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The activity of saccharolytic enzymes in Collembola is associated with species affinity for caves

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Abstract: The activity of enzymes associated with digestion can reflect food availability and feeding preferences of invertebrates in a particular habitat. Caves are mostly nutrient-poor habitats lacking primary production. In the present study the enzymatic activity of cellulases, trehalases and chitinases was measured in eight collembolan species differently associated with the cave environment: the troglobionts (obligate cave species) *Pseudacherontides spelaeus* and *Protaphorura janosik*; the eutroglophiles *Ceratophysella denticulata*, *Folsomia candida* and *Heteromurus nitidus*; the subtroglaphiles *Hypogastrura aequipilosa* and *Orthonychiurus rectopapillatus*; and the troglaxene (not associated with caves) *Megaphorura arctica*. Qualitative enzymatic patterns and quantitative differences in species activity were considered in terms of the taxonomic, feeding and ecological classifications of Collembola. Activity of the tested enzymes was confirmed in all species. Cellulolytic and chitinolytic activity seemed to play a crucial role for the discrimination of guilds within all categories. An increasing trend of chitinolytic activity was observed in Collembola associated with the subterranean environment and deeper soil layers, while cellulolytic activity decreased towards more adapted cave forms. Variability in enzymatic activity in cave-dwelling species indicated food specialization across sub- and eutroglophiles and troglobionts, respectively. The results of this study point out that enzymatic activity varies between groups of the cave fauna with different degrees of association to subterranean habitats (cave guilds).

Keywords: enzymatic activity, cellulase, trehalase, chitinase, Collembola, cave guild

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INTRODUCTION

Feeding ecology among soil Collembola reflects the high heterogeneity of the soil environment (Chahartaghi et al., 2005). Collembola are generally classified as decomposers (Rusek, 1998), feeding unselectively on a wide variety of food materials. However, the trophic niches of species vary among different collembolan orders and families, indicating a pronounced phylogenetic signal that supports the trophic-niche conservatism hypothesis (Potapov et al., 2016).

Food resources in caves are depleted when compared with surface ecosystems, which obtain energy and organic carbon from photosynthesis by

green plants. Microbial communities represent the base of the food pyramid in caves (Camassa, 2004), and they rely almost entirely on surface organic resources actively or passively transported into the caves. The most available food sources in caves are animal cadavers, faeces, animal eggs and plant debris (leaf litter, rotten wood). Generally, Collembola that occupy environments with both plant and fungal food sources available are able to alternate herbivory and fungivory (Endlweber et al., 2009).

While collembolan communities diverge vertically in above-ground ecosystems, their horizontal distribution is distinct in caves. Troglaxenes and subtroglaphiles are abundant in entrance zones illuminated during the day (euphotic zone) and characterized by the

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presence of algae, bryophytes and phanerogams, while eutroglophiles and troglobionts live permanently in an environment devoid of fresh plant food sources (Culver and Pipan, 2009; Kováč et al., 2014).

Food composition in Collembola has been studied using various techniques, such as analyses of gut content (e.g. Ponge, 2000; Castaño-Meneses et al., 2004; Fiera, 2014), activity of digestive enzymes (Zinkler, 1971; Urbášek & Rusek, 1994; Berg, 2004), lipid composition (Ruess et al., 2007) or stable isotopes (e.g. Ruess et al., 2004; Chahartaghia et al., 2005; Endlweber et al., 2009; Potapov et al., 2016). Each method has its own limitations, and a combination of several approaches seems to be the proper way to better understand feeding ecology and to refine the functional ecological classification of soil mesofauna.

Regarding the usual interpretation of digestive enzymes, the presence of cellulase activity in the digestive tract of an animal suggests the ability to digest algae and plant materials and indicates herbivory. On the other hand, the presence of trehalase activity indicates the ability to digest the cell contents but not the cell walls of lichens and microbes, while chitinases enable digestion of the hyphae contents and cell walls of fungi (Siepel & De Ruiter-Dijkman, 1993; Berg et al., 2004). Along with digestion, potential non-digestive roles of enzymes also appear. Invertebrate chitinases can subserve arthropod molting (Merzendorfer & Zimoch 2003), defense/immunity and pathogenicity (Arakane & Muthukrishnan, 2010). Trehalose is a haemolymph transport sugar of insects having a role in the regulation of food intake and stress protection (Thompson, 2003). In Collembola, trehalose has an important role as a cryoprotective agent as well (Sinclair & Sjørnsen, 2001), and its activity can vary seasonally, especially in arctic species.

Moreover, an association is assumed between the specific enzymatic activity of Collembola and their vertical stratification in the soil profile (life form) (Urbášek & Rusek, 1994). Vertical migration induced by seasonal changes in habitats and subsequent seasonal alternation of food sources is common in some collembolan species (e.g. Hishi et al., 2007).

The level of enzymatic activity depends on the type of food consumed prior to sampling and phase of the life cycle (Berg et al., 2004). Moreover, the activity of some enzymes may significantly differ in the same species at two different sites (Berg et al., 2004).

The high morphological and ecological heterogeneity of Collembola has resulted in the proposal of different types of ecological classifications. The traditional classification of life forms is based on the general body plan (Gisin, 1943; Rusek, 2007), while enzymatic activity and the stable isotope ratio have been considered in more recent studies (Berg et al., 2004; Potapov et al., 2016). Based on qualitative differences in enzymatic equipment, several functional groups were defined in soil mesofauna (Siepel & DeRuiter-Dijkman, 1993), representing functional variability within the group. Berg et al. (2004) found qualitative differences in cellulolytic, trehalolytic and chitinolytic equipment between collembolan species and sorted

them into 4–5 principal feeding guilds (Siepel & DeRuiter-Dijkman, 1993).

It is expected that enzymatic equipment may also differ considerably between cave-dwelling animals, depending on their nutritional ecology, but these studies have not been conducted yet.

The present study is one of the first attempts aimed at analyzing enzymatic activity in subterranean arthropods, specifically qualitative and quantitative differences in the activity of trehalase, cellulase and chitinases between Collembola with different affinity to the cave environment (cave guilds). We hypothesized that non-cave-adapted species living in an environment with more heterogeneous food sources would have higher activity of some enzymes, especially cellulase, compared to obligate cave species.

MATERIALS AND METHODS

Tested species and categorical variables

Specimens were collected in the field together with substrate and organic material from collection sites and transported to the laboratory (Table 1). Seven collembolan species with various affinity to cave habitats were examined: *Ceratophysella denticulata*, *Folsomia candida*, *Heteromurus nitidus*, *Hypogastrura aequipilosa*, *Orthonychiurus rectopapillatus*, *Protaphorura janosik* and *Pseudacherontides spelaeus*. For comparison, *Megaphorura arctica* was tested as an example of a species that does not have any association with the cave environment; therefore, we consider it as “trogloxene”. For practical purposes, we use the term “cave guild” to designate groups of species with different affinities to the cave environment, namely troglloxenes, subtroglloxiles, eutroglloxiles and troglloxionts (Sket, 2008). Soil life forms after Rusek (2007) and feeding guilds after Potapov et al. (2016) were used as other categorical variables to reveal potential trophic niche specialization. Body length, number of eyes and total protein content were used as functional traits, with the body weight as a covariable.

Evaluation of enzymatic activity

Cellulase, trehalase and chitinase (glucosaminidase, chitobiase, endo-chitinase) activity was measured in eight collembolan species (see above). From 8 to 15 individuals of each species were weighed and washed with 0.6% cholic acid. Each group of collembolans was placed into a small glass mortar filled with 0.25 ml of Britton-Robinson (BR) buffer (pH = 6) and pulverised with a glass pestle until no body parts were recognizable. The mixture was transferred to a plastic Eppendorf tube placed in crushed ice. The mortar and pestle were rinsed with 0.25 ml of BR buffer, and this volume was added to the Eppendorf tube. The homogenate was centrifugated at 6,000 rpm/7 min., and the supernatant was stored at -18°C until used for enzyme activity assay. The activity of each enzyme was measured in 4–6 replications. In *P. janosik*, enzymatic activity in specimens from laboratory culture was compared with those collected in the field and pulverised immediately after transporting to the laboratory.

Table 1. Collembola species and their life forms (LF) after Rusek (2007), cave forms (CF) after Sket (2008) and feeding guilds (FG) after Potapov et al. (2016).

Species	Abb.	Family	LF	CF	FG	bl	S	FH	Original locality	RC
<i>Ceratophysella denticulata</i> (Bagnall, 1941)	<i>Cde</i>	Hypogastruridae	Bb1	EU	EPAM	1.8	L	A	Čertova díera Cave (SK)	s, y
<i>Folsomia candida</i> (Willem, 1902)	<i>Fca</i>	Isotomidae	Bc2a	EU	HEM	1.7	L	A	Domica Cave (SK)	s, y
<i>Heteromurus nitidus</i> (Templeton, 1835)	<i>Hni</i>	Entomobryidae	Bc1a	EU	HEM	3.0	L	A	Domica Cave (SK)	s, y
<i>Hypogastrura aequipilosa</i> (Stach, 1949)	<i>Haq</i>	Hypogastruridae	Bb1	SUB	EPAM	2.0	L	A	Čertova díera Cave (SK)	s, y
<i>Megaphorura arctica</i> Tullberg, 1876	<i>Mar</i>	Onychiuridae	Bc1b	TX	EPPM	3.5	L	N	Svalbard, soil, moss under bird cliffs (N)	s, -
<i>Orthonychiurus rectopapillatus</i> (Stach, 1933)	<i>Ore</i>	Onychiuridae	Bc1b	SUB	EUM	1.3	L	A	Domica Cave (SK)	s, y
<i>Protaphorura janosik</i> Weiner, 1990	<i>Pja</i>	Onychiuridae	Bc1b	TGB	EUM	3.8	F	N	Jaskyňa pod Spišskou Cave (SK)	-
<i>Protaphorura janosik</i> Weiner, 1990	<i>Pja2</i>	Onychiuridae	Bc1b	TGB	EUM	3.8	L	A	Jaskyňa pod Spišskou Cave (SK)	s, y
<i>Protaphorura janosik</i> Weiner, 1990	<i>Pja4</i>	Onychiuridae	Bc1b	TGB	EUM	3.8	F	N	Duča Cave (SK)	-
<i>Pseudacherontides spelaeus</i> (Ionesco, 1922)	<i>Psp</i>	Hypogastruridae	Bc2b	TGB	EUM	0.7	L	N	Fănațe Cave (RO), guano	g, -

Codes: bl – body length (mm) (literature data); S – sample: L – laboratory, F – field; FH – food history: A – artificial (inactivated yeasts with malt extract), N – natural substrate; RC – rearing conditions: s – cave sediment/ substrate from collecting site, g – bat guano from collecting site, y – inactivated yeast; EU – eutroglophile, SUB – subtroglophile, TGB – troglobiont, TX – troglone; Bb1 – upper hemiedaphobiont, Bc1a – large euedaphobiont with furca, Bc1b – large euedaphobiont with missing/reduced furca, Bc2a – medium euedaphobiont with furca, Bc2b – medium euedaphobiont with missing/reduced furca; EUM – euedaphic microorganisms consumers, EPPM – epigeic plant and microorganisms consumers, EPAM – epigeic animal and microorganisms consumers, HEM – hemiedaphic microorganisms consumers.

Cellulase and trehalase activity was measured by quantifying the rate of glucose production from substrates. For samples, 39 μ l of homogenate (supernatant) were pipetted into 1.5 ml plastic Eppendorf tubes filled with 39 μ l of substrate solution: carboxymethyl-cellulose (0.02g/ml) or trehalose (0.06 g/ml). Two technical replications for each sample were tested. A homogenate blank containing homogenate and 260 μ l of trichloroacetic acid (TCA) and substrate blanks containing 1 ml of substrate were incubated together with the sample for 24 h at 37°C. Before incubation, a drop of toluene was added as a bactericide. After incubation, 260 μ l of TCA was added to the sample and 39 μ l of incubated substrate blank solution was added to the homogenate blank and mixed. Samples and blanks were centrifugated at 6,000 rpm/7 min., and 50 μ l of supernatant of samples and blanks were added into separated wells in a microtitration plate filled with 250 μ l of GLU GOD 250 Lachema reagent to determine the glucose concentration. The microtitration plate was incubated for 20 min at 37°C. Then the absorbance was measured at 495 nm using a microplate reader (Synergy 2, BIO TEK). A glucose solution was used for the calibration.

Chitinase activity (glucosaminidase, chitobiase, endo-chitinase) was measured fluorometrically by quantifying the amount of transformed specific fluorogenic substrates 4-Methylumbelliferyl N-acetyl- β -D-glucosaminide (MUNAGA), 4-Methylumbelliferyl N,N'-diacetyl- β -D-chitobioside (MUCHB) and 4-Methylumbelliferyl β -D-N,N',N''-triacetylchitotriose, respectively (MUCHT), using the Chitinase Assay Kit, Fluorimetric (Sigma-Aldrich CS1030). For blanks, 100 μ l of diluted (1:79 by BR buffer) substrate

solutions of MUNAGA, MUCHB and MUCHT were pipetted onto a black microtitration plate. For samples, 90 μ l of the same diluted substrate solutions and 10 μ l of homogenate were used for each of the 3 substrates. The microtitration plate was incubated for 60 min at 37°C. After incubation, 200 μ l of 0.4 M sodium carbonate (Na_2CO_3) was added into each hollow. Fluorescence was then measured using a microplate reader (Synergy 2, BIOTEK, Excitation = 360/40, Emission = 460/40); 4-Methylumbelliferone solution was used for calibration.

The content of total soluble proteins was determined in each homogenate by adding 100 μ l of the homogenate to 100 μ l of Bradford reagent (Sigma, B6916) and measuring the absorbance at 595 nm (Synergy 2, BIOTEK) after incubation at room temperature for 5-45 min. A solution of bovine serum albumin (Sigma, P 7656) was used as the protein standard for calibration.

Statistical analyses

For statistical evaluation of differences in the enzymatic activities among species One-Way ANOVA with HSD post-hoc comparisons were used (software Statistica v6.0). Relationships between body mass and total soluble protein content and between both these parameters and enzymatic activity were tested using multidimensional regression (Statistica v6.0).

The variability of the enzymatic activity was analyzed by applying direct analysis with linear response associated using the CANOCO 5.03 multivariate software package. Constrained partial RDA was performed with species mean body weight as the covariable. We assessed the proportion of the variability explained by different taxonomical categories, morphological traits and environmental

variables. The forward selection method (FW) showed variables explaining the highest proportion of enzyme variability in a given species. The significance of the environmental variables and categories was specified using the Monte Carlo test with 9999 randomizations.

RESULTS

The presence of the tested enzymes (trehalase, cellulase and chitinases) from the whole-body homogenates was confirmed in all species under study.

Enzymatic activity in relation to mean body mass and total soluble protein content

Collembola species differed significantly in their mean body mass (One-way ANOVA, $F(7,75) = 62.42$, $p < 0.001$), with *P. spelaeus* as the smallest species and *M. arctica* and *P. janosik* as the largest ones (Table 2).

The content of the soluble proteins in one milligram of homogenate increased with decreasing mean body mass of the Collembola groups used for the homogenizations ($r = 0.33$, ANOVA, $F(1,48) = 5.87$, $p < 0.02$). Considering mean body mass of species, the highest concentration of proteins was measured in the smallest species, *P. spelaeus*, and the lowest in *H. nitidus*. Differences in the content of soluble proteins among species were significant (ANOVA, $F(7, 43) = 4.5265$, $p < 0.001$). The mean value of this parameter differed significantly in *P. spelaeus* from *H. nitidus*, *F. candida*, *M. arctica*, *O. rectopapillatus* and *P. janosik* (HSD post-hoc comparison, $p < 0.03$, Table 2).

However, the differences in enzymatic activity among species did not simply follow the protein content pattern. The species means of mass-specific enzyme

activity of trehalase and chitinase showed increasing trends, with an increasing mean total soluble protein content (r from 0.02 to 0.56), although linear regression models were not significant (ANOVA, $p < 0.19$). Therefore, we preferred to compare enzymatic activities based on protein-specific values. In contrast to other enzymes, the mass-specific activity of cellulase increased insignificantly with a decreasing of the body protein content ($r = -0.33$).

The interspecific differences in protein-specific trehalolytic activity were not significant (ANOVA, $F(7, 33) = 1.97$, $p = 0.089$), with the highest activity observed in *H. nitidus*. Trehalolytic activity in this species differed insignificantly from the lowest activity measured in *M. arctica* and *C. denticulata* (HSD post-hoc comparison, $p < 0.19$, Table 2). The values expressed in mass-specific units showed a similar pattern, with the exception of the overestimated value observed in *P. spelaeus* (Table 2).

Cellulolytic activity showed significant interspecific differences for both mass-specific and protein-specific values, with the highest activity measured in *M. arctica* (ANOVA, $F(7, 32) = 4.31$, $p = 0.0019$ and $F(7, 32) = 4.2951$, $p = 0.0019$). Activity of the protein-specific cellulase in *M. arctica* was significantly higher compared to *H. aequipilosa*, *F. candida*, *P. spelaeus* and *P. janosik* (HSD post-hoc comparisons, $p < 0.035$; Table 2).

Chitinolytic activity expressed in protein-specific units displayed significant differences between species. *O. rectopapillatus* and *H. aequipilosa* showed the highest activity of glucosaminidase compared to other species and significantly higher levels of all chitinolytic enzymes (ANOVA, $F(7, 35) = 5.58$, $p = 0.0002$, HSD post-hoc comparison, $p < 0.05$).

Table 2. Mean body mass, total soluble protein content and enzymatic activities of Collembola species (whole body homogenates). Enzymatic activities expressed as mass-specific (U.g^{-1}) and protein-specific (U.gP^{-1}). U = unit of enzyme activity defined as $\mu\text{Mol.min}^{-1}$. Means $\pm$ standard errors are presented; small letters indicate significant differences among means at $p = 0.05$.

Species	Body mass $\mu\text{g.ind.}^{-1}$	Proteins mg.g^{-1}	Enzyme activity			
			Trehalase		Cellulase	
			U.g^{-1}	U.gP^{-1}	U.g^{-1}	U.gP^{-1}
<i>P. spelaeus</i>	17.98 ± 0.78 c	13.84 ± 2.97 b	0.431 ± 0.364	0.036 ± 0.022	0.021 ± 0.011 ab	0.001 ± 0.001 b
<i>F. candida</i>	127.86 ± 15.84 c	4.37 ± 1.41 a	0.067 ± 0.023	0.018 ± 0.009	0.035 ± 0.010 b	0.007 ± 0.002 b
<i>C. denticulata</i>	133.91 ± 13.45 bc	7.66 ± 1.67 ab	0.109 ± 0.049	0.014 ± 0.005	0.134 ± 0.026 a	0.017 ± 0.003 ab
<i>O. rectopapillatus</i>	134.37 ± 10.71 bc	5.22 ± 0.31 a	0.282 ± 0.172	0.050 ± 0.028	0.098 ± 0.018 ab	0.019 ± 0.004 ab
<i>H. aequipilosa</i>	127.28 ± 6.15 bc	7.43 ± 1.48 ab	0.186 ± 0.060	0.031 ± 0.015	0.061 ± 0.015 ab	0.008 ± 0.002 b
<i>H. nitidus</i>	253.95 ± 32.46 b	4.00 ± 0.43 a	0.292 ± 0.056	0.081 ± 0.012	0.081 ± 0.026 ab	0.020 ± 0.006 ab
<i>P. janosik</i>	590.58 ± 105.97 a	4.54 ± 0.53 a	0.255 ± 0.087	0.064 ± 0.021	0.041 ± 0.024 b	0.009 ± 0.005 b
<i>M. arctica</i>	620.27 ± 12.59 a	4.77 ± 0.89 a	0.065 ± 0.002	0.014 ± 0.001	0.131 ± 0.013 a	0.027 ± 0.003 a
	Enzyme activity					
	Glucosaminidase		Chitinase		Endochitinase	
	U.g^{-1}	U.gP^{-1}	U.g^{-1}	U.gP^{-1}	U.g^{-1}	U.gP^{-1}
<i>P. spelaeus</i>	0.955 ± 0.921	0.062 ± 0.055 b	0.425 ± 0.421	0.026 ± 0.026 ab	0.218 ± 0.214 b	0.014 ± 0.013 b
<i>F. candida</i>	0.156 ± 0.118	0.036 ± 0.027 b	0.080 ± 0.024	0.020 ± 0.007 b	0.077 ± 0.021 b	0.019 ± 0.006 b
<i>C. denticulatus</i>	9.712 ± 6.092	0.087 ± 0.018 b	0.212 ± 0.056	0.032 ± 0.009 b	0.153 ± 0.034 b	0.023 ± 0.005 b
<i>O. rectopapillatus</i>	2.035 ± 0.343	0.414 ± 0.086 a	0.581 ± 0.146	0.117 ± 0.030 a	0.863 ± 0.183 a	0.177 ± 0.046 a
<i>H. aequipilosa</i>	1.840 ± 0.266	0.273 ± 0.071 ab	0.509 ± 0.149	0.071 ± 0.022 ab	0.388 ± 0.152 ab	0.054 ± 0.024 b
<i>H. nitidus</i>	0.644 ± 0.101	0.161 ± 0.023 b	0.144 ± 0.026	0.035 ± 0.006 b	0.148 ± 0.030 b	0.036 ± 0.006 b
<i>P. janosik</i>	0.766 ± 0.279	0.183 ± 0.060 b	0.180 ± 0.083	0.040 ± 0.018 b	0.144 ± 0.062 b	0.033 ± 0.013 b
<i>M. arctica</i>	0.602 ± 0.176	0.126 ± 0.037 b	0.088 ± 0.027	0.018 ± 0.006 b	0.078 ± 0.026 b	0.016 ± 0.005 b

The activity of chitobias in *O. rectopapillatus* was higher than in other species, with an exception of *H. aequipilosa* and *P. spelaeus* (ANOVA, $F(7, 35) = 3.77$, $p = 0.0038$, HSD post-hoc comparison, $p < 0.05$), while the endochitinase activity in this species was higher than in all other species (ANOVA, $F(7, 35) = 7.0734$, $p = 0.00003$, HSD post-hoc comparison, $p < 0.002$).

Enzymatic activity and Collembola categories

Considering the taxonomical categories, the highest proportion of enzymatic variability was explained at the species level (Table 3). The degree of association with caves (cave guilds) was more important for the collembolan enzymatic equipment than feeding guilds or life forms (Table 4).

Direct RDA ordination (Fig. 1) placed *F. candida* and *P. spelaeus* in the same quadrant of the ordination space, contrary to *M. arctica* and *O. rectopapillatus*. All populations of *P. janosik* had similar enzymatic

equipment, but one with distinctly higher chitinase activity.

Among categorical variables, the largest variability was explained by cave affinity (cave guilds), less by life forms and feeding guilds (Table 5). Functional traits were not significant, but body length was evaluated as the most important. The activity of digestive enzymes explained by cave guilds showed an increasing trend in cellulolytic activity from troglobionts to troglonexes (Fig. 2A). However, all the categories partially overlapped, and subtroglaphiles seemed to be the most diverse in enzymatic activity (Fig. 2B).

Enzymatic activity within the feeding guilds (Fig. 3A) indicated an increasing trend of cellulase activity from “epigeic animal and microorganism consumers” (EPAM) to “epigeic plant and microorganism consumers” (EPPM). Chitinolytic activity decreased from “euedaphic microorganism consumers” (EUM) to “hemiedaphic microorganism consumers” (HEM). But the more detailed view, including the variability within guilds, showed that EUM represented the most variable group, while variability within EPPM, HEM and EPAM was smaller (Fig. 3B). Enzymatic activity explained by life forms showed a pattern similar to that of the feeding guilds (Fig. 4). Euedaphobionts (Bc) showed higher variability in contrast to hemiedaphobionts (Bb, identical to EPAM guild).

DISCUSSION

Enzymatic equipment in cave-dwelling Collembola

The presence or absence of certain enzymes associated with digestion can reflect the ability of animals to utilize various food sources. The proportions of digestive enzymes may indicate what amount of different food sources may be potentially digested by the animal; however, the potential and actual diet may differ. Quantitative proportions of

Table 3. Enzymatic variability (partial RDA) explained by systematic categories. The significance of the first canonical axis was tested.

F – Fisher statistics, P – significance level, ND – not determined.

Categories	explained variability %	F	P
Order	4.6	ND	
Family	19.3	9.6	0.025
Species	53.0	26.0	0.001
Order + Family + Species	53.8	18.6	0.002

Table 4. Enzymatic variability (partial RDA) explained by ecological categories. The significance of the first canonical axis was tested.

F – Fisher statistics, P – significance level.

Categories	explained variability %	F	P
Feeding guilds	25.1	10.2	0.006
Life forms	14.5	5.7	0.009
Cave forms	35.7	14.9	0.001

Table 5. Enzymatic variability explained by categorical variables and functional traits; variables that explain the highest proportion of enzymatic variability using the forward selection method (FW) are marked. F – Fisher statistics, P – significance level, P (adj) – significance with Holm correction P-values in bold are significant. Significance based on Monte Carlo tests with 9999 randomizations. Covariable: body weight; analysis: interactive forward selection with covariate.

	Explained variability %	F	P	P(adj)	FW selection
Categorical variables					
Cave dwelling – troglonexe	16.3	3.9	0.019	0.235	✓
Cave dwelling - subtroglaphile	54.8	16.6	0.000	0.001	✓
Cave dwelling - eutroglaphile	16.9	4.1	0.014	0.183	
Cave dwelling - troglobiotic	10.6	2.5	0.070	0.630	
Life form – Bb1	2.3	0.5	0.660	1	
Life form – Bc1a	5.6	1.3	0.251	1	
Life form – Bc1b	28.3	7.3	0.001	0.025	
Life form – Bc2a	8.8	2.0	0.098	0.778	
Life form – Bc2b	8.4	1.9	0.110	0.778	
Feeding guilds – epam	2.3	0.5	0.664	1	
Feeding guilds – eppm	16.3	3.9	0.022	0.242	
Feeding guilds – hem	9.2	2.1	0.097	0.778	
Feeding guilds – eum	27.4	5.8	0.002	0.039	
Functional traits					
Body length	13.7	3.3	0.035	0.357	
Proteins (Bradford)	4.0	0.9	0.389	1	
Eyes	2.1	0.5	0.701	1

Fig. 1. Variability of the protein-specific enzymatic activities of digestive enzymes in tested Collembola explained by differences among species. Red triangles indicate centroids. Direct RDA ordination. Cde – *Ceratophysella denticulata*; Fca – *Folsomia candida*; Hni – *Heteromurus nitidus*; Haq – *Hypogastrura aequopilosa*; Mar – *Megaphorura arctica*; Ore – *Orthonychiurus rectopapillatus*; Pja – *Protaphorura janosik*; Psp – *Pseudoscorpionella spelaeus*.

enzyme activity may respond to the actual food more sensitively than qualitative ones.

In the present study, the species collected in caves were examined to reveal their enzymatic equipment in relation to specific conditions of subterranean habitats. Urbášek & Rusek (1994) found only slight differences in the enzymatic activity between Collembola life forms. Similarly, we confirmed the presence of all tested enzymes in body homogenates of the species involved in this study. The enzymatic equipment corresponded to herbo-fungivorous grazers, the most prevalent collembolan feeding guild (Berg et al., 2004). Although all species examined were qualitatively uniform in digestive enzymes, the activities of enzymes exploiting the same substrate

quantitatively differed between species. The quantitative differences of enzymatic activities did not simply follow concentrations of soluble proteins; thus, an effect of the genetically fixed infraspecific proportions of certain digestive enzymes is indicated. This may reflect food specialization determined by evolutionary adaptation or non-genetic regulation of synthesis or activation of different enzymes.

We observed significant differences in cellulolytic and chitinolytic activity between the cave species, confirming the key importance of these enzymes in the indication of their feeding habits. The majority of species displayed very low cellulolytic activity compared to other enzymes. The increasing trend of cellulase activity towards troglaxenes and subtroglaxophiles confirmed our hypothesis. Thus, the degradation of fresh plants and wood is non-relevant for collembolans occupying a deeper cave environment, which is primarily oligotrophic (Culver & Pipan, 2009). Along with cellulase produced in the midgut or by salivary glands, some insects contain cellulases released by symbiotic bacteria (Fisher et al., 2013) or may have horizontally transferred genes for cellulases involved in carbohydrate metabolism and cellulose degradation (Faddeeva-Vakhrusheva et al., 2016). In general, it is difficult to specify whether the enzymes responsible for the breakdown of food items originate from the animal itself or are excreted in the alimentary tract by the microflora when deriving the enzymatic activity from whole-body homogenates (Berg et al., 2004; Fisher et al., 2013). An endogenous cellulolytic protein has been confirmed in only one collembolan species, *Cryptopygus antarcticus* (Song et al., 2017).

Rearing conditions can influence the enzymatic equipment as well, especially quantitatively. Despite the fact that most of the species examined in the present study came from a laboratory culture feeding with yeast, significant interspecific differences were observed. This suggests that these differences are not associated with the current physiological responses in

Fig. 2. Enzymatic activity in Collembola explained by cave guilds (A) and variability within cave guilds (B). Red triangles indicate centroids. Direct RDA ordination. Abbreviations: EU – eutroglophile; SUB – sub-troglophile; TGB – trogllobiont; TX – troglaxene.

Fig. 3. Enzymatic activity of Collembola explained by feeding guilds (A) and variability within particular categories (B). Red triangles indicate centroids. Direct RDA ordination. Abbreviations: EUM – euedaphic microorganisms consumers; EPPM – epigeic plant and microorganisms consumers; EPAM – epigeic animal and microorganisms consumers; HEM – hemiedaphic microorganisms consumers.

relation to artificial food but reflect the species-specific enzymatic equipment. Two natural populations of *P. janosik* did not display any differences from one another in enzymatic activity. In contrast, a distinct pattern in the activity of digestive enzymes was found in comparing natural populations with a long-term laboratory culture reared on substrate from the sampling locality and feeding on yeast. Thus, the enzymatic equipment of the laboratory population characterized by high activity of chitinases probably reflects the artificial yeast-enriched diet. However, only one sample from the culture was available; thus, the increasing trend towards chitinolytic activity in the population feeding on yeast could not be statistically supported. The influence of the above-mentioned factors on enzymatic equipment of the cave collembolans requires further study. Unfortunately,

the difficult access to subterranean environments and mostly dispersed distribution of Collembola specimens in caves make the collection of the material for study of digestive enzymes highly demanding.

Enzymatic activity of Collembola and affinity to cave environment (cave guild)

Trogloxene species *M. arctica* is classified as a euedaphobiotic life form (Rusek, 2007) and an epigeic plant and microorganisms consumer (Potapov et al., 2016). We observed high cellulolytic activity and very low chitinolytic activity in this troglloxene species, which is in accordance with findings of Hodkinson et al. (1994) that it feeds predominantly on living and dead bryophytes, detritus and algal cells.

As to the trogllophiles, both subtrogllophile species, *O. rectopapillatus* and *H. aequipilosa*, differed in the proportion of enzyme activities, indicating variability of feeding preferences inside this cave guild. Generally, subtrogllophiles were established as secondary decomposers based on analyses of stable isotopes (Chahartaghia et al., 2005), grazing on saprophytic, endomycorrhizal and ectomycorrhizal fungi. This corresponds to the relatively high activity of chitinases in *O. rectopapillatus*. On the other hand, we registered low activity of all tested enzymes in *H. aequipilosa*, which is classified as an animal and microorganisms consumer (Potapov et al., 2016). Subtrogllophiles appear to be food generalists rather than specialists (Maraun et al., 2003). This is likely the case of the guild occupying the cave entrances, the habitat enriched by heterogeneous dead organic material colonized with subtrogllophiles.

The category “eutrogllophile” is represented in our study by *F. candida* and *H. nitidus*, both classified also as hemiedaphic microorganism consumers, and *C. denticulata*, also classified as an epigeic plant and animal consumer and fungal feeding secondary decomposer (Ruess et al., 2007; Chahartaghia et al., 2005; Potapov et al., 2016). Such variability in feeding preferences in this cave guild is indicated by

Fig. 4. Enzyme activity of Collembola explained by life forms. Red triangles indicate centroids. Direct RDA ordination. Abbreviations: Bb1 – upper hemiedaphobiont; Bc1a – large euedaphobiont with furca; Bc1b – large euedaphobiont with missing/reduced furca; Bc2a – medium euedaphobiont with furca; Bc2b – medium euedaphobiont with missing/reduced furca.

variable mouthparts morphology and trophic niche preferences (Malcicka et al., 2016; Potapov et al., 2016) and results in significant differences in the enzymatic equipment shown in the recent study.

Troglobionts *P. spelaeus* and *P. janosik* differed in enzymatic activity, which reflects their different food specialization associated with microhabitat selection. *P. spelaeus* is a strict guanobiont, while *P. janosik* occurs in a wider spectrum of cave microhabitats, including cave sediment and rotten wood. Generally, there is a difference in guano-colonizing organisms preferring guano deposits of different age. Raw (acid) guano is colonized by microfungi, while in older guano bacteria dominate (Nováková et al., 2005). The lower chitinolytic and higher trehalolytic activity in *P. spelaeus* suggests its specialized feeding on the cell content of metabolically active fungal hyphae (Berg et al., 2004).

Enzymatic activity in cave-adapted fauna in evolutionary approach

The edaphic lifestyle was proposed as the evolutionary ancestral state of Collembola (D'Haese, 2002). Considering the evolution of cave-adapted fauna from preadapted relatives (Holsinger, 2000) and the lack of primary production in caves, we assumed a shift of enzymatic activity from cellulases in edaphic forms towards chitinases in obligate cave forms. On the other hand, incidental and relatively poor food sources in caves may suggest the evolutionary pressure to maintain the ability to utilize various sources of energy. It is also conceivable that diversification of species generally leads to the colonization of various food niches during their evolution and is associated with more rapid changes in enzyme activities compared to body or mouthparts morphology. In the present study, higher variability was observed at the species level compared to families. This suggests that affiliation to higher taxonomical categories, and thus similar mouthparts morphology as well as general body plan, does not fully designate species to an equal ecological role. This is supported by the different enzymatic activities found in species within the same family. The above-mentioned disproportions between enzymatic activity and available categorizations point to the existence of food-specialists, regardless of the life-form or cave guild.

Enzymatic activity as an adaptive feature in obligate (troglobiotic) cave Collembola is still poorly known. It is necessary to examine a wider spectrum of such species and several populations within each cave-adapted form to investigate interspecific differences in enzymatic equipment as well as differences within the same species. Moreover, the molecular approach, especially identification of genes responsible for production or activation of particular enzymes, could explain the mechanisms leading to different physiology of digestion in subterranean Collembola.

CONCLUSIONS

Activity of all tested enzymes was confirmed across all cave guilds, probably as a result of the evolutionary

pressure to maintain the ability to utilize various food sources. The quantitative differences of enzymatic activities did not simply follow concentrations of soluble proteins; thus, an effect of the genetically fixed infraspecific proportions of certain digestive enzymes is indicated. Cellulolytic and chitinolytic activity showed a key role in the indication of species feeding habits. Different enzymatic activity was found within the same family, supporting the statement that the ecological role of the species is not determined by its taxonomical category. Quantitative interspecific differences were not associated with the current physiological responses in relation to artificial food, thus reflecting the species-specific enzymatic equipment. The study confirmed our hypothesis that there is a shift of enzymatic activity from cellulases in edaphic forms towards chitinases in obligate cave forms of Collembola.

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