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Using video microscopy to improve quantitative estimates of protozoal motility and cell volume

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ABSTRACT

The objective of this study was to apply digital imaging to improve quantification of rumen protozoal biomass and distinguish treatment differences in cell motility and volume among ruminal protozoa. Observations of protozoa in rumen fluid treated with essential oils (CinnaGar, CIN; Provimi North America, Brookville, OH) or an ionophore (monensin, MON) indicated possible cell shrinkage. We hypothesized that MON would decrease protozoal motility and interact with CIN on cell volume. In addition, we hypothesized that analysis of still frames from video of swimming protozoa would improve volume prediction accuracy. Flocculated rumen fluid was incubated in batch culture dosed with N-free feed only (control), MON, CIN, or a combination of MON+CIN. Samples were taken at 0, 3, or 6 h post-treatment and wet-mounted on a microscope fitted with a high-definition camera. At 3 h post-inoculation, there was a treatment interaction for average speed such that CIN attenuated the effect of MON, with treatment means of 243, 138, 211, and 183 μm/s for control, MON, CIN, and MON+CIN, respectively. At 6 h post-inoculation, MON decreased average speed by 79.2 μm/s compared with the main effect mean without MON. We measured both minimum and maximum diameters (depth and width, respectively) perpendicular to the longitudinal axis of swimming protozoa, yielding a 3-dimensional estimate of protozoal volume. The ellipsoid formula $\left(4/3\right)\pi abc$, where $a = 1/2$ length, $b = 1/2$ width, and $c = 1/2$ depth, was compared with previously published volume estimations using genera-specific coefficients (genera-specific coefficient × length × width^2). Residuals (genera-specific coefficients − ellipsoid) were plotted against predicted (ellipsoid) and centered to the mean $\left(X_i - \bar{x}\right)$ to evaluate both mean and slope biases. For *Entodinium* spp., $Y = 0.248\ (\pm 0.037)\ (X_i - 7.98 \times 10^4) + 1.97 \times 10^4\ (\pm 1.48 \times 10^3)$; $n = 100$; r^2 [coefficient of determination (squared correlation coefficient)] = 0.31, with significant slope and mean biases. For family *Isotrichidae*, $Y = -0.124\ (\pm 0.068)\ (X_i - 2.54 \times 10^6) - 1.21 \times 10^4\ (\pm 4.86 \times 10^4)$; $n = 32$; $r^2 = 0.10$, where slope tended to be different from zero but with no mean bias. For *Epidinium* spp., $Y = 0.375\ (\pm 0.056)\ (X_i - 2.45 \times 10^5) + 6.65 \times 10^4\ (\pm 0.28 \times 10^4)$; $n = 64$; $r^2 = 0.43$, with both mean and slope biases. The present regression analyses demonstrate that the genera-specific coefficient-based method more likely overestimates volume for *Entodinium* and *Epidinium* than for the teardrop-shaped *Isotrichidae*. Based on simulations derived from previous literature reporting treatments that depress protozoal populations or among-animal changes in protozoal population structures, our proposed ellipsoid method offers potential to advance the prediction of treatment effects on protozoal volume and to shift focus from the number of cells present to the diversity, function, and biomass of protozoa under various treatment conditions.

Key words: rumen protozoa, protozoal volume, protozoal motility, monensin

INTRODUCTION

Rumen protozoa contribute to wasteful intraruminal N recycling resulting from predation of bacteria (Firkins et al., 2007) and associate with symbiotic methanogens (Vogels et al., 1980; Krumholz et al., 1983). Because of their hydrogenosomes or mitosomes (Hackstein and Tielens, 2010), ruminal protozoa represent a readily available source of H_2 or formate to ecto- or endosymbiotic methanogens. Defaunation (the removal of protozoa from the rumen) has been associated with decreased methanogenesis (Eugène et al., 2004; Newbold et al., 2015), presumably resulting from disruption of methanogen symbiosis. Yet, those studies often reported depressed NDF digestibility, whereas protozoa are credited with improved ruminal fiber digestibility resulting from rumen pH stabilization in diets with rapidly

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Table 1. Equations for protozoal volume estimation referenced in this article

Equation[1]	Family or genera	Reference
$0.8099 \times W^3$	All	Harmeyer and Hill (1964)
$\pi \times (L/4)^2 \times L$	All	Teather et al. (1984)
$\pi \times (W/2)^2 \times L$	All	Dehority (2010), Belanche et al. (2012)
$0.45 \times W^2 \times L$	*Entodinium*	Clarke et al. (1982)
$0.48 \times W^2 \times L$	*Isotrichidae*	Clarke et al. (1982)
$0.61 \times W^2 \times L$	*Epidinium*	Clarke et al. (1982)
$4/3 \times \pi \times a \times b \times c$	All	Ellipsoid (proposed herein)

[1] L = cell length, W = cell width, a = 1/2 cell length, b = 1/2 cell width, and c = 1/2 cell depth.

available starch and sugar (Jouany and Ushida, 1999), oxygen consumption (Firkins et al., 2007), and physical shredding of fiber particles (Dehority, 2010). Newbold et al. (2015) noted that predation and fiber degradation were more active for order *Entodiniomorphida*, but members of family *Isotrichidae* need to be recognized for different associated methanogens (Belanche et al., 2015) and physiology, especially glycogenesis (Denton et al., 2015) and motility (Diaz et al., 2014a).

Not all protozoal cells contribute equally to their hydrolytic activity in the rumen, leading researchers to group protozoal counts by relative size (Eadie et al., 1970; Whitelaw et al., 1984; Williams and Coleman, 1992). Whitelaw et al. (1984) chose not to count protozoa because cell size varied so much and was expected to give a misleading representation of their activity. Although Newbold et al. (2015) noted that predation activity is likely associated positively with cell size within *Entodiniomorphida*, cell size per se oversimplifies this relationship because larger *Isotrichidae* apparently have much less predation activity compared with small *Entodinium*. Thus, quantitative measurement of biomass per se will not necessarily represent ecological differences among a range of protozoal populations seen within the rumen (Kittelmann et al., 2016).

A method combining ready ease of distinction based on taxonomy, typically either by standard distinction of generic cell counts or 18S rRNA gene copies (Kittelmann et al., 2015), with protozoal volume offers potential to better explain variation in studies designed to assess interactions between protozoa, bacteria, and archaea. Quantitative videographic approaches also reduce bias toward increased protozoal biomass associated with bacterial and archaeal contamination compared with protozoal standards isolated from the rumen (Sylvester et al., 2005). Moving from discrete and somewhat subjective scoring protocols, measurement of protozoal volume has been limited in efficacy to date because of its reliance on 2-dimensional measurements and an assumed third dimension (depth). Microscopic observation of live cells indicates a flattened or tapered morphology, revealing limitations of previously published volume estimation formulas (Table 1) using common cylindrical derivations (Teather et al., 1984; Dehority, 2010). Although derived to account for this discrepancy, even genera-specific equations use an assumed (rather than directly measured) depth (Clarke et al., 1982). Thus, it would be ideal to capture images of live protozoa to measure depth. Although their speed has previously been considered a deterrent to still image quality (Nam et al., 2009), image resolution has advanced enough since then to reconsider this limitation.

Protozoa of family *Isotrichidae* migrate in search of sugars and small starch granules, and their motility and chemotaxis have been known for some time (Dehority, 2003). In contrast, those within order *Entodiniomorphida* also have chemotaxis (but without the migration ecology) that should help maintain their association with the particulate phase in the rumen (Firkins et al., 2007). Both groups have complex but differing chemosensory signaling pathways targeting both glucose and peptides (Diaz et al., 2014a). Assessment of protozoal motility has been the simplest metric of chemotactic or chemorepellant (peptides are sometimes chemorepellant to *Isotrichidae*) response to treatment, traditionally using some form of transparency tracing from computer or video monitors (Leick et al., 1997; Diaz et al., 2014a). However, the labor associated with these techniques limits the duration of observation (Leick et al., 1997) or sample size (Diaz et al., 2014a). High-throughput analyses for protozoal motility would likely advance our understanding of how differing protozoal inhibition methods influence ruminal activity, thus potentially explaining variable responses to protozoal suppression approaches (Hristov et al., 2013).

Ye (2013) dosed continuous culture fermentors with monensin (**MON**) with or without an essential oil combination of cinnamaldehyde and garlic oil (**CIN**; CinnaGar, Provimi North America, Brookville, OH), noting that CIN did not change protozoa counts; in contrast, estimates of protozoal volume using the cylindrical formula (Teather et al., 1984; Dehority, 2010) were increased by CIN whereas N:cell ratio decreased. Typically, cell size has been positively correlated with N:cell ratio (Weller and Pilgrim, 1974). However, a potential role for the apparent mode of action of CIN

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