Activity of glycogen catabolism enzymes in *Contracaecum rudolphii* (Anisakidae)

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Accepted for publication 30 November 2008

Summary. Extracts of *Contracaecum rudolphii* L3 and L4 larvae, females and males were assayed for carbohydrate content and activities of enzymes that decompose glycogen and disaccharides. The sugar content was about 6-8% of nematode wet weight. In terms of their content, the saccharides assayed could be arranged in the following order: glycogen, glucose and trehalose. All carbohydrate concentrations were greater in females than in males. *C. rudolphii* shows activity of enzymes that decompose glycogen *via* phosphorolysis and hydrolysis. A high activity of glycogen phosphorylase (2.89 mmol mg^{-1}) was recorded in the L3 larvae; the activity observed in the adults was lower by half. The amylase activities in all the forms of the parasite were relatively high and practically the same (0.163-0.175 U mg^{-1} and 0.93-1.03 μmol mg^{-1} for α-amylase and glucoamylase, respectively). Among disaccharidases (maltase, saccharase, lactase), the greatest activity was shown by maltase. It was particularly high (2.67 μmol mg^{-1}) in the L3. The saccharase activity in these larvae was twice that of the adults. Lactase had the lowest activity (0.26-0.49 μmol mg^{-1}).

Key words: Anisakidae, *Contracaecum rudolphii,* enzymes, metabolism of glycogen.

Contracaecum rudolphii is a parasite of piscivorous birds. In Europe, it is most common in the cormorants, sea ducks and gaviforms. The parasite was found to be particularly hazardous for the young cormorants (Kuiken *et al.*, 1999; Torres *et al.*, 2005). The third-stage larvae (L3), developing in intermediate hosts, *i.e.*, fishes of fresh, brackish, and marine waters (Szostakowska & Fagerholm, 2007), may cause economic losses in fisheries (Yang *et al.*, 2000). Studies of Szostakowska & Fagerholm (2007) confirmed the suggestion of Rokicki (2005) and Dziekońska-Rynko & Rokicki (2007) that *C. rudolphii* can complete its life cycle in north-western Poland. Therefore, the metabolism of the parasite merits a detailed study. The available literature contains only a few papers focusing on *C. rudolphii* metabolism (Kračmar *et al.*, 2000; Dziekońska-Rynko & Rokicki, 2005).

Most parasites use carbohydrates as the major energy source (Van Brand, 1979). Among these, glycogen is the major energy-storing material. Glycogen break-down proceeds both *via* phosphorolysis, catalysed by glycogen phosphorylase, and *via* hydrolysis, carried out by α-amylases aided by glucoamylases and other α-glycosidase. Glycogen metabolism in parasitic nematodes is best known from *Ascaris suum*. Enzymes associated with its glycogen synthesis and decomposition had been purified and characterised (Donahue *et al.*, 1981; Yacoub *et al.*, 1983; Ženka & Prokopič, 1984; Żółtowska, 2001a, b). Glycogen metabolism was also described from many other parasitic nematodes, including the fish parasites *Hysterothylacium aduncum, Cystidicola farionis* and *Anisakis simplex* (Żółtowska *et al.*, 2001; 2002; Łopieńska-Biernat *et al.*, 2008). By contrast, not much is known about the glycogen metabolism in the bird gastrointestinal nematode *C. rudolphii*. Our preliminary study made it possible to describe the basic properties of hydrolases associated with carbohydrate metabolism in *C. rudolphii* (Żółtowska *et al.*, 2005). The presence of hydrolases in the secretion/excretion fluid is in agreement with results of the test described by Dziekońska-Rynko & Rokicki (2005). However, no information is available on glycogen phosphorylase, an enzyme involved in the phosphorolytic glycogen decomposition pathway. Therefore, this study aimed to determine activity of this enzyme and to undertake parallel assays of sugar-hydrolysing enzymes. It was decided to link

these tests with analyses of concentration of individual sugars in different growth forms of *C. rudolphii* parasitising the black cormorant in the Masuria (north-western Poland).

MATERIAL AND METHODS

The study was carried out on L3 and fourth-stage larvae (L4) and adults of *Contracaecum rudolphii*. The nematodes were isolated from stomachs of the cormorants (*Phalacrocorax carbo sinensis*) (n = 10) shot in early April 2007 on Lake Bełdany (the Masurian Lake District). The nematodes, cleaned of the cormorant stomach contamination, were rinsed in 0.9% NaCl and divided into age groups based on morphological criteria (Baruš *et al.*, 1978; Moravec, 1994; Bartlet, 1996; Baruš *et al.*, 2000). Prior to the assays, the weighed nematodes were kept at -80°C.

Enzyme extraction. The nematodes were homogenised in a glass Potter homogenizer with 0.9% physiological salt (1 ml salt 100 mg^{-1} nematodes). The homogenate was centrifuged (2000 *g*) for 10 min at 4°C and the supernatant was used for assaying enzyme activity and sugar and protein contents. Protein was determined by method of Bradford (1976).

Sugar assays. Soluble carbohydrates (trehalose, glucose and fructose) were analysed with HPLC in an SCL-10A Shimadzu (Kyoto, Japan) chromatograph equipped with a RID 10A refractometric detector. Separation was carried out at 35°C in a Waters (The Netherlands) High-Performance Carbohydrate cartridge column (4.6 x 250 mm). The mobile phase consisted of 3:2 acetonitryl: water, at 1.0 ml x min^{-1} flow rate. The data were analysed using the Chromax 2005 software (POL-LAB, Warszawa, Poland). The total sugar content was determined with the anthron technique (Mokrasch, 1954). Glycogen was determined with the micromethod of Sølling & Esmann (1975).

Enzyme activity assays. Activity of α-amylase was assayed as described by Caraway (1955) and expressed in U/mg; activities of the glycosidases: glucoamylase, maltase, lactase, and saccharase were determined as in Dahlqvist (1968). Activity of glycogen phosphorylase was measured in terms of glycogen decomposition by determining, with the enzymatic technique of Michal (1984), the amount of glucose-1-phosphate formed (G-1-P). Enzyme activities were expressed in mol G-1-P per mg protein. Enzyme activities were assayed at optimum pH as determined in an earlier study

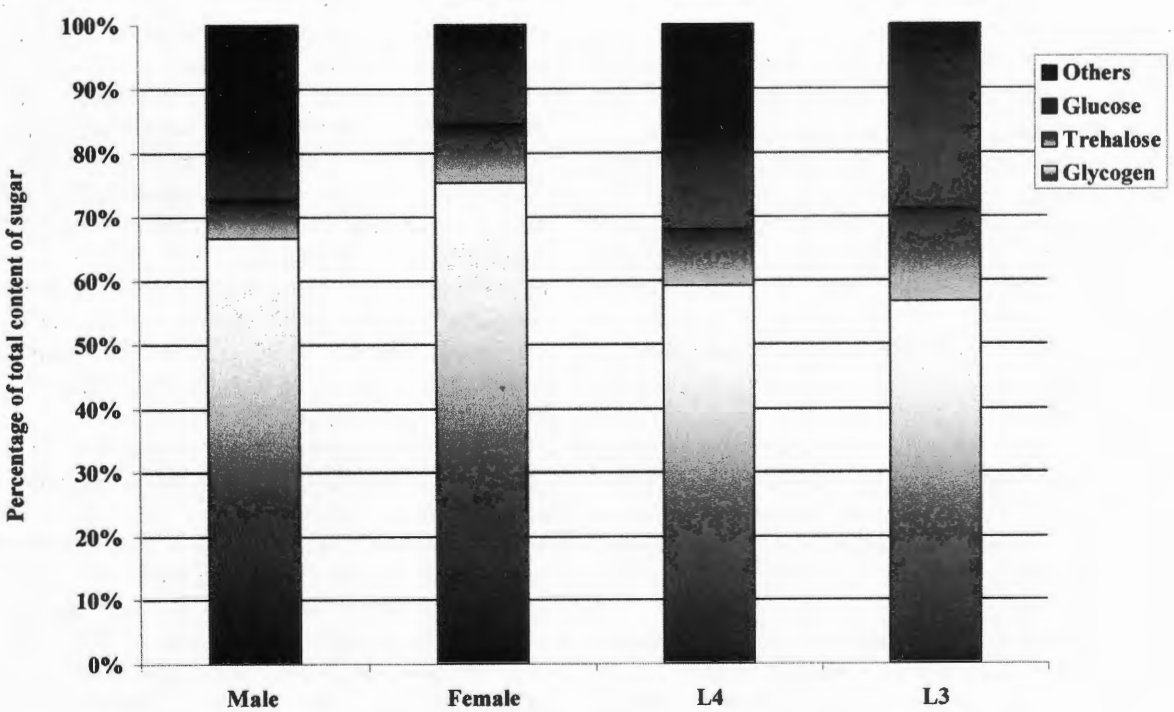

Fig. 1. Percentage contributions of individual sugars to the total carbohydrate content (the total sugar content from Table 1 is assumed as 100%).

(Żółtowska *et al.*, 2005). The following buffers were used: 0.15 M veronal/acetate (pH 6.1 and 6.9 for α-amylase and glycogen phosphorylase, respectively); 0.2 M acetate buffer (pH 3.6 for glucoamylase, pH 5.3 for saccharase, and pH 5.6 for maltase and lactase). The data were subjected to statistical treatment involving analysis of variance (ANOVA) and Tukey's test.

RESULTS

All the ten cormorants were found to be infested by *Contracaecum rudolphii* (100% prevalence). The mean infestation intensity was 35.33 *C. rudolphii* individuals per bird.

The total sugar content varies within 6-8% of wet weight. In terms of their content, the carbohydrates assayed were: glycogen > glucose > trehalose (Table 1). The greatest carbohydrate content was in L4; L3 had the lowest values, which differed significantly from the mean concentrations found in L4 and females (Table 1). The sugar content in females was significantly greater than that in males. A similar pattern was observed with glycogen, a carbohydrate dominant in terms of its content. The glycogen contribution to total carbohydrates was greatest in females (75.35%) and somewhat lower in males (66.11%); in the larvae, glycogen accounted for 59.21 and 56.69% of the carbohydrates in L4 and L3, respectively (Fig. 1). The glycogen level in L3 was significantly lower than that in the adults. The total sugars and glycogen contents in females were significantly higher than that in males. The concentrations of trehalose and glucose in males were lower than those in the other stages (Table 1). It is interesting that males showed trace concentrations of fructose.

Contracaecum rudolphii contains both phosphorolytic and hydrolytic enzymes that break down glycogen (Table 1). A particularly high activity of glycogen phosphorylase was observed in the larvae. Activity in the adults was half that in L3 and was almost identical in males and females. Activities of α-amylase and glucoamylase were relatively high and almost the same in all larvae and adult stages (Table 1). Among disaccharidases, maltase gave the greatest activity (Table 1). It was particularly high in L3, which differed significantly from all the other stages of the parasite. L3 and L4 also had the highest efficiency of sucrose hydrolysis. The activity of this enzyme in L3 was twice that found in the adults. Lactase had the lowest activity among the assayed disaccharidases in all *C. rudolphii* stages (Table 1).

Table 1. Sugar concentrations and activities of glycogen catabolism enzymes and disaccharidases in *Contracaecum rudolphii*.

Stage	Male a	Female b	Larvae	
			L4 c	L3 d
Carbohydrates (mg g^{-1} tissue)				
Total	63.6 ± 1.8[1]	70.2 ± 4.9 ^{a,c}	78.2 ± 4.1	59.8 ± 3.3 ^{a,c}
Glycogen	42.2 ± 1.8	52.9 ± 2.5 ^{a}	46.3 ± 2.9 ^{a,b}	33.9 ± 3.3 ^{a,b,c}
Trehalose	3.9 ± 1.4 ^{c,d}	6.4 ± 2.5	6.9 ± 1.1 ^{a}	8.7 ± 1.8 ^{a}
Glucose	7.5 ± 1.0 ^{b,c,d}	11.9 ± 1.0 ^{a,d}	13.6 ± 1.3 ^{a}	16.6 ± 2.2 ^{a}
Fructose	0.4 ± 0.2	-	-	-
Enzyme activity (μmol mg^{-1})				
Glycogen phosphorylase	1.37 ± 0.50	1.38 ± 0.24	2.06 ± 0.55 ^{a,b}	2.89 ± 0.65 ^{a,b,c}
α-amylase[2]	0.175 ± 0.028	0.163 ± 0.028	0.175 ± 0.012	0.167 ± 0.015
Glucoamylase	0.93 ± 0.08	1.03 ± 0.25	1.03 ± 0.11	1.03 ± 0.08
Maltase	1.72 ± 0.08	1.65 ± 0.07	1.74 ± 0.09	2.67 ± 0.09 ^{a,b,c}
Lactase	0.37 ± 0.07 ^{c}	0.26 ± 0.03 ^{a,c}	0.49 ± 0.05 ^{a,b}	0.33 ± 0.06 ^{b,c}
Saccharase	0.67 ± 0.09	0.62 ± 0.08	0.87 ± 0.14 ^{a,b}	1.26 ± 0.14 ^{a,b,c}

[1]mean ± SD, [2]activity of α-amylase in U mg^{-1}. Letters indicate significant differences ($p<0.05$) between the actual mean and those in columns marked by corresponding letters (a, b, c, d).

DISCUSSION

Parasitological indices determined in this study confirmed that *C. rudolphii* is a common nematode parasite of cormorants in Poland (Kanarek *et al.*, 2002; Kanarek & Rokicki, 2005; Szostakowska & Fagerholm, 2007). All the birds examined were infested with *C. rudolphii*. This also occurred with cormorants from another Masurian lake, the Selment Wielki (Żółtowska *et al.*, 2007).

The two cormorant populations differed in the intensity of infestation; infestation of the Bełdany cormorants (34.7 per bird) was half that in the Selment Wielki (69.4 per bird). This is may be a result of seasonal fluctuations in the parasite population size, as the birds from the Bełdany were shot in spring, whilst those from the Selment Wielki were obtained in late summer of the same year.

As in many other parasitic nematodes, glycogen is the main carbohydrate energy reserve in *C. rudolphii* and accounted for about 60-70% of the carbohydrate pool in adult nematodes and the L4. Glycogen contribution to the carbohydrate pool in L3 was found to be lower (55%), which may be associated with a higher content of trehalose and glucose, hence greater contributions of those two carbohydrates to the total sugar content. An age-dependent increase in the glycogen content had already been reported from some other parasitic gastrointestinal nematodes, *e.g.*, *A. suum, A. simplex* and *H. aduncum*, as well as from *C. farionis*, a fish swim bladder parasite. The increase of glycogen content with age was accompanied by a progressive reduction in the trehalose content. We observed that higher concentrations of trehalose occur in younger rather than in older nematodes (Żółtowska *et al.*, 2001; 2002; Dmitryjuk *et al.*, 2006; Łopieńska-Biernat *et al.*, 2006). It may be that, as nematodes develop, the relationship between glycogen and trehalose metabolism changes. Trehalose synthesis from glucose in nematodes has been described by Behm (1997).

The concentrations of all carbohydrates were greater in *C. rudolphii* females than in males. This is relatively common in helminths, and is associated with higher energy losses incurred by females, resulting from their reproductive function (Van Brand, 1979). We presume that reproductive function may also be invoked to explain the presence of fructose in males. Fructose is an important energy source for vertebrate spermatozoa (Strzeżek, 1998). Unfortunately, knowledge on carbohydrate composition of the invertebrate sperm plasm is very scant (Barnes, 1962).

The higher content of glycogen in the adult *C. rudolphii* could have resulted from a lower activity of glycogen phosphorylase in that stage compared to the larval stages. Due to the high activity of glycogen phosphorylase, glycogen phosphorolysis in the larvae, particularly in L3, has to be very intensive. A similar relationship was reported from *C. farionis* (Żółtowska *et al.*, 2001). A reverse situation was described in *H. aduncum*, the adults of which were more efficient than larvae in phosphorolysing glycogen (Żółtowska *et al.*, 2002).

No such age-dependent variability was observed in the two amylases. Their activities were almost identical in all the age groups examined. The results are similar to *H. aduncum*, where amylase activities in larvae and adults did not differ (Żółtowska *et al.*, 2002). However, of note is the very high α-amylase activity in *C. rudolphii*, 100 times that in *H. aduncum*. This difference is difficult to explain on account of the host's diet.

Analysis of our results may suggest that amylases of *C. rudolphii,* efficiently aided by α-glucosidases and in particular by maltase, supply the parasite's haemolymph with large amounts of glucose. This suggestion seems to be confirmed by the high content of glucose in *C. rudolphii*, which is greater than that of trehalose. The *C. rudolphii* extract also hydrolysed trehalose (Żółtowska *et. al.*, 2007) and saccharose, the weakest hydrolysis occurring in lactose. We presume that this effect results from a low specificity of the parasite's α-glucosidases rather than from the presence of separate enzymes that have a strict substrate specificity. We reported a similar situation from *A. suum* and *A. simplex* (Dmitryjuk & Żółtowska, 2004; Łopieńska-Biernat *et al.*, 2008).

Intensity of carbohydrate metabolism in *C. rudolphii,* with respect to both glycogen and trehalose, described in an earlier paper (Żółtowska *et al.*, 2007), is developmental stage-dependent. Carbohydrate catabolism in the L3 (invasive for birds) is significantly higher than that in any other growth form that appears during the parasite's development in birds.

ACKNOWLEDGEMENT

The study was supported by the Polish Ministry of Science and Higher Education grant No. P04C02428.

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K. Żółtowska, E. Łopieńska-Biernat, J. Rokicki. Активность энзимов катаболизма гликогена у *Contracaecum rudolphii* (Anisakidae).

Резюме. Экстракты личинок третьей и четвертой стадии, самок и самцов *Contracaecum rudolphii* были исследованы на содержание углеводородов и активность энзимов, участвующих в разложении гликогена и дисахаридов. Содержание сахаров было около 6-8% от сырого веса нематод. В порядке убывания содержания сахара могут быть расположены следующим образом: гликоген, глюкоза, трегалоза. Концентрации всех углеводородов были выше у самок, чем у самцов. У *C. rudolphii* была выявлена активность энзимов, разлагающих гликоген в процессе фосфоролиза и гидролиза. Высокая активность гликоген-фосфорилазы (2.89 mmol mg^{-1}) была отмечена у личинок третьей стадии, тогда как активность этого фермента у взрослых нематод была вдвое ниже. Амилазная активность у всех стадий паразитов была сравнительно высокой и мало отличалась у разных стадий (0.163-0.175 U mg^{-1} и 0.93-1.03 μmol mg^{-1} для α-амилазы и глюкозамилазы, соответственно). Среди энзимов метаболизма дисахаридов (мальтаза, сахараза, лактаза), наивысшая активность отмечена для мальтазы, которая была особенно высока у личинок третьей стадии (2.67 μmol mg^{-1}). Сахаразная активность у этих личинок была в два раза выше, чем у взрослых нематод. Лактаза характеризовалась самой низкой активностью (0.26-0.49 μmol mg^{-1}).