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Digestive enzymes in *Rhinolophus euryale* (Rhinolophidae, Chiroptera) are active also during hibernation

Edita Maxinová[1,2*], Vladimír Šustr[3], Marcel Uhrin[1]

[1]*Department of Zoology, Faculty of Science, Pavol Jozef Šafárik University in Košice, Šrobárova 2, 04180 Košice, Slovakia*
**Corresponding author, E-mail: edita.maxinova@gmail.com*

[2]*Department of Zoology and Animal Cell Biology, Faculty of Science and Technology, University of the Basque Country UPV/EHU, Barrio Sarriena s/n, 48940 Leioa, Bizkaia, Basque Country*

[3]*Institute of Soil Biology, Biology Centre of the Czech Academy of Sciences, Na Sádkách 702/7, 37005 České Budějovice, Czech Republic*

ABSTRACT

During the winter, bats use hibernation as a means of surviving the period of low prey offer. However, the Mediterranean horseshoe bat (*Rhinolophus euryale*) arouses from torpor quite frequently. Based on the actual climatic conditions, it can profit from occasional foraging oportunities, when they occur. We analysed faeces collected on four nights during the period from November 2012 to February 2013 from the Domica-Baradla cave system (Slovakia and Hungary). In mid-November, the largest proportion of faecal contents were from Lepidoptera. Later on, the proportion of non-consumptive mass in the faeces increased and prey remnants disappeared. We analysed the activity of digestive enzymes (amylase, chitobiase, endochitinase and glukosaminidase) in faeces. The activity of these enzymes was detected in fresh faeces throughout the whole winter. The faecal activity of the chitinases was relatively stable during the monitored period, whilst the activity of amylase was highest during late November and December. Some level of active digestive enzymes during the winter could be an adaptation to occasional winter foraging.

KEYWORDS

bats; winter season; faecal matter; diet analysis; amylase; chitinases

INTRODUCTION

The gastrointestinal tract of bats seems to be highly specialised to their prey, for example, to arthropods in the conditions of the temperate zone. It is known that bats have fast metabolism, with an intake of prey up to one half of their body weight per day (Encarnação & Dietz 2006). The maximum retention time (or transit time) of food has been measured in several studies, and it is clear that it is very short, in general lasting from 132 to 309 min in vespertilionid bats (Roswag et al. 2012). According to the rapid transit time, the assimilatory process must be extremely efficient to cover the high metabolic rate. Nevertheless, in bats, few specialities have been found in the gastrointestinal tract, for example, the absence or dramatic reduction of the caecum and appendix in most bat species was confirmed (Park & Hall 1951, Klite 1965, Roswag et al. 2012). Although Buchholz (1958) confirmed a normal spectrum of digestive enzymes, some authors have also confirmed the activity of special enzymes. In North American vespertilionid bats, Whitaker et al. (2004) confirmed the activity of the enzyme chitinase, which was also found earlier in *Rhinolophus ferrumequinum* from Europe (Jeuniaux (1961). Strobel et al. (2013) later confirmed that European vespertilionid bat species have acidic mammalian chitinase (AMCase), which is produced in gastric glands in the stomach. Whilst chitin is characterised as a major structural component of pathogens, in the diet of mammals, two basic functions of mammalian chitinase are found, namely, as a component of innate immunity and for the digestion of food (Boot et al. 2005). Chitinolytic systems of insectivorous bats act primarily to break up the insect cuticle, thus permitting easier access by digestive enzymes to lipoproteins and carbohydrates embedded within the chitin matrix and to the soft body tissues (Webb et al. 1993). Although chitinase does not appear to affect the harder chitinous body parts in summer, it would potentially have more time during periods of lower temperatures, whilst bats were in hibernation in winter (although the metabolic rate would be much lower). Whitaker

et al. (2004) confirmed the activity of chitinases in both summer and winter but found significantly lower levels in winter. Another crucial enzyme, animal α-amylase, is the salivary and pancreatic α-amylase (1,4-α-D-glucan glucanohydrolase EC 3.2.1.1), which hydrolyses the α-1,4-glucosidic bonds of oligosaccharides and polysaccharides (e.g. starch and glycogen) in irregular parts by reducing the size of the molecule. Pancreatic amylase hydrolyses the α-1,4-glucosidic bonds of oligosaccharides present in the lumen of the intestine. Given the central role of food on exocrine pancreatic function and the limited food sources during winter (the limited possibility of hunting) during the few months of hibernation, a rapid change in pancreatic enzyme activity can be assumed (Janeček 1997).

In this study, we use *Rhinolophus euryale* as a model bat species active during hibernation. Recurrent arousals from torpor are in general energetically expensive in bats, causing 75–90% consumption of their stored fat (Thomas et al. 1990; Dunbar & Tomasi 2006; Jonasson & Willis 2012). These winter arousals can lead in bats to foraging or drinking behaviour (Avery 1985; Brigham 1987; Thomas & Cloutier 1992; Thomas & Geiser 1997) and to commuting flights between a hibernaculum and foraging areas and thus could be considered as consumptive arousals (see also Maxinová et al. 2017). This occurs mainly when meteorological conditions improve, that is, when the temperature increases. For example, there is evidence that *R. ferrumequinum* or *Myotis nattereri* also feed on mild winter nights (Ransome 1968, 1971; Hope et al. 2014). Non-consumptive arousals are also known, and bats produce faeces without prey remains during such arousals (Miková et al. 2013; Maxinová et al. 2017).

Based on the winter activity of *R. euryale* and occasional foraging, we further hypothesise that the presence of digestive enzymatic activity is independent of food intake or starvation during hibernation as an adaptation to the activity and foraging of this species during the winter.

1. MATERIAL AND METHODS

1.1. Study site

The research was carried out in the Slovak Karst region (SE Slovakia) and the Aggtelek Karst (NE Hungary) in the caves Čertova diera pri Domici, Domica and Baradla (48.48°, 20.46° / 48.48°, 20.47° / 48.47°, 20.50°; Gaál & Gruber 2014). Long-term monitoring was conducted at all the studied localities, and the horseshoe bat aggregations under study were confirmed as monospecific (Uhrin et al. 2012). Sampling nights were as follows: (1) 16–17 November 2012, Domica cave; (2) 27–28 November 2012, (3) 19–20 December 2012 and (4) 20–21 February 2013, all in the Baradla cave.

1.2. Faecal sampling and analysis

Faecal samples were collected from plastic foil placed under the hibernating aggregation of *R. euryale* during the four collection nights. The foil was cleaned up in the evening and pellets were collected on the following morning; thus, the samples were one night (ca. 12 h) old. Pellets (60–120 each night) were collected randomly from the foil surface. The samples in the field were immediately put on ice into a thermos and then stored frozen at −20 °C in a laboratory and later used for morphological and enzymatic analyses.

A total of 80 pellets (20 for each date) were morphologically analysed under a binocular magnifier. Prey categories were identified with the help of comparative slides, methodological studies and entomological keys (Chinery 1977; McAney et al. 1991; Whitaker et al. 2009). Percentage volume (vol%) was used for the presentation of the results.

For enzyme analyses, we used 2.6 g of faecal pellets. Right before the enzyme analyses, faeces were homogenised in 3 ml of MQ H_2O in a glass Potter-Elvehjem homogeniser, with ice cooling. The homogenate was centrifuged (MPV-65R Mechanica Warzsawa, Poland) at 4,600 *g* (7 min), and the supernatant was quickly divided into aliquot volumes for each enzyme test and stored in a refrigerator at −20 °C.

Soluble proteins in fresh faeces were determined by modification of the Bradford method using a 96-well plate (Bradford 1976): 7 µl of homogenate was add to 210 µl of Bradford reagent (Sigma, B6916), and after 5–45 min of incubation at room temperature, absorption was measured at 595 nm (Synergy 2 microplate reader, BioTek Instruments, Inc). A solution of bovine serum albumin (Sigma, P 7656) was used as a protein standard for calibration. Three repetitions and two measurements were made for each sample, and the results were then averaged. The volume of soluble proteins was expressed in micrograms of soluble protein per milligram of homogenised sample fresh weight (µg mg^{-1}).

The faecal activity of glucosaminidase, chitobiase (EC 3.2.1.52) and endochitinase (EC 3.2.1.14) was determined using a fluorimetric chitinase assay kit (Sigma, CS1030) based on the release of 4-methylumbelliferone (4MU). 4MU *N*-acetyl-β-D-glucosaminide, 4MU *N,N'*-diacetyl-β-D-chitobioside and 4MU β-D-*N,N',N''*-triacetylchitotrioside were used as substrates for glucosaminidase, chitobiosidase and endochitinase, respectively. 10 µl of faeces homogenate were added to 90 µl of substrate solution (5 mg of substrate for 250 µl DMSO diluted 40× in Britton–Robinson (BR) buffer (pH 5.0). The reaction mixture was then incubated for 1 h at 37 °C; the reaction was then interrupted by adding 200 µl Na_2CO_3 (0.0424 g/ml) and fluorescence was measured in a Synergy 2 microplate reader (BioTek Instruments, Inc) using 360/40 excitation and 460/40 emission filters. An enzyme unit (U) was defined as the amount of enzyme producing 1 µmol of 4MU per minute. Enzyme activity was expressed in units per gram of fresh weight (U g^{-1}) or units per gram of soluble protein (U g^{-1}).

Alpha-amylase activity was determined by chromogenic tablet assay (Alpha-amylase test, SPOFA, Czech Republic). The homogenate was incubated with tablets containing a substrate (a blue dye covalently bound to starch) and BR buffer (pH 6.0) for a few hours at 37 °C. The reaction was terminated by a solution of acetone with Na_2CO_3 (900 ml H_2O, 100 ml ac-

etone, 10 g Na_2CO_3). The acetone with Na_2CO_3 was added into the blank solution earlier than the homogenate prior to incubation. A drop of toluene was then added to each sample as a bactericide. The samples and the blank were then centrifuged at 6,000 *g* for 7 min, and the absorption of the supernatant was measured at 620 nm. Enzyme activity was expressed in units per gram of fresh weight or per gram of soluble protein (U g^{-1}).

Given the small number of replications, the differences amongst the enzymological values from different collection dates were tested using simple non-parametric tests (Kruskall–Wallis analysis of variance from non-parametric statistics in Statistica v 6.0 software). The dependency of the weight of specific enzyme activities on the concentration of soluble proteins was verified using linear regression in Statistica v 6.0.

2. RESULTS

Faeces from night 1 contained 92 vol% of Lepidoptera remnants, the rest was composed of non-consumptive mass (6 vol%) and hair (1 vol%). On night 2, the bats produced faeces that contained 5 vol% of Lepidoptera remnants only, whilst the main part was formed by non-consumptive mass (82.2 vol%) and the rest was hair. Faeces with no Lepidoptera at all occurred in the night during deep hibernation (night 3), and it consisted of non-consumptive mass (87.5 vol%) and hair only (12.5 vol%). On the February night (night 4), hair parts decreased to 3.15 vol% and the rest was non-consumptive mass.

Fresh faecal pellets contained soluble proteins during the whole monitored period. Changes in their concentration were not statistically significant between the collection dates (Kruskal–Wallis ANOVA, $p = 0.37$). However, the data showed an evident decreasing trend during the investigated period (Table 1).

We demonstrated the presence of amylase, glucosaminidase, chitobiase and endochitinase in fresh faeces during the whole winter period (Table 1). The differences in chitobiase ($p < 0.05$) and weight-specific expressed endochitinase ($p < 0.04$) between the collection dates were confirmed by Kruskal–Wallis ANOVA. Changes in the measured enzyme activities between the collection dates seemed to maintain a similar trend typical for each enzyme without respect to expression in weight-specific or protein-specific units.

Amylolytic activity was dominant, was very variable and showed a trend with higher values on the two intermediate dates, surprisingly rather inverse to the course of total soluble proteins. In contrast, glucosaminidase followed the trend of soluble proteins, rather decreasing from November to February (the positive correlation between weight-specific glucosaminidase activity and total faecal soluble protein content was almost significant ($r = 0.6$, $p = 0.05$)). The remaining chitinolytic activities (chitobiose and endochitinase) rather increased in faeces during the winter independently of the total protein concentration.

Table 1: Activity of enzymes (AM, amylase; GA, glucosaminidase; CHB, chitobiase; ECH, endochitinase) provided as units per gram of fresh weight (U g−1) and units per gram of soluble protein (U(gP)−1) and total protein content expressed as micrograms of soluble protein per milligram of homogenised sample fresh weight (FW) with average ± standard error.

Date	Enzyme			Proteins
		U g^{-1}	U $(gP)^{-1}$	µg P $(mg\ FW)^{-1}$
1	AM	1.34 ± 1.51	32.29 ± 82.66	29.50 ± 4.46
	GA	0.30 ± 0.03	11.20 ± 2.07	
	CHB	0.03 ± 0.01	1.21 ± 1.00	
	ECH	0.02 ± 0.01	0.79 ± 0.82	
2	AM	4.61 ± 1,85	224.3 ± 101.24	19.13 ± 5.46
	GA	0.15 ± 0.03	8.21 ± 2.54	
	CHB	0.08 ± 0.01	4.36 ± 1.22	
	ECH	0.09 ± 0.01	5.21 ± 1.00	
3	AM	4.60 ± 1.51	355.14 ± 82.66	15.04 ± 4.46
	GA	0.06 ± 0.03	4.83 ± 2.07	
	CHB	0.10 ± 0.01	7.42 ± 1.00	
	ECH	0.06 ± 0.01	4.42 ± 0.82	
4	AM	1.11 ± 1.51	55.68 ± 82.66	20.02 ± 4.46
	GA	0.13 ± 0.03	6.87 ± 2.07	
	CHB	0.14 ± 0.01	7.02 ± 1.00	
	ECH	0.09 ± 0.01	4.82 ± 0.82	

3. DISCUSSION

We analysed faeces from the hibernation period morphologically and biochemically. If bats foraged, they hunted Lepidopteran species only during the winter. The highest proportion of Lepidoptera content was recorded on the first night in mid-November. Later on, at the end of November, some Lepidoptera remains were recorded, but from that time on, non-consumptive faeces predominated. There is also evidence that rhinolophid as well as vespertilionid bats feed on mild winter nights (Williams et al. 2010; Hope et al. 2014; Zahn & Kriner 2016).

A relatively stable content of soluble proteins was found in winter faeces. In view of the fact that we analysed relatively fresh faeces (12 h in maximum), it is probable that most of these proteins as well as the enzymatic activity detected originated from the bat digestive tract. In non-consumptive faeces, proteins may be contained in the mucous layer of excrements and may originate from mucous secretions of gut intestinal epithelium; they may represent digestive enzymes secreted into the gut lumen or may originate from remains of damaged epithelial cells liberated into the excrements. The presence of some traces of active enzymes in faeces may indicate the continual secretion of digestive enzymes into the gut lumen or a loss of tissue enzymes from parts of damaged intestinal epithelium liberated into excrements. Total protein seems to decrease after stopping of feeding in late November, but the

decrease is not significant and did not continue in February, thus indicating that protein being liberated into excrements is not fully dependent on feeding status.

Surprisingly, amylase showed high activity during the whole winter period. Amylase may be deposited more in the acinar cells during hibernation than in summer because of the absence of stimuli related to food intake, which are the primary signals for the release of pancreatic enzymes into the small intestine. This idea is supported by similar conditions in the hibernating rodent *Muscardinus avellanarius*, in which amylase is accumulated during torpor in the rough endoplasmic reticulum and zymogen granules of pancreatic acinar cells (Malatesta et al. 1998). Although amylase may not be synthesised during deep torpor following the global suppression of transcription and translation (van Breukelen & Martin 2001, 2002), the synthesis of a basal level of amylase can be always present during arousals despite the absence of food. Thus, an increase in the synthesis of amylase before hibernation, minimal degradation during the winter months and basal synthesis during arousals help to maintain relatively high levels of amylase despite the long winter period. Maintenance of basal levels of pancreatic amylase during hibernation can contribute to the survival of an individual in the spring, when prey offer is limited and available food is of a lower quality. The possibility of hydrolysing carbohydrates from food in the lumen of the intestine after awakening, without earlier synthesis of new amylase, can save energy reserves for other functions, such as restoration of organs and reproduction. Reduction of the volume of pancreatic mass and protein content during hibernation (Bauman et al. 1987; Bauman 1990) as well as a decrease in the level of pancreatic amylase by 40–50% may be advantageous in terms of saving energy for hibernants. Our results showed that liberating amylase into faeces is independent of the defecation rate of most soluble proteins, indicating different mechanisms of output of different proteins from the intestine into faeces. Amylase activity in faeces does not seem to be positively correlated with feeding. Probably, the presence of some food in the intestine prevents the washing out of digestive enzymes into faeces in early November in comparison with late Novemer and December. In February, the proposed pool of intestinal (pancreatic) amylase may then be depleted.

Changes in glucosaminidase activity in faeces showed a different pattern of changes during the winter, better coinciding with changes in total soluble protein.

The other chitinolytic enzymes (chitobiase and endochitinase) appeared to show a surprising increase but an insignificant trend in winter excrements coinciding rather positively with starvation. Balslev-Clausen et al. (2003) suggest that hibernants rely on the power of fatty acids from fat reserves during hibernation and the ability to hydrolyse nutrients from food is not necessary to support metabolism. On the other hand, Whitaker et al. (2004) assumed the degradation of food consumed earlier to replenish energy losses during the winter and thus explained the presence of highly active chitinase. They assumed that chitinase can degrade chitin remnants from summer hunting and thus supply energy. Currently, there are two views on the presence of chitinolytic activity in bats: it is possibly due to microorganisms in the small intestine, thus microbial enzymes help to digest chitin, or bats are able to produce AMCase, so that the production of this enzyme is endogenous (Stevens 1988; Sugita et al. 1996; Boot et al. 2001; Šimůnek et al. 2001; Eurich et al. 2009; Strobel et al. 2013). Two basic functions of mammalian chitinases have been found, namely, as a component of innate immunity and for the digestion of food (Boot et al. 2005). The possible chitinolytic activity of enzymes playing a role in immunity protection may explain the presence of this activity during the hibernation period. On the other hand, the high activity of both chitinases and amylase may help bats take advantage of potential foraging opportunities that appear during the winter. We assume that chitinases remain active as an adaptation of the species to arousals and occasional foraging during the whole winter. Unfortunately, we are not able to confirm whether the origin of chitinases is endogenous or microbial.

To conclude, we were able to analyse digestive enzymes from faeces using a non-invasive method that proved to be comparable to invasive ones. The remaining activity of digestive enzymes could be an adaptation to occasional foraging during the winter, and the activity of chitinases may be connected with the high need for an immune response during hibernation as well as with degradation of chitinous remains from earlier foraging – and the replenishing of energy. The activity of digestive enzymes is independent of food intake or starvation during the winter.

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