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An analysis of the relationship between plasma urea and ammonia concentration in dairy cattle fed a consistent diet over a 100-day period

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Measurement of plasma urea concentration is often used to identify a risk of dietary nitrogen-associated infertility. However, the use of plasma urea concentration in this way relies on it being an effective predictor for other potential toxic products associated with nitrogen metabolism (such as plasma or uterine ammonia). Recent research has shown that dietary nitrogen-associated infertility can be produced by diets which elevate plasma ammonia concentration without markedly increasing plasma urea concentration. Thus for cattle on different diets plasma urea concentration cannot be used to predict plasma ammonia concentration. This study evaluated whether plasma urea concentration could be used to predict plasma ammonia concentration in cattle kept on consistent diets. Data were analysed from a study where 42 cattle had been fed a control diet or the control diet plus 250 g urea per cow per day and had had weekly measurements of post-prandial plasma urea and ammonia concentrations. This analysis found that over a 100-d period, plasma urea concentration was relatively constant and unaffected by time while plasma ammonia concentration was significantly more variable, being affected by time since the study started, and whether cows began the study in the first or second group. Correlation between plasma ammonia and urea was limited; plasma urea concentration explained only 3·8% of the variation in plasma ammonia concentration. These data suggest that, even in cows on consistent diets, plasma urea concentration is not a good predictor of plasma ammonia, and that a simple urea threshold may not accurately identify the risk of dietary nitrogen-associated infertility.

Keywords: Ammonia, urea, correlation, cattle.

In order to achieve its milk yield potential the modern dairy cow is dependent on a high intake of dietary nitrogen (Zimmerman et al. 1991). Nevertheless such diets, particularly those with high levels of effective rumen degradable protein (ERDP), have also been associated with decreased fertility (Laven & Drew, 1999). However, the evidence for an effect of high intakes of ERDP on fertility is inconclusive, particularly for cattle fed normal commercial diets, with many studies showing little or no effect of increased dietary nitrogen on fertility (Laven et al. 2007).

There are two potentially toxic by-products of protein metabolism: urea and ammonia (Visek, 1984; Ocon & Hansen, 2003). Urea is commonly suggested to be the primary toxic by-product (e.g. Rhoads et al. 2006). However, Laven et al. (2007) concluded that much of the effect of increased intakes of degradable protein was probably mediated by ammonia rather than urea, and that in such cases plasma ammonia concentration was more closely correlated with reduced fertility than plasma urea concentration. There were two main reasons for this conclusion. The first was a comparison of two studies which investigated the effect of intakes of quickly degradable protein (in the form of urea) on the yield and quality of embryos. Dawuda et al. (2002) fed a diet that markedly increased plasma urea concentration (to 9 mM) and moderately increased postprandial plasma ammonia concentration (to 70 μM), while Sinclair et al. (2000) moderately increased plasma urea concentration (to 7 mM) and greatly increased postprandial plasma ammonia concentration

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(to >300 μM). Whereas Sinclair et al. (2000) reported that feeding their diet for 13 d prior to oocyte collection significantly reduced oocyte cleavage and blastocyst production, Dawuda et al. (2002) reported that feeding their diet for 17 d prior to embryo collection had no effect on either the number or the quality of embryos recovered, thus suggesting that plasma ammonia concentration was more important than urea concentration in determining whether a diet had a significant effect on embryo quality. Rhoads et al. (2006) suggested that Dawuda et al. (2002) would have seen an impact of their diet if they had implanted their recovered embryos into recipients. However, this conclusion does not explain why Sinclair et al (2000) found a significant impact on embryo quality of a high-protein diet which elevated plasma ammonia concentration without markedly elevating urea concentration. The second rationale stated by Laven et al. (2007) was the lack of evidence, in cows fed diets based on grazed grass, for high dietary nitrogen intakes (particularly in the form of nitrates) having a deleterious effect on fertility (Kenny et al. 2001; Laven et al. 2002; Ordonez et al. 2007). Laven et al. (2007) concluded that, although such diets were associated with high urea concentrations in milk and plasma, the relatively constant feed intake associated with grazing resulted in only a limited rise in systemic ammonia concentration. They then suggested that itwas the absence of this ammonia rise that prevented fertility from being affected.

Despite these findings, there has been little use of ammonia as a measure of the likely risk of dietary nitrogen-associated infertility outside of scientific studies because of the inherent difficulty of measurement of blood ammonia (Butler et al. 1996) and because of its significant association with time since feeding (Sinclair et al. 2000a). This has meant that urea, which is easier to measure and less variable during the day (Sinclair et al. 2000a), has been commonly used as the predictor for dietary nitrogen-associated infertility. This is reflected in the development by Butler et al. (1996) of a blood urea threshold of 6·8 mM above which dietary nitrogen-associated infertility should be suspected.

Clearly, if, as suggested by Laven et al. (2007) and Sinclair et al. (2000), plasma ammonia concentration is a better predictor for ERDP-associated infertility than urea concentration, we need to understand better the factors that influence urea and ammonia concentration and the relationship between them in order to identify any potential problems associated with using urea rather than ammonia as a predictor for reduced fertility.

As part of a study which investigated the effect of high quickly degradable nitrogen on follicular development and embryo growth (Laven et al. 2004), multiple contemporaneous blood samples were taken and analysed for ammonia and urea. The present study used those data to investigate the factors affecting plasma urea and ammonia and the relationship between these two metabolic by-products.

Table 1. Constituents of the diet fed to the control group

	g/kg feed
Grass silage	222
Maize silage	222
Wheat	153
Rapeseed meal	160
Molassed sugar beet	79
Cane molasses	65
Soyabean meal	59
Protected fat	32
Vitamins and mineral mix	8
Metabolizable energy	273 MJ/d
Crude protein	3981 g/d

Materials and Methods

Animals and their allocation

Forty-two Holstein dairy cows were selected from a dairy herd in southern England using the following criteria: (1) third or subsequent lactation, (2) calved in the previous 16 weeks and (3) deemed suitable for re-breeding following veterinary examination. Cows were ranked according to calving date and 9–18-d milk yield (mean 37·2 ± 0·98 kg/d). Sequential pairs of cows within the ranking order were then identified. Within each pair, one cow was allocated randomly to each treatment (control or high-urea diet). Allocation to treatment occurred on two occasions; 22 cows were allocated to treatment on the first occasion and 20 cows 3 weeks later.

Management, feeding and treatments

All cows were housed in the same cubicle building and were bedded daily with access to fresh water at all times. They were milked twice daily and yields recorded automatically on each milking occasion.

The control diet was formulated to meet metabolizable energy and metabolizable protein requirements for early lactation cows according to UK recommendations (AFRC, 1993). The high-urea diet differed from the control diet only in the inclusion of 250 g urea per cow per day (670 g Provitlic120 per cow per day; Dallas Keith Ltd, Witney, Oxon, UK). The diets were fed to appetite once a day as a total mixed ration (TMR). All cows were fed the control diet for an initial acclimatization period of 3 weeks prior to allocation to treatment. The diets fed to the cows allocated at each of the two timepoints were identical. The main constituents of the control diet are shown in Table 1. After allocation to treatment cattle were synchronized using a PRID® (CEVA Animal Health, Chesham, Bucks, UK) for 8 d with an injection of 25 mg of dinoprost (Synthetic PGF_2; Enzaprost® CEVA Animal Health), given the day before PRID® removal. Artificial insemination was carried out at 48 h and 72 h after PRID® removal. Subsequent inseminations were carried out as a result of observed oestrus supported by information from ovarian

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scanning and milk progesterone analysis. Cows were inseminated for a maximum of three cycles. Further details of diets and management are published in Laven et al. (2004).

All experimental procedures were carried out in accordance with the UK Animal Scientific Procedures Act (1986).

Measurements

Blood was collected weekly from all cows until 7 weeks post conception into EDTA-impregnated tubes (Greiner Labortechnik Ltd, Gloucestershire, UK) 3–4 h after feeding. The timing of the samples was designed to match the peak ammonia results found in a pre-study pilot. The blood was centrifuged at 1500 ***g*** at 4 °C for 15 min and plasma recovered and stored at −18 °C. Urea concentrations were determined in an auto-analyser using the method of Talke & Schubert (1965). Plasma ammonia concentrations were determined using phase 2 of the same method, with decreased absorbance at 340 nm reflecting plasma ammonia concentrations (Mondzac et al. 1965).

Statistical analyses

To compare the change in plasma ammonia concentration over the duration of the study with that of plasma urea concentration, models were created using study data which described how the two parameters changed over time. The data included in the model were individual milk yield, treatment group (control or high urea) and time of study start (whether cows were allocated to study on the first or second occasion). The data structure consisted of repeated measures taken over time on individual cows blocked within treatment groups. The variability of the data structure was preserved by building a mixed effects two level hierarchical model using the SAS PROC MIXED procedure (Littell et al. 1998; Singer, 1998) with the following model:

$$Y_{ijk} = R_{j(i)} + \alpha_i + \beta_1 t_{jk} + \beta_2 t_{jk}^2 + \beta_3 t_{jk}^3 + e_{ijk}$$

where Y_{ijk} is a vector of ammonia or urea responses at time k on the jth cow within treatment i; μ is the intercept, $R_{j(i)}$, is the random effect of the jth cow nested within the ith treatment (normally distributed with mean zero and variance σ_d^2); α_i, is the fixed effect of the ith treatment; $\beta_1 t_{jk}$, $\beta_2 t_{jk}^2$, $\beta_3 t_{jk}^3$, are the fixed effects of time (β_1) with cubic (β_2) and quadratic (β_3) polynomials; e_{ijk}, is the random error of the jth cow at time k on treatment I (normally distributed with mean zero and variance σ_e^2).

In a mixed effects model the fixed effects enter the model through the mean and the random effects enter the model through the variance.

$$\text{Estimate } (Y_{ijk}) = \mu + \alpha_1 + \beta_1 t_{jk} + \beta_2 t_{jk}^2 + \beta_3 t_{jk}^3$$

$$\text{Variance } (Y_{ijk}) = \sigma_d^2 + \sigma_e^2$$

$$\text{Covariance } (Y_{ijk}, Y_{ijl}) = \sigma_d^2 + \text{cov}(e_{ijk}, e_{ijl})$$

The covariance matrix of the responses was explored using different correlation structures for the repeated measures (Littell et al. 2000). Six covariance structures (compound symmetry (CS); first order autoregressive (AR(1)); first order autoregressive with random effects (AR(1)+RE); unstructured (UN); toeplitz (toep); and autoregressive moving average (ARMA(1,1)) were fitted to the data and the best model was selected based on the smallest values of fit statistics for Akaike information criteria (AIC), AIC corrected (AICC), and Bayesian information criteria (BIC). In the event of conflict the more parsimonious model was selected.

The AR(1)+RE covariance structure was proposed by Diggle (1988) and in contrast to AR(1) the covariance between lags only decreases to a common contribution from the random effect of cow. In data sets, such as this, with a long series of repeated data the correlation from AR(1) can decrease almost to zero, which in many cases can be an inappropriate covariance structure.

The analysis was based on data from the first 102 d after the allocation of the first group of cattle to the high-urea diet. Seventeen repeated measurements were available for urea and 16 for ammonia. The missing data were assumed to be missing at random. When analysing unbalanced data in PROC MIXED, Spilke et al. (2005) recommended the use of REML to estimate the variance component and the Kenward–Roger (1997) procedure for approximating the degrees of freedom. Initial data exploration included fitting separate kernel smoothed regression lines (bandwidth = 10·75 d) to scatter plots of the ammonia, and urea responses against time for each of the two treatment groups. Fixed effects were tested using maximum likelihood (ML) and retained in the model at $P<0{\cdot}05$ based on the type 3 tests of effects. Pre-planned treatment and start date comparisons were made with the probability of difference (PDIFF) option in the LSMEANS statement and declared significant at $P<0{\cdot}05$.

The methods of Hamlett et al. (2003) and Roy (2006), which estimate correlation whilst adapting for the presence of repeated measures, were adapted to obtain a correlation coefficient between urea and ammonia.

Results

Urea

The covariance structure which best fitted the data was AR(1)+RE. There was no significant effect of days on trial, milk yield or start date and no significant interactions. The only significant effect found was that of treatment ($P<0{\cdot}001$), with cattle on the high-urea diet having a mean plasma urea concentration 1·66 mM greater than cattle on the control diet. The intra-class correlation (ICC) was 0·43, indicating that urea was highly correlated within cow.

Model

$$\text{Urea} = 6{\cdot}45 + 1{\cdot}66 * \text{Treatment}$$

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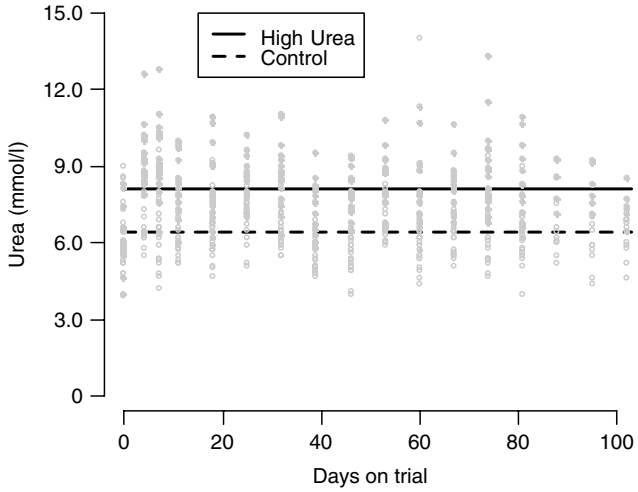

Fig. 1. Change with time in the concentration of plasma urea, with fitted regression line for each treatment group. Results from cattle given the control diet are represented using open circles, those from cattle given supplementary urea using filled circles.

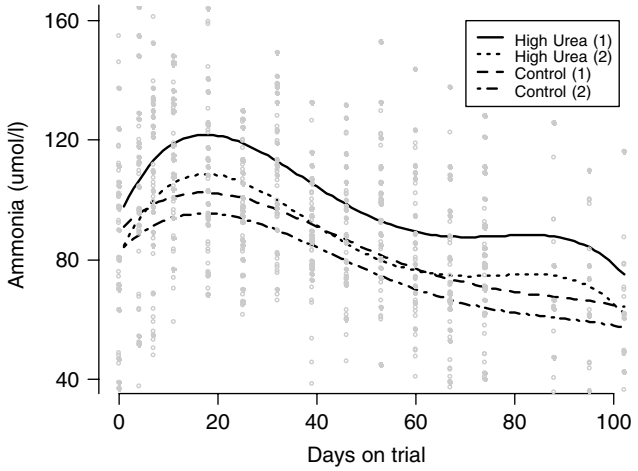

Fig. 2. Change with time in the concentration of plasma ammonia, with fitted polynomial trend line for each treatment group for each start date. Results from cattle given the control diet are represented using open circles, those from cattle given supplementary urea using filled circles. Separate equations for the treatment and control groups were produced by re-running the final mixed effects model without an intercept.

Ammonia

As with urea an AR(1)+RE model gave the best fit. However, in contrast to urea, factors other than treatment were found to have a significant effect on plasma ammonia concentration, with days on trial and start date included in both models. No significant interactions were found, nor was there a significant effect of milk yield.

Model

High urea

$$\begin{aligned}\text{Ammonia} = {} & 81{\cdot}1 + 3{\cdot}66 * (\text{days on trial}) - 0{\cdot}152 \\ & * (\text{days on trial})^2 + 0{\cdot}00203 * (\text{days on trial})^3 \\ & - 8{\cdot}94\text{E}{-}6 * (\text{days on trial})^4 + 13{\cdot}1 * \text{start}\end{aligned}$$

Control

$$\begin{aligned}\text{Ammonia} = {} & 82{\cdot}624 + 1{\cdot}72 * (\text{days on trial}) \\ & - 0{\cdot}0691 * (\text{days on trial})^2 \\ & + 0{\cdot}000801 * (\text{days on trial})^3 \\ & - 3{\cdot}06\text{E}{-}6 * (\text{days on trial})^4 + 6{\cdot}89 * \text{start}\end{aligned}$$

The intra-class correlation (ICC) was 0·28 indicating that the ammonia results were less highly correlated within cow than urea.

Prediction of ammonia from urea results

An AR1+RE correlation structure was used to model the covariance of the repeated measures.

Model

$$\begin{aligned}\text{Ammonia} = {} & 32{\cdot}3 + 7{\cdot}69 * (\text{Urea}) - 0{\cdot}100 \\ & * (\text{Urea} * \text{days on trial}) \\ & + 1{\cdot}64 * (\text{days on trial}) - 0{\cdot}0289 * (\text{days on trial})^2 \\ & + 0{\cdot}000169 * (\text{days on trial})^3 + 10{\cdot}3 * \text{start}\end{aligned}$$

The correlation (r) between urea and ammonia at any one time point measurement was 0·195 ($r^2 = 0{\cdot}038$). This implies that, because of the variation of ammonia with time since the trial start, on its own urea explained only 3·8% of the variability of ammonia.

Discussion

This analysis clearly shows that for cattle fed a consistent diet the relationship between plasma urea and plasma ammonia concentration varied significantly with time. This has been previously shown for different diets (Sinclair et al. 2000a; Laven et al. 2007); however, this analysis is the first to show this for cows on a consistent diet. So for cows on the same diet, as well as for cows on different diets, the same plasma urea concentrations can be associated with significantly different plasma ammonia concentrations. The correlation between the two parameters over the 102 d of this study was not significant, and for the cows in this study plasma urea concentration was a poor predictor for plasma ammonia concentration. As the control diet fed in this study was a conventional diet similar to that fed to many housed lactating cows, it is highly likely that these conclusions can be extrapolated to the field situation.

The present study was not designed to investigate how or why the factors that affect plasma ammonia or urea

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concentration produce their effect. Thus the models used in this analysis were developed to compare how plasma ammonia and urea concentrations changed during this study. The output from these models (Figs 1 and 2) clearly shows that the reason for the poor correlation between plasma urea and plasma ammonia concentration seen in this study was that plasma urea was unaffected by any of the factors included in the model (time after study start, milk yield, time of study start and treatment group) except for treatment group, whereas plasma ammonia concentration was affected by treatment group, time after study start and time of study start.

Clearly the present results cannot identify the reasons for the different behaviour over time of plasma ammonia and plasma urea, but the data strongly suggest that whereas the primary determinant of urea concentration was total crude protein intake (consistent with the findings of Butler et al. 1996), the effect of crude protein intake on plasma ammonia was markedly influenced by other factors. The underlying causes of the changes in plasma ammonia seen in this study are likely to be a combination of extrinsic factors (e.g. changes in protein degradability of the diet) and intrinsic factors (e.g. increased rate of ammonia capture by the rumen micro-organisms). Further research is required to establish the most important factors.

Conclusions

If, as Laven et al. (2007) and Sinclair et al. (2000) both suggest, increases in plasma ammonia lead to the reduced fertility associated with increased ERDP intake, then plasma urea concentration cannot be used as a proxy for plasma ammonia concentration when investigating fertility problems.

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