Modeling the distribution of ciliate protozoa in the reticulo-rumen using linear programming

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ABSTRACT

The flow of ciliate protozoa from the reticulo-rumen is significantly less than expected given the total density of rumen protozoa present. To maintain their numbers in the reticulo-rumen, protozoa can be selectively retained through association with feed particles and the rumen wall. Few mathematical models have been designed to model rumen protozoa in both the free-living and attached phases, and the data used in the models were acquired using classical techniques. It has therefore become necessary to provide an updated model that more accurately represents these microorganisms and incorporates the recent literature on distribution, sequestration, and generation times. This paper represents a novel approach to synthesizing experimental data on rumen microorganisms in a quantitative and structured manner. The development of a linear programming model of rumen protozoa in an approximate steady state will be described and applied to data from healthy ruminants consuming commonly fed diets. In the model, protozoa associated with the liquid phase and protozoa attached to particulate matter or sequestered against the rumen wall are distinguished. Growth, passage, death, and transfer of protozoa between both pools are represented. The results from the model application using the contrasting diets of increased forage content versus increased starch content indicate that the majority of rumen protozoa, 63 to 90%, are found in the attached phase, either attached to feed particles or sequestered on the rumen wall. A slightly greater proportion of protozoa are found in the attached phase in animals fed a hay diet compared with a starch diet. This suggests that experimental protocols that only sample protozoa from the rumen fluid could be significantly underestimating the size of the protozoal population of the rumen. Further data are required on the distribution of ciliate protozoa in the rumen of healthy

animals to improve model development, but the model described herein does indicate that the attached protozoal population is a significant component of the total rumen protozoal community.

Key words: linear programming, protozoa, reticulo-rumen, sequestration

INTRODUCTION

Protozoa constitute an important fraction of the microbial population in the ruminal ecosystem, although their function in the reticulo-rumen is not entirely clear (Firkins et al., 2007). Extensive bacterial predation reduces the flow of microbial protein to the duodenum but protozoa have a stabilizing effect on the entire microbial ecosystem, especially on starch-rich diets (van Zwieten et al., 2008). The contribution of ciliate protozoa to total flow of microbial dry matter is generally much smaller than would be expected from the amounts of protozoa in the reticulo-rumen (Jouany et al., 1988), although this has been questioned by others (Sylvester et al., 2005). Protozoal biomass as a proportion of total microbial biomass, measured by real-time PCR, was found to be similar between the rumen and duodenum (Sylvester et al., 2005). Selective retention of protozoa within the rumen is essential to allow their survival in this organ. Attachment of protozoa to fresh rumen digesta (Orpin, 1985) or sequestration on the reticulo-rumen wall (Abe and Iriki, 1989) might play a significant role in their retention in the rumen. Sequestration is defined as the method by which “protozoa maintain their numbers within the rumen against the washout effect associated with the flow of ingesta” (Abe et al., 1981). Protozoa are also known to adjust their generation time in response to altered passage rate or substrate, which could assist in maintaining a rumen community (Dehority, 1998, 2004; Firkins et al., 2007). However, whereas numbers of protozoa in the liquid phase are frequently reported, numbers of protozoa in the nonliquid phase remain largely unknown, although protozoa in the rumen solids phase have been quantified previously (Dehority, 1984; Hook et al., 2011). Mathematical models of microbial metabolism in the

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Figure 1. Diagrammatic representation of rumen protozoa model showing the fractional rate constant associated with each flow.

rumen have helped integrate data and concepts to improve understanding of the microbial contribution to rumen function (Dijkstra et al., 2002). Czerkawski (1987) developed a model of protozoal flows within the rumen and into the omasum and the lower gut. He suggested that the protozoal outflow from the rumen to the omasum is considerably greater than flow into the abomasum, because of loss of protozoa in the omasum. However, evidence reported by others (e.g., Imai et al., 1981; Michalowski and Harmeyer, 1983; Coleman, 1988; Leng, 1989; Towne and Nagaraja, 1990) does not appear to support the suggested lysis of protozoa in the omasum. In addition, Czerkawski (1987) assumed a uniform distribution of ruminal protozoa in the liquid phase and a fractional outflow rate of protozoa in the phase equal to the fractional outflow rate of liquid. However, protozoal numbers in rumen fluid samples taken from the dorsal or caudal rumen were higher than numbers in samples taken from reticulum fluid (Michalowski and Harmeyer, 1983; Yang and Varga, 1989). These results bring into question the assumption of uniform distribution and outflow. Furthermore, Czerkawski (1987) assumed the generation time of protozoa to be much lower than the retention time of the

liquid phase and hence the assumed net growth rate of protozoa in the liquid phase to be zero. However, mean protozoal generation times on a range of diets have been shown to vary between 6.0 and 55.6 h, with corresponding fluid passage rates varying between 0.041 and 0.173/h (Hodgson and Thomas, 1975; Leng et al., 1984; Okine et al., 1989; Ankrah et al., 1990). These considerations indicate that the model of Czerkawski (1987) may not be wholly appropriate. Therefore, the aim was to develop a simple alternative to the model of Czerkawski (1987), having similar complexity to that of France et al. (1990), to quantify protozoal dynamics in the reticulo-rumen.

MATERIALS AND METHODS

Model Development

The compartmental scheme adopted for representation of protozoal dynamics in the rumen is shown in Figure 1, and the mathematical notation is defined in Table 1. The protozoal pools are defined as P_l for the protozoa found in the liquid phase and P_s for the protozoa of the attached phase. The attached phase consists of both protozoa attached to feed particles and

Table 1. Definition of entities used in the rumen protozoa model

Entity	Unit	Definition
P_l	Dimensionless	Proportion of total protozoa in the rumen fluid
P_s	Dimensionless	Proportion of total protozoa attached to solids or associated with rumen epithelium
P	Dimensionless	Total protozoa in the rumen (equals unity)
μ_l	h^{-1}	Fractional growth rate of protozoa in pool l
μ_s	h^{-1}	Fractional growth rate of protozoa in pool s
d_l	h^{-1}	Fractional death rate of protozoa in pool l
d_s	h^{-1}	Fractional death rate of protozoa in pool s
k_l	h^{-1}	Fractional rate of passage of protozoa from pool l to omasum
k_s	h^{-1}	Fractional rate of passage of protozoa from pool s to omasum
$\bar{k}_l$	h^{-1}	Fractional rate of passage of liquid from rumen to omasum
$\bar{k}_s$	h^{-1}	Fractional rate of passage of solids from rumen to omasum
r_l	h^{-1}	Fractional rate of transfer from pool l to pool s
r_s	h^{-1}	Fractional rate of transfer from pool s to pool l
$\bar{r}_l$	h^{-1}	Fractional rate of protozoal transfer from liquid to particles
σ_l	h^{-1}	Fractional (i.e., specific) sequestration rate of protozoa in pool l
T_l	h	Mean retention time for protozoa in pool l
T_s	h	Mean retention time for protozoa in pool s

protozoa sequestered on the wall of the reticulo-rumen, due to a lack of quantitative experimental data to support separate pools.

Assume the system is close to steady state, then

$$\begin{aligned}\frac{dP_l}{dt} &= (\mu_l - r_l - k_l - d_l)P_l + r_s P_s \approx 0 \text{ and} \\ \frac{dP_s}{dt} &= (\mu_s - r_s - k_s - d_s)P_s + r_l P_l \approx 0.\end{aligned}$$

Let

$$\begin{aligned}x_{l1} &= \mu_l P_l; \quad x_{l2} = r_l P_l; \quad x_{l3} = k_l P_l; \quad x_{l4} = d_l P_l \text{ and} \\ x_{s1} &= \mu_s P_s; \quad x_{s2} = r_s P_s; \quad x_{s3} = k_s P_s; \quad x_{s4} = d_s P_s, \\ \text{where all } x_{ij} &\geq 0.\end{aligned}$$

Therefore,

$$\begin{aligned}x_{l1} - x_{l2} - x_{l3} - x_{l4} + x_{s2} &\approx 0 \text{ and} \\ x_{s1} - x_{s2} - x_{s3} - x_{s4} + x_{l2} &\approx 0.\end{aligned}$$

These 2 approximate equalities can be changed to exact equalities by the addition of slack variables:

$$\begin{aligned}x_{l1} - x_{l2} - x_{l3} - x_{l4} + x_{s2} + y_l &= 0 \text{ and} \\ x_{s1} - x_{s2} - x_{s3} - x_{s4} + x_{l2} + y_s &= 0.\end{aligned}$$

The slack variables y_l and y_s can be negative, zero, or positive, so let

$$\begin{aligned}y_l &= y_l^+ - y_l^- \text{ and} \\ y_s &= y_s^+ - y_s^-, \\ \text{where } y_l^+, y_l^-, y_s^+, y_s^- \text{ all } &\geq 0.\end{aligned}$$

Then,

$$\begin{aligned}x_{l1} - x_{l2} - x_{l3} - x_{l4} + x_{s2} + y_l^+ - y_l^- &= 0 \text{ and} \\ x_{s1} - x_{s2} - x_{s3} - x_{s4} + x_{l2} + y_s^+ - y_s^- &= 0.\end{aligned} \quad [1a, b]$$

Define $z = y_l^+ + y_l^- + y_s^+ + y_s^-$. Our problem is now reduced to minimizing the function z subject to equations [1a, b].

The entities P_l and P_s are fractions representing the proportion of rumen protozoa associated with the liquid phase and those attached to particulate matter or sequestered against the rumen wall, respectively. Therefore, the following additional restrictions apply:

$$\begin{aligned}P_l + P_s &= 1, \\ 0 \leq P_l, P_s &\leq 1.\end{aligned}$$

The fractional rate constants are bounded accordingly (see Parameterization section for derivation of numerical values):

$$\begin{aligned}0.018 &\leq \mu_l, \mu_s \leq 0.167, \\ 0.206 &\leq r_l \leq 4.06, \\ 0.025 &\leq r_s \leq 1.5, \\ 0.0025 &\leq k_l \leq 0.111, \\ 0.00062 &\leq k_s \leq 1.42, \text{ and} \\ 0.002 &\leq d_l, d_s \leq 0.067.\end{aligned}$$

These bounds yield the following constraints:

$$\begin{aligned}0.018P_l &\leq x_{l1} \leq 0.167P_l, \\ 0.018P_s &\leq x_{s1} \leq 0.167P_s, \\ 0.206P_l &\leq x_{l2} \leq 4.06P_l, \\ 0.025P_s &\leq x_{s2} \leq 1.5P_s, \\ 0.0025P_l &\leq x_{l3} \leq 0.111P_l, \\ 0.00062P_s &\leq x_{s3} \leq 1.42P_s, \\ 0.002P_l &\leq x_{l4} \leq 0.067P_l, \text{ and} \\ 0.002P_s &\leq x_{s4} \leq 0.067P_s.\end{aligned}$$

Mean ruminal retention time (T) for protozoa in the liquid phase and for those attached to particulate matter or sequestered against the rumen wall are defined by, respectively,

$$\begin{aligned}T_l &= \frac{1}{d_l + k_l + r_l} \text{ and} \\ T_s &= \frac{1}{d_s + k_s + r_s}.\end{aligned}$$

These retention times are bounded accordingly (see Parameterization section for derivation of numerical values):

$$\begin{aligned}4.17 &\leq T_l \leq 23.3 \text{ and} \\ 7.14 &\leq T_s \leq 500; \\ \text{that is,} \\ 23.3^{-1} &\leq T_l^{-1} \leq 4.17^{-1} \text{ and} \\ 500^{-1} &\leq T_s^{-1} \leq 7.14^{-1}.\end{aligned}$$

These bounds yield the additional constraints:

$$\begin{aligned}23.3^{-1}P_l &\leq x_{l2} + x_{l3} + x_{l4} \leq 4.17^{-1}P_l \text{ and} \\ 500^{-1}P_s &\leq x_{s2} + x_{s3} + x_{s4} \leq 7.14^{-1}P_s.\end{aligned}$$

In summary, the protozoa fractions and associated values of the rate constants when the protozoal popula-

tion of the rumen is at or close to equilibrium can be found by solving the linear program

$$\text{Minimize } z = y_l^+ + y_l^- + y_s^+ + y_s^-,$$

subject to

$$x_{l1} - x_{l2} - x_{l3} - x_{l4} + x_{s2} + y_l^+ - y_l^- = 0 \text{ and}$$

$$x_{s1} - x_{s2} - x_{s3} - x_{s4} + x_{l2} + y_s^+ - y_s^- = 0,$$

$$P_l + P_s = 1,$$

$$0.018P_l \leq x_{l1} \leq 0.167P_l,$$

$$0.018P_s \leq x_{s1} \leq 0.167P_s,$$

$$0.206P_l \leq x_{l2} \leq 4.06P_l,$$

$$0.025P_s \leq x_{s2} \leq 1.5P_s,$$

$$0.0025P_l \leq x_{l3} \leq 0.111P_l,$$

$$0.00062P_s \leq x_{s3} \leq 1.42P_s,$$

$$0.002P_l \leq x_{l4} \leq 0.067P_l, \text{ and}$$

$$0.002P_s \leq x_{s4} \leq 0.067P_s,$$

$$23.3^{-1}P_l \leq x_{l2} + x_{l3} + x_{l4} \leq 4.17^{-1}P_l \text{ and}$$

$$500^{-1}P_s \leq x_{s2} + x_{s3} + x_{s4} \leq 7.14^{-1}P_s,$$

$$P_l, P_s \geq 0,$$

$$x_{l1}, x_{l2}, x_{l3}, x_{l4}, x_{s1}, x_{s2}, x_{s3}, x_{s4} \geq 0, \text{ and}$$

$$y_l^+, y_l^-, y_s^+, y_s^- \geq 0.$$

Parameterization

A thorough examination of the literature was performed to obtain bounds for each parameter. Studies of cattle and sheep fed commonly consumed diets were considered, and all of the values obtained can be found in Table 2. The lower bound on parameter μ_l and μ_s (0.018/h) originates from a trial with sheep by Leng et al. (1984) in which sheep were fed a mix of oat chaff and lucerne hay with molasses and casein. The upper bound of 0.167/h is from Ankrah et al. (1990), in which the generation time of protozoa was estimated from steers fed chopped first-cut alfalfa hay. The specific death rates of protozoa in the attached, d_s , and free-living, d_l , phases are bounded by rates of 0.067/h and 0.002/h. The upper bound of 0.067/h is a measure of lysis rate in the rumen of cattle fed freshly cut ryegrass (Leng et al., 1986). The lower bound of 0.002/h represents the lysis rate in the rumen of cattle fed whole sugar cane and wheat bran (Leng et al., 1981). The

fractional passage rate of rumen fluid leaving the rumen to the omasum, $\bar{k}_l$, is bounded by the rates of 0.041 and 0.173/h. The upper bound of 0.173/h is from cattle fed chopped lucerne hay and sampled 0 to 2 h following feeding (Okine et al., 1989). The lower bound of 0.041/h indicates the rumen liquid outflow rate in sheep fed ground barley, ground hay, and flaked maize (Hodgson and Thomas, 1975). For the fractional rate of passage of particles from the rumen to the omasum, $\bar{k}_s$, the upper bound of 0.073/h was found by Thornton and Minson (1972) in sheep fed a dried chaffed panicum grass diet, and the lower bound of 0.015/h is a measure of particle transfer in sheep fed medium-quality hay (Kaske and von Engelhardt, 1990). The specific rate of transfer of protozoa from the liquid to particulate matter, $\bar{r}_l$, was found to be bounded above by a rate of 4/h from an in vitro study by Orpin (1985) of protozoa from sheep fed chopped hay and rolled oats. The lower bound of 0.167/h is from an in vivo study conducted by Ankrah et al. (1990) in steers consuming chopped first-cut alfalfa hay. The upper bound of 1.5/h for specific rate of transfer from attached to liquid phase, r_s , is from an experiment by Ankrah et al. (1990) in which steers were fed chopped first-cut alfalfa hay. The lower bound of 0.025/h is from an in vivo experiment involving sheep fed lucerne stem in a nylon bag, which was removed and examined at intervals for protozoal attachment, and attachment time was found to be greater than 40 h (Bauchop, 1979). The mean retention time of protozoa in the liquid phase of the rumen, T_b , was bounded by 4.17 and 23.3 h. These limits were calculated on the premise that death and passage are obligatory whereas transfer is not, and death and passage assume their extreme observed values. The upper bound is therefore the inverse of the sum of the lower bounds on d_l and $\bar{k}_l$ (0.002/h and 0.041/h, respectively), and the lower bound is the inverse of the sum of the upper bounds on d_l and $\bar{k}_l$ (0.067/h and 0.173/h, respectively). For the mean retention time of protozoa in the attached phase of the rumen, T_s , the lower bound of 7.14 h is the inverse of the sum of upper bounds on d_s and $\bar{k}_s$ (0.067/h and 0.073/h, respectively). The upper bound of 500 h is the inverse of d_s at a value of 0.002/h. The lower bound uses the premise that all P_s are attached to particles, death and passage are obligatory but transfer is not, and d_s and $\bar{k}_s$ assume their maximum observed values. The upper bound uses the premise that all P_s are sequestered, death is obligatory but transfer is not, and d_s assumes its minimum observed value.

To account for sequestration of protozoa within the reticulo-rumen, the following steps were taken. From the literature, it was established that the fractional pas-

Table 2. Parameter values (see Table 1 for definitions of parameters) obtained from literature review

Parameter	Bounds	Literature value (/h)	References
μ	Minimum	0.018	Hungate, 1966; Potter and Dehority, 1973; Coleman, 1979; Leng, 1982, 1984; Leng et al., 1984, 1986; Ffoulkes and Leng, 1988; Krebs et al., 1989; Ankrah et al., 1990; Williams and Withers, 1993; Dijkstra, 1994; Dehority, 2004, 1998; Karnati et al., 2007, 2009; Sylvester et al., 2009
	Maximum	0.167	
d	Minimum	0.002	Leng et al., 1981, 1984, 1986; Leng, 1982; Ffoulkes and Leng, 1988; Ankrah et al., 1990
	Maximum	0.067	
$\bar{k}_l$	Minimum	0.041	Hodgson and Thomas, 1975; Hartnell and Satter, 1979; Poppi et al., 1981; Mudgal et al., 1982; Sharp et al., 1982; Uden et al., 1982; Whitelaw et al., 1984; McCollum and Galyean, 1985; Leng et al., 1986; Ushida et al., 1986; Caton et al., 1988; Ffoulkes and Leng, 1988; Jouany et al., 1988; Argyle and Forster, 1989; Okine et al., 1989; Yang and Varga, 1989; Malcolm and Kiesling, 1990; Casper et al., 1999; Sylvester et al., 2005
	Maximum	0.173	
$\bar{k}_s$	Minimum	0.015	Thornton and Minson, 1972; Grovum and Williams, 1977; Hartnell and Satter, 1979; Colucci et al., 1982; Mudgal et al., 1982; Uden et al., 1982; Ushida et al., 1986; Punia et al., 1987; Caton et al., 1988; Jouany et al., 1988; Okine et al., 1989; Kaske and von Engelhardt, 1990; Okine and Mathison, 1991; Casper et al., 1999
	Maximum	0.073	
$\bar{r}_l$	Minimum	0.167	Hungate, 1966; Bauchop and Clarke, 1976; Amos and Akin, 1978; Orpin and Letcher, 1978; Bauchop, 1979; Orpin, 1985; Newbold, 1989; Ankrah et al., 1990
	Maximum	>4	
r_s	Minimum	0.025	Bauchop and Clarke, 1976; Amos and Akin, 1978; Bauchop, 1979; Abe et al., 1981; Orpin, 1985; Ankrah et al., 1990
	Maximum	1.5	

sage rate of the liquid phase, $\bar{k}_l$, is bounded by 0.041/h (Hodgson and Thomas, 1975) and 0.173/h (Okine et al., 1989). Because protozoal counts in the omasal fluid are 6 to 64% of the rumen fluid counts (Weller and Pilgrim, 1974; Collombier et al., 1984; Punia et al., 1984, 1992; Punia and Leibholz, 1984; Michalowski et al., 1986), the actual specific passage rate of the protozoa in the liquid phase, k_l , becomes bounded by 0.0025/h and 0.111/h. Assume that the fractional passage rate for the liquid phase is equal to the sum of the specific passage rate of the protozoa in the liquid phase and the specific sequestration rate of protozoa in the liquid phase; that is, $\bar{k}_l = k_l + \sigma_l$. Therefore, by difference, we find that the specific sequestration rate of protozoa in the liquid phase, σ_l , is bounded by 0.0385/h and 0.0623/h.

The specific rate of attachment of protozoa to particles, $\bar{r}_l$, is bounded by 0.167/h (Ankrah et al., 1990) and 4/h (Orpin, 1985). Thus, if the specific rate of attachment of protozoa to the nonliquid phase is equal to the sum of the specific rate of attachment of protozoa to particles and the specific sequestration rate of protozoa in the liquid phase; that is, $r_l = \bar{r}_l + \sigma_l$, then the specific rate of attachment of protozoa to the nonliquid phase, r_b , is bounded by 0.206/h and 4.06/h. The protozoa in the P_s pool are composed of protozoa attached to particles, $P_{s,att}$, and protozoa that have been sequestered. Outflow from the protozoa solids pool is represented by the fractional rate of passage of rumen particles with

attached protozoa; that is, $k_s P_s = \bar{k}_s P_{s,att}$. If we assume that the ratios $P_{s,att}/P_s$ and $\bar{r}_l/r_l$ are equal, then $k_s P_s = \bar{k}_s \bar{r}_l P_s / r_l$ gives $k_s = \bar{k}_s \bar{r}_l / r_l$. Having determined bounds for k_s , $\bar{r}_l$, and r_l , bounds on k_s can be computed. The specific rate of passage of protozoa in the solid phase, k_s , is therefore, calculated to be bounded by 0.00062/h and 1.42/h.

RESULTS AND DISCUSSION

Model Application

The model was solved using Premium Solver (Frontline Systems, Incline Village, NV) in Excel (Office 2007; Microsoft Corp., Redmond, WA), giving a solution $z = 0$, $P_l = 10.1\%$, and $P_s = 89.9\%$ for the linear program stated in the summary of Model Development section. Ranges of P_l and P_s were determined using the technique of compromise programming (Ignizio and Cavalier, 1994). Two objectives were considered for this:

$$\begin{aligned} &\text{Minimize } z_1 = y_l^+ + y_l^- + y_s^+ + y_s^-, \text{ and} \\ &\text{Optimize } z_2 = P_l, \end{aligned}$$

and assigned arbitrary weights of 1.0 and 0.1, respectively. Optimize reads as minimize when seeking the lower limit on P_l and as maximize when seeking the upper limit. Note that maximize z_2 is equivalent to

minimize $-z_2$. Thus the compromise objective function used for finding the lower limit on P_l was

$$\text{Minimize } z = z_1 + 0.1z_2.$$

Minimizing z subject to the constraints identified in Model Development gave the solution $z_1 = 0$, $P_l = 10.1\%$, and $P_s = 89.9\%$, which is the solution obtained previously. Similarly, the compromise objective function used for finding the upper limit on P_l was

$$\text{Minimize } z = z_1 - 0.1z_2,$$

which gave the solution $z_1 = 0$, $P_l = 37.3\%$, and $P_s = 62.7\%$. Thus, an optimal range was found for P_l of 10.1 to 37.3%, with a range for P_s of 62.7 to 89.9%. Therefore, the majority of protozoa in the reticulo-rumen are found in the attached phase. The attached phase includes protozoa attached to feed particles, as well as those sequestered on the epithelial surface. Although information for entodiniomorphids is largely lacking, evidence exists that vestibuliferids attach to particles in the rumen (Orpin and Hall, 1977) and sequester against the rumen wall (Abe et al., 1981; Dehority and Tirabasso, 1989). However, the evidence is limited to qualitative studies. For example, Abe et al. (1981) noticed a thick protozoal mass on the wall of the reticulum of Holstein steers slaughtered after overnight starvation, but such information does not allow separation of attached protozoa into protozoa attached to particles or to rumen wall. Lack of data also makes it difficult to separate the rumen protozoa in the model into vestibuliferids and entodiniomorphids. Although vestibuliferids are the protozoa more commonly associated with sequestration on the rumen epithelium, a study by Abe et al. (1981) found that approximately 22% of the protozoa on the reticulum wall were entodiniomorphids. As for whether protozoa "attach" to the wall, a recent study by Steele et al. (2011) found that following 20 washes in PBS and fixation for scanning electron microscopy of rumen papillae, vestibuliferids were still observed on the surface of the papillae, suggesting a strong association. Clarke (1965) and Abe et al. (1981), as well as others, observed a diurnal pattern in the number of protozoa found in the rumen fluid, and Abe et al. (1981) suggested that the vestibuliferids are able to sequester themselves on the wall of the reticulum after feeding. Based on the evidence presented, sequestration would likely account for a significant proportion of protozoa found in the attached phase in this model.

Model application was conducted to determine how the effect of different diets on passage rate and retention time in the reticulo-rumen would affect the proto-

zoal partitioning (Table 3). By increasing the proportion of forage in the diet, the lower bound of the fractional passage rate of rumen fluid, $\bar{k}_l$, would increase, as calculated herein by the lower bound multiplied by $1 + \Delta$, where Δ is an increment between 0 and 1. In turn, the upper bound of the fractional passage rate of rumen solids, $\bar{k}_s$, would decrease, as calculated by the upper bound multiplied by $1 - \Delta$. By increasing starch in the diet, the upper limit of the fractional passage rate of rumen fluid, $\bar{k}_l$, would decrease, as determined herein by the upper limit multiplied by $1 - \Delta$. Alternatively, the lower limit of the fractional passage rate of the rumen solids, $\bar{k}_s$, would increase, as determined by the lower limit multiplied by $1 + \Delta$. Such directional adjustment is consistent with findings of Seo et al. (2006). This change in the fractional passage rate limits would also change the retention time of the liquid and solids, as well as the specific rate of attachment of protozoa, as calculated in the Parameterization section. In general, the diet effect on the model favors a solution for P_l between 10 and 35%, with slightly more protozoa in the liquid phase with increasing starch (Table 3). However, when Δ is greater than 0.13 for the starch diet, the model solves to 0% P_l , which has no biological rationale. The results are qualitatively in agreement with a recent in vivo study that found significantly increased numbers of protozoa in rumen fluid samples collected from sheep when the proportion of concentrate in the feed was increased from 30 to 70% (Martinez et al., 2010). Another in vivo experiment found the number of protozoa in the liquid phase to increase as cattle were transitioned from a hay diet to a 65% mixed grain diet, and then decline as cattle were returned to the hay diet (Hook et al., 2011). This result was also seen by Brosard et al. (2003), where sheep limit-fed a high starch diet of 60% wheat and 40% hay had an increased number of protozoa in the rumen fluid, especially entodiniomorphids, compared with sheep fed 100% hay.

An additional application of the model was performed to test unique limits on specific growth rates, μ , and specific death rates, d . Only sparse data exist in the literature for specific growth and death rates in distinct phases and, therefore, it was assumed that the growth rate and the death rates of the liquid phase were equal to the rates of the attached phase. To test the effect of varying specific growth rates, we supposed that the lower bound on the specific growth rate of protozoa in the attached phase, μ_s , would increase, as calculated by the lower bound multiplied by $1 + \Delta$. In turn, the upper bound on specific growth rate of protozoa in the rumen fluid, μ_l , would decrease, as calculated by the upper bound multiplied by $1 - \Delta$. When Δ was 0.25, the model solved with an optimal range on P_l of 10.1 to 36.4%, and when Δ was 0.50, the range became 10.1

Table 3. Model application to test modification of fractional passage rate due to diet (forage diet or starch-rich diet) on protozoa partitioning (P_l is proportion of protozoa in liquid phase)

Δ^1	Forage P_l (%)	Starch P_l (%)
0 ²	10.1 to 37.3	10.1 to 37.3
0.05	10.1 to 36.3	10.5 to 37.3
0.10	10.1 to 35.4	10.9 to 37.3
0.20 ³	10.1 to 33.4	0

¹ Δ is an increment between 0 and 1.

²At 0 Δ , no distinction could be made between forage and starch diets in the model.

³Greater than 0.13 Δ resulted in a 0% starch P_l .

to 35.5%. For the specific death rate, we assumed that the lower bound of specific death rate of protozoa in the liquid phase, d_l , would increase, as determined by the lower bound multiplied by $1 + \Delta$. This would be accompanied by a decrease in the upper bound of the specific death rate of protozoa in the attached phase, d_s , as determined by the upper bound multiplied by $1 - \Delta$. The change in the limits of d_l and d_s also influenced the mean retention time of the model, as calculated in Parameterization. When Δ was 0.25, the model solved with an optimal range on P_l of 10.1 to 33.9%, and when Δ was 0.50, the model solved with an optimal range on P_l of 10.1 to 30.2%. Combining an altered growth and death rate with Δ of 0.25 gave a range on P_l of 10.1 to 32.9%, and a Δ of 0.50 gave a range on P_l of 10.1 to 28.0%. Changes in the specific death rate appeared to have a more significant effect on the model solution than changes in the specific growth rate, but this was largely due to the inclusion of d_l and d_s in the calculation of the limits for T_l and T_s , respectively.

In 1989, Leng (1989) asserted that the majority of protozoa die in the rumen, as opposed to the omasum, which was the hypothesis stated by Czerkawski (1987). Leng (1989) estimated that 65 to 85% of protozoa die within the rumen, likely due to autolysis. To test this hypothesis within our model, the “majority of protozoa die in the rumen” constraints:

$$d_l P_l + d_s P_s \geq 0.5(\mu_l P_l + \mu_s P_s)$$

and

$$k_l P_l + k_s P_s \leq 0.5(\mu_l P_l + \mu_s P_s);$$

that is,

$$x_{l3} + x_{s3} \leq 0.5x_{l1} + 0.5x_{s1} \text{ and}$$

$$x_{l4} + x_{s4} \geq 0.5x_{l1} + 0.5x_{s1}$$

were added, but no effect was observed on any optimal solution obtained previously. This shows that Leng’s assertion is not refuted by the model.

The last application of the model was a test of the assertion that the majority of protozoa exiting the

reticulo-rumen leave in the liquid phase. As protozoa are known to attach to particles, as well as sequester on the epithelium, the protozoa that do leave the rumen are likely in the liquid phase (Czerkawski, 1987). To test this hypothesis, the constraint

$$k_l P_l \geq k_s P_s;$$

$$\text{that is, } x_{l3} \geq x_{s3},$$

was included in the model, but no effect on any optimal solution was found. Therefore, the hypothesis that the majority of protozoa leaving the rumen leave in the liquid phase was not rejected by the model.

Model Critique

This paper represents a novel approach to synthesizing experimental data on rumen microorganisms in a quantitative and structured manner. The metabolic activity of microorganisms in the rumen is the major determinant of rumen fermentation and ultimately of the profile of nutrients available for absorption. The main nutrients available for absorption include volatile fatty acids produced in the rumen from fermentation of various substrates (Bannink et al., 2006; Dijkstra et al., 2008) and amino acids derived from microbial protein synthesized in the rumen (Dijkstra et al., 1998a). Knowledge of certain protozoal characteristics is poor compared with that of bacteria. Previously, a detailed mechanistic model of protozoal metabolism was developed (Dijkstra, 1994) that helped to quantify the contribution of protozoa to fermentation processes that are difficult or impossible to measure in vivo. The model helped quantify the contribution of protozoa to fiber degradation in the rumen in various dietary situations (Dijkstra and Tamminga, 1995) and was used to indicate the considerable effect of protozoal metabolic activity on recycling of N within the rumen (Dijkstra et al., 1998b). However, this model did not distinguish protozoa attached to particles, sequestered, or free in rumen fluid.

The amount and rate of protozoal flow to the lower gut is the subject of much debate. It has been suggested that the amount of protozoa flowing in the lower gut is much lower than that which could be expected from the amount of protozoa in the rumen (see review by Jouany et al., 1988). More recently, a review by Firkins et al. (2007) discussed data obtained using real-time PCR, which indicated that the ratio of protozoal biomass to total microbial biomass in the rumen is comparable to that found in the duodenum (Sylvester et al., 2005). Several methods have been proposed and applied to measure protozoal outflow. Comparisons between pro-

tozoal numbers in the rumen and omasal fluid indicated that omasal counts were only 6 to 64% of rumen fluid counts (Weller and Pilgrim, 1974; Collombier et al., 1984; Punia et al., 1984, 1992; Punia and Leibholz, 1984; Michalowski et al., 1986). However, numbers of protozoa in rumen fluid are unreliable indicators of protozoal biomass and outflow, because a proportion of the protozoa are associated with plant particles or the reticulo-rumen wall (Amos and Akin, 1978; Abe et al., 1981). The use of 2-aminoethylphosphonic acid (AEPA) as a protozoal marker has been questioned because of its presence in bacteria and in feed (Ling and Buttery, 1978; Whitelaw et al., 1984). Protozoal outflow as measured by use of a general microbial marker (e.g., ^{15}N , ^{35}S) and a specific bacterial marker (2,6-diaminopimelic acid, DAPA) yielded highly variable estimates of protozoal outflow and sometimes even negative estimates due to intraruminal digestion of bacterial cell walls (Ling, 1990). Steinhour et al. (1982) applied a ^{15}N rate of incorporation method and estimated that 33 to 51% of microbial outflow was of protozoal origin. Protozoal contribution to total microbial flow obtained by phosphatidyl choline was much lower (9 to 19% in John and Ulyatt, 1984; 6% in Ivan et al., 1992; 6 to 11% in Ivan et al., 1993).

Czerkawski (1987) suggested, in his model of protozoal flow from the rumen, that the greatest loss of protozoa is in the omasum, which would result in the low protozoal numbers found in the hindgut. This has since been shown by Leng (1989) and Towne and Nagaraja (1990) to not be the case. Therefore, the current model presented here is intended to provide a more accurate representation of protozoa of the reticulo-rumen, considering the more recent literature on distribution, sequestration, and generation times.

The current model has limitations due to a lack of available data in the literature and may warrant further investigation. First, the authors were unable to find experimental values for specific growth rate of protozoa in the attached phase. Values for the specific growth rate in the free-living phase, as well as values for an undefined phase, were available and, therefore, the specific growth rate in the attached phase was assumed equal to the growth rate in the free-living phase. Previous experiments by Dehority (1998, 2004) and Sylvester et al. (2005) indicate that protozoa adjust their generation times as a response to passage rates and substrate changes, so the lack of attached growth data may not be especially important for this model. Data are lacking regarding the specific death rate of protozoa in general, and only a small number of values for the death rate in the free-living phase were obtained. Again, the specific death rate of protozoa in the attached phase was assumed equal to that of the free-living phase. The lack

of data for specific death rate may be of more concern than the lack of data for specific growth rate because the model was more sensitive to change in the latter than in the former. The last limitation that was encountered was a lack of quantification studies that have enumerated the protozoa in the rumen fluid, attached to rumen solids and particulates, and those sequestered on the rumen wall, simultaneously. Without this information, it is difficult to assess the validity of the relative proportions of protozoa in the attached and free-living phases determined in the current model application.

The model predicts proportions of protozoa in the attached and free-living phases of the reticulo-rumen in a quasi-steady state. It suggests that the protozoal contribution to rumen function is not highly sensitive to changes in several of the variables tested and that the relative contribution of the liquid phase is small. Bounds for each input parameter were determined through a thorough examination of the literature, with a focus on rumen protozoa of healthy sheep and cattle consuming common diets. An *in vitro* experiment by Czerkawski and Breckenridge (1979a,b) found that protozoa sequestered in solid digesta of a fermentation system and the concentration of protozoa found in the effluent was only 10 to 20% of that found in the attached phase. The study by Czerkawski and Breckenridge (1979a,b) lends weight to the findings of the current model in terms of proportion of protozoa in the attached phase, and they also suggest that the reason the hay diet results in a greater proportion of protozoa in the attached phase is due to the greater amount of solid digesta available for attachment. Finally, it is difficult to predict the effect that time of sampling will have on these proportions, although the research by Abe et al. (1981) suggests that the greatest proportion of protozoa in the attached phase would occur a few hours postfeeding.

The assumptions and conclusions made in this article are rooted in and supported by historical records. Observations of Hungate (1966) state that, "the protozoa, especially certain types with slow division rates, may settle or collect around components moving slowly from the rumen and maintain themselves without being washed out." Abe and Kumeno (1973) suggested that the protozoal population of the rumen may maintain its population within the rumen by passing into the lower gut at a slower rate than the rumen fluid phase. Finally, Bauchop and Clarke (1976) were able to show the attachment of ciliates to feed particles in the rumen using scanning electron microscopy, lending weight to the idea that protozoa were able to sequester within the rumen and exit the rumen at a slower rate than that of the rumen fluid. Overall, the results of the current

model application indicate that the majority of protozoa are found in the attached phase, independent of the 2 diets compared. Therefore, the protozoa of the rumen are able to sequester against washout by associating with particulates and the rumen wall, consistent with protozoal research.

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