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Rumen ciliated protozoa decrease generation time and adjust 18S ribosomal DNA copies to adapt to decreased transfer interval, starvation, and monensin[1]

J. T. Sylvester,*† S. K. R. Karnati,* B. A. Dehority,*‡ M. Morrison,*§ G. L. Smith,* N. R. St-Pierre,* and J. L. Firkins*[2]
*Department of Animal Sciences, The Ohio State University, Columbus 43210
†Current position: Buckeye Nutrition, 330 E. Schultz Ave., Dalton, OH 44618
‡Ohio Agricultural Research and Development Center, The Ohio State University, Wooster 44691
§CSIRO Livestock Industries, Queensland Biosciences Precinct, University of Queensland, 306 Carmody Road, St Lucia, Queensland, Australia 4067

ABSTRACT

Defaunation studies have documented decreased ammonia concentrations associated with reduced microbial protein recycling and wastage of dietary protein, whereas many methods to suppress protozoa can reduce feed intake or depress ruminal organic matter or fiber digestibility. Therefore, more research is needed to optimize dietary conditions that improve protozoal growth and ruminal outflow relative to autolysis and recycling. Response in growth rate to ruminal outflow was simulated by abrupt changes in transfer interval of batch cultures, and substrate availability was evaluated by feeding without or with abrupt addition of monensin, which was postulated to inhibit digestive vacuole function. In experiment 1, *Entodinium caudatum*, a mix of *Entodinium* species, *Epidinium caudatum*, or *Ophryoscolex caudatus* cultures rapidly adjusted their generation times to approach respective changes in transfer interval from 3 to 2 or 1 d (cultures were always fed at 24-h intervals). Monensin (0.25 μM) consistently delayed this response. To evaluate a metabolic upshift associated with feeding or a downshift associated with substrate depletion, experiment 2 used real-time PCR to quantify protozoal 18S rRNA gene (rDNA) copies that were expressed relative to cell numbers or to the cellular constituents N and nucleic acids after feeding without or with monensin (0.5 μM). The 18S rDNA copies per milligram of nucleic acids were least for *Ophryoscolex* compared with the other cultures. When averaged over cultures (no culture × treatment interaction), 18S rDNA copies per unit of nucleic acids decreased at 16 h when cultures were starved but increased with feeding unless monensin uncoupled availability of consumed substrate. Rumen protozoal growth increased in response to decreased transfer interval in experiment 1. Substrate availability appeared to initiate metabolic responses preparing for cell growth, explaining how cultures could rapidly adjust to decreasing transfer interval in experiment 2. Because feeding was not coupled with transfer in experiment 2, however, a metabolic control probably arrested cell division to prevent overgrowth relative to substrate availability.

Key words: rumen protozoal growth, protozoal generation time, monensin, protozoal 18S ribosomal DNA copies

INTRODUCTION

Rumen protozoa can stabilize fermentation by engulfing and slowing the rate of starch degradation, metabolizing lactic acid, and contributing to and promoting ruminal fiber degradation (Williams and Coleman, 1992; Hristov and Jouany, 2005). However, these benefits are potentially diminished because protozoa promote proteolysis of feed, predate upon bacteria, and release protein degradation fragments into the rumen (Walker et al., 2005). Unpredictable protozoal metabolism either decreases the supply of microbial protein to the animal (Hristov and Jouany, 2005) or necessitates increased protein intake (Firkins et al., 2006). In that latter review and the subsequent review of Firkins et al. (2007), we have elaborated that only partial protozoal suppression might balance the benefits and detriments of protozoal ecology. Compared with most studies with ruminants at low ME intakes, we reasoned that a faster ruminal passage rate in the dairy cow should simultaneously increase outflow rate of protozoal cells to the small intestine while decreasing their generation time; faster passage rates and more efficient protein synthesis should reduce the negative consequences of protozoa-mediated N recycling in dairy cows.

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[2]Corresponding author: firkins.1@osu.edu

Potter and Dehority (1973) deduced that protozoal counts in the rumen of sheep were maintained by decreasing generation time in response to faster ruminal passage rate and to a greater level of feeding. Yet, to fully evaluate protozoal generation time under different dietary conditions, in vivo studies are limited by large animal-to-animal differences in protozoal populations (Sylvester et al., 2005; Yáñez-Ruiz et al., 2006), variability in cell counting (Firkins et al., 2007), and challenges associated with isolating specific factors influencing protozoal generation time. Although improving the ability to standardize and isolate conditions, the RUSITEC semicontinuous culture system used by some researchers documents protozoal attraction to particles but only allows protozoal overflow with the liquid phase (Czerkawski, 1984), whereas Karnati et al. (2007) proposed that protozoa swim in the fluid but are chemotactically attracted to particles based on the finding that protozoal outflow rate from the rumen approximated the rumen particulate passage rate. Continuous culture systems that have a separate particulate phase need a moderately long residence time to maintain protozoal numbers (Hoover et al., 1976). Consequently, to meet our objectives to alter generation time by manipulating outflow rate, we chose to use single protozoal cultures.

Cryopreservation should improve the ability to mechanistically assess protozoal growth by using more robust cultures that presumably are more hardy than aged cultures or allows improved repeatability among studies (Kisidayova et al., 2005). Cultures of the amylolytic *Entodinium caudatum* and the fibrolytic *Epidinium caudatum* can grow at generation times as low as 12 h (Dehority, 1998), whereas the fibrolytic *Ophryoscolex* species grow much more slowly (30 h or greater; Dehority, 2004), so these cryopreserved cultures were chosen to provide diversity in niche, size, and growth rate. To our knowledge, the growth rates of cryopreserved protozoa have not been compared against those of fresh isolates, so we isolated a mixture of 4 *Entodinium* species as a positive control.

Dehority (2004) reported that increasing substrate provision to a culture of *Epi. caudatum* did not affect generation time. They suggested that there was a balance between protozoal biomass and the amount of nutrients from bacteria that maintained protozoal numbers. However, 2 other explanations could exist: 1) there was increasing growth and concomitant increasing autolysis (Dijkstra et al., 1998), or 2) transfer rate already was limiting cell numbers, so increasing substrate was held in check by some metabolic control to maintain an optimal protozoal biomass (the underlying hypothesis in the current study). In addition to comparing feeding versus starvation, we abruptly introduced the feed additive monensin. This ionophore, which is approved for use with dairy cattle, inhibits rumen protozoa initially until they adapt (Nagaraja et al., 1997). Although a mode of action is not clearly established for rumen protozoa, in the nonrumen ciliate *Paramecium*, monensin inhibits proteolysis within storage vesicles (Fok and Ueno, 1987) and inhibits lysosomal fusion of the food vacuole (Gautier et al., 1994). Only those *Paramecium* cells capable of forming a food vacuole were able to be subsequently rescued from nutrient deprivation (Maruyama and Takagi, 1997). Given the physiological similarities of these ciliates to rumen ciliates, monensin was assumed to similarly uncouple the availability of substrate already consumed.

Sylvester et al. (2004) developed a real-time PCR assay that targets protozoal 18S rRNA genes (**rDNA**). Relying on a harvested community protozoal sample to quantify total protozoal biomass in the rumen and outflow to the intestine, Sylvester et al. (2005) noted that the 18S rDNA copies expressed per protozoan cell ranged from about 1,000 to 6,000. These differences were attributed to differences in cell sizes of populations in the community samples or to varying growth rates. The majority of rDNA copies in ciliates probably are of macronuclear origin and already processed for transcription for rapid vegetative growth, rather than from the micronucleus, which contains DNA (including genes with introns) that is archived until replication during conjugation (Prescott, 2000). Iwamoto et al. (2004) suggested that *Tetrahymena thermophila* (a nonrumen ciliate) senses its cell volume or macronuclear DNA concentration and coordinates this regulation with nutrient supply and transfer. If not coordinated with transfer (and sudden dilution of cell numbers relative to substrate provision), checkpoints in the cell cycle should subsequently arrest cell division (Berger, 2001). Thus, while verifying reasons for changes in 18S rDNA copies among protozoal standards for use as a biomass marker, 18S rDNA also was hypothesized to be an ideal indicator to evaluate shifts in growth rates among rumen ciliates in cultures undergoing different dietary treatments.

In experiment 1, our objectives were to evaluate changes in generation time associated with transfer interval in cultures fed daily without or with monensin to test the hypothesis that abruptly decreasing transfer interval would stimulate protozoal growth rate (cell division) to maintain population density relative to substrate supply, whereas the simultaneous administration of monensin would retard changes in growth rate. To test this hypothesis, a new procedure to estimate generation time was derived. Our aim in experiment 2 was to monitor the biomass of diverse cultures on d 2 of a 3-d transfer regimen (but fed once daily) during star-

vation, after feeding, or after being fed with monensin to test the hypothesis that feeding would stimulate cellular responses needed to initiate cell division based on substrate supply unless monensin addition disrupted substrate availability and triggered a metabolic downshift.

MATERIALS AND METHODS

Culturing Media

Two protozoal media were used: medium SP for *O. caudatus* and medium M for all other species (Dehority, 1998). Substrate for cultures was a suspension containing 1.5% (wt/vol) starch and 1% (wt/vol) alfalfa prepared in distilled water and gassed with O_2-free CO_2. Starch was a combination of 50% (wt/vol) ground wheat and 50% (wt/vol) rice flour; forage was 60% (wt/vol) ground alfalfa hay and 40% (wt/vol) ground fresh alfalfa. Fresh alfalfa was harvested, frozen immediately, ground to a fine powder, and stored at −80°C until thawing for each day's use (Ribeiro and Eastridge, 2006). Other ingredients were ground at room temperature using a Wiley mill (Arthur H. Thomas, Philadelphia, PA) to pass through a 1-mm screen. All cultures were fed once daily at 0900 h at a feeding rate of 0.12 mL of substrate per 10 mL of culture (i.e., 2.7 mg of DM/d).

Protozoal Cultures

Epidinium caudatum and *O. caudatus* were purchased from the European Rumen Ciliate Culture Collection (provided by C. J. Newbold, University of Wales, Aberystwyth, UK). The *Ent. caudatum* was isolated from the rumen of a steer (Dehority, 1998). These 3 frozen ciliate cultures were revived by thawing tubes in a 39°C water bath for 5 min. Protozoal aliquots (100 μL) were transferred under a constant stream of O_2-free CO_2 into 16- × 150-mm tubes containing prewarmed media (10 mL) and substrate (0.12 mL). The fresh culture containing mixed *Entodinium* species was isolated from a lactating dairy cow fed a TMR of 43, 8, and 49% of the DM as corn silage, cottonseed, and concentrate, respectively. Ruminal fluid (~500 mL) was collected and incubated in a 39°C water bath for 45 min. Two drops of fluid containing sedimented protozoa were transferred to a culture tube containing 10 mL of media. The mixed culture was maintained for 3 mo before being identified as *Ent. caudatum, Ent. simplex, Ent. exiguum*, and *Ent. longinucleatum*, using the method described by Dehority (1993).

Experiment 1

The volume of protozoal culture was progressively doubled from 10 to 20, 40, 80, 160, and finally 320 mL by successively transferring each culture with an equal volume of fresh medium. All cultures were fed daily at the corresponding rate (0.12 mL of substrate solution per 10 mL of culture) and maintained in 500-mL Pyrex glass bottles sealed with number 4 rubber stoppers. All cultures were maintained for at least 4 transfers at a 3-d interval before experiments started. On d 0 of the experiment, stock cultures were gently mixed, and 5 mL were removed from their respective stock culture using a wide-mouth glass pipette and placed into 16- × 150-mm tubes containing the treatment, fresh media, and substrate.

In a preliminary experiment, we evaluated 0, 0.25, 0.5, 1.0, and 2.5 μ*M* (final concentration) of monensin sodium (Sigma, St. Louis, MO), which was dissolved in 75% (vol/vol) ethanol and sterilized using a 0.45-μm filter. Before transfer, monensin was added to each tube to achieve the desired final concentration, maintaining a constant ethanol volume (15 μL). The 0.25 μ*M* concentration of monensin was deemed the most appropriate for experiment 1 because it decreased protozoal cell count by 90 and 50% for the second transfer of the *Ent. caudatum* and *Epi. caudatum* cultures, respectively, whereas greater concentrations suppressed growth to such an extent that measurement of growth kinetics would not be possible.

The initial cultures (5 mL) were removed from the main inoculum (after careful mixing) using a sterile wide mouth glass pipette and placed into new tubes containing 5 mL of fresh media and substrate. One milliliter of formalin (50% vol/vol) was added to the remaining 5 mL of stock solution for time zero measurements and stored at 4°C until protozoal enumeration was completed. Tubes were tilted on a 10° slant during the experiment.

Protozoal Enumeration and Species Identification. Smaller *Entodinium* species (both in the single and Mix cultures) were counted in a Sedgewick-Rafter counting chamber using the method described previously by Dehority (1993), with the following modification: after staining protozoal cultures, 1.5 mL was pipetted into 2.0-mL microcentrifuge tubes and centrifuged at 5,000 × *g* for 2 min. One milliliter of supernatant was removed and replaced with 1 mL of 30% glycerol. Larger and less numerous protozoal species (i.e., *Epi. caudatum* and *O. caudatus*) were counted by transferring 2 replicate, 0.5-mL aliquots of stained culture in 10 separate 50-μL spots onto a 25- × 10-cm piece of clear acrylic plastic and covered with glass coverslips. Total protozoal cells were counted at 20× magnification using a dissecting microscope.

Calculation of Generation Time. Generation time is defined as the time needed to double cell numbers and therefore is a net of cell division and cell lysis.

In a constant transfer regimen, cell count will halve with transfer of one-half the volume into an equal volume of medium but will reach the pretransfer count before the next transfer (Dehority, 1998). We evaluated an equation to evaluate changes in generation time at each transfer (Fondevila and Dehority, 2001). However, this procedure biased the estimated change in generation time in a previous study (Dehority, 2004) and in the current one. Any underestimation in cell count at one time point would underestimate the increase in cell number from the preceding interval while overestimating the increase in cell count in the succeeding interval (and vice versa for overestimation of counts). To minimize the bias from these magnified deviations, we derived a new procedure that would calculate a variable response in generation time after an abrupt change in transfer interval based on regression over all time points. Total protozoal counts were determined at each of the transfers, summed to yield total cumulative cells, and transformed to logarithm base 2, following Prescott (1957). The transformed mean cell counts were plotted for each transfer interval and shown in Figure 1. From these plots, the most appropriate regression model would need to be determined for best fit in a systematically objective procedure. Backward elimination regression was performed using PROC GLM of SAS (1999) so that the sequential removal of terms from the highest to the lowest order polynomial was terminated when the LACKOFIT option deemed the simplest model to be the best fit. After choosing the most appropriate regression model, that model was then fit using PROC REG of SAS (1999) to force the intercept through the common inoculation counts (time 0 in Figure 1). These resulting best-fit equations (reported in Table 1) were plotted as continuous functions (represented by solid and dashed lines) in Figure 1. As discussed by Prescott (1957), a change of one logarithm unit (base 2) is one doubling (i.e., one generation) on the y-axis of Figure

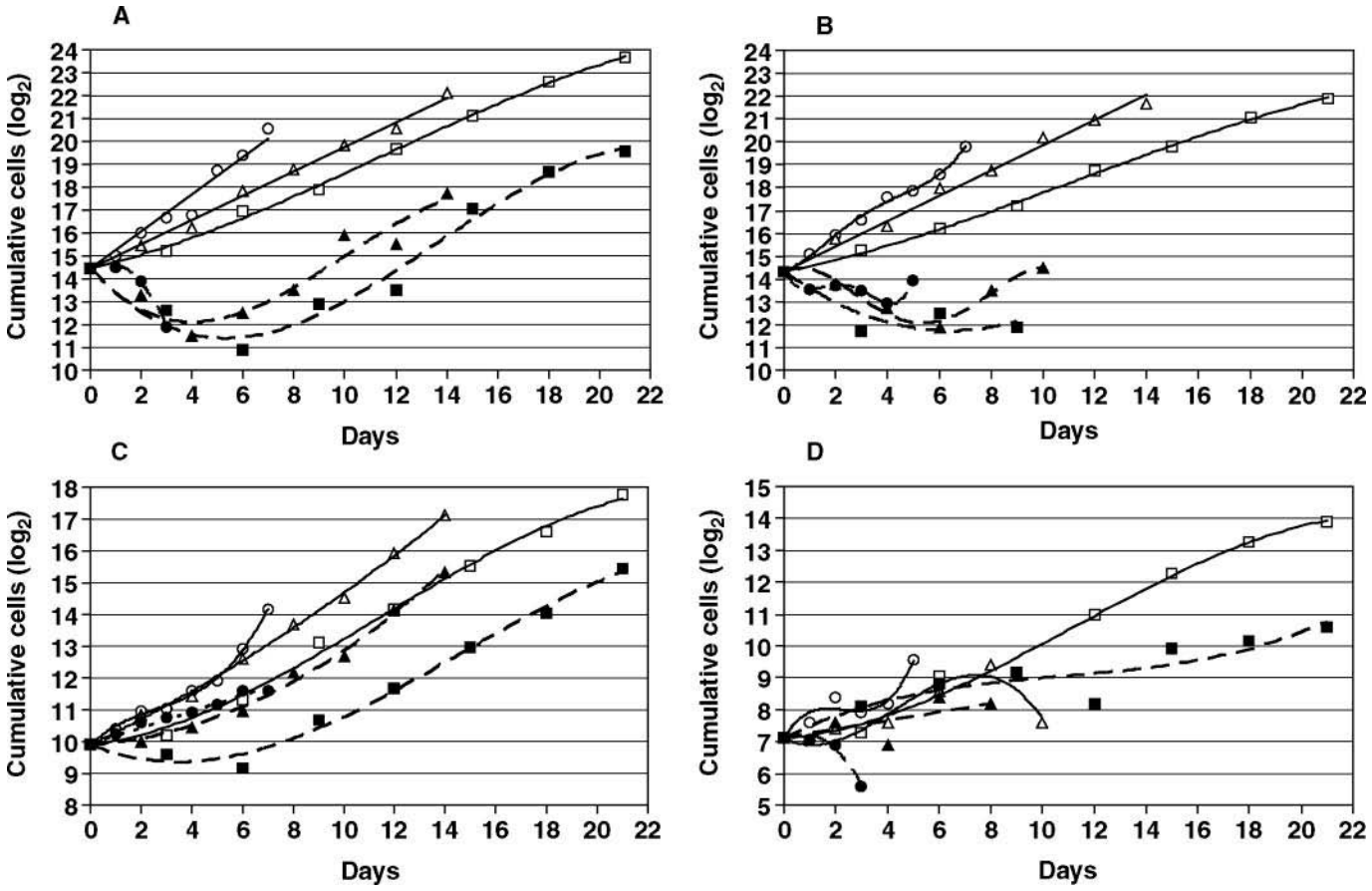

Figure 1. Cumulative cell growth (logarithm base 2) for *Entodinium caudatum* (A), a mix of *Entodinium* species (B), *Epidinium caudatum* (C), and *Ophryoscolex caudatus* (D) after abruptly changing transfer interval in experiment 1. All cultures were previously fed every 24 h and transferred every 3 d. At time 0 of the experiment, half of the cultures were maintained without (—) monensin but transfer intervals were 1 d (○), 2 d (△), or 3 d (□); the other half of the cultures were administered 0.25 μ*M* of monensin (—) and transfer intervals were 1 d (●), 2 d (▲), and 3 d (■). Corresponding best-fit equations are depicted in Table 1.

1, so the slope is the proportion of one generation per day (i.e., the increment of the x-axis). If the regression best-fit equation was linear, then there would be a constant slope over the entire incubation. However, if the best-fit equation in Table 1 was nonlinear, then the changing slope could be determined by taking the first derivative of the polynomial regression equation and solving for any value of X, which was restricted to the day of transfer corresponding with the respective cell counts. The reciprocal of the slope at each day of transfer was its predicted generation time. In Figure 2, these respective generation times were plotted (but symbols suppressed) against transfer day, and curves were spline-fit by Excel.

Experiment 2

Protozoal culture volumes were increased from 10 to 400 mL as described previously except that cultures were maintained in Corning 500-mL flat plastic cell culture flasks and tubes were not tilted on a 10° slant as in experiment 1. Culture volumes were allowed to increase until each species had 9 flasks containing 400 mL. The culture vessels were randomly assigned to a treatment, and all species and replicates were randomly distributed in a 39°C incubator. The experiment was repeated, and data were analyzed separately as 2 blocks.

Treatments and Sampling. Treatments consisted of not feeding (**NF**) the cultures, feeding the cultures at the normal rate (**Fed**), and feeding the cultures with the addition of monensin (**Fed + M**) at 0.5 μM (final concentration). In a preliminary experiment described previously, the cell counts were decreased by 96 and 73% by the second transfer for 0.50 μM of monensin for the *Ent. caudatum* and *Epi. caudatum* cultures (data not shown). In contrast with experiment 1 in which multiple transfers were planned after monensin introduction, the objectives of experiment 2 were to study abrupt changes in cell composition after a single transfer, so the 0.50 μM concentration was chosen to ensure treatment response within the 24-h feeding cycle. In a preliminary experiment in which cultures were counted at 2-h intervals, *Entodinium* cultures had a nearly constant number of dividing forms, but *Epi. caudatum* had approximately 5% of counts in dividing forms at 6, 8, 10, and 12 h after feeding but had few detectable forms at other times (data not shown). Consequently, experimental treatments started 24 h posttransfer after which samples were taken at 0, 6, and 16 h (i.e., 24, 30, and 40 h posttransfer and feeding for NF).

After inverting the flasks 2 times, samples were taken from cultures using a 25-mL wide-mouth pipette. Culture (133 mL; i.e., 1/3 of the original volume) was removed from 3 replicate flasks containing each species on 3 respective treatments at 0 h and combined to give a composite 400-mL sample for analyses; the remaining culture was further incubated until 1/3 was removed at 6 h and the final 1/3 volume taken at 16 h. Composite samples were treated with formalin [1.0% (vol/vol) final concentration] to fix protozoal cells and prevent cell disruption during subsequent procedures. After centrifugation at 13,000 × g for 10 min, the supernatant was removed via vacuum aspiration, and the sedimented protozoa were then separated from bacteria using the filtration method of Sylvester et al. (2004) that was modified by using distilled water instead of saline to wash the protozoa. Those authors reported that fixation with 1% (vol/vol) formalin and using repeated washes with mild agitation allowed virtually complete recovery of mixed protozoal cells (largely comprised of *Entodinium* species). Bacterial cells in the filtrate were collected by centrifugation (13,000 × g for 10 min at 4°C). Both isolated protozoal and bacterial cells were re-suspended in approximately 10 mL of water, weighed, and stored at 4°C.

All samples were analyzed for DM and Kjeldahl N according to AOAC (1990). Harvested protozoal cells were analyzed to determine the protozoal 18S rDNA copies using an assay developed by Sylvester et al. (2004). Briefly, total genomic DNA was extracted from 0.25 mL of culture using modifications of the Puregene isolation kit (Gentra Systems, Minneapolis, MN). The DNA was amplified using PCR with protozoa-specific primers to generate the corresponding sample-derived standards and the individual real-time PCR standard curves for each culture. We standardized 18S rDNA copies per amount of total nucleic acids (**NA**) to account for varying cell volume, consumed feed, or storage polysaccharide. Protozoal samples were analyzed for total purines with standardization to a RNA basis (Sylvester et al., 2005). This standardized value does not represent total NA but was maintained because this assay is the most common microbial marker used in ruminant nutrition, containing approximately 22% purine bases (Firkins et al., 2006). Although standardized to an RNA basis, it was referred to as NA to avoid confusion that purines also are contributed by DNA.

Statistical Analysis

Cultures were preincubated and transferred to increase the number of flasks of cultures needed to be randomly assigned to the 4 treatments and 3 incubation times. Minor differences in resultant cell numbers among replicate flasks were covariate-adjusted to a common initial mean at 0 h. Protozoal cells per incubation flask followed a normal distribution, but data for 18S rDNA copies and the ratios of 18S rDNA copies/cell

Table 1. Regressions of cumulative cell growth of 4 protozoal cultures with different transfer intervals or administered 0.25 μ*M* of monensin (experiment 1)[1]

				Linear		Quadratic		Cubic		Quartic		
Culture	Transfer interval (d)	Monensin	Intercept	Coefficient	SE	Coefficient	SE	Coefficient	SE	Coefficient	SE	RMSE
Ento	1	−	14.5	0.809	0.036							0.428
Ento	2	−	14.5	0.531	0.009							0.210
Ento	3	−	14.5	0.250	0.0530	0.0232	0.0075	−0.00067	0.00025			0.183
Ento	1	+	14.5	0.680	0.131	−0.506	0.049					0.115
Ento	2	+	14.5	−1.27	0.31	0.198	0.064	−0.00656	0.00324			0.710
Ento	3	+	14.5	−1.25	0.18	0.145	0.026	−0.00353	0.00085			0.630
Mix	1	−	14.3	0.447	0.228	0.336	0.170	−0.0940	0.0394	0.00755	0.00284	0.126
Mix	2	−	14.3	0.552	0.011							0.283
Mix	3	−	14.3	0.223	0.037	0.0173	0.0052	−0.000509	0.000174			0.129
Mix	1	+	14.3	−2.15	0.08	1.96	0.09	−0.648	0.028	0.0679	0.0028	0.026
Mix	2	+	14.3	0.638	0.466	−0.611	0.244	0.103	0.039	−0.00481	0.00197	0.292
Mix	3	+	14.3	−0.806	0.292	0.0630	0.0367					0.769
Epi	1	−	9.91	0.736	0.093	−0.176	0.039	0.0226	0.00032			0.107
Epi	2	−	9.91	0.383	0.020	0.00941	0.00175					0.118
Epi	3	−	9.91	0.0908	0.0676	0.0341	0.0095	−0.00099	0.00032			0.233
Epi	1	+	9.91	0.260	0.009							0.103
Epi	2	+	9.91	0.0501	0.0275	0.0244	0.0024					0.161
Epi	3	+	9.91	−0.326	0.079	0.0535	0.0110	−0.00122	0.00037			0.271
Oph	1	−	7.11	1.34	0.43	−0.623	0.249	0.0902	0.0343			0.299
Oph	2	−	7.11	−0.422	0.265	0.221	0.077	−0.0173	0.0053			0.369
Oph	3	−	7.11	0.0574	0.1047	0.0340	0.0147	−0.00101	0.00049			0.361
Oph	1	+	7.11	0.0406	0.202	−0.296	0.076					0.178
Oph	2	+	7.11	0.136	0.041							0.452
Oph	3	+	7.11	0.402	0.153	−0.0307	0.0215	0.000946	0.00071			0.528

[1]Ento = *Entodinium caudatum*; Mix = a mix of *Entodinium* species; Epi = *Epidinium caudatum*; and Oph = *Ophryoscolex caudatus*. The polynomial regressions correspond to the best-fit lines plotted in Figure 1. The intercept was forced to the initial values, so root mean square error (RMSE) is shown rather than r^2.

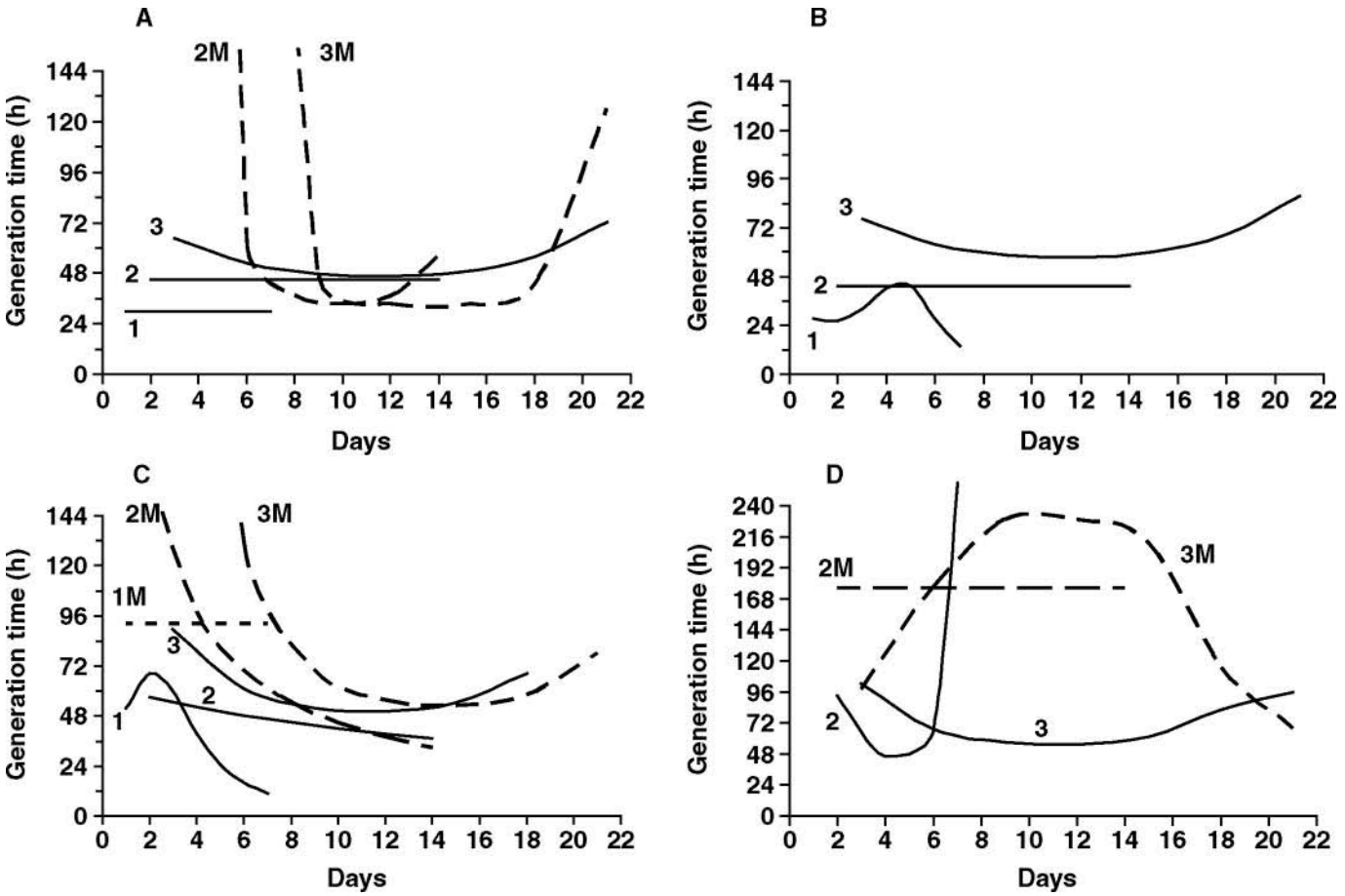

Figure 2. Calculated generation times for *Entodinium caudatum* (A), a mix of *Entodinium* species (B), *Epidinium caudatum* (C), and *Ophryoscolex caudatus* (D) over time of incubation after abruptly changing transfer interval in experiment 1. From the corresponding regressions of cumulative cell yields (Table 1), the first derivative was derived to calculate a generation time at each respective transfer interval (1, 2, or 3 for each curve to represent transfer d; M = monensin). The smoothed lines pass through these points (symbols were removed) and represent cultures that were maintained without (—) or with 0.25 μM monensin (—) administered at time 0. Predicted generation times are not shown when there were poor or highly variable growth responses for the monensin 1-d transfer (A), all transfer intervals for monensin (B), and both control and monensin 1-d transfers (D).

and 18S rDNA copies per mg of NA were transformed to logarithm base 10 to normalize the data distributions. Data were analyzed as a split-plot design using PROC MIXED of SAS (1999). The model contained the dependent variable, the covariate term, and the fixed effects of block (2 incubations), species (4 cultures), treatment (NF, Fed, and Fed + M), incubation time (0, 6, and 16 h), and all 2- and 3-way interactions among species, treatment, and time. All 3-way interactions were $P > 0.10$; hence, 2-way interactions were illustrated in graphical form as main effect means of species (averaged over treatments) or treatment (averaged over species) at each incubation time.

Variance homogeneity between different species [i.e., N (0, σ^2_e)] was tested using the COVTEST option. When variances between parameters were deemed heterogeneous ($P < 0.20$ was the cutoff, but all were $P < 0.05$), the variance for *O. caudatus* was determined separately from the pooled variance from the other 3 cultures; when variances were homogeneous ($P > 0.20$), the standard errors were pooled among all cultures. Main effect means were compared using ANOVA, and means were separated using Fisher's *F*-test-protected least significant difference. On all graphs, main effect means are separated by capital letters (X, Y, or Z) above groups of bars that would need to be averaged among sampling times to calculate the respective main effect mean. When there were interactions with sampling time, the simple means within culture or within treatment are separated by lower case letters (a, b, or c) above single bars. Unless reported, any interactions were $P > 0.10$.

RESULTS

Experiment 1

Compared with normally growing cells, protozoa harvested from cultures on the monensin treatments were shrunken and translucent and were morphologically similar to the NF treatment except for numerous dividing forms with abnormal morphology (data not shown). Both of these responses to monensin were relatively consistent among cultures.

The number of cells from the *Entodinium caudatum* culture started at 22,569 (transformed to 14.5 on the y-axis; i.e., $2^{14.5}$ cells) on d 0 when all cells had been adjusted to 3-d transfers (Figure 1A). As transfer interval decreased to 2 d or 1 d, the average generation time (the time to change 1 log unit) progressively decreased. When monensin was added, those being transferred at 1 d declined to extinction after the fourth transfer, whereas the cultures being transferred every 2 or 3 d recovered their initial cell numbers by the fourth transfer (Figure 1A). As with the *Ent. caudatum* culture, the cumulative cell growth of the *Entodinium* Mix also increased more rapidly with decreasing transfer interval without monensin (Figure 1B). When monensin was added, the cumulative cell numbers never recovered to their initial cell values (Figure 1B). Decreasing transfer interval from 3 to 1 d successively decreased the time to accumulate similar cell yields for *Epidinium caudatum*, and the pattern was more delayed when monensin was added (Figure 1C). The culture of *Ophryoscolex caudatus* had difficulty growing at transfer intervals shorter than 3 d, and cell yields for cultures with monensin at this transfer interval were less than that of the control (Figure 1D).

With the initiation of the experiment, the generation time of *Ent. caudatum* on the 3-d transfer without monensin slightly decreased around d 10 to 12 and then increased back up to about 72 h (i.e., its transfer interval; Figure 2A). The generation times of *Ent. caudatum* on the 2- and 1-d transfers were predicted to be constant at 45 and 30 h, respectively, which approximate the transfer intervals of 48 and 24 h (Figure 2A). By the third transfer after monensin was added to *Ent. caudatum* cultures, the generation time was predicted to be below 60 h, but it again increased to exceed the transfer interval in the 2- and 3-d transfer regimens (Figure 2A). As with *Ent. caudatum*, the generation times of the *Entodinium* Mix without monensin also tended to approach the same time as the transfer interval for the 3-d transfer and the 2-d transfer, the latter of which was predicted to have a constant generation time of 43 h (Figure 2B). The generation time for the 1-d transfer of the *Entodinium* Mix culture increased and then decreased (Figure 2B). However, if the fit was forced to be linear (data not shown), the reciprocal of the linear regression coefficient (0.756/d) would yield a 32-h generation time for Mix, which again approximated the constant 30-h value of *Ent. caudatum*. The addition of monensin inhibited growth in all transfer regimens for *Entodinium* Mix. For *Epi. caudatum*, the generation time also decreased concomitantly as the transfer interval decreased (Figure 2C). With monensin, there was a constant generation time of 92 h for the 1-d transfer, which means that cell numbers would eventually decline to extinction if the transfers would have been extended longer. However, when transfer intervals were 2 or 3 d, generation time gradually decreased with increasing incubation time until it approached the same generation times as their respective controls without monensin (Figure 2C). As with other cultures, the generation time for the 3-d transfer of *O. caudatus* had a similar slight drop below the transfer interval as it adjusted to the experimental protocol followed by gradual readjustment back up to approach or slightly exceed the transfer interval (Figure 2D). The generation time for the 2-d transfer of *O. caudatus* dropped below 72 h, but it rapidly approached infinity by the fourth transfer as net cell growth ceased (Figure 2D). The generation time for monensin-treated *O. caudatus* cultures was predicted to be a constant 176 h for the 2-d transfers, so the populations would decline to extinction with extended time (Figure 2D). However, the generation time for the 3-d transfer interval of *O. caudatus* with monensin increased and then converged near the generation time for its 3-d control (Figure 2D).

Experiment 2

The simple means of total protozoal cells per culture are shown at each treatment combination to depict the entire treatment structure (Table 2). However, there were no main effects of treatment, interactions with treatment, or interactions with hour. The main effect means for species (averaged over all treatments and times) were all different ($P < 0.05$) from each other. The *Ent. caudatum* culture always maintained the greatest cell concentrations, followed by the *Entodinium* Mix, *Epi. caudatum*, and *O. caudatus* cultures, respectively. There also was a main effect of time, in which the 16-h means (averaged for all species and treatments) were less ($P < 0.05$) than respective main effect means at 0 or 6 h. Except for the *Entodinium* Mix culture, though, the cell numbers were all greater for Fed than NF or Fed + M.

The decrease ($P = 0.10$, but no statistical inferences are shown in Figure 3A) in recovered N at 16 h than 0 or 6 h generally coincides with decreases in cell recovery

Table 2. Mean cells recovered from 4 protozoal cultures at different times after feeding with or without monensin in experiment 2[1]

	Not Fed				Fed				Fed + M			
Hour	Ento	Mix	Epi	Oph	Ento	Mix	Epi	Oph	Ento	Mix	Epi	Oph
	Cells × 10^5 400 mL^{-1} culture											
0	25.4	10.3	3.4	0.031	25.4	10.3	3.4	0.031	25.4	10.3	3.4	0.031
6	26.2	9.4	3.6	0.052	23.5	10.0	4.8	0.067	21.2	10.1	3.5	0.066
16	14.4	7.0	2.8	0.042	25.2	5.6	3.8	0.086	13.0	5.6	2.0	0.047

[1]*Entodinium caudatum* (Ento), mixed *Entodinium* species (Mix), *Epidinium caudatum* (Epi), and *Ophryoscolex caudatus* (Oph) cultures were transferred every 72 h but fed every 24 h. After adaptation and 24 h after the last transfer, cultures were not fed (Not Fed) at the normal interval or were fed without (Fed) or with (Fed + M) monensin, 0.5 μM final concentration. Cultures were sampled at 0, 6, or 16 h after commencement of treatments. There were no interactions ($P > 0.20$) among main effects. The main effect of species was $P < 0.05$, with main effect means for Ento (22.2), Mix (8.7), Epi (3.4), and Oph (0.050) all $P < 0.05$ from each other (SE for main effect means for species = 0.954). The main effect of hour was $P < 0.05$, with main effect means for 0 h (9.78) and 6 h (9.35) not different ($P > 0.20$), and both were $P < 0.05$ from 16 h (6.66); the SE for main effect means for hour = 0.83.

(Table 2). When averaged over treatments, the log-transformed copies of 18S rDNA were less at 16 h than 0 h for both the *Ent. caudatum* and the *Entodinium* Mix cultures, but *O. caudatus* had the fewest copies at 0 h and the greatest at 6 h (Figure 3B). The main effect mean for *O. caudatus* also was less ($P < 0.01$) than those for the other cultures. In contrast, when expressed per cell (Figure 3C), the main effect means were greatest for *O. caudatus* ($10^{4.73}$). The main effect means were $10^{3.28}$ and $10^{3.38}$ for the *Ent. caudatum* and *Entodinium* Mix cultures ($P > 0.10$ from each other) and $10^{3.99}$ for *Epi. caudatum* ($P < 0.05$ from either of the *Entodinium* cultures; Figure 3C). For *O. caudatus*, the log copies/cell increased at 6 and 16 h compared with 0 h, explaining the species × time interaction ($P < 0.01$; Figure 3C). In contrast with Figure 3C but consistent with Figure 3B, the 18S rDNA copies per mg of NA were less for *O. caudatus* (Figure 3D). For that species, this ratio increased at 6 h and then declined at 16 h to be between the 0- and 6-h values, explaining the species × time interaction ($P < 0.01$; Figure 3D).

There were no effects of treatment or treatment × time interaction for protozoal N in the cultures (Figure 4A). Total 18S rDNA copies had a treatment × time interaction (Figure 4B). For cultures that were not fed, total 18S rDNA copies did not change at 6 h (i.e., 30 h since the last feeding) and then dropped at 16 h. For main effect means for Fed, the copies numerically increased at 6 and 16 h (i.e., did not decrease as in NF). For Fed + M, the copies increased ($P < 0.05$) at 6 h and then decreased ($P < 0.05$) at 16 h compared with 0 h. There was a main effect of treatment ($P < 0.05$) for 18S rDNA copies/cell (Figure 4C). Although there was not a treatment × time interaction ($P > 0.10$), the values for 0 h were covariate-adjusted to be the same across treatments; consequently, the lower ($P < 0.07$) main effect mean for NF than the main effect means for Fed or Fed + M was explained by the copies/cell decreasing at 16 h for NF but increasing with Fed or staying the same with Fed + M (Figure 4C). Compared with 0 h ($10^{8.38}$ for all treatments), the 18S rDNA copies per mg NA decreased ($P < 0.09$) at 16 h for NF ($10^{7.96}$) but increased ($P < 0.09$) for the Fed treatment ($10^{8.79}$, Figure 4D). Adding monensin (Fed + M) resulted in values at 16 h ($10^{8.43}$) being similar to those at 0 h. The difference from the 0-h values were – 0.42, + 0.41, and + 0.05 log units, respectively. When expressed on an actual basis, the 0-h value is 263% of the 16-h value for NF, the 16-h value for Fed is 257% of the 0-h value, and the 16-h value for Fed + M is 112% of the 0-h value.

DISCUSSION

Measurement of Protozoal Growth

Our aim in experiment 1 was to study the change in generation time after abruptly adapting from a 3-d transfer interval to 2 or 1 d. Because protozoal growth is enhanced by live (rather than autoclaved) bacteria, standard practice is to feed cultures at 1-d intervals to maintain an appropriate balance to optimize protozoal biomass relative to the viable bacteria in the culture (Fondevila and Dehority, 2001; Dehority, 2004), so transfer intervals also were kept in daily increments. To our knowledge, this is the first depiction of this quantitative method, but the mathematical assumptions are comparable with those described previously (Prescott, 1957). The predicted generation times are not depicted as symbols because they are not measured data, but the smoothed lines pass through each prediction. When the fits were linear, we verified the same generation times as using established formulas (Dehority, 1998, 2004).

Presumably, decreased generation time based on cell yields could be a result of an increase in cell division rate or a decrease in lysis rate or both. A constant rate of change of generation time (a linear fit) indicates that the relationship between cell division and lysis is not

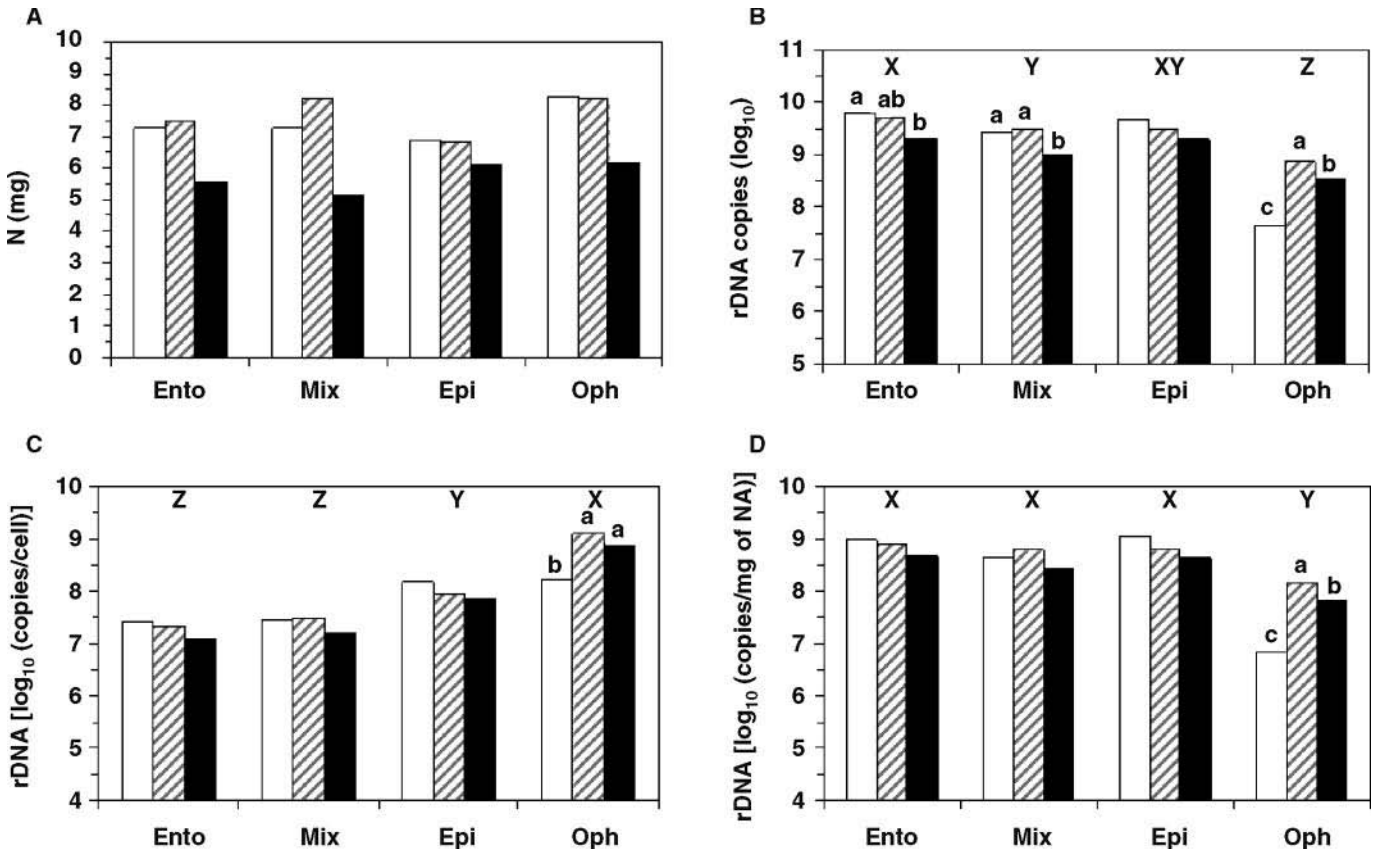

Figure 3. Main effect means (averaged across treatments) for cultures of *Entodinium caudatum* (Ento), a mix of *Entodinium* species (Mix), *Epidinium caudatum* (Epi), or *Ophryoscolex caudatus* (Oph) that were recovered on 10-μm filters at 0 (open bars), 6 (hatched bars), or 16 (filled bars) h of incubation in experiment 2. Measurements include A) total N per culture flask (SE at each time for Ento, Mix, and Epi were 1.01; and for Oph, 2.05), B) logarithm (base 10) of total protozoal 18S rDNA copies (The SE at each time for Ento, Mix, and Epi were 0.17; and Oph, 0.073), C) logarithm (base 10) of total protozoal 18S rDNA copies/cell (the SE at each time for Ento, Mix, and Epi were 0.18; and Oph, 0.10), and D) logarithm (base 10) of total protozoal 18S rDNA copies/mg of nucleic acids (NA; the SE for Ento, Mix, and Epi at each time were 0.20; and Oph, 0.10). Differences ($P < 0.05$) among main effect means for culture (i.e., averaged over the 3 sampling times) are depicted by unlike capital letters (X, Y, and Z). When culture interacted with time, differences among times within cultures ($P < 0.05$) are depicted by unlike lower-case letters (a, b, and c).

changing. Consequently, cells would presumably adjust their growth rate after the first transfer but before the second for a linear fit that differed from that in the adaptation period using a 3-d transfer regimen. However, especially with increasing polynomial terms for the cell count data, there can be a very sensitive change in generation time, so the combined approach of using the GLM and REG procedures is recommended to minimize subjectivity in choosing the appropriate model. At a few early transfers when the calculated generation time approached infinity (2- and 3-d transfers with monensin in Figure 2A and 3-d transfers with monensin in Figure 2C), generation times were nominally set to 500 h (off the scale of the y-axis) to illustrate a virtually instantaneous decline in generation time. Although arbitrary, increasing the value made no visual difference, whereas a large decrease in generation time would not be demonstrated if those points were simply omitted. Another limitation of our procedure is when the cell yields declined over time in some treatments. To avoid confusion in the interpretation of the negative generation time, curves were not plotted for the 1-d transfer with monensin in Figure 2A, all monensin transfers in Figure 2B, and the 1-d transfers without or with monensin in Figure 2D. With the exception of these plots, the consistent patterns of adaptation of generation time among cultures to converge to approximate the new transfer intervals agrees well with results from prior studies in which generation time successively decreased with decreasing transfer interval until cell yields were not maintained (Dehority, 1998, 2004). In contrast, our procedure does not rely on cell extinction, which occurs after the adaptation phase that was to be studied in our experiment.

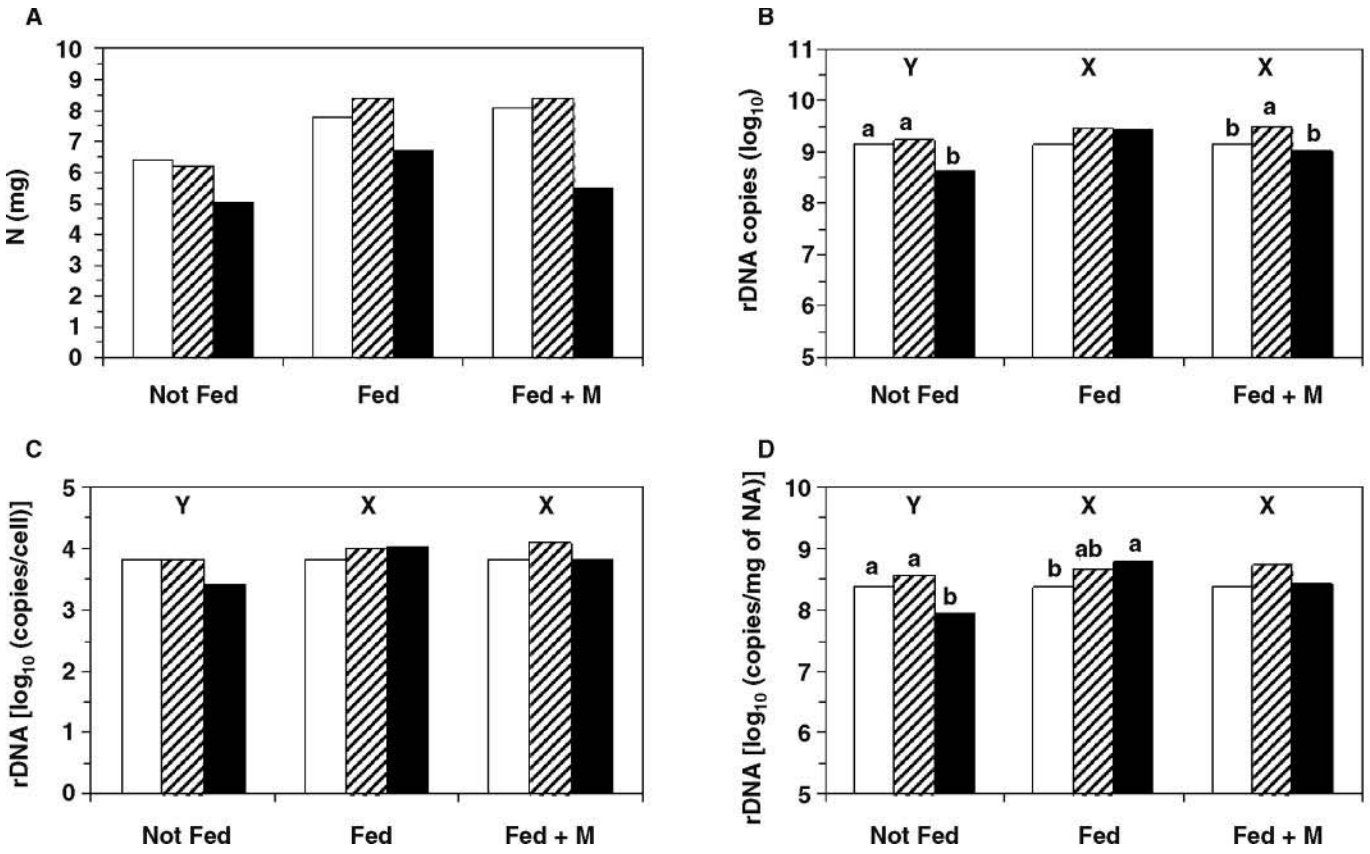

Figure 4. Main effect means (averaged among cultures) at 0 (before the treatment; open bars), 6 (hatched bars) or 16 (filled bars) h after the cultures were not fed, fed, or fed plus administered monensin (Fed + M) for the protozoal fraction that was recovered on 10-μm filters in experiment 2. Measurements include A) N recovered per culture flask (pooled SE of 1.09 for each bar), B) logarithm (base 10) of total protozoal 18S rDNA copies (pooled SE of 0.13 for each bar), C) logarithm (base 10) of total protozoal 18S rDNA copies/cell (pooled SE of 0.14 for each bar), and D) logarithm (base 10) of total protozoal 18S rDNA copies/mg of nucleic acids (NA; pooled SE of 0.16 for each bar). Differences among main effect means for treatment (i.e., averaged over the 3 sampling times) are depicted by unlike capital letters (X, Y, and Z). When treatment interacted with time, differences among times within cultures are depicted by unlike lower-case letters (a, b, and c). All *P*-values were <0.05 except for C and D ($P < 0.09$).

Monensin and Substrate Availability

The concentrations of monensin found to be inhibitory in our experiments are less than those previously used in vitro (Dennis et al., 1986). However, those researchers used ruminal fluid, which contains much greater bacterial numbers and DM each of which have a high binding affinity for monensin (Chow et al., 1994).

After abrupt monensin administration, protozoal morphology was similar to that of starved cells: microscopically, cells were much more shrunken and translucent (i.e., not filled with storage polysaccharide) than cells that were fed without monensin. In addition, we also noted abnormal morphology during cell division when monensin was abruptly added. In *Paramecium* species, which has been extensively evaluated as a model for ciliate protozoa, nutrient digestion is a sequential multi-step cycle (Ching and Berger, 1986). Each step of digestion is distinct, with earlier phases condensed when nutrients become limiting (Fok et al., 1984). Abrupt monensin addition inhibits the proteolysis within storage vesicles in *Paramecium*, disrupting nutrient digestion and decreasing growth rate (Gautier et al., 1994). Because the eukaryotic cell cycle is highly conserved, we assume that rumen ciliates regulate growth similar to *Paramecium*. For these latter ciliates, a commitment to cell division is made about 25% through the cell cycle, regardless of generation time; after this checkpoint, a sudden interruption in nutrient availability will not arrest cell division until the next cycle (Berger, 2001). A likely explanation to our consistent visual observation of aberrant dividing forms for all cultures during initial adjustment to monensin is that these cells were committed to division before monensin was added; however, subsequent adaptation to monensin (potentially mediated through a change in membrane structure or in membrane trafficking) probably allowed cell division to return to normal.

Transfer Interval and Generation Time (Experiment 1)

We expected to have a rapid adjustment to transfer interval for both *Ent. caudatum* and *Epi. caudatum*, given that Dehority (1998) calculated that minimum generation times for both approached 12 h. Both the *Entodinium* Mix and *Epi. caudatum* cultures with the 1-d transfers originally increased generation time until they finally decreased their generation time. Dehority (2004) suggested that this delay in adaptation was a result of a lag in protozoal growth rate or an indirect response from equilibrating with bacterial populations in the cultures (balancing the need for adequate bacterial growth factors needed by protozoa without allowing bacterial overgrowth). *Ophryoscolex purkynjei* was reported to have generation times of 29 h (Dehority, 2004) to 48 h (Dehority, 2003), which is somewhat less than the approximately 55-h generation time of *O. caudatus* in the present study. Because the 2 species are closely related (Miltko et al., 2006), differences probably are a result of unknown culture conditions. Dehority (2004) noted that the concentration of protozoal cells or substrate fed per cell did not affect generation time, whereas changing transfer interval had a major response. Subsequent work with varying amounts of spent medium has ruled out end product inhibition under our conditions (B. A. Dehority, unpublished data).

Monensin was more inhibitory for the *Entodinium* Mix than for the other cultures, including *Ent. caudatum*. This might have been a result of some more sensitive entodinia species in Mix or perhaps because clone cultures that are hardy enough to remain viable after cryopreservation also might have been initially selected for the ability of membranes to adapt rapidly to monensin. These explanations need further verification.

Adaptation to Substrate Availability (Experiment 2)

18S rDNA Copies as a Metabolic Indicator. Compared with the micronucleus, which contains archived copies of the genome, the macronucleus of nonrumen ciliates (and assumedly those in the rumen) contains thousands of gene copies actually used in transcription (Prescott, 2000). In ciliated protozoa, a process for control of the number of gene copies in the macronucleus is regulated, but the mechanism is not systematically controlled and is not fully known (Morrison et al., 2005). Ciliates can induce or repress the number of gene copies in their macronuclei during periods of ample or insufficient nutrient supply, respectively (Prescott, 2000; Iwamoto et al., 2004). Presumably, an increase or decrease in 18S rRNA gene copies would be amplified through increased 18S rRNA abundance, but other increased macronuclear genes also would increase mRNA abundance. Consequently, the total DNA and RNA, as estimated using the purine procedure, was deemed an appropriate measurement to standardize 18S rDNA copies. The DNA:RNA ratio in rumen protozoa is approximately 1:4 (Czerkawski, 1976), and to our knowledge, there is no description of how this ratio might change under different growth conditions for rumen ciliates. Previously, Sylvester et al. (2005) documented minimal contamination of bacterial NA in washed protozoal samples. Feed NA are in lower concentration than those in microbes and are rapidly degraded (Calsamiglia et al., 1996). In support of the conclusion that feed NA did not bias our results, feed NA contamination should be greatest after feeding (which should cause an opposite trend in the 18S rDNA copies/NA ratio) and should be comparable for the Fed or the Fed + M treatments.

Differences Among Cultures. Despite the large difference among species in cell numbers per culture, the major difference in 18S rDNA copies among cultures was due to *O. caudatus* having about 3 logarithm units less than that of the other cultures (which were all similar). All cultures had similar N recovered, so the total 18S rDNA copies per cell was a function of cell size, with the larger *Epi. caudatum* and even larger *O. caudatus* containing proportionately more copies per cell. Ribosomal concentration is proportional to protein synthesis rate (Berger, 2001), so rRNA amount per cell should increase with increasing cell volume. Mean protozoal cell volumes for *Ent. caudatum* and *Epi. caudatum* are approximately 980 and 4,708 μm^3, respectively (Dehority, 1993). The same 5:1 ratio in volume holds for the mean 18S rDNA copies per cell (1,900 and 9,800 for *Ent. caudatum* and *Epi. caudatum*; data are from Figure 3C averaged over time). Sylvester et al. (2005) reported about 6,000 copies per cell for a mixed protozoal population containing 85% *Entodinium* species (which includes several large species) and 15% *Epidinium* species. Expressing the 18S rDNA copies per unit of NA reduced differences among the *Ent. caudatum* or *Entodinium* Mix cultures and *Epi. caudatum*, but it still reflected a lower ratio for the slower growing *O. caudatus*.

Differences Among Treatments. The lesser N recovered in the protozoal fraction at 16 h for NF probably was a result of cell lysis from starvation (Figure 4). Compared with NF, the loss from 6 to 16 h for Fed probably was a result from degradation of feed N that could not be removed from the filters during the protozoal separation procedure. The loss from 6 to 16 h for Fed + M is likely a combination of feed degradation and protozoal lysis. The current results for N recovered in the protozoal fraction probably are not due to changes

in bacterial biomass; Fed + M decreased bacterial DM only by 11%, and bacterial DM was increased only by 9 and 6% at 6 and 16 h compared with 0 h (data not shown).

Both the rDNA copies per culture tube or rDNA copies expressed per unit of NA substantially decreased from 0 to 16 h for NF but increased substantially for Fed, with the response for Fed + M being intermediate. Assuming monensin prevents adequate digestion of feed and partially decreases availability of nutrients, as discussed previously, this intermediate response among treatments at 16 h supports the monitoring of nutrient availability by signal transduction mechanisms that await discovery in rumen protozoa. When substrate is depleted, a metabolic downshift and conservation of nutrients also has been well described for *P. tetraurelia* as a survival mechanism to reduce maintenance energy expenditure (Ching and Berger, 1986). More recently, secretory processes and high-affinity receptor kinetics have been documented for protein growth factors (typically using insulin but also other peptide hormones) for *Paramecium* and *Tetrahymena* (Christensen et al., 2003; Leondaritis et al., 2005; Csaba et al., 2007), documenting how these populations monitor and maintain population density to stimulate growth or potentially even cause programmed death. Although there is much less study in rumen ciliates, our preliminary work (Diaz et al., 2008) supports similar responses in metabolic control of cell numbers in *Ent. caudatum* and *Epi. caudatum* cultures using insulin-like signal transduction. Kisidayova and Varadyova (2005) also documented a dramatic increase in VFA production rate when insulin was added as a growth factor to a culture of *Ent. caudatum*, although our current results from experiment 1 suggest that their long (4- or 5-d) transfer intervals explain their lack of increase in cell numbers. Insulin-like signal transduction has been demonstrated ubiquitously among microbial domains (Lenard, 1992).

CONCLUSIONS

When feeding every 24 h in experiment 1, generation time of *Ent. caudatum*, *Entodinium* Mix, and *Epi. caudatum* cultures, but not the slow growing *O. caudatus*, rapidly decreased to consistently coincide with decreased transfer interval (in 24-h increments) to maintain an appropriate density of protozoal cells, whereas abrupt introduction of monensin prevented or delayed this response in generation time. In experiment 2, the 18S rDNA copies per cell varied in proportion to cell volume of protozoal species, but the lack of a treatment × culture interaction supports the utility of 18S rDNA copies expressed per unit of NA as a useful index to evaluate how ciliate populations coordinate growth rate relative to nutrient supply. The decreased 18S rDNA copies per unit of NA for Fed + M compared with Fed supports the conservation of nutrients when nutrient availability from consumed feed is disturbed. We reasoned that feeding on d 2 of a 3-d transfer regimen in experiment 2 would initiate metabolic signals to initiate cell division, including an increase in 18S rDNA copies. However, a greater existing biomass (for feeding without transfer compared with feeding combined with transfer) would rapidly deplete substrate, which should signal the subsequent arrest of the cell division cycle. In contrast, if increasing upshift in nutrients were concomitant with increasing outflow of protozoa, there would likely be a greater proportion of completed protozoal divisions with less opportunity for protozoal autolysis and greater outflow of bacteria consumed by protozoa. Increasing DMI in high producing dairy cattle will obviously increase supply of substrate for protozoal growth but also probably increases ruminal outflow of protozoa. Therefore, future studies are needed to characterize the cellular mechanisms regulating division and lysis of rumen protozoa to evaluate their impact on the efficiency of protein usage by high producing dairy cattle.

REFERENCES

Association of Official Analytical Chemists. 1990. Official Methods of Analysis. 15th ed. AOAC, Arlington, VA.

Berger, J. D. 2001. Riding the ciliate cell cycle–A thirty-five-year prospective. J. Eukaryot. Microbiol. 48:505–518.

Calsamiglia, S., M. D. Stern, and J. L. Firkins. 1996. Comparison of nitrogen-15 and purines as microbial markers in continuous culture. J. Anim. Sci. 74:1375–1381.

Ching, A. S., and J. D. Berger. 1986. Control of cell division in *Paramecium tetraurelia*. Effects of abrupt changes in nutrient level on accumulation of macronuclear DNA and cell mass. Exp. Cell Res. 167:191–202.

Chow, J. M., J. S. Van Kessel, and J. B. Russell. 1994. Binding of radiolabeled monensin and lasalocid to ruminal microorganisms and feed. J. Anim. Sci. 72:1630–1635.

Christensen, S. T., C. F. Guerra, A. Awan, D. N. Wheatley, and P. Satir. 2003. Insulin receptor-like proteins in *Tetrahymena thermophila* ciliary membranes. Curr. Biol. 13:R50–R52.

Csaba, G., P. Kovacs, and E. Pallinger. 2007. Effect of starvation on insulin production and insulin binding in *Tetrahymena*. Cell Biochem. Funct. 25:473–477.

Czerkawski, J. W. 1976. Chemical composition of microbial matter in the rumen. J. Sci. Food Agric. 27:621–632.

Czerkawski, J. W. 1984. Microbial fermentation in the rumen. Br. J. Nutr. 43:101–108.

Dehority, B. A. 1993. Laboratory Manual for Classification and Morphology of Rumen Ciliate Protozoa. CRC Press Inc., Boca Raton, FL.

Dehority, B. A. 1998. Generation times of *Epidinium caudatum* and *Entodinium caudatum*, determined in vitro by transferring at various time intervals. J. Anim. Sci. 76:1189–1196.

Dehority, B. A. 2003. Rumen Microbiology. Nottingham Univ. Press, Nottingham, UK.

Dehority, B. A. 2004. In vitro determination of generation times for *Entodinium exiguum, Ophryoscolex purkynjei*, and *Eudiplodinium maggii*. J. Eukaryot. Microbiol. 51:333–338.

Dennis, S. M., T. G. Nagaraja, and A. D. Dayton. 1986. Effect of lasalocid, monensin and thiopeptin on rumen protozoa. Res. Vet. Sci. 41:251–256.

Diaz, H. L., J. L. Firkins, M. A. Lyons, and J. R. Knapp. 2008. Chemotaxis toward glucose and xylose by mixed ruminal protozoa and dose-responsive insulin recovery from wortmannin inhibition by entodiniomorphid cultures. J. Dairy Sci. 91(Suppl. 1):334 (Abstr.)

Dijkstra, J., J. France, and S. Tamminga. 1998. Quantification of the recycling of microbial nitrogen in the rumen using a mechanistic model of rumen fermentation processes. J. Agric. Sci. 130:81–94.

Firkins, J. L., A. N. Hristov, M. B. Hall, G. A. Varga, and N. R. St-Pierre. 2006. Integration of ruminal metabolism in dairy cattle. J. Dairy Sci. 89(E. Suppl.):E31–E51.

Firkins, J. L., Z. Yu, and M. Morrison. 2007. Ruminal nitrogen metabolism: Perspectives for integration of microbiology and nutrition for dairy. J. Dairy Sci. 90(E. Suppl.):E1–E16.

Fok, A. K., S. S. Leung, and R. D. Allen. 1984. Modulation of the digestive-lysosomal system in *Paramecium caudatum*. I. Effects of temperature. Eur. J. Cell Biol. 34:265–270.

Fok, A. K., and M. S. Ueno. 1987. Ionophores and weak bases inhibit phagolysosomal proteolysis in *Paramecium*. Eur. J. Cell Biol. 45:145–150.

Fondevila, M., and B. A. Dehority. 2001. In vitro growth and starch digestion by *Entodinium exiguum* as influenced by the presence or absence of live bacteria. J. Anim. Sci. 79:2465–2471.

Gautier, M. C., N. Garreau de Loubresse, L. Madeddu, and L. Sperling. 1994. Evidence for defects in membrane traffic in *Paramecium* secretory mutants unable to produce functional storage granules. J. Cell Biol. 124:893–902.

Hoover, W. H., B. A. Crooker, and C. J. Sniffen. 1976. Effects of differential solid-liquid removal rates on protozoa numbers in continuous cultures of rumen contents. J. Anim. Sci. 43:528–534.

Hristov, A. N., and J.-P. Jouany. 2005. Factors affecting the efficiency of nitrogen utilization in the rumen. Pages 117–166 in Nitrogen and Phosphorus Nutrition of Cattle and Environment. A. N. Hristov and E. Pfeffer, ed. CAB Int., Wallingford, UK.

Iwamoto, M., T. Sugai, and Y. Nakaoka. 2004. Cell division induced by mechanical stimulation in starved *Tetrahymena thermophila*: Cell cycle without synthesis of macronuclear DNA. Cell Biol. Int. 28:503–509.

Karnati, S. K. R., J. T. Sylvester, S. M. Noftsger, Z. Yu, N. R. St-Pierre, and J. L. Firkins. 2007. Assessment of ruminal bacterial populations and protozoal generation time in cows fed different methionine sources. J. Dairy Sci. 90:798–809.

Kisidayova, S., and Z. Varadyova. 2005. Effect of insulin on in vitro fermentation activity of microorganism community of rumen ciliate *Entodinium caudatum* culture. Cell Biol. Int. 29:147–152.

Kisidayova, S., Z. Varadyova, T. Michalowski, and C. J. Newbold. 2005. Regeneration of cryoresistance of in vitro rumen ciliate cultures. Cryobiology 51:76–84.

Lenard, J. 1992. Mammalian hormones in microbial cells. Trends Biochem. Sci. 17:147–150.

Leondaritis, G., A. Tiedtke, and D. Galanopoulou. 2005. D-3 phosphoinositides of the ciliate Tetrahymena: Characterization and study of their regulatory role in lysosomal enzyme secretion. Biochim. Biophys. Acta 1745:330–341.

Maruyama, C., and Y. Takagi. 1997. Survival or extinction of *Paramecium multimicronucleatum* cultured with *P. tetraurelia* is associated with the ability or inability to form food vacuoles. Eur. J. Protistol. 33:274–283.

Miltko, R., T. Michalowski, P. Pristas, P. Javorsky, and J. H. P. Hackstein. 2006. Factors influencing morphological variability of rumen ciliates from the genus *Ophryoscolex*. J. Anim. Feed Sci. 15:35–38.

Morrison, T. L., J. S. Yakisich, D. Cassidy-Hanley, and G. M. Kapler. 2005. TIF1 represses rDNA replication initiation, but promotes normal S phase progression and chromosome transmission in *Tetrahymena*. Mol. Biol. Cell 16:2624–2635.

Nagaraja, T. G., C. J. Newbold, C. J. Van Nevel, and D. I. Demeyer. 1997. Manipulation of rumen fermentation. Pages 523–632 in The Rumen Microbial Ecosystem. P. N. Hobson and C. S. Stewart, ed. Chapman and Hall, London, UK.

Potter, E. L., and B. A. Dehority. 1973. Effects of changes in feed level, starvation, and level of feed after starvation upon the concentration of rumen protozoa in the ovine. Appl. Microbiol. 26:692–698.

Prescott, D. M. 1957. Relation between multiplication, rate and temperature in *Tetrahymena pyriformis*, strains HS and GL. J. Protozool. 4:252–256.

Prescott, D. M. 2000. Genome gymnastics. Natl. Rev. 1:191–198.

Ribeiro, C. V. D. M., and M. L. Eastridge. 2006. Short communication: Effect of number of extractions on percentage of long-chain fatty acids from fresh alfalfa. J. Dairy Sci. 89:3505–3507.

SAS. 1999. What's New in SAS® Software for Version 7 and the Version 8 Developer's Release. Release 7 ed. SAS Inst. Inc., Cary, NC.

Sylvester, J. T., S. K. R. Karnati, Z. Yu, M. Morrison, and J. L. Firkins. 2004. Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. J. Nutr. 134:3378–3384.

Sylvester, J. T., S. K. R. Karnati, Z. Yu, C. J. Newbold, and J. L. Firkins. 2005. Evaluation of a real-time PCR assay for measuring the ruminal pool and duodenal flow of protozoal nitrogen. J. Dairy Sci. 88:2083–2095.

Walker, N. D., C. J. Newbold, and R. J. Wallace. 2005. Nitrogen metabolism in the rumen. Pages 71–115 in Nitrogen and Phosphorus Nutrition of Cattle. E. Pfeffer and A. Hristov, ed. CABI Publ., Cambridge, MA.

Williams, A. G., and G. S. Coleman. 1992. The Rumen Protozoa. Springer-Verlag, New York, NY.

Yáñez-Ruiz, D. R., N. D. Scollan, R. J. Merry, and C. J. Newbold. 2006. Contribution of rumen protozoa to duodenal flow of nitrogen, conjugated linoleic acid and vaccenic acid in steers fed silages differing in their water-soluble carbohydrate content. Br. J. Nutr. 96:861–869.