, 2002; Ruiz et al., 2002; Marini and Van Amburgh, 2003; Gozho et al., 2008; Wickersham et al., 2008, 2009a,b; Brake et al., 2010, 2011; Ouellet et al., 2010; Bailey et al., 2012a,b; Titgemeyer et al., 2012; Chibisa and Mutsvangwa, 2013; Davies et al., 2013; Spek et al., 2013; Recktenwald et al., 2014; Holder et al., 2015; Batista et al., 2016; Mutsvangwa et al., 2016) representing 96 treatment means. The only selection criterion was that the work was conducted in cattle and used the double-labeled urea approach to measure urea kinetics. Nine of the studies were conducted using dairy cattle and 12 studies were conducted using beef cattle. The observed data are summarized in Table 1.

Table 1. A summary of observed nutrient intake, ruminal metabolism, ruminal N outflow, and urea transfers from the meta-data

Item	N	Mean	SD	Minimum	Maximum
BW, kg	96	401.3	209.8	139.0	768.0
Intake, kg/d					
DM	96	10.9	8.5	2.9	28.7
OM	57	8.0	7.3	2.7	26.9
ADF	17	3.5	2.2	0.9	6.3
NDF	12	7.9	2.6	4.3	10.1
Starch	13	3.9	0.8	2.9	5.6
Nitrogen	58	0.3	0.3	0.1	0.8
Rumen fermentation					
pH	54	6.2	0.3	5.6	6.9
Ammonia, mM	63	5.6	4.0	0.1	15.9
Total VFA, mM	50	96.8	25.2	52.2	143.3
Acetate, % of VFA	43	68.6	9.5	41.2	90.0
Propionate, % of VFA	42	20.8	8.0	12.0	46.5
Butyrate, % of VFA	43	9.6	3.4	5.3	19.0
Ruminal N outflow, g/d					
Total N	38	210.2	232.1	40.8	967.0
Microbial N	38	136.7	156.1	25.3	651.0
Nonammonia, nonmicrobial N	30	89.2	76.0	15.3	281.0
N metabolism					
BUN, mg/dL	81	10.4	5.9	0.7	27.0
BUN entry, g/d	96	214.1	142.3	17.6	504.0
Gut urea-N entry, g/d	96	150.1	101.5	17.4	390.0
Urea-N returned to the ornithine cycle, g/d	80	65.0	74.7	2.8	280.0
Urea-N utilized for anabolism, g/d	76	47.0	43.8	8.8	213.8
Urinary urea-N, g/d	90	64.4	46.9	0.1	201.6
Urinary N, g/d	96	113.4	76.3	13.4	291.1
Fecal N, g/d	96	107.8	71.8	14.7	262.0
Fecal N from urea, g/d	76	5.4	4.1	0.3	14.8
Retained N, g/d	92	36.4	23.7	-8.3	111.0

Figure 1. A schematic diagram of nitrogen metabolism in the Molly cow model. NANMN = nonammonia, nonmicrobial nitrogen; NA = nucleic acids. Boxes with dashed lines are compartments; boxes with solid lines represent pools; arrows indicate fluxes.

Model Description

The mechanistic and dynamic functions of the Molly cow model pertaining to ruminal urea-N recycling and urinary urea elimination have been described by Baldwin (1995). A schematic of the key elements is presented in Figure 1. As an overview, dietary insoluble protein degradation in the rumen is defined as the balance between rates of degradation and passage. Microbes in the rumen utilize a portion of the soluble protein and AA and ammonia to support microbial growth, and they catabolize an additional portion of the protein and AA, resulting in release of ammonia. Urea entering the rumen is quantitatively converted to ammonia. Mass action functions are used to simulate insoluble protein degradation, microbial metabolism of soluble N, ruminal ammonia absorption, intestinal protein digestion, AA absorption, hepatic urea synthesis, and urinary urea excretion. Microbial growth and urea recycled to the rumen through ruminal epithelium, and utilization for anabolic purposes are represented using Michaelis-Menten equations. Conversions among urea, ammonia, and AA are calculated stoichiometrically with adherence to mass balance principles. Concentrations of the key metabolites are predicted at any point in time as state variables in a system of ordinary differential

equations, which are numerically integrated over time. State variables include ammonia, soluble protein, AA, insoluble protein, and microbial mass in the rumen, as well as urea and AA in the blood. These mathematical equations were encoded in and solved using ACSL (ver. 3.1.4.2, Aegis Technologies Group Inc., Huntsville, AL).

Model inputs were prepared as previously described (Li et al., 2018) with some modifications. Briefly, dietary nutrient composition (DM, protein, ADF, NDF, lignin, crude fat, starch, and ash) was generated for each treatment by summation of feed values according to the dietary formulas. If the nutrient composition of the constituent ingredients was reported, those values were used; otherwise, tabular values from an integrated feed library were used in place of the missing values. The feed library was generated from the NRC (2001) feed library, a library derived from commercial laboratory data (collected by the National Animal Nutrition Program; www.animalnutrition.org; Tran et al., 2016), and the Cornell Net Carbohydrate and Protein System (CNCPS) feed library. Order of use to construct the integrated library gave preference to the commercial library, followed by the NRC (2001) library, and the CNCPS library. Mean nutrient bias was calculated for each nutrient (OM, protein, ADF, NDF, crude fat, and starch) across diets within each

study as the difference between calculated dietary nutrient inputs and those reported in the publication when available (Hanigan et al. (2013)). The relative bias for the study was used to adjust the nutrient content of each ingredient weighted for the contribution of that ingredient to the dietary nutrient sum for the study. This retained the ingredient level information while reducing dietary mis-specifications and avoiding the introduction of nutrient bias. Rumen protein and starch degradation were calculated following White et al. (2017a,b). Rumen ADF degradation was generated using the same data set and statistical approach as White et al. (2017b):

$$f_{RdADF} (\%, \text{DM}) = 47.5 - 16.3 \times St + 0.02 \times St^2 + 0.91 \times DMI, \quad [1]$$

where f_{RdADF} represented rumen ADF degradation (% of DM); St was dietary starch concentrations (% of DM); and DMI was expressed as kilograms per day. The concordance correlation coefficient (CCC) for this equation was 0.91, and the root mean squared error (RMSE) was 11% of mean.

Model Modifications

Model modifications were made to the version of Molly described by Gregorini et al. (2015) following conservation of mass principles. For purposes of this description, state variables, generally nutrients, metabolites, or the volume of a pool such as rumen liquid (kg, L, or mol), were denoted as $Q_{A,Y}$, where A denoted the nutrient or metabolite in compartment Y ; $C_{A,Y}$ represented the concentration of A in compartment Y (kg/L or mol/L; primarily in ruminal fluid or blood); the flux of nutrients within the system was denoted as $F_{AB,YZ}$ (kg/d or mol/d), where A denoted a substrate, B a product, Y the location of the conversion, and in the case of transfer from one compartment to another with or without interconversion, Z represented the receiving compartment; $f_{AB,YZ}$ represented a stoichiometric constant for conversion of substrate A to product B ; $K_{AB,YZ}$ represented a rate constant (variable units) for conversion of A to B , and $X_{AB,YZ}$ denoted a sensitivity exponent for conversion of A to B (variable units); J_A represented an inhibition constant for inhibitor A . Specification of a single entity (A) or pool location (Y) was used when no conversion or location change occurred.

Initial model evaluations indicated that residuals for ruminal microbial N outflow were positively correlated with ruminal ammonia concentrations, suggesting that

microbial N flow from the rumen was underpredicted as ruminal ammonia concentrations increased. Therefore, an exponential factor (X_{EAm}) was added to the microbial growth equation to allow adjustment of sensitivity to ruminal ammonia concentrations if required ($F_{MiG,Rum}$, kg/d).

$$F_{MiG,Rum} = \left(F_{ATPF,Rum} - F_{ATPM,Rum} \right) \times \left(0.012 + \frac{K_{YATP,Rum}}{1 + K_{AaMi,Rum}/C_{Aa,Rum}} \right) \times \left(\frac{1}{1 + \left(K_{AmMi,Rum}/C_{Am,Rum} \right)^{X_{EAm}}} \right) \times \left(1 + \frac{C_{Lipid,Fd}}{C_{Fat,Fd} \times K_{Fat,Fd}} \right), \quad [2]$$

where $F_{ATPF,Rum}$ represented ATP production generated from nutrient fermentation in the rumen (mol/d); $F_{ATPM,Rum}$ represented ATP used for microbial maintenance (mol/d); $K_{YATP,Rum}$ was an efficiency variable defining the yield of microbial DM per mole of ATP (kg/mol); $K_{AaMi,Rum}$ and $K_{AmMi,Rum}$ were rate constants for the effect of ruminal concentrations of AA and ammonia on microbial growth (L/mol); and $C_{Aa,Rum}$ and $C_{Am,Rum}$ represented ruminal concentrations of AA and ammonia (mol/L); $C_{Lipid,Fd}$ was the dietary concentration of endogenous lipid (%), intrinsic plant lipids excluding storage oils and fats); $C_{Fat,Fd}$ represented the dietary concentration of added fat (%); and $K_{Fat,Fd}$ was the rate constant for the effect of added fat on microbial growth (%/%).

Ammonia passage to the small intestine ($F_{Am,RumInt}$, mol/d) was simulated as a mass action function of the ruminal ammonia pool size ($Q_{Am,Rum}$, mol) and rumen liquid outflow rate ($K_{Liq,RumInt}$, d⁻¹):

$$F_{Am,RumInt} = Q_{Am,Rum} \times K_{Liq,RumInt}. \quad [3]$$

The differential equations describing the change in $Q_{Am,Rum}$ with respect to time, ruminal total N outflow (kg/d), and ruminal NANMN outflow (kg/d) were altered to include consideration of $F_{Am,RumInt}$.

Ammonia flow from the rumen was assumed completely absorbed and converted to urea in the liver, as was previously assumed for ammonia absorbed from the rumen:

$$F_{AmUr,Vis} = \left(F_{Am,RumBld} + F_{Am,RumInt} \right) \times f_{AmUr}, \quad [4]$$

where $F_{AmUr,Vis}$ was ammonia conversion to urea (mol/d); $F_{Am,RumBld}$ represented ruminal ammonia absorption (mol/d); and f_{AmUr} was the stoichiometric coefficient for conversion of ammonia to urea (0.5 mol of NH_3 /mol of urea).

Urea recycling to the rumen is represented as the sum of transfer across the ruminal epithelium ($F_{Ur,BldRum(Trans)}$, mol/d) and salivary delivery ($F_{Ur,BldRum(Sal)}$, mol/d; Baldwin, 1995). Those 2 fluxes were summed to provide an overall ruminal urea entry rate ($F_{Ur,BldRum}$, mol/d), which could be compared with the measured urea entry rates:

$$F_{Ur,BldRum} = F_{Ur,BldRum(Trans)} + F_{Ur,BldRum(Sal)}. \quad [5]$$

Lapierre and Lobley (2001) demonstrated that gut urea entry was composed of ruminal and intestinal urea-N entry. To estimate intestinal urea entry rate from a single measured gut entry rate ($F_{Ur,BldGut}$, mol/d) requires an assumption of the fractional transfer into the rumen and the intestine. The true split is driven by tissue mass, the transport activity per unit of mass, and blood flow. The latter is proportional to tissue activity and fermentation rate in the lumen (Storm et al., 2011, 2012), and thus it was assumed that ruminal urea entry across rumen epithelium and postruminal urea entry was proportional to DM disappearance in each compartment ($f_{DMDis,Rum}$, kg/kg):

$$F_{DMDis,Rum} = F_{DM,FdRum} - F_{DM,RumInt}, \quad [6]$$

$$F_{DMDis,Int} = F_{DM,RumInt} - F_{DM,IntFec}, \quad [7]$$

$$f_{DMDis,Rum} = \frac{F_{DMDis,Rum}}{F_{DMDis,Int}}, \quad [8]$$

$$F_{Ur,BldInt} = \frac{F_{Ur,BldRum(Trans)}}{f_{DMDis,Rum}}, \quad [9]$$

$$F_{Ur,BldGut} = F_{Ur,BldRum} + F_{Ur,BldInt}, \quad [10]$$

where ruminal DM disappearance ($F_{DMDis,Rum}$, kg/d) was calculated by subtracting ruminal DM outflow ($F_{DM,RumInt}$, kg/d) from DMI ($F_{DM,FdRum}$, kg/d); postruminal disappearance DM ($F_{DMDis,Int}$, kg/d) was equal to ruminal DM outflow minus fecal DM outflow ($F_{DM,IntFec}$, kg/d); and $F_{Ur,BldInt}$ was the intestinal urea entry rate (mol/d). This likely overestimates postruminal entry

because a fraction of the DM disappearance is not from fermentative action. However, given that urea freely equilibrates across cell membranes, the presence of DM in the small intestine will draw urea in, even though it may not be converted to ammonia until it passes to the large intestine. Thus, assuming that no urea enters the small intestine would have also resulted in bias. Additional data are required to address this assumption.

Urea recycled to the lower gut was assumed to be converted to ammonia, and then captured by microbes as microbial N in the hind gut ($F_{UrMi,Int}$, mol/d), excreted in feces ($F_{UrAm,IntFec}$, mol/d), or reabsorbed ($F_{UrAm,Int}$, mol/d). Lapierre and Lobley (2001) indicated that the contribution of urea-N to support microbial protein synthesis was minor in the lower gut compared with that in the rumen. It seems likely that capture by microbes would be proportional to fermentation rate, and given that DM disappearance was used to define entry, we further assumed that 25% of the amount entering the intestine was captured in microbial N, thus approximating proportionality with fermentative activity:

$$F_{UrMi,Int} = 0.25 \times F_{Ur,BldInt}, \quad [11]$$

$$F_{UrAm,IntFec} = K_{Ur,IntFec} + K_{SlpUr,IntFec} \times F_{Ur,BldInt}, \quad [12]$$

$$F_{UrAm,Int} = \frac{F_{Ur,BldInt} - F_{UrMi,Int} - F_{UrAm,IntFec}}{f_{AmUr}}, \quad [13]$$

where $K_{SlpUr,IntFec}$ and $K_{Ur,IntFec}$ were parameters used to estimate fecal ammonia-N excretion.

The representation of urea synthesis ($F_{AmUr,Liv}$, mol/d) was modified to include the contribution from ammonia absorbed from the hind gut:

$$F_{AmUr,Liv} = F_{AaUr,Vis} + F_{AaUr,Gest} + F_{AmUr,Vis} + F_{UrAm,Int} \times f_{AmUr}, \quad [14]$$

where $F_{AaUr,Vis}$ represented deamination of AA in the viscera. Viscera is assumed to be the primary site of AA degradation, with the remainder occurring in the gravid uterus ($F_{AaUr,Gest}$, mol/d), as described by Hanigan et al. (2009).

Urinary urea excretion ($F_{Ur,BldUrin}$, mol/d) was originally described as a mass action function of blood urea concentrations (Baldwin, 1995). However, as Li et al. (2018) pointed out, blood urea concentrations do not scale with body mass, and thus flux was biased with respect to body size. This is a deviation relative to

all other equations of this type in the model, which are driven by pool size rather than concentration. Conceptually, renal urea clearance is a function of blood flow, the concentration of urea in renal blood, and the activity of the kidney (Stevens and Levey, 2005). However, blood flow is not represented in the model. It was assumed that blood flow was proportional to tissue mass and activity with the tissues grouped as high (viscera) and low (body) activities (Baldwin, 1995). It was also assumed that blood volume was proportional to BW, which is a well-documented relationship. Given these relationships, blood volume, blood flow, and tissue activity are all proportional to BW, and blood flow $\times$ blood concentration is also proportional to blood volume $\times$ concentration, which is the definition of the pool size. Thus, the pool size represents renal urea supply, and because renal capacity also scales with BW, we can represent glomerular filtration as a function of the pool size, but not as a function solely of concentration. Thus, the prior representation was a mistake, which was corrected by altering the equation to be driven by the blood urea pool size ($Q_{Ur,Bld}$, mol), which scales appropriately with BW:

$$F_{Ur,BldUrin} = Q_{Ur,Bld} \times K_{Ur,BldUrin}, \quad [15]$$

where $K_{Ur,BldUrin}$ represents the mass action constant for urea excretion by the kidney.

Digested microbial NPN was assumed to be composed entirely of purines and pyrimidines and entirely excreted in the urine after derivatization in the liver. This is a deviation from the earlier model, which did not represent absorption of nucleic acids from microbes. Thus, urinary N ($F_{N,Urin}$, kg/d) consisted of urinary urea-N ($F_{UrN,BldUrin}$, kg/d) plus purine derivative-N ($F_{Nn,MiUrin}$, kg/d):

$$F_{Nn,MiUrin} = F_{Mi,RumInt} \times f_{MiNnNn} \times K_{Pi,MiFec} \times 0.16, \quad [16]$$

$$F_{UrN,BldUrin} = \frac{F_{Ur,BldUrin}}{f_{AmUr}} \times K_{MwtN}, \quad [17]$$

$$F_{N,Urin} = F_{UrN,BldUrin} + F_{Nn,MiUrin}, \quad [18]$$

where $F_{Mi,RumInt}$ was the ruminal microbial protein outflow (kg/d); f_{MiNnNn} represented the fraction of total microbial N that was NPN; $K_{Pi,MiFec}$ represented the intestinal digestion coefficient for microbial protein, which was 0.75 (Baldwin, 1995); and K_{MwtN} was the N molecular mass of 0.014 kg/mol.

Fecal N excretion ($F_{N,IntFec}$, kg/d) was updated to reflect absorption of nonprotein microbial N and urea recycled into the hind gut:

$$F_{N,IntFec} = \left(F_{Pi,MiFec} + F_{Nn,MiFec} + F_{Pi,IntFec} + \left(F_{UrMi,Int} / f_{AmUr} \right) + \left(F_{UrAm,IntFec} / f_{AmUr} \right) \right) \times K_{MwtN}, \quad [19]$$

where $F_{Pi,MiFec}$ was undigested microbial protein N (mol/d); $F_{Nn,MiFec}$ was undigested nonprotein microbial N (mol/d); $F_{Pi,IntFec}$ represented undigested insoluble feed protein N (mol/d); $F_{UrMi,Int}$ referred to microbial N captured from recycled urea-N in the hindgut (mol/d); $F_{UrAm,IntFec}$ was ammonia-N excreted in feces (mol/d) that was derived from blood urea; f_{AmUr} was a stoichiometric parameter (0.5 mol/mol) for conversion of ammonia to urea.

Infusion Variables

Several studies were conducted that used infusions of casein, glucose or VFA (Wickersham et al., 2008, 2009b; Bailey et al., 2012a,b; Titgemeyer et al., 2012; Batista et al., 2016). To represent their effects, consideration of infused nutrients was added to the model as follows. Ruminally infused VFA were added as additional fluxes to each of the VFA differential equations ($dQ_{Ac,Rum}/dt$, $dQ_{Pr,Rum}/dt$, $dQ_{Bu,Rum}/dt$, mol/d):

$$\frac{dQ_{Ac,Rum}}{dt} = F_{Ac,FdRum} + F_{CsAc,Rum} + F_{AaAc,Rum} + F_{LaAc,Rum} + F_{InfAc,Rum} - F_{Ac,RumBld} - F_{Ac,RumInt}, \quad [20]$$

$$\frac{dQ_{Pr,Rum}}{dt} = F_{CsPr,Rum} + F_{AaPr,Rum} + F_{LaPr,Rum} + F_{InfPr,Rum} - F_{Pr,RumBld} - F_{Pr,RumInt}, \quad [21]$$

$$\frac{dQ_{Bu,Rum}}{dt} = F_{Bu,FdRum} + F_{CsBu,Rum} + F_{AaBu,Rum} + F_{InfBu,Rum} - F_{Bu,RumBld} - F_{Bu,RumInt}, \quad [22]$$

where $F_{Ac,FdRum}$ and $F_{Bu,FdRum}$ represented fluxes of dietary acetate and butyrate from silage (mol/d); $F_{CsAc,Rum}$, $F_{CsPr,Rum}$, and $F_{CsBu,Rum}$ referred to acetate, propionate, and butyrate derived from carbohydrate fermentation in the rumen (mol/d); $F_{AaAc,Rum}$, $F_{AaPr,Rum}$, and $F_{AaBu,Rum}$ were conversions of AA to each of the VFA

(mol/d); $F_{LaAc,Rum}$ and $F_{LaPr,Rum}$ represented conversions of lactate to acetate and propionate (mol/d); $F_{InfAc,Rum}$, $F_{InfPr,Rum}$, and $F_{InfBu,Rum}$ represented ruminal VFA infusion rates (mol/d) of each; $F_{Ac,RumBld}$, $F_{Pr,RumBld}$, and $F_{Bu,RumBld}$ represented absorptions of each of the VFA (mol/d); and $F_{Ac,RumInt}$, $F_{Pr,RumInt}$, and $F_{Bu,RumInt}$ referred to VFA passage from the rumen (mol/d).

Ruminally infused glucose was added as a flux to the soluble carbohydrate differential equation (mol/d):

$$\frac{dQ_{Cs,Rum}}{dt} = F_{ScTCs,Rum} + F_{StCs,Rum} + F_{HaCs,Rum} + F_{HcCs,Rum} + F_{CeCs,Rum} + F_{InfGl,Rum} - F_{CsFv,Rum} - F_{CsMi,Rum} - F_{Cs,RumInt}, \quad [23]$$

where $F_{ScTCs,Rum}$ and $F_{StCs,Rum}$ represented dietary intake of total soluble carbohydrate expressed in 6-carbon equivalents and soluble starch (mol/d); $F_{HaCs,Rum}$, $F_{HcCs,Rum}$, and $F_{CeCs,Rum}$ referred to ruminal degradations of starch, hemicellulose, and cellulose to soluble carbohydrate (mol/d); $F_{InfGl,Rum}$ was ruminal glucose infusion (mol/d); $F_{CsFv,Rum}$, $F_{CsMi,Rum}$, and $F_{Cs,RumInt}$ represented soluble carbohydrate degradation, utilization for microbial growth, and passage from the rumen (mol/d).

Abomasally infused glucose was assumed to be completely absorbed and thus added to the absorbed glucose flux ($F_{Gl,IntBld}$, mol/d):

$$F_{Gl,IntBld} = F_{HaGl,Int} + F_{Cs,RumInt} + F_{MiGl,Int} + F_{MiLGl,Int} + F_{InfGl,Int}, \quad [24]$$

where $F_{HaGl,Int}$ was dietary starch digestion (mol/d); $F_{MiGl,Int}$ represented microbial starch digestion in the lower gut (mole/d); $F_{MiLGl,Int}$ referred to a flux that equals 2 units of glycerol from digested microbial lipid (mol/d); and $F_{InfGl,Int}$ was postruminal glucose infusion.

Ruminally infused casein was incorporated as a component of the diet with the assumption that it was 100% soluble. Postruminally infused casein was also considered as a dietary ingredient with zero solubility and a zero rate of ruminal degradation so that all of it passed from the rumen.

Model changes were verified for mathematical consistency using a reference observation from the data set. Predictions of ruminal ammonia, microbial mass, and blood urea pool size (mol), with respect to simulation time, were assessed before and after model modifications to determine if the changes inappropriately altered overall model balance. The reference inputs were a 598-kg lactating Holstein dairy cow consuming 26.3 kg of DM/d of a diet containing CP, fat, starch, NDF, and ADF of 16.6, 5.5, 33.7, 29.8, and 18.9% of DM.

Simulation Settings, Parameter Estimation, and Model Evaluations

The model was solved using a fourth-order, variable-step Runge-Kutta integration algorithm with a maximum step size of 0.005 d. Model stability and time to steady state were assessed by simulating for 14 d using the reference inputs, followed by alteration of the rate constant for ruminal ammonia absorption ($K_{Am,RumBld}$) from 10.44 d⁻¹ to 20 on d 14, continuation of the run for another 14 d, and then returning the rate constant to 10.44, and continuation of the simulation for an additional 14 d (Figure 2A). This process was repeated after model modification and parameter estimation using a starting and ending value of 5.76 d⁻¹ for $K_{Am,RumBld}$ (Figure 2B). Pool sizes of ruminal ammonia, microbial mass, and blood urea were recorded by day and plotted with respect to time to verify model stability and code accuracy, and to assess model behavior and time to steady state.

Model parameters were estimated simultaneously by fitting to the observed data using the Direction Set optimization algorithm (Press, 2007) provided in ACSL to maximize the log-likelihood function. Predicted values were sampled at steady state on d 14 (Figure 2) for comparison with observed values.

Model accuracy and precision were assessed based on the log-likelihood function, RMSE; the proportions of mean squared error (**MSE**) associated with mean bias, slope bias, and dispersion (Bibby and Toutenburg, 1977); and CCC (Lin, 1989). Residual errors were also visually appraised to identify any additional patterns that may have been resident in the data.

Following model modifications and parameter estimation, simulations were conducted to assess N efficiency using 2 different feeding scenarios. The simulated animal was initialized as a Holstein cow weighing 600 kg at d 70 of lactation and consuming 20 kg of DM per day. For feeding strategy 1, there were 11 dietary CP concentrations ranging from 10 to 20% of DM. The diets all contained fat, starch, soluble starch, NDF, ADF, and ash concentrations of 6, 30, 9, 30, 20, and 6% of DM, respectively; thus, CP was substituted for residual OM, which is primarily pectins and organic acids. Dietary RUP concentration was set to 6% of DM for all diets, with variation in RDP being the sole contributor to varying dietary CP. This resulted in dietary RDP concentrations from 4 to 14% of DM. For feeding strategy 2, dietary CP was held constant at 16% of DM and 9 different concentrations of RUP ranging from 10 to 90% of CP were used. In this case, RUP was substituted for RDP given a constant CP content. The remaining nutrient concentrations were the same as for feeding strategy 1. Model predictions of total ru-

Figure 2. Ruminal ammonia ($Q_{Am,Rum}$, mol), microbial mass ($Q_{Mi,Rum}$, kg), and blood urea ($Q_{Ur,Bld}$, mol) pools with respect to simulation time for the initial model (A) and the modified model (B). The rate constant for ruminal ammonia absorption ($K_{Am,RumBld}$, d^{-1}) was set to 10.44 at the start of the simulation for the initial model, changed to 20 on d 14, and returned to 10.44 on d 28. $K_{Am,RumBld}$ was set to 5.01 at the start of the simulation for the modified model, set to 20 on d 14, and returned to 5.01 on d 28.

ruminal N outflow ($F_{N,RumInt}$, kg/d), microbial N outflow ($F_{MiN,RumInt}$, kg/d), NANMN outflow ($F_{NANMN,RumInt}$, kg/d), ammonia-N converted to ruminal microbial N ($F_{AmMi,Rum}$, kg/d), ammonia generated in the rumen from recycled urea ($F_{UrAm,Rum}$, mol/d), total ammonia production ($F_{Am,Rum}$, mol/d), blood urea entry rate ($F_{AmUr,Liv}$, mol/d), gut urea entry rate ($F_{Ur,BldGut}$, mol/d), and urinary urea excretion ($F_{Ur,BldUrin}$, mol/d) were collected. The proportion of microbial N synthesized from ammonia-N was calculated as $F_{AmMi,Rum}$ divided by $F_{MiN,RumInt}$. The proportion of total ammonia generated from recycled urea was calculated as $F_{UrAm,Rum}$ divided by $F_{Am,Rum}$.

Regression Analyses

Regressions were conducted using a mixed linear model with study as a random effect using the lmer function of the lme4 package (version 1.1-17; Bates et al., 2015) in R (version 3.3.0; R Development Core Team, 2015) to quantify relationships between observed dietary N intake and observed ruminal N outflow, observed blood urea entry, and relationships between observed blood urea entry and observed gut urea entry or urinary urea excretion. Coefficients of determination were defined as follows: R^2_m is the marginal R^2 , which represents the proportion of variance that can be explained by the fixed effect, and R^2_c is the conditional R^2 , which indicates the proportion of variance that can be explained by the fixed effect plus the random effect.

RESULTS AND DISCUSSION

Steady State and Model Verifications

Ruminal ammonia (mol), ruminal microbial mass (kg), and blood urea (mol) pool sizes with respect to simulation time before and after perturbations of the ruminal ammonia absorption constant are shown in Figure 2. After 10 d of model simulation, changes in pool sizes of these 3 variables were negligible, suggesting that all the variables associated with these N pools had reached steady state. When challenging the model by changing the rate constant for ruminal ammonia absorption ($K_{Am,RumBld}$, d^{-1}) on d 14 and 28, the model returned to apparent steady state in 7 d, supporting selection of a simulation time of 14 d to represent steady-state values. The model was found to be stable and returned to initial pool sizes after perturbation when the parameter values were returned to the initial values on d 28, suggesting the code was acting as expected.

Increasing $K_{Am,RumBld}$ on d 14 greatly decreased ruminal ammonia pool size in the modified model (Figure 2). As a consequence, microbial pool size was decreased

and blood urea pool size was increased (Figure 2B), which contrasts with the very small changes in microbial and blood urea pool sizes for the initial model (Figure 2A). Thus, the altered model and associated parameters resulted in greater microbial growth sensitivity to ruminal ammonia concentrations, which was consistent with our previous observations of model behavior (Li et al., 2018).

Ruminal Ammonia Metabolism

Ruminal ammonia, as a major end-product of ruminal fermentation, is a vital indicator of ruminal N metabolism. Ammonia is produced from microbial degradation of dietary AA, dietary NPN, and salivary and blood urea-N entering the rumen, and it is utilized by microbes to synthesize protein, absorbed from the rumen, or passed from the rumen in the liquid fraction (Figure 1). After model improvements and refitting, the RMSE for ruminal ammonia concentrations decreased from 53.1 to 43.7%, with a decrease in mean bias from 16.4 to 7.7% and a decrease in slope bias from 13.2 to 8.4%. These results support our hypothesis regarding the representation of these transactions; however, precision was still relatively poor, suggesting that the observed ammonia concentrations were highly variable. Nolan (1975) demonstrated that the ammonia pool can completely turn over in less than 2 h. Thus, time of sampling relative to a meal and the size of the meal are important determinants of ruminal ammonia concentrations that are rarely reported. In most cases, the animals were fed frequently to minimize this problem, but even with 2-h feeding intervals, ammonia concentrations at sampling could vary if the diet is highly degradable. Sampling location is another source of variation. Wohlt et al. (1976) reported that concentrations of ruminal ammonia were greatest in the dorsal region of the rumen and were lower in samples obtained by stomach tube compared with samples taken via rumen cannula. Thus, variation in observed values also likely exists due to sampling location. Neither of these potential sources of variation can be reproduced in the model as currently formulated, thus contributing to the lack of precision.

Substantial amounts of ammonia are generated from microbial degradation of dietary protein in the rumen. On average, ruminal NANMN outflow accounted for 30% of incremental dietary N intake (Figure 3C), and thus 70% of dietary protein was degraded in the rumen. Intercept and slope scalars ($K_{RUP,Rum}$ and $K_{SlpRUP,Rum}$) were introduced by Hanigan et al. (2013) to scale in situ measures of ingredient CP degradation rates so the model predictions more closely aligned with observed extent of degradation. In theory, if the in situ measures

and the model were perfect representations of reality, the intercept would be 0 and the slope would be 1. Given the needed adjustments of in situ assessments, we must conclude that either protein degradation rates determined in situ are biased or the model structure is biased. In the revised model, $K_{RUP,Rum}$ and $K_{SlpRUP,Rum}$ moved toward 0 and 1, respectively, compared with earlier estimates, implying that model structure was at least part of the problem, although ingredient protein degradation rates were also updated (White et al., 2017a). The revised estimates of the in situ adjustment parameters slightly decreased RMSE for ruminal NANMN from 28.8 to 26.2%, with mean bias decreased from 12.8 to 10.6 and slope bias decreased from 8.7 to 7.9% (Table 2; Figure 4).

A portion of ammonia comes from microbial hydrolysis of recycled urea either directly transferred from blood across ruminal epithelium or via saliva. In the model, urea flowing into the rumen via saliva is calculated from blood urea concentrations multiplied by saliva volume, and urea passing across rumen epithelium is simulated using a Michaelis-Menten equation driven by blood urea concentrations and inhibited by ruminal ammonia concentrations (Baldwin, 1995). Refitting the maximal rate of blood urea conversion to ruminal ammonia ($Vm_{UrAm,Rum}$) and the inhibition constant for the effect of ammonia on blood urea conversion to ruminal ammonia (J_{Am}) resulted in an increase in the maximal rate ($Vm_{UrAm,Rum}$) from 56.7 to 84 ± 3.4 mmol/d (Table 3), and an increase in the ruminal ammonia inhibition constant (J_{Am}) from 0.003 to $0.0074 \pm 1.1 \times 10^{-5}$ L/mol. The revised parameters resulted in decreased RMSE for predictions of gut urea-N entry from 70.5 to 26.7%, primarily due to substantially decreased mean bias from 57.5 to 0.8%. A larger $Vm_{UrAm,Rum}$ indicates greater maximal rates of transfer of blood urea into the rumen. At ruminal ammonia concentrations of 5, 10, and 15 mM, 76, 62, and 52% of the maximal urea transfer rate would be achieved with J_{Am} at 0.0074, supporting the inhibitory effect of ruminal ammonia on urea transfer.

Ammonia is an essential substrate for microbial protein synthesis. Microbial N outflow represents approximately 57% of the incremental total dietary N intake (Figure 3B). Model simulation results indicated that 65 to 76% of microbial N was derived from ammonia depending on the diet, which was consistent with estimates of the contribution of total ammonia-N to bacterial N ranging from 64 to 80% (Pilgrim et al., 1970; Nolan and Leng, 1972). Fitting the maximal rate of growth of microbes from AA ($K_{YATP,Rum}$) resulted in a value of 0.064 ± 0.002 kg/mol compared with the prior value of 0.03. The affinity constant for microbial growth stimulation by ammonia ($K_{AmMi,Rum}$) was

Figure 3. Relationships between observed dietary N intake and ruminal N, microbial N, and nonammonia, nonmicrobial N (NANMN) outflow. The solid lines represent the results of linear regression for individual studies. The dotted line indicates the global regression for the full data set. Study effect was included as a random variable in the global regression model. (A) Ruminal N outflow (g of N/d) versus dietary N intake (g of N/d): $Y = 47.04 + 0.88X$ ($R_m^2 = 0.78$ and $R_c^2 = 0.99$); (B) ruminal microbial N outflow (g of N/d) versus dietary N intake (g of N/d): $Y = 28.50 + 0.57X$ ($R_m^2 = 0.72$ and $R_c^2 = 0.99$); (C) ruminal NANMN outflow (g of N/d) versus dietary N intake (g of N/d): $Y = 14.77 + 0.30X$ ($R_m^2 = 0.88$ and $R_c^2 = 0.90$), where R_m^2 is marginal R^2 , which represents the proportion of variance that can be explained by the fixed effect, and R_c^2 is conditional R^2 , which indicates the proportion of variance that can be explained by the fixed effect plus the random effect.

estimated at 6.8 ± 0.3 mM compared with the prior value of 0.2 mM (Hanigan et al., 2013), and the newly introduced ammonia sensitivity exponent (X_{EAm}) was estimated at 8.55 ± 0.23 mol/mol; $K_{AmMi,Rum}$ indicates the concentration of ruminal ammonia when microbial growth is half of the maximal rate. Although the work of Satter and Slyter (1974) demonstrated that microbial growth was maximized at an ammonia concentration of 1.5 mM, Reynal and Broderick (2005) suggested that ammonia concentrations >8.4 mM might be needed to maximize microbial protein synthesis. Hanigan et al. (2013) previously solved for a half-maximal concentration of 0.2 mM, but the ammonia concentrations predicted previously by the model were very biased, leading to the current work. The derived value for X_{EAm} was much greater than expected, indicating that there is a robust sensitivity of ammonia concentrations to microbial growth and it was better defined by the data, resulting in improved predictions of microbial growth. At ammonia concentrations of 5 and 10 mM, 67.3 and 96.4% of maximal growth would be achieved with $K_{AmMi,Rum}$ at 6.8 mM using the revised equation, which was consistent with the result reported by Reynal and Broderick (2005). Fitting the data herein decreased prediction errors of ruminal microbial N outflow from an RMSE of 43.5 to 32.3%, because of a reduced slope bias from 13.1 to 1.8% (Table 2). However, the prediction error for ruminal microbial N outflow exhibited

by the unmodified model was greater than observed by Hanigan et al. (2013), possibly because of the limited observations herein. Additional parameterization work should be undertaken using a larger data set.

The mass action rate constant ($K_{Am,RumBld}$) used to represent ammonia absorption (Baldwin, 1995) solved for a value of 5.76 d^{-1} compared with the prior value of 10.44 d^{-1} , which reduced the mean prediction bias. The model simulation indicated that predicted ruminal ammonia N absorption rates averaged 28% of incremental dietary N intake with a range from 15 to 45%. Lapierre et al. (2005) and Firkins and Reynolds (2005) reported that net portal-drained viscera ammonia-N absorption represented between 30 and 42% of dietary N intake. The reduction in the rate constant was partially due to consideration of ammonia outflow from the rumen in the liquid fraction. In the absence of such a consideration, all ammonia leaving the rumen was by absorption. Ammonia-N passage averaged 0.9 mol/d, which is 20% of the total ammonia-N flux and 6% of total N outflow. This was consistent with observed proportions of 4 to 10% of total N flow from the rumen (Chan et al., 1997; Zhu et al., 1997). This change in representation had no effect on total ammonia absorption because it was assumed that ruminal ammonia outflow was completely absorbed (Smith, 1969).

The RMSE for total ruminal N outflow (a sum of microbial N, undegraded feed protein N, ammonia-N

Table 2. Residual error analyses for predictions of urea-N recycling and ruminal N outflows in Molly¹

Item	N	Observed mean	Predicted mean		RMSE, % mean		Mean bias, % MSE		Slope bias, % MSE		CCC	
			Initial	Modified	Initial	Modified	Initial	Modified	Initial	Modified	Initial	Modified
Ruminal outflow, g/d												
Total N	38	210.2	214.1	246.3	38.4	29.8	0.24	13.5	36.3	6.6	0.94	0.97
Microbial N	38	136.7	149.4	165.0	43.5	32.3	4.6	11.0	13.1	1.8	0.93	0.97
Nonammonia, nonmicrobial N	30	89.2	80.0	89.8	28.8	26.2	12.8	10.6	8.7	7.9	0.94	0.95
N metabolism												
Ruminal ammonia, mmol/L	63	5.6	4.4	4.9	53.1	43.7	16.4	7.7	13.2	8.4	0.73	0.84
BUN, mg/dL	81	10.4	8.1	10.2	72.4	39.6	9.6	0.2	19.3	2.5	0.40	0.77
BUN entry, g/d	96	214.1	253.6	233.0	26.5	17.0	48.4	26.9	25.0	0.8	0.94	0.97
Gut urea-N entry, g/d	96	150.1	69.8	146.6	70.5	26.7	57.5	0.8	29.3	22.0	0.46	0.91
Urinary urea-N, g/d	90	64.4	183.1	87.9	243.5	69.0	59.5	27.8	37.3	14.5	0.35	0.77
Urinary N, g/d	96	113.4	183.1	116.6	82.5	25.4	55.3	1.2	36.7	17.7	0.71	0.94
Fecal N, g/d	96	107.8	121.0	107.9	21.1	17.1	33.6	0.26	2.9	4.9	0.75	0.80
Fecal N from urea, g/d	76	5.4	NA	5.9	NA	82.7	NA	1.5	NA	0.1	NA	0.44
Retained N, g/d	92	36.4	-0.8	29.3	118.1	98.8	68.6	3.9	5.1	39.7	0.30	0.52

¹RMSE = root mean square error; MSE = mean square error; CCC = concordance correlation coefficient; NA = not applicable.

and AA-N) was reduced from 38.4 to 29.8% after model changes and parameterization, and CCC increased from 0.94 to 0.97 (Table 2). The mean bias was increased from 0.24 to 13.5%. However, the slope bias was substantially decreased from 36.3 to 6.6%. Several high-leverage points were observed by examination of the residuals' plots, which contributed to the moderate mean bias (Figure 4).

Blood Urea Metabolism

The RMSE of blood urea concentrations was substantially decreased from 72.4 to 39.6% and CCC increased from 0.40 to 0.77, suggesting that both accuracy and precision of predicted blood urea concentrations were enhanced. Mean bias and slope bias were decreased from 9.6 to 0.2%, and 19.3 to 2.5%, primarily due to improved representations of ruminal ammonia metabolism and blood urea metabolism.

Predictions of blood urea-N entry exhibited an RMSE of 17.0% (26.9% mean bias and 0.8% slope bias) and a CCC of 0.97, indicating that the model represented the mechanisms of hepatic ureagenesis properly. Blood urea-N entry accounted for approximately 64% of incremental dietary N intake (Figure 5), suggesting that hepatic ureagenesis is a major crossroad in terms of whole-body N exchange. In the current work, we added representations of intestinal urea entry, which altered urea distribution and ammonia absorption from the digestive tract. Previous studies indicated that some blood urea was recycled to the lower gut and captured in microbial protein (Nolan and Leng, 1972; Bailey et al., 2012a). Considering there is less fermentable energy for microbial growth in the hindgut than in the rumen, we assumed that 25% of recycled urea to the intestine was utilized for microbial protein synthesis. The remainder was excreted in feces as ammonia or reabsorbed from the hindgut. Changing the assumption from 25 to 50% resulted in a 4% decrease in blood urea entry and gut urea entry and a 6% increase of fecal N output, indicating that the model was not tremendously sensitive to this assumption. Model simulation results indicated that approximately 48 to 67% of the urea recycled to the gut flows into the rumen largely depending on diets, suggesting that the addition of intestinal urea entry was important in that it allowed contributions to N cycling without requiring entry of urea into the rumen.

Amino acid catabolism is another source of hepatic ureagenesis. Approximately 15 to 35% of absorbed AA are utilized as an energy source by gut tissues on a daily basis (Lobley and Lapierre, 2003; Arriola Apelo et al., 2014). Daily use of EAA by the liver can be as high as 35 to 50% of that absorbed when high-protein

diets are fed or milk production is low (Lapierre et al., 2005). Of those cleared by the splanchnic tissues, the majority are catabolized and converted to ammonia and carbon skeletons (Hanigan et al., 2004; Lapierre et al., 2005). To prevent hyperammonemia, ammonia is converted to urea in the liver. Although the mechanisms of AA catabolism are simulated in the model (Baldwin, 1995; Hanigan et al., 2009), limited data are available to evaluate the accuracy of AA-N contributions to urea synthesis. Retained N reported by the investigators represents the fraction of absorbed N that is converted to lean body tissue growth or lost in scurf. Comparison of these values to lean body tissue deposition predicted by the model indicated an RMSE of 118.1% with a mean bias of 68.6% and a slope bias of 5.1% before the modifications and reparameterizations (Table 2). The predicted retained N was -0.8 g/d, suggesting the animals were essentially in N balance during the experimental periods. However, the mean of the observed N retention was 36.4 g/d. We addressed this problem simultaneously with urea recycling by fitting the maximal rate of the AA conversion to body protein ($V_{m_{AaOth, Vis}}$, mol/d) to the data. This resulted in an increase in the parameter from 223 to 273.8 g/d, resulting in a decreased prediction error for retained N to an RMSE of 98.8% with a mean bias of 3.9% and slope bias of 39.7%, which was an improvement; however, the slope bias suggests that the representation of overall N balance systematically deviates from observed values.

This error might be due to the accumulation of errors in measured N balance. Unexplained N losses, including spilled feed, incomplete recovery of fecal material, and gaseous forms of N or nitrate formation, contribute to overestimates of N balance (Firkins and Reynolds, 2005). Additional robust data such as BW gain and body compositions are needed to provide a closer estimate to true N retention than balance studies.

Reynolds and Kristensen (2008) concluded that increasing dietary N intake was associated with increased blood urea entry (Figure 5) but the effect of N intake on gut urea entry was small. However, that is not consistent with the observations herein. As shown in Figure 6, observed gut urea entry linearly increased with dietary N intake (Figure 6A) and N intake relative to metabolic BW (Figure 6B). Approximately 64% of synthesized urea was recycled to the gut lumen (Figure 7A). Model simulations indicated that predicted urea-N recycled to the rumen account for 30 to 54% of total ruminal ammonia production depending on dietary CP content and RDP proportions, thus representing an important contribution to overall ruminal N balance.

Observed urinary urea excretion also increased linearly with increased dietary N intake (Figure 8A) and N intake relative to metabolic BW (Figure 8B). Approximately 32% of synthesized urea was eliminated in urine (Figure 7B). Reynolds and Kristensen (2008) observed that fractional gut urea entry and urinary urea-N excretion varied with dietary protein concen-

Figure 4. (A) Observed (●, black) or residual (▲, red) ruminal nonammonia, nonmicrobial N (NANMN) outflows (g/d) versus predicted flows using the modified model; (B) observed (●, black) or residual (▲, red) ruminal microbial N outflow (g/d) versus predicted values using the modified model; and (C) observed (●, black) or residual (▲, red) ruminal total N outflow (g/d) versus predicted values using the modified model. The dotted lines represent the line of unity.

trations in a reciprocal manner. Similar results were found in the current work (Figure 6C and Figure 8C), although the relationship was not strong. However, the relations between the fraction of gut urea entry or urinary urea excretion with dietary protein concentration are easily misleading, because high dietary protein concentration does not necessarily equate to high protein intake. When the fractional rates were regressed on dietary N intake with respect to metabolic BW, gut entry decreased quadratically, whereas urinary excretion increased quadratically (Figure 6D and Figure 8D), but these relationships are poorly defined by the data. Thus, it seems the best indicator of N recycling to the gut is simply the amount of urea entering blood. But this overall rate will be modified to the extent that ruminal ammonia concentrations control urea entry. Diets that are low in degradable N and high in fermentable carbohydrate can be expected to pull a larger fraction of synthesized urea back into the rumen compared with those generating high ruminal ammonia concentrations and with low fermentability.

Figure 5. Relationship between observed dietary N intake (g of N/d) and observed urea-N entry rate (g of N/d). Solid lines represent the results of linear regression for individual studies. The dotted line indicates the global regression for the full data set. Study effect was included as a random variable in the global regression model. The regression model for the whole data set was $Y = 0.23 + 0.64X$ ($R^2_m = 0.93$ and $R^2_c = 0.98$), where R^2_m is marginal R^2 , which represents the proportion of variance that can be explained by the fixed effect, and R^2_c is conditional R^2 , which indicates the proportion of variance that can be explained by the fixed effect plus the random effect.

Table 3. Model parameter estimates when the Molly cow model was fitted to the observed data

Model parameter	Description	Previous value ¹	Final estimate	SE
$K_{RUP,Rum}$	Intercept scalar to adjust in situ determined ruminal protein degradation rates (mol^{-1})	2.17	1.52	0.06
$K_{SPRUP,Rum}$	Slope scalar to adjust in situ determined ruminal protein degradation rates (mol^{-1})	0.03	0.016	0.003
$K_{Am,RumBld}$	Rate constant for ammonia absorption (d^{-1})	10.44	5.76	0.58
$K_{YATP,Rum}$	Efficiency constant defining the yield of microbial DM per mole of ATP (g/mol)	0.03	0.064	0.002
$K_{AmMl,Rum}$	Rate constant for the effect of ammonia on microbial growth (L/mol)	2.0×10^{-4}	0.0068	0.0003
X_{EAm}	Exponential factor to adjust the effect of ammonia on microbial growth (mol/mol)	NA ²	8.55	0.23
J_{Am}	Rate constant for the inhibitory effect of ammonia on blood urea conversion to ruminal ammonia (L/mol)	0.003	0.0074	1.1×10^{-5}
$Vm_{UrAm,Rum}$	Maximal rate of the blood urea conversion to ruminal ammonia (mol/d)	0.0567	0.084	3.4×10^{-3}
$K_{SpUr,IntFec}$	Slope scalar to predict fecal urea excretion (mol^{-1})	NA	0.028	0.003
$K_{Ur,IntFec}$	Intercept scalar to predict fecal urea excretion (mol^{-1})	NA	0.14	0.03
$Vm_{AdOth,Vts}$	Maximal rate of the AA conversion to lean body tissue (mol/d)	223.2	273.8	15.4
$K_{Ur,BldUrm}$	Rate constant for urea excretion by the kidney (mol^{-1})	2.134 ³	1.86	0.05
$K_{Protein,IntFec}$	Intestinal digestion coefficient for protein (g/g)	0.68	0.81	0.03

¹ K_{RUP} , K_{SPRUP} , $K_{Am,RumBld}$, and $K_{YATP,Rum}$ were refitted to a data set reported by Hanigan et al. (2013) after revising water kinetics; $K_{AmMl,Rum}$ and $K_{Protein,IntFec}$ were from Hanigan et al. (2013); $K_{Ur,BldUrm}$ was from Hanigan et al. (2009); J_{Am} , $Vm_{UrAm,Rum}$, and $Vm_{AdOth,Vts}$ were from the original model described by Baldwin (1995).

²Not applicable.

³The unit for $K_{Ur,BldUrm}$ in the initial model was liters per mole.

Figure 6. Relationships between observed dietary protein and observed gut urea entry. The solid lines represent the results of linear regression for individual studies. The dotted line indicates the global regression for the full data set. Study effects were included as a random variable in the global regression model. (A) Gut urea-N entry rate (g of N/d) versus dietary N intake (g of N/d): $Y = 9.49 + 0.41X$ ($R_m^2 = 0.90$ and $R_c^2 = 0.97$); (B) gut urea-N entry rate (g of N/d) versus dietary N intake relative to metabolic BW (g/d per kg of $BW^{0.75}$): $Y = 4.49 + 44.90X$ ($R_m^2 = 0.61$ and $R_c^2 = 0.95$); (C) fractional gut urea entry rate (GER) of blood urea entry rate (UER) (%) versus dietary CP (%): $Y = 70.17 + 0.027X^3 - 1.0X^2 + 8.45X$ ($R_m^2 = 0.49$ and $R_c^2 = 0.88$); and (D) fractional GER of blood UER (%) versus dietary N intake relative to metabolic BW (g/d per kg of $BW^{0.75}$): $Y = 101.83 + 1.83X^2 - 18.54X$ ($R_m^2 = 0.34$ and $R_c^2 = 0.88$), where R_m^2 is marginal R^2 , which represents the proportion of variance that can be explained by the fixed effect, and R_c^2 is conditional R^2 , which indicates the proportion of variance that can be explained by the fixed effect plus the random effect.

Figure 7. Relationships between observed blood urea entry rate (g of N/d) and observed gut urea entry rate or urinary urea-N excretion (g of N/d). The solid lines represent the results of linear regression for individual studies. The dotted line indicates the global regression for the full data set. (A) Gut urea entry rate versus blood urea entry: $Y = 10.03 + 0.64X$ ($R_m^2 = 0.94$ and $R_c^2 = 0.99$); (B) urinary urea-N excretion versus blood urea entry rate: $Y = -3.68 + 0.32X$ ($R_m^2 = 0.82$ and $R_c^2 = 0.96$), where R_m^2 is marginal R^2 , which represents the proportion of variance that can be explained by the fixed effect, and R_c^2 is conditional R^2 , which indicates the proportion of variance that can be explained by the fixed effect plus the random effect. The study effect was considered as a random variable.

Urinary N Excretion

Urinary N is mainly derived from urea at 55 to 62% of urinary N (Gonda and Lindberg, 1994), with lesser contributions from purine derivatives and ammonia. Altering the representation of urea excretion to be a function of pool size rather than concentration and refitting to the data resulted in a fractional excretion rate of 1.86 d^{-1} , a substantial reduction in RMSE from 243.5 to 69.0%, and an increase in CCC from 0.35 to 0.77, suggesting a considerable improvement associated with the change in representation. However, the precision of predicted urinary urea was still poor. As urinary N is likely collected with minimal error and not subject to variation in sampling, it seems likely that a significant proportion of the remaining variance reflects biological mechanisms and is thus potentially explainable. Several studies have indicated that renal processes such as the glomerular filtration rate and urea recycling from the glomerular filtrate are affected by CP (Rabinowitz et al., 1973; Eriksson and Valtonen, 1982) and NaCl (Godwin and Williams, 1984). Residual analyses indicated that urinary urea residuals were positively correlated with CP intake ($P < 0.05$), suggesting that

urinary urea excretion was underestimated when high-protein diets were fed. Spek et al. (2013) demonstrated that urinary urea excretion tended to be negatively related to the renal urea reabsorption ratio for high-protein diets, whereas no significant effect was observed for low-protein diets. Our results were consistent with their report, suggesting that the addition of inhibitory effects of protein on urea reabsorption might improve the representations of urinary urea excretion. There was no correlation between urinary urea residuals and ash intake, which was consistent with previous studies, as the effects of dietary NaCl on urinary urea excretion were not significant (Spek et al., 2012, 2013). However, the amount of explained variation in urinary urea excretion increased 8% when NaCl was included in the model (Spek et al., 2013). Sorting out these potential effects will require a concerted effort to collect such information to build a more complicated model to reflect the effects of CP and NaCl on renal mechanisms of urea absorption and excretion.

Adding representations of the excretion of purine derivatives in urine improved both accuracy and precision of urinary N predictions from a CCC of 0.71 to 0.94 and substantially decreased RMSE from 82.5 to 25.4%.

Figure 8. Relationships between dietary protein and urinary urea excretion. The solid lines represent the results of linear regression for individual studies. The dotted line indicates the global regression for the full data set. Study effect was included as a random variable in the global regression model. (A) Urinary urea-N excretion (g of N/d) versus dietary N intake (g of N/d): $Y = -3.44 + 0.21X$ ($R_m^2 = 0.79$ and $R_c^2 = 0.96$); (B) urinary urea-N excretion (g of N/d) versus dietary N intake relative to metabolic BW (g/d per kg of $BW^{0.75}$): $Y = -16.74 + 27.60X$ ($R_m^2 = 0.72$ and $R_c^2 = 0.95$); (C) fractional urinary urea excretion (UUE) of blood urea entry rate (UER) (%) versus dietary CP (%): $Y = 16.06 - 0.014X^3 + 0.54X^2 - 3.60X$ ($R_m^2 = 0.42$ and $R_c^2 = 0.86$); and (D) fractional UUE of blood UER (%) versus dietary N intake relative to metabolic BW (g/d per kg of $BW^{0.75}$): $Y = 1.03 - 2.20X^2 + 18.85X$ ($R_m^2 = 0.26$ and $R_c^2 = 0.85$), where R_m^2 is marginal R^2 , which represents the proportion of variance that can be explained by the fixed effect, and R_c^2 is conditional R^2 , which indicates the proportion of variance that can be explained by the fixed effect plus the random effect.

The improvement was largely due to removal of mean bias and slope bias, which decreased from 55.3 to 1.2% and from 36.7 to 17.7%, respectively.

Fecal N Excretion

Compared with the initial model, several nitrogenous components were considered in the feces, including undigested microbial nucleic acids, microbial protein synthesized in the hindgut, and ammonia generated from fecal urea hydrolysis. Considering that there is no urea pool in the gut, fecal N generated from urea hydrolysis was simulated as a linear function of intestinal urea entry rate. An intercept scalar ($K_{Ur,IntFec}$) and a slope scalar ($K_{SlpUr,IntFec}$) were derived to predict fecal excretion of N derived from urea based on the observed values. The endogenous urea-N loss in feces ($K_{Ur,IntFec}$) was 0.14 and the fractional transfer of urea-N entering the gut to feces ($K_{SlpUr,IntFec}$) was 0.028. The negligible slope estimate suggests that there was not a linear relationship between intestinal urea entry and fecal N from catabolized urea, which implies fecal urea-N excretion was constant among diets as N intake varied. Because the model does not consider endogenous protein losses, and we fitted these parameters against both fecal urea excretion and total fecal N output, $K_{Ur,IntFec}$ could be used to represent total endogenous N loss in feces, which includes urea, sloughed cells, and enzyme excretions into the gut. As a result, consideration of these additional N compounds in feces allowed the intestinal digestion coefficient ($K_{Protein,IntFec}$) to more closely reflect true protein digestion at 0.81 compared with the prior value of 0.68. This digestion coefficient was consistent with studies from monogastric animals. The true ileal digestibility of N was 76.9 to 78.2% in growing rats (Donkoh and Moughan, 1994), 75 to 83% in growing pigs (Furuya and Kaji, 1989; Hess et al., 2000), and 75 to 86% in broiler chickens (Huang et al., 2005; Gabriel et al., 2008). The RMSE for fecal N excretion was slightly decreased from 21.1 to 17.1%, with substantially decreased mean bias from 33.6 to 0.26%, and CCC was increased from 0.75 to 0.80, suggesting that the changes did improve the representation of total fecal N output.

Model Simulations

Having fitted the revised model to the data, we used the model to simulate the effects of several dietary scenarios on N metabolism. When RUP N intake was held constant at 192 g/d and RDP N intake was increased from 128 to 448 g/d, predicted microbial N outflow increased from 136 to 356 g/d and, consequently, predicted total N outflow increased from 361 to 617

g/d (Figure 9A). This led to an increase in predicted blood urea-N entry from 136 to 408 g/d and increased predicted urinary urea-N excretion from 37 to 195 g/d (Figure 9B). Although predicted gut urea-N entry rate increased as dietary RDP N intake increased from 99 to 207 g/d (Figure 9B), the proportions of predicted total ammonia that were generated from recycled urea in the rumen decreased continuously from 41 to 30% (Figure 9C), primarily because of the increased ammonia production rate and the inhibitory effect of ammonia on urea transfer from blood to rumen. With increased RDP intake, more AA and peptides are theoretically available for microbial protein synthesis in the rumen, which contributed to decreased proportions of predicted microbial protein N from ruminal ammonia-N (71 to 65%; Figure 9C). Under this feeding strategy, both the proportion of ammonia degraded from recycled urea and the proportion of ammonia-N captured into microbial N were decreased, resulting in decreased apparent ruminal N efficiency from 113 to 96% (Figure 9D).

When N intake was held constant at 512 g/d, increasing RDP proportions from 10 to 90% resulted in decreased predictions of ruminal NANMN outflow from 369 to 134 g/d and increased predictions of ruminal microbial N outflow from 196 to 351 g/d. However, the decreased NANMN flow was slightly greater than the increase in microbial N flow, leading to small linear decreases in predicted total ruminal N outflow from 566 to 488 g/d (Figure 10A). As a result, predicted blood urea-N entry was slightly decreased from 309 to 298 g/d (Figure 10B), suggesting that blood urea-N entry was relatively insensitive to RDP. When the proportion of RDP increased from 10 to 90%, ruminal ammonia was sufficient to support microbial protein synthesis despite decreased flux of urea-N into the rumen and increased urinary urea-N excretion (Figure 10B). In this scenario, proportions of predicted ammonia production incorporated from recycled urea decreased from 54 to 29%, as the inhibitory effect of ammonia on ruminal urea entry was exerted, and proportions of predicted rumen microbial N captured from ammonia-N decreased from 76 to 66% (Figure 10C). These changes contributed to decreased apparent ruminal N efficiency from 110 to 94% (Figure 10D).

CONCLUSIONS

Major improvements in predictions of ruminal N metabolism, blood urea metabolism, and urinary N excretion were achieved, including removal of slope bias for predictions of ruminal microbial N outflow, blood urea concentrations, and fecal N excretion, and reductions in mean bias for predictions of ruminal ammonia concentrations and urinary N excretion. Predicted blood

Figure 9. Effects of varying dietary RDP concentrations on model behavior given a constant RUP proportion (6% of DM); DMI was constant among diets. $F_{N,RumInt}$ represents ruminal total N outflow; $F_{MiN,RumInt}$ represents ruminal microbial N outflow; $F_{NANMN,RumInt}$ is ruminal nonammonia, nonmicrobial N outflow; $F_{AmUr,Liv}$ represents blood urea entry rate; $F_{Ur,BldGut}$ is gut urea entry rate; $F_{Ur,BldUrin}$ represents urinary urea excretion; $F_{AmMi,Rum}$ is a flux of ammonia-N converted to microbial protein; $F_{UrAm,Rum}$ is flux of ammonia generated from recycled urea in the rumen; $F_{Am,Rum}$ is total ammonia production rate; $F_{N,FdRum}$ is total dietary N intake.

urea entry and gut urea-N entry exhibited negligible mean bias and slope bias, implying the representation captured the main mechanisms of ruminal urea-N recycling. Hepatic ureagenesis is a major crossroad of

whole-body N exchange. Urea-N entry into blood accounts for approximately 64% of dietary N intake, of which 64% is recycled to the gut lumen. Depending on the diet, model simulation results indicated that urea

Figure 10. Effects of varying dietary RDP proportions on model behavior given a constant CP concentration (16% of DM); DMI was constant among diets. $F_{N,RumInt}$ represents ruminal total N outflow; $F_{MiN,RumInt}$ represents ruminal microbial N outflow; $F_{NANMN,RumInt}$ is ruminal nonammonia, nonmicrobial N outflow; $F_{AmUr,Liv}$ represents blood urea entry rate; $F_{Ur,BldGut}$ is gut urea entry rate; $F_{Ur,BldUrin}$ represents urinary urea excretion; $F_{AmMi,Rum}$ is a flux of ammonia-N converted to microbial protein; $F_{UrAm,Rum}$ is flux of ammonia generated from recycled urea in the rumen; $F_{Am,Rum}$ is total ammonia production rate; $F_{N,FdRum}$ is total dietary N intake.

recycled to the gut accounted for 29 to 54% of total ruminal ammonia production, and between 65 and 76% of this ammonia-N was captured in microbial protein. This equates to 41% of N intake returning to the gut,

with 60% entering the rumen. Of this, approximately 70% is captured in microbial protein, which represents 17% of N intake. Thus, this is an important component of the ruminal N system. Our simulation results sug-

gested that although feeding moderately low CP diets with low RDP proportions might decrease microbial protein synthesis in the rumen, this feeding strategy allows greater proportions of feed protein to escape the rumen, greater capture of recycled urea-N by rumen microbes, and reduced urinary urea excretion. The combination of these resulted in increased total metabolizable protein supply. These predicted outputs would suggest that reducing RDP proportions in a moderately low CP diet would lead to a substantial change in apparent ruminal N efficiency, which could improve animal performance in terms of lean growth or milk output.

ACKNOWLEDGMENTS

This work was funded, in part, by the National Research Initiative Competitive Grants Program from the USDA National Institute of Food and Agriculture (Washington, DC; grant no. 2007-35206-17848) and the Virginia Agricultural Experiment Station and the Hatch Program of the National Institute of Food and Agriculture, USDA; the College of Agriculture and Life Sciences Pratt Endowment at Virginia Tech; and the Alberta Livestock and Meat Agency Ltd. (Edmonton, Alberta, Canada; grant 2015F055R).

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