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Author: Grzegorz Bełżecki Neil R. McEwan Barbara Kowalik Tadeusz Michałowski Renata Miltko

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Effect of *Entodinium caudatum* on starch intake and glycogen formation by *Eudiplodinium maggii* in the rumen and reticulum

Grzegorz Bełżecki[1*] g.belzecki@ifzz.pl, Neil R McEwan[2], Barbara Kowalik[1], Tadeusz Michałowski[1], Renata Miltko[1]

[1]The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Instytucka Street 3, 05-110 Jabłonna, Poland

[2]Institute of Biological, Environmental & Rural Sciences, Aberystwyth University, Aberystwyth, SY23 3DA, Wales, United Kingdom

[*]Corresponding author at: The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Instytucka Street 3, 05-110 Jabłonna, Poland. Tel.: +48 22 765 33 01; fax: +48 22 765 33 02.

Abstract

This study aimed to quantify the engulfed starch and reserve α-glucans (glycogen) in the cells of the ciliates *Eudiplodinium maggii*, as well the α-glucans in defaunated and selectively faunated sheep. The content of starch inside the cell of ciliates varied from 21 to 183 mg/g protozoal DM relative to the rumen fauna composition whereas, the glycogen fluctuated between 17 and 126 mg/g DM of this ciliate species. Establishment of the population *Entodinium caudatum* in the rumen of sheep already faunated with *E. maggii* caused a drop in both types of quantified carbohydrates. The content of α-glucans in the rumen of defaunated sheep varied from 4.4-19.9 mg/g DM and increased to 7.4-29.9 or 11.8-33.9 mg/g DM of rumen contents in the presence of only *E. maggii* or *E. maggii* and *E. caudatum*, respectively. The lowest content of the carbohydrates was always found just before feeding and highest at 4h thereafter. The α-glucans in the reticulum varied 7.5-40.1, 14.3-76.8 or 21.9-106.1 mg/g DM of reticulum content for defaunated, monofaunated or bifaunated sheep, respectively. The

results indicated that both ciliate species engulf starch granules and convert the digestion products to the glycogen, diminishing the pool of starch available for amylolytic bacteria.

Keywords: Engulfment; α-glucans; Glycogen; Rumen protozoa; Starch

Introduction

Starch is one of the most important components of a ruminant's diet (Gonzáles-García et al. 2009; Mendoza et al. 1993). The main site of its degradation in the digestive tract is the rumen. The amount of starch digested here can reach up to 90% of the daily ration (Owens and Soderlund 2006; Moharrery et al. 2014), due to activity of microorganisms such as: bacteria, fungi and protozoa inhabiting the rumen (McAllister et al. 1990; Trinci et al. 1994; Williams and Coleman 1992).

Following degradation of starch, the products obtained are fermented or used as a reserve of α-glucans by microorganisms. In the case of ciliates with intracellular digestion, degradation of insoluble food particles is preceded by ingestion. The process of digestion of starch and characterisation of enzymes involved in its digestion has been the subject of numerous studies (e.g. Bełżecki et al. 2007; Coleman 1986). They indicated that all of the ciliate species examined exhibit amylolytic activity (Williams and Coleman 1992). This includes even the small vestibuloferid protozoan *Dasytricha ruminantium* which does not ingest starch (Williams 1986). Conversely, relative to the studies cited above the quantification of starch engulfment by rumen protozoa and its fermentation and/or conversion to the cellular reserve carbohydrates has rarely been the objective of comprehensive studies, although examples of such studies exist (Abou Akkada and Howard 1960; Jouany and Ushida 1999.

The involvement of ciliates in starch degradation in the rumen is controversial. For example, Mendoza et al. (1993) found that the establishment of a ciliate population

(faunation) reduced ruminal starch digestion, whereas Veira et al. (1983) observed the opposite effect. In addition to these publications, Meyer et al. (1986) did not find differences between digestible of starch in the rumen of faunated and defaunated (i.e. ciliate-free) animals. The ruminants examined by these authors possessed either a mixed protozoal fauna or ciliates of the genus *Entodinium* which belong to "small ophryoscolecids", a group which use starch as their main source of energy. The most common and most typical representative of this group is *Entodinium caudatum* (Williams and Coleman 1992). The second group of rumen ciliates comprises the "large ophryoscolecids" consisting of the following genera: *Eudiplodinium, Metadinium, Polyplastron, Elytroplastron, Epidinium* and *Ophryoscolex.* Numerous species from this group exhibit fibrolytic activity and contribute to the digestion of structural carbohydrates (Coleman 1986; Dehority 1993; Michałowski et al. 2003; Miltko et al. 2006; Miltko et al. 2014). *Eudiplodinium maggii* is a typical example species of this group. This species of protozoan, apart from having the ability to degrade structural plant polysaccharides, possesses amylolytic properties (Bełżecki et al. 2007). However, knowledge of the contribution of ciliates such as *E. maggii* to starch metabolism in the rumen is limited to data obtain by Bełżecki and Michałowski (2001) or Mendoza et al. (1993) and the information on the effect of ciliates on the content of the mentioned carbohydrates in the digesta reticulum to Bełżecki and Michałowski (2005).

To address the dearth of knowledge in this area, the objectives of these studies were:

1). to quantify the engulfed starch and the deposition of reserve carbohydrates in the protozoa cells living in the rumen;

2). to determine the influence of the established ciliate population on the content of α-glucans in the rumen and in the reticulum

3). to determine the effect of coexistence of *E. maggii* with the starch preferring ciliate species *E. caudatum* on these characteristics.

Material and Methods

Feed and feeding

Three Polish Merino rams weighing 63-73 kg were used to perform these studies. The animals were fitted with permanent 100 mm ID rumen cannulas for about 2 months before the start of the experiment. They were kept in separate pens and fed daily on a diet of 1500 g meadow hay and 260 g ground barley (the chemical composition of the diet is shown in Table 1). Each of the dietary components was divided into two equal portions and offered to the animals at 08:00 and 8 pm20:00. Drinking water was available *ad libitum*.

Experimental design

The study comprised three periods. In the first (period I) the animals were protozoa free, in the second (period II) the sheep possessed a population of *E. maggii,* and in the third (period III), the protozoal fauna consisted of *E. maggii* and *E. caudatum* (period III). Each period was composed of two sub-periods. The first sub-period always lasted for 3 weeks and allowed the establishment of microbial populations (Jouany and Ushida, 1990) due to defaunation (period I) or refaunation (periods II and III) of sheep. In the second sub-period, the material required to perform the analysis was collected as described below.

Defaunation and refaunation of sheep

The sheep were defaunated according to Michałowski et al. (1999). Briefly, the reticulo-rumen was emptied, the digesta contents was frozen at -20 °C. The walls of both chambers were washed with a warm (40 °C) solution of Artificial Rumen Fluid (Michałowski et al. 1999). The washing procedure was repeated two times a day for the next three days. On

the last day of the defaunation procedure the frozen digesta was thawed, then heated to 70 °C for 15 min to eliminate the protozoa, and finally after cooling to 40 °C the content was returned to the rumen. After the defaunation procedure the sheep were fed a gradually increasing amount of hay and ground barley.

The ciliate-free sheep were faunated by addition of *E. maggii* and next bifaunated by further addition *E. caudatum*. The protozoa use as the inoculum were isolated from the natural rumen fauna, identified according to Dogiel (1927) and single-species populations were cultured *in vitro* as described Miltko et al. (2015).

Sampling design

Collection of material for quantification of α-glucans in the rumen

The sampling was performed just before the morning feeding and at 4, 8 and 12 h thereafter. The collections before morning and evening feedings were always followed by evacuation of rumen content. The digesta was weighted, thoroughly mixed and sampled and then immediately placed back to the rumen. To obtain representative samples at 4 and 8 h after feeding, the rumen contents was always taken from 5 different places in the rumen, pooled together and thoroughly mixed. The weight of the pooled material was about 1 kg. The following samples were collected: for the counting of protozoa (2 × 5 g) and to quantify α-glucans (2 × 70 g). The remaining content was immediately placed back in the rumen. The samples for counting of protozoa were fixed by adding an equal volume of a 4% aqueous solution of formalin and stored at room temperature whereas samples of rumen contents were lyophilised, weighed and stored at -20 ˚C. The sampling was repeated three times on three different days. However, no more than 2 collections were obtained weekly from a single animal. This was done in order to minimize any possible changes in the rumen metabolism.

Collection of material for quantification of α-glucans in the rumen protozoa

Samples of rumen contents were used to prepare the purified suspensions of protozoa. The ciliates were separated from plant material and external bacteria by a repeated sedimentation according to Bełżecki et al. (2007) and Miltko et al. (2014). Briefly, 1 kg samples of rumen contents were diluted with 2 L warm (40 °C) of "*caudatum*" type salt solution (Coleman et al. 1972) and strained through a sieve of pore size 0.9 mm. The filtrate was poured into a large separating funnel and incubated for 30 min at 40 °C. At this stage, tiny particles of food resulting from fermentation due to microbial activity migrated to the top whereas the ciliates formed a white-grey layer at the bottom of the funnel. To prevent any degradation of starch and/or the cellular glycogen during purification of ciliates in the next steps the sediment were resuspended in cold (4 °C) "*caudatum*" salt solution and sedimented again. The sedimentation procedure was repeated 2 - 3 times until one free of external bacteria suspension was obtain. This method allowed a 76% protozoal recovery to be obtained. Finally the suspension of purified ciliates was adjusted to 100 mL with the cold (4 °C) *"caudatum"* salt solution. A sample (5 mL) of this suspension was fixed with 4% formaldehyde solution and stored at room temperature to determine the concentration of protozoa, remaining in the part that was sedimented. The sediment of ciliates was suspended in cold distilled water, lyophilised, weighted and stored at -20 °C.

A similar procedure was used for isolation of *E. maggii* from rumen bifaunated sheep (period III), the only difference was filtration of suspended ciliates by use of a filter on a mesh of 40 μm which allowed separation of *E. caudatum* from *E. maggii*. After separation protozoa were fixed and lyophilised as described above.

The procedure of protozoa purification was conducted just before morning feeding of sheep and at 2, 4, 6, 8 and 10 h thereafter. The sampling was repeated three times on three different

days. However, no more than 2 collections were obtained weekly from a single animal. This was done in order to minimize any possible changes in the rumen metabolism.

Collection of material for quantification of α-glucans in the reticulum

Reticulum content (approximately 80 mL) was collected via the rumen cannula just before morning feeding and 1, 2, 3, 4, 6, 8, 10 and 12 h thereafter. The reticulum content was thoroughly mixed and divided in the following manner: to determine the density of the protozoal population (2 × 5 mL), samples were fixed in a 4% aqueous solution of formalin at a ratio 1:1 (V/V) and stored at room temperature; to determine α-glucans in reticulum content (2 × 20 mL), samples were frozen at -20 °C before being lyophilised, weighed and stored at -80 °C. The materials were collected three times on three different days of each period of research. However, no more than two samples were collected from any animal per week.

Analytical methods

Chemical analysis

Chemical composition of diet was analysed according to protocols of the Association of Official Analytical Chemists (AOAC, 2000).

Ciliate counting

The ciliates in the fixated samples from rumen and reticulum content, as well as the purified protozoa were counted microscopically as described by Miltko et al. (2015).

Quantification of α-glucans

The α-glucans were quantified in barley grain, rumen and reticulum contents as well as in the cells of protozoa. To obtain homogeneous material the barley grain and

lyophilised rumen and reticulum contents, were ground in an electric grinder. From the material obtained portions of barley grain (30 mg), reticulum content (30 mg), and rumen content (100 mg) were taken and suspended in a mixture of methanol-chloroform-water in the proportion of 15:3:2 (V/V/V) according to Rose et al. (1991).

The suspension of protozoa was further homogenised in a glass homogeniser equipped with a Teflon pestle. This homogenisation was necessary because lyophilisation alone did not damage of the ciliates cells sufficiently to permit further analysis.

The suspensions of the barley grain and rumen and reticulum contents as well as the protozoal homogenate were incubated at room temperature for 15 min, and then centrifuged for 15 min at 4 °C and 11,000 × *g*. The sediment was collected, evaporated and re-suspended in 2 mL of 0.1 M NaOH. The suspension obtained was incubated overnight at 50 °C. The next day, the mixture was acidified to pH 5.0 with 0.4 M acetic acid and 0.5 mL amyloglucosidase (Sigma Aldrich 10115) at a concentration of 0.05 mg/mL was added followed by incubation for 24 h at 50 °C. After the incubation, the glucose released was measured using an enzymatic method (see below).

Quantification of glucose

The glucose concentration was determined by an enzymatic method using a Glucose-Oxy kit (Pointe Scientific), and was performed according to the manufacturer's protocol. The glucose was determined by measuring at 505 nm using a HACH LANGE DR 6000 UV VIS Spectrophotometer. Glucose standards were measured simultaneously and used to calculate the content of glucose in the samples studied.

Quantification of amylose

The measurement of amylose in samples was carried out according to the method of Gibson et al. (1997) and Morison and Laingnelet (1983). This method was based on the specific selective formation and precipitation of the branched complexes of amylopectin with Concanavalin A (Con A type VI). The scheme of α- glucans analysis is presented in Fig. 1. The concentration of amylose were measured in barley grain and lyophilised protozoal cells. Briefly, samples of lyophilised protozoa (30 mg) or barley grain (30 mg) were homogenised and suspended in a mixture composed of methanol-chloroform-water in proportion 15:3:2 (V:V:V), centrifuged for 15 min at 4 °C and 11,000 × *g*. The sediment was evaporated and re-suspended in 2 mL of 0.1 M NaOH, then incubation and acidified with 0.4 M acetic acid as described above. The solution obtained was than centrifuged for 15 min at 4 °C and 11,000 × *g*. The sediment was dried and re-suspended in a 2 mL solution composed of 0.6 M urea (Sigma-Aldrich U5378) and Dimethyl sulfoxide (DMSO) (Sigma-Aldrich 41640) at a 1:9 ratios (V/V). The mixture was incubated in boiling water until the starch dissolved completely. Next, it was cooled to room temperature and acidified to pH 6.4 by use of 0.4 M acetic acid. Finally, the volume of the sample was adjusted to 25 mL by addition of a solution consisting of the following quantities per liter: 5.2 g NaCl; 21.1 g CH_3COONa; 200 mg $CaCl_2 \times 2H_2O$; 300 mg $MgCl_2 \times 6H_2O$; 30 mg $MnCl_2 \times 4H_2O$ and used to quantification the α-glucans and amylose. The concertation of amylose content was measured by mixing 0.75 mL of the solution of starch-urea-DMSO with an equal volume of a suspension composed of Concanavalin A Type VI (Sigma Aldrich C2010). The mixture obtained was incubated for 60 min at room temperature then centrifuged for 15 min at 4 °C and 20,000 × *g*. The sediment was discard whereas the supernatant (1 mL) was mixed with 1 mL of 0.2 M acetate buffer and incubated in boiling water for 5 min then centrifuged at 4 °C and 15,000 × *g* for 15 min. The supernatant obtained after hydrolysis of the amyloglucosidase solution (see above) was used to quantify the glucose.

Verification of quantification methods for α-glucans analyses

Determination of accuracy of measurements of glycogen and starch

Samples (4, 8, 12, 16, and 20 mg) of maize amylopectin (Sigma -Aldrich 10120), as well as bovine liver glycogen (Sigma-Aldrich G0885) and oyster glycogen (Sigma-Aldrich G8751) and starch (Sigma-Aldrich S7260) were hydrolysed in triplicate as described above and the glucose released was measured using the same enzymatic method. The quantity of products was calculated according to Volenec (1986).

Determination recovery of amylopectin

Samples (2, 4, 10, 12 and 15 mg) of potato amylose (Sigma Aldrich A0512) were mixed separately with maize amylopectin with bovine liver or oyster glycogen. The mixtures were treated in accordance with the method of determination of starch in the protozoa cells using Con A type VI to separate amylopectin and glycogen. The content of amylose in the mixtures was calculated on the basis of glucose released from the hydrolysed substrates.

Determination of proportion of amylose to amylopectin in the cell of protozoa

It was assumed that the ratio of amylose to amylopectin in barley grain is constant and that amylose in protozoal cells originates exclusively from starch. To verify this supposition the following experiment was conducted. The digestive sample about 0.25 kg, was taken from the rumen before the morning feeding of sheep. After sampling the ciliates were isolated by the method of sedimentation as described above. Sedimented ciliates was suspended in a warm (40 °C) "*caudatum*" salt solution and separated into 40 mL Erlenmeyer flasks. Then 0.5 g of starch containing only trace amounts of amylose (S9679 Sigma-Aldrich) was added and incubated for 4 h at 40 °C with a continuous flow of carbon dioxide (60 cm^3/min). After

incubation protozoa were separated from the remaining starch granules in the fluid by filtration through a sieve of 20 μm pore diameter. The ciliate residue on the filter was washed with "*caudatum*" salt solution, then suspended in water, lyophilised, and stored at -80 °C. The samples of protozoa obtained were used to determine a ratio of amylose to amylopectin and the concentration of α-glucans the same methods as described above.

Comparison of degradation rate of amylose and amylopectin by *E. maggii*

It was assumed that amylose is degraded by *E. maggii* at the same constant rate as glucan/amylpectin. To verify this supposition the *E. maggii* were isolated from the rumen of monofaunated sheep, using the method of sedimentation in 40 °C "*caudatum*" salt solution described above. The samples obtained were incubation at 40 °C and the protozoa sediment was re-suspended in cold (4 °C) "*caudatum*" salt solution, then sedimented and homogenised in a Potter homogeniser equipped with a Teflon pestle. In order to maintain the low temperature of the homogenisation process, the homogeniser was placed in an ice bath. The homogenate obtained was centrifuged at 4°C and 20,000 × *g* for 25 min. The supernatant was collected and used as a crude enzyme preparation.

The reaction mixture consisted of: 700 μL 0.4% solution of amylopectin (Sigma A-8515) or amylose (Sigma A-92665), 100 μL crude enzyme preparation and 200 μL 0.02 M phosphate buffer at pH 6.0. The mixture obtained was incubated at 40 °C for 1 h then was stopped by addition of 1250 μL dinitrosalicylic acid reagent (Miltko et al. 2016) and boiled for 5 min. The sample was cooled and the absorbance was measured at 560 nm using a HACH LANGE DR 6000 UV VIS Spectrophotometer. The content of reducing sugars released during hydrolysis, was calculated using the results of measuring the absorbance of the control samples and standard solutions of glucose.

The measurements were repeated three times using three different crude enzyme preparations. The protein content was measured with the use of a Microprotein kit (Pointe Scientific HP782-400).

Calculations

Estimation of barley starch and glycogen in the ciliates

Based on the assumption that the amylose present in the cells of protozoa is barley in origin, the amount of starch barley engulfed by ciliates was calculated:

Equation 1: $\mathbf{S_{Ba}} = 3.92 \cdot A$

where: $\mathbf{S_{Ba}}$- the content of barley starch; 3.92 - the coefficient of amylose to amylopectin in barley starch; A - the content of amylose in barley starch.

Based on the Equation 1, concentration of barley starch in protozoa cells was determined which allowed calculation of the amount of glycogen inside the protozoan cells:

Equation 2: $\mathbf{S_G} = \mathbf{S_T} - \mathbf{S_{Ba}}$

where: $\mathbf{S_G}$ - reserves α- glucans (glycogen) in protozoa cell; $\mathbf{S_T}$ - α-glucans in the ciliate cells; $\mathbf{S_{Ba}}$ - the content of barley starch.

Statistical analysis

Statistical analysis was performed using the Statistica 10.0 software package (StatSoft Inc. Tulsa, OK, USA). The results obtained were expressed as mean values with standard error of mean (SEM). The normality of distribution of variables was tested by the Shapiro-Wilk test, and all variables were found to be normally distributed. Statistical significance was calculated using three periods (I- defaunated; II- monofaunated with *E. maggii*; III- bifaunated with *E. maggii* and *E. caudatum*) by four time points (0, 4, 8 and 12 h - to determine the concentration of ciliates, and α-glucans in the rumen), or by six time points (0, 2, 4, 6, 8 and 10 h - to determine the concentration of α- glucans, in the cells of protozoa) or by nine time points (0,

1, 2, 3, 4, 6, 8, 10 and 12h to determine the concentration of α-glucans in the reticulum) by 3 days (3 analogical analysis for each animal on 3 different days) with repeated measures of analysis of variance (ANOVA) followed by Tukey's HSD post-hoc. The effects were considered to be significant at $P \leq 0.05$ or $P < 0.001$.

Results

Verification of quantification methods for α-glucans analyses

Assaying the accuracy of the α-glucans quantification method (Fig. 2) showed a positive straight line relationship between the quantity of amylopectin, glycogen and starch in hydrolyzed samples and its quantity could calculated on the basis of released glucose. The lowest recovery was obtained for glycogen from oysters (93.7%) whereas the highest for starch (96.5%).

The effectiveness of amylopectin recovery from mixed samples (amylose + amylopectin; amylose + glycogen) also indicated a straight relationship ranged from 95.9% to 99.5% (Fig. 3).

The results of determining amylose in ciliates carbohydrates indicated a lack of these carbohydrates. Moreover the results confirmed also that all amylopectin present in protozoa cells was completely bound with Con A type VI and precipitated.

Comparison of the degradation rate of amylose and amylopectin by the crude enzyme preparation obtained from *E. maggii* cells revealed that both substrates (amylose and amylopectin) were degraded. However the digestion rate was lower for amylose released 7.0 ± 0.14 μM glucose/mg protein per min, and higher for amylopectin released 7.6 ± 0.17 μM glucose/mg protein per min.

Starch intake and glycogen deposition in protozoa cell

The estimation of the concentration of α-glucans in single protozoa cell is presented in Fig. 4. The mean content of α-glucans inside *E. maggii* during the period II was 39.1 ng per cell. Inoculation of *E. caudatum* into monofaunated sheep decreased the amount of these carbohydrates by about 36%. The concentration of α-glucans inside the *E. caudatum* was 6 times lower relative to *E. maggii* in period II and 4 times – compared to period III. Both species, independent of the experimental period, showed the same pattern of diurnal variation. The highest concentration of α-glucans was observed 2 h after feeding sheep and then decreased over time.

The results of starch quantification in the *E. maggii* cells are presented in Fig. 5. The amount of this polysaccharide varied between 21 and 183 mg/g DM of protozoa relative to the time after feeding and presence or absence of *E. caudatum* in the rumen. The lowest concentration was observed immediately prior to feeding whereas the highest was at 4 h thereafter ($P<0.001$). The establishment of *E. caudatum* in the rumen of sheep resulted in a significant decrease ($P<0.001$) of starch engulfment by *E. maggii* in the range of 40-73%.

The concentration of reserve glycogen varied between 17 and 126 mg/g DM of ciliates in relation to the time after feeding and presence or absence of *E. caudatum* in the rumen (Fig. 6). The lowest value was found at 2 h after feeding whereas the highest was at 6 or 10 h later during periods II and III, respectively. In general, the development of *E. caudatum* in the rumen of sheep resulted in a drop in the quantity of glycogen in the cells of *E. maggii* by 16-80%.

The content of α-glucans in the rumen

The total concentration of α-glucans in the rumen of defaunated sheep varied between 6.2 and 19.9 mg/g DM of rumen content (Table 2) and increased after establishment of the

population of *E. maggii*. No differences between periods were noted immediately prior to feeding of sheep. The largest difference was found at 4 h after feeding (Table 2) when the concentration of α-glucans in the monofaunated (period II) and bifaunated sheep (period III) was 33% and 41% higher than in the ciliate-free, respectively. The establishment of *E. caudatum* in the rumen of sheep already possessing a developed population of *E. maggii* had no effect on the pool size of α-glucan.

The diurnal variations indicated that the concentration of α-glucans in the rumen of defaunated sheep (period I) increased over 3 fold in the first 4 h after feeding then decreased almost 3 times at 8 h and 4.5 times at 12 h after feeding. The increased content of α-glucans 4 h after feeding was also observed during periods II and III. However, the opposite was seen in defaunated sheep where a decrease in the content of these carbohydrates was observed 12 h after feeding of sheep.

The numbers of *E. maggii* varied between 25.1 and 34.7 $\times$ 10^3 per g of rumen contents during period II. The inoculation of *E. caudatum* into the rumen with a developed population of *E. maggii* resulted in a drop in the concentration of *E. maggii* by about 40%. The number of *E. caudatum* was over 11 times higher than *E. maggii* in period III and varied between 182 and 230 $\times$ 10^3 per g of rumen content dependent on the time after feeding (Table 3).

The protozoal α-glucans inside the *E. maggii* cells in the rumen of monofaunated sheep (period II) varied from 5.0 to 13.5 mg/g DM of rumen content with respect to the time after feeding (Table 4). The establishment of *E. caudatum* decreased the pool size of these carbohydrates in the cells of *E. maggii* by 2-5 times and diminished to 4-18% of their contribution in the total ruminal pool size. The undigested starch inside the protozoa varied from 0.5 to 5.5 mg/g DM of rumen content in relation to the time after feeding and experimental period. The development of the *E. caudatum* population during period III in the rumen impacted negatively on the content of cellular starch in *E. maggii* with a 37-53%

reduction. The diurnal variation of starch and protozoal α-glucans indicated the same pattern independent of their origin. The highest concentration was observed 4 h after feeding and then a slow decrease up to 12 h after feeding was observed.

Concentration of ciliates and α-glucans in the reticulum

The ciliates were not present in the reticulum of sheep during period I. Their mean concentration during periods II and III are presented in Table 5. The number of *E. maggii* in the reticulum content varied between 1.9 and 8.1 × 10^3 cells/g digesta in relation to the time after feeding and experimental period. Development of the population of *E. caudatum* during period III resulted in a drop in the concentration of *E. maggii*, and it was observed that the number of *E. caudatum* was an order of magnitude larger than that of *E. maggii*.

The concentration of α-glucans in the reticulum of sheep fluctuated between 8 and 107 mg/g DM of reticulum content in relation to period and the time after feeding (Fig. 7). The lowest concentration was found just before feeding and the highest at one hour after feeding. The α-glucan content increased by 3-5 times during the 1 h after feeding, and was followed by a continuous decrease. In general, the establishment of *E. maggii* in the rumen of defaunated sheep increased the concentration of α-glucans by about 50%. The establishment of the second species of ciliates was followed by a 35% increase in the concentration of measured carbohydrates.

Discussion

Determination of the starch in ground barley indicated that the amount of this carbohydrate was 63.5 ± 1.58% of the DM of crush barley, which did not differ from the typical value reported previously (Shu and Rasmunssen 2014).

The quantity of starch engulfed by ciliates was calculated on the basis of the content of amylose in barley starch and in ciliate cells using the method of Gibson et al. (1997). This method enabled separate monitoring of changes in the quantity of the engulfed starch and of the glycogen in the cells of the protozoa. No such discrimination was possible when either an isotopic (Coleman 1969) or an enzymatic method (Fondevila and Dehority 2001) were used, because they both enabled only the quantification of the α-glucans present in the examined material. It is also noteworthy to emphasize that we verified the accuracy of the method used before its application and demonstrated that it was suitable for quantitative analysis of our material. The accuracy of α-glucans measurements showed a positive straight line relationship between the quantity of amylopectin in hydrolyzed samples and its quantity calculated on the basis of released glucose. The calculated mean recovery was 94.9% of the weight of the hydrolyzed samples of amylopectin. In the case of the amylose present in the mixed samples (amylose + amylopectin, amylose + glycogen) this was even greater at 98%. Verification of the quantification methods of α-glucans indicated a lack of amylose inside the protozoal cells. The comparison of degradation of amylose and amylopectin by using a crude enzyme preparation indicated that degradation of amylopectin was 8.8% higher than amylopectin. Such a relationship was also observed by Brewer et al. (2012).

The amount of α-glucans inside the cells of *E. maggii* in the rumen of monofaunated (period II) and bifaunated sheep (period III) was lowest just before the morning feeding and highest 4 h thereafter, followed by a continuous decrease until the next feeding (Fig. 4). This increase of α-glucans was presumably caused by intensive engulfment of starch granules by protozoa (Michałowski et al. 2003) whereas a decrease reflected the digestion of the α-glucans (Bełżecki et al. 2007) and the use of the released glucose as a substrate for synthesising reserve α-glucans or for covering energy requirements or both.

The results indicated that cellular glycogen of *E. maggii* in monofaunated sheep decreased during the first 2 h after feeding from over 80 mg/g DM to 35 mg/g DM of protozoa suggesting their intense utilization by protozoa. However, during the next 8 h the level of this glucan increased over three fold. This increase suggested the possibility of utilization of some of the other starch sources of glucose such as structural carbohydrates: cellulose and/or hemicellulose present in hay for synthesis of storage glycogen.

This suggestion is in accordance with the finding of Coleman (1992). Surprisingly, no significant effect was found after refaunation with the starch-preferring ciliates *E. caudatum* (Abou Akkada and Howard 1960).

The highest concentration of α-glucan inside the protozoa cells was observed 4h after feeding and was over 24% of the DM of *E. maggii*. This value is lower than that reported by Jouany and Ushida (1999) which estimated the contribution of these carbohydrates as over 40% of DM of ciliates. This disagreement could be caused by different amounts of starch in the diet of the sheep; the diet used by Jouany and Ushida contained 63% starch whereas in the current studies they did not exceed 15% DM of diet. This difference could also perhaps be explained by a restriction in the engulfment of starch by ciliates resulting from the lower concentration of starch granules in the rumen fluid of our sheep compared to the animals used in the previous work.

The concentration of total α-glucans in the rumen of sheep varied significantly relative to the presence of ciliates in the rumen and time of sampling. Their content in defaunated sheep fluctuated between 4 and 20 mg/g DM of rumen contents and increased after inoculation of the population *E. maggii* but did not change significantly after further faunation with *E caudatum* (period III). The observed increase could be the result of a drop in the number of amylolytic bacteria after establishment of a ciliate population (Denton et al. 2015). Moreover, the elongation time of polysaccharide digestion inside protozoal cells should also

be taken into account (McAllister and Cheng 1996; Mendoza et al. 1993). It should, however, be emphasized that the results obtained by some authors did not confirm the stimulatory effect of protozoa on the content of α-glucans in the rumen. For example Veira et al. (1983) found a decrease in the content of these carbohydrates following the establishment of ciliates in the rumen of sheep. Conversely, Meyer et al. (1986) observed no effect. This suggests that some other factors may contribute to an explanation of the role of protozoa in starch metabolism in the rumen. One of these might be the composition and abundance of rumen fauna. The highest number of ciliates present in the rumen of sheep examined by Mendoza et al. (1993), Meyer et al. (1986) and Veira et al. (1983) were generally no more than 77×10^3 individuals (mainly *Entodinium* spp.) per gram of rumen content. The concentration of *E. maggii* in the current studies was lower in comparison to that cited above. However, in spite of lower numbers, 45-68% of the total α-glucans originated from their cells. The reason for this was the fact that these ciliates belong to the "large ophryoscolecids". Due to this they were able to engulf numerous granules of starch diminishing the pool size of this carbohydrate for colonization, thus restrict their metabolism in the rumen (Denton et al. 2015; Towne and Nagaraja 1990).

Despite the lack of effect of fauna population on starch concentration in rumen contents between periods II and III, differences were found in the starch and glycogen inside the cells of *E. maggii*. The reason for this was the presence of *E. caudatum* in the rumen content what suggest competition between both of the ciliate species for starch. Inoculation of *E. caudatum* into the rumen of sheep resulted in a significant decrease in the quantity of starch engulfed by *E. maggii*, by about 55%, and also a decrease in their numbers by about 40%. The reduction in the contribution of these ciliates to starch metabolism in the rumen was caused by starch engulfing by *E. caudatum.* It diminished the quantity of starch available to *E. maggii* during period III as almost 75% of starch present was in *E. caudatum* whereas only 25% was in cells of *E. maggii*.

The results of the experiment concerning the effects of ciliates on the content of α-glucans in the reticulum indicated that establishment of *E. caudatum* caused a decrease in the density of the *E. maggii* population by about 50% in the reticulum content.

The concentration of the carbohydrates examined varied from about 8 to over 107 mg/g DM of reticulum content in relation to time after feeding of sheep and experimental period. The data obtained (Fig. 7) showed that the concentration of α-glucans in reticulum content exhibited the well-established rhythm of fluctuations characterised by the lowest concentration being before feeding and the highest 1 h later. The results obtained showed that the *E. maggii* in the reticulum constituted only 12-19% of that seen in the rumen. In comparison to this, the concentration of *E. caudatum* in the reticulum content constituted 70-73% of their numbers in the rumen. These differences perhaps result from the long residence time of large ciliates in the rumen (Michalowski et al. 1986). They also seem to characterise the potential roles of large and small protozoa as a direct source of α-glucans for the host.

Conclusion

The studies performed support an involvement of the ciliate species examined in starch metabolism in the rumen. They engulf starch granules and convert the digestion products to reserve carbohydrates. Starch metabolism positively affected the content of α-glucans, diminishing the pool of starch available for other microorganisms, mainly for amylolytic bacteria. The results of the enumeration of the representatives of both ciliate species in the rumen and reticulum of sheep seems to suggest different roles for *E. maggii* and *E. caudatum* as a direct source of the α-glucans for the host due to difference in their residence time in the rumen.

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Figure Captions

Fig. 1. Schematic diagram to show the stages of α-glucans analysis.

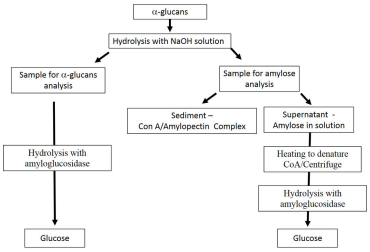


Fig. 2. The relationship between the hydrolysed α-glucans: Gb - bovine liver glycogen; Am - maize amylopectin, Go - oyster glycogen; S - starch and calculated on the basis of glucose.

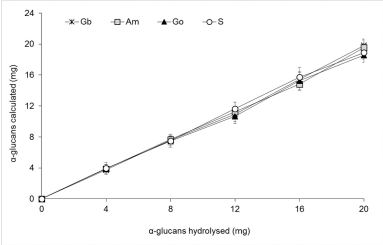


Fig. 3. The relationship between the quantity of amylose in the hydrolyzed samples of a mixture amylose with: bovine liver glycogen (A + Gb); maize amylopectin (A + Am); oyster glycogen (A + Go) and calculated on the basis of glucose.

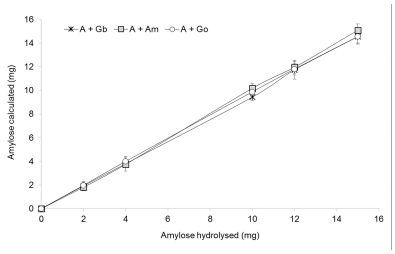


Fig. 4. The changes in the quantity of α- glucans in the cells of protozoa: period II - *E. maggii* (■), period III- *E. maggii* (▲) and *E. caudatum* (□) in relation to the time after feeding. Error bars denote SD values; different superscripts (A, B, C and a, b) indicate differences ($P<0.001$ and $P<0.05$, respectively).

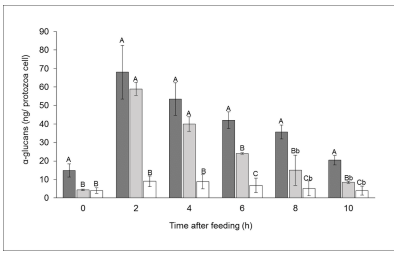


Fig. 5. Changes in the quantity of starch in the cells of ciliates *E. maggii* during period II (■) and period III - (■) in relation to the time after feeding. Error bars denote SD values; different superscripts (A, B and a, b) indicate differences ($P<0.001$ and $P<0.05$, respectively).

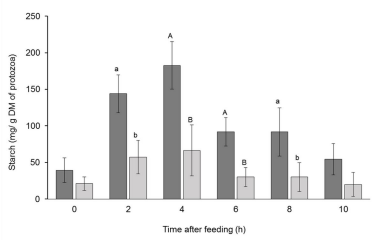


Fig. 6. Changes in the quantity of glycogen in the cells of ciliates *E. maggii* during period II (■) and period III - (■) in relation to the time after feeding. Error bars denote SD values; different superscripts (A, B and a, b) indicate differences ($P<0.001$ and $P<0.05$, respectively).

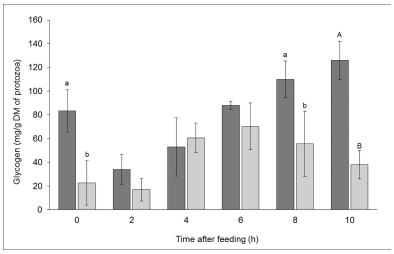
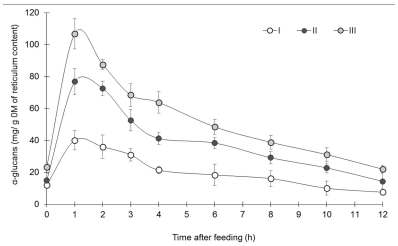


Fig 7. The changes in the concentration of α-glucans in the reticulum content of sheep during period I – defaunated (○), period II – monofaunated with *E. maggii* (●) and period III – bifaunated sheep with *E. maggii* and *E. caudatum* (▽) in relation to the time after feeding. Error bars denote SD values.



Tables

Table 1. Ingredients and chemical composition of the diet of sheep.

Components (% DM)		Chemical composition, (% DM)	
Meadow hay	82.9	Organic matter	93.8
Crushed barley	14.4	Ash	6.2
Vitamin-mineral premix*	2.8	Crude protein	11.5
		Crude fiber	26.9
		Ether extract	2.0
		NDF	60.3
		ADF	33.9
		ADL	4.9

* Premix (POLFAMIX O-K, TROUW NUTRION, POLAND) contents per 1 kg: vit. A 300 000 IU, vit. D_3 30 000 IU, vit. E 1500 mg, Mn 3000 mg, Zn 2500 mg, I 50 mg, Co 15 mg, Se 3 mg, Ca 24%, Na 6% , P 12%, Mg 6.5%; DM – dry matter; NDF – neutral detergent fibre, ADF – acid detergent fibre, ADL – acid detergent lignin.

Table 2. The concentration of α-glucans (mg/g DM of rumen content) in the rumen of defaunated (period I), monofaunated *E. maggii* (period II), or bifaunated *E. maggii* and *E. caudatum* sheep (period III).

Time after feeding (h)	Period (P)			SEM	H	P	H×P
	I	II	III				
0	6.2_{Y}	7.4_{X}	11.8_{Y}	1.20	<0.001	<0.001	0.037
4	19.9_{X}^{Aa}	29.9_{Yy}^{b}	33.9_{X}^{B}	2.32			
8	6.8_{Y}^{Aa}	21.3_{Yy}^{b}	21.8_{Y}^{B}	2.72			
12	4.4_{Y}^{a}	9.6_{x}	12.1_{Y}^{b}	1.32			

Upper different superscripts between rows (A, B and a, b) indicate differences (P<0.001 and P<0.05, respectively) between periods (I, II, III). Lower different subscripts beetwen columns (X, Y and x, y) show differences (P<0.001 and P<0.05, respectively) between hours after feeding (0, 4, 8, 12 h); SEM – standard error of mean; P – main effect of periods; H - main effect of hours after feeding; H×P –hours after feeding × periods.

Table 3. The concentration of ciliates in the rumen content ($\times 10^3$/g) of sheep faunated with: *E. maggii* (period II) or *E. maggii* and *E. caudatum* (period III) in relation to the time after feeding.

Time after feeding (h)	Period (P)			H	P	H×P
	II	III				
	E. maggii	*E. maggii*	*E. caudatum*			
0	33.5	19.6	221.8	0.211	0.023	0.891
4	25.1[a]	15.4[b]	181.7			
8	27.6[a]	17.0[b]	201.4			
12	34.7	20.1	230.2			
SEM	2.34	0.97	9.07			

Values marked with different superscripts differ significantly ($P<0.05$) between periods (I, II, III); SEM – standard error of mean; P – main effect of periods; H - main effect of hours after feeding; H×P –hours after feeding × periods

Table 4. The changes in the concentration pool of cellular α-glucans in the rumen of monofaunated sheep with *E. maggii* - period II and bifaunated with *E. maggii* and *E. caudatum* - period III in relation to the time after feeding.

Specification	Time after feeding (h)	Period (P)			H	P	H×P
		II	III				
		E. maggii	*E. maggii*	*E. caudatum*			
α-glucans	0	5.0^{A}	$0.9_{Y}{}^{B}$	8.9	0.001	0.002	0.687
	4	13.5	6.2_{Xx}	16.1			
	8	9.9^{a}	$2.6_{y}{}^{b}$	10.5			
	12	5.5	1.1	8.5			
SEM		1.42	0.73	1.13			
Barley starch	0	1.0_{Y}	0.5_{y}	1.7	0.056	<0.001	0.399
	4	5.5_{Xx}	3.0_{x}	16.0			
	8	2.7	1.0	10.5			
	12	2.0_{y}	0.6	8.6			
SEM		0.58	0.35	1.62			

Upper different superscripts between rows (A, B and a, b) indicate differences (P<0.001 and P<0.05, respectively) between periods (I, II, III). Lower different subscripts between columns (X, Y and x, y) show differences (P<0.001 and P<0.05, respectively) between hours after feeding (0, 4, 8, 12 h); SEM – standard error of mean; P – main effect of periods; H - main effect of hours after feeding; H×P –hours after feeding × periods.

Table 5. The numbers of ciliates in the reticulum content (× 10^3/mL) of sheep faunated *E. maggii* (period II) or *E. maggii* and *E. caudatum* (period III).

Hours after feeding (h)	Period (P)			H	P	H×P
	II	III				
	E. maggii	*E. maggii*	*E. caudatum*			
0	8.1[a]	3.7[b]	158.5	0.182	0.031	0.964
4	5.4[a]	1.9[b]	126.9			
8	5.9[a]	2.4[b]	143.3			
12	7.4	3.5	167.1			
SEM	0.70	0.34	15.36			

Values marked with different superscripts differ significantly (P<0.05) between periods (I, II, III); SEM – standard error of mean; P – main effect of periods; H - main effect of hours after feeding; H×P –hours after feeding × periods.