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An Electrophoretic Study of Caecal Proteins of Clinostomum Marginatum (Trematoda)

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ABSTRACT – Whole-worm extracts of Clinostomum marginatum were subjected to polyacrylamide gel electrophoresis. Gels of early stage metacercariae, late-stage metacercariae and adult worms contained 26,24, and 27 protein bands respectively. The protein band patterns of the three stages were similar except for 1) a hemoglobin band in gels of adult extracts and 2) a large increase in the quantity of four anodic bands of late-stage gels. The proteins constituting these four bands were present in the caecal contents of late-stage metacercariae. These same proteins were also found in metacercarial cysts as part of an exuded material left behind by the fluke after excystment.

The appearance of the caeca of *Clinostomum marginatum* (syn.*C. schizothoraxi*) metacercariae can vary considerably (Kaw 1950), and consequently has been discounted as a valid taxonomic feature (Pandey and Baugh 1970). Published diagrams of *C. marginatum* metacercariae include those with narrow wrinkled caeca (Osborn 1912, Cort 1913, and Van Cleave and Mueller 1934) and those picturing obviously distended caeca (Amin 1969 and Grabda-Kazubska 1974).

The literature also contains a variety of descriptions regarding the color of *C. marginatum* metacercariae, having been described as being brillant white or yellow (Davis 1965) orange or salmon color (Linton 1911), or grey-white (Reichenback-Klinke and Elkan 1965).

Since the metacercariae of *C. marginatum* are capable of remaining in the encysted state for extended periods of time (Edney 1940), the caecal distension and color changes may be due to an accumulation of material in the caeca. In this study, polyacrylamide gel electrophoresis was used to analyze the proteins of both the whole-worm extracts and caecal contents of *C. marginatum.*

C. marginatum metacercariae were obtained from yellow perch, *Perca flavescens,* which were taken with a half-inch trap net or by angling from Buck Lake (S. 15, T. 147N, R. 32W, Beltrami Co., Minnesota). After removal from the host, metacercariae were either excysted mechanically with watchmakers forceps or allowed to excyst in a pepsin-Ringers -HC1 solution (Fried et al. 1970). After excystation, metacercariae were rinsed three times in cold-blooded Ringers solution and designated as either early-stage or late stage metacercariae based on worm size and the degree of caecal distension.

Adult flukes were taken from pharynges of juvenile Great Blue Herons, *Ardea herodias,* captured in a rookery located two miles from Buck Lake. After extraction, adult flukes were rinsed three times in bird-Ringers solution.

Whole-worm extracts of early-stage, late-stage and adult worms were prepared by homogenizing 300 plus or minus 2 mg of live worms (excess Ringers solution blotted on absorbent tissue) with 1.0 ml of a 0.7% NaCL solution in a precooled glass tissue homogenizer equipped with a motor-driven pestle for two 1-minute periods. The homogenate was centrifuged at 3,000 rpm in an International Centrifuge, Model CS, for 15 minutes and the supernatant was used for electrophoresis.

Samples of caecal contents were obtained from late-stage metacercariae. Each worm was placed in a small spot-plate depression and two or three punctures were made in each caecum with fine needles while viewed under a dissection microscope. Caecal material was then expressed from the caeca with fine needles and collected in a 5-ul micropipette. Control samples were collected from other late-stage metacercariae by making two 1-mm incisions in the region between the worm's caeca. Parenchymal fluids and any excretory material which may have come from severed excretory vessels were expressed from the incisions and collected in a 5-ul micropipette.

Samples of the regurgitant remaining in the cyst after excystment in pepsin-Ringers-HC1 solution were collected from cysts of late-stage metacercariae by the following precedure: Encysted metacercariae were dissected from the musculature of fish so that each cyst was surrounded by a 1-cc block of host tissue. These were placed in individual petri dishes containing 30 ml of a 1% pepsin-Ringers-HC1 solution, pH 2.3, and incubated at 40 C. After excystment, the blocks of tissue were rinsed three times in cold-blooded Ringers. A 5-ul pipette was inserted into the cyst through the site of excystment and the regurgitant was collected.

Polyacrylamide gel electrophoresis was carried out according to Davis (1964) using chemicals from Ames Co. (Division of Miles Laboratories, Elkhart, Indiana). Preparation of sample gels varied depending on the source of protein. Sample gels of caecal materials and their controls were made by layering 10 ul of sample between two 20-ul portions of acrylamide solution. Sample gels of whole-worm extracts and their Versatol serum controls (General Diagnostics, Morris Plains, New Jersey) were mixed with acrylamide solution to yield protein concentrations of 400 ug per gel and 200 ug per gel respectively, as determined by the method of Lowry et al. (1951). Electrophoresis was carried out at 2^{o}-4^{o} C. at 4 mA per tube for the first ten minutes and 2 MA per tube thereafter until separation was complete. The gels

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TABLE I. Mobilities of proteins from C. marginatum whole-worm extracts.

Band #	early stage			late stage			adult		
	%occurrence	E_f(mean)	SD(x 10^{-3})	%occurrence	E_f(mean)	SD(x 10^{-3})	%occurrence	E_f(mean)	SD(x 10^{-3})
1	100%	0.012	6.36	100%	0.012	2.00	100%	0.016	6.35
2	60%	0.028	3.72	40%	0.045	21.59	90%	0.046	6.91
3	100%	0.099	9.60	100%	0.104	5.47	90%	0.077	4.55
4	50%	0.125	9.91	---	---	---	50%	0.116	10.60
5	---	---	---	---	---	---	100%	0.124	7.31
6	100%	0.145	6.56	100%	0.144	4.64	80%	0.134	6.83
7	70%	0.182	6.12	---	---	---	90%	0.181	6.76
8	100%	0.199	6.21	100%	0.198	4.47	100%	0.193	6.00
9	100%	0.241	7.48	90%	0.231	5.45	100%	0.230	6.53
10	100%	0.277	6.34	100%	0.257	4.85	70%	0.241	4.24
11	100%	0.308	7.53	100%	0.299	5.51	100%	0.267	6.20
12	80%	0.336	11.19	90%	0.321	5.22	100%	0.302	5.66
13	100%	0.389	9.51	100%	0.407	6.07	100%	0.371	6.72
14	100%	0.431	7.59	50%	0.432	14.10	100%	0.419	9.64
15	---	---	---	---	---	---	100%	0.480	7.20
16	100%	0.487	9.00	50%	0.484	7.62	---	---	---
17	100%	0.522	8.81	80%	0.521	5.97	---	---	---
18	100%	0.547	11.05	100%	0.554	6.29	100%	0.535	8.38
19	100%	0.594	10.34	100%	0.593	6.80	100%	0.581	7.52
20	100%	0.662	12.76	100%	0.635	4.72	100%	0.636	7.46
21	50%	0.671	18.51	100%	0.674	4.89	100%	0.687	9.61
22	100%	0.710	13.24	100%	0.723	4.92	100%	0.715	10.60
23	100%	0.766	11.04	100%	0.763	5.20	70%	0.742	7.67
24	100%	0.803	10.97	100%	0.798	5.30	100%	0.792	11.40
25	---	---	---	---	---	---	60%	0.819	8.69
26	100%	0.881	11.09	80%	0.846	9.36	100%	0.850	12.60
27	80%	0.932	8.61	20%	0.933	7.78	30%	0.947	10.15
28	60%	0.957	11.96	40%	0.957	6.32	30%	0.971	3.21
29	100%	1.000	0.00	100%	1.000	0.00	100%	1.000	0.00

were stained by boiling in 1% Amido-Schwartz for ten minutes, destained electrophoretically and stored in 7% acetic acid. A Beckman Model 25 spectrophotometer was used to scan the ten best gels of each extract and the mobility (ef) of each protein band relative to the marker dye was calculated from the resulting graphs. Each electropheretic separation consisted of ten experimental gels and two Versatol standards. All separations were done in triplicate to insure their validity.

Morphological Data

The average dimensions of the flukes were 1.88 mm (taken at the posterior testes) x 6.06 mm for early-stage metacercariae, 3.05 mm for adult flukes. Early-stage metacercariae, late-stage metacercariae and adult flukes had average live weights of 3.07 mg, 9.57 mg, and 4.54 mg respectively.

A major morphological difference between the three developmental stages of *Clinostomum marginatum* described in this study is the degree of distension of the caecal branches. Caeca of early-stage metacercariae appear empty and narrow while those of late-stage metacercariae are greatly distended and filled with a yellow material. Caeca of adults are narrow, crenated, and filled with a reddish material.

Results of Polyacrylamide Electrophoresis

Figures 1 (A-C) are composite densiometric tracings of protein band patterns of early, late, and adult stages respectively. A total of 29 different bands appear in the three graphs. Early-stage, late-stage, and adult gels have 26, 24, and 27 protein bands respectively. Of these, 19, 15, and 17 respectively, occurred in 100% of the gels examined.

Figure 1D is a densiometric tracing of the electrophoretic pattern of caecal material collected by puncturing the fluke's caeca. The tracing displays bands 20-23 in the same relative quantities as in tracings of late-stage metacercarial gels. A wide band (E_f = 0.266) and two smaller slow-moving bands occur near the cathodic end of the pattern.

The caecal control (Fig. 1E) has a thin band (E_f = 0.302) near the cathodic end of the pattern and a group of small fast-moving proteins near the anodic end of the gel. This group of fast-moving protein bands does not display the spatial relationship characteristic of bands 20-23 in either of the metacercarial patterns.

Figure 1F is the densiometric tracing of the electrophoretic pattern of material collected from cysts after excystation in pepsin solution. Bands 20-23 are present in their characteristic pattern.

Interpretation of Protein Patterns

The morphological similarities between early and late-stage metacercariae are mirrored in the protein band patterns of the two stages. Bands 1, 3, 6, and 8 were prominent in gel patterns of both metacercarial stages, and the electrophoretic mobilities of the bands were very similar in each case. The most obvious difference between the two metacercarial patterns was the large increase in the densities of bands 20-23 in late-stage metacercariae. The proteins constituting these four bands are found in the caecal branches of late-stage metacercariae (Fig. 1D), and their accumulation appears to correspond to an increase in the quantity of caecal material and the distension of the caeca.

Protein patterns of adult *C. marginatum* extracts contain all 29 different bands except numbers 16 and 17. Bands 20-23 are found in quantities smaller than in either of the metacercarial stages. As these proteins are found as part of an exuded material left in the cyst after excystation (Fig. 1F) the reduction of bands 20-23 in the adult may be the result of disgorgement by the fluke during or shortly after excystation, a phenomenon often observed in *in vitro* conditions during the course of thepresent study.

Bands 5, 15, and 25 are peculiar to adult gels, the former being the most obvious difference between the adult and metacercarial gels. This band was identified as hemoglobin as determined by its characteristic pigmentation and its reaction with benzidine reagent (Sercy 1969). The hemoglobin apparently came from the heron upon which adult flukes actively feed. Both bands 16 and 17 were absent in adult gels and it may be that the relatively large hemoglobin band had a blanketing effect on the two smaller bands (i.e. because of their similar mobilities and the broadness of the hemoglobin band, the proteins constituting bands 16 and 17 though present, may have migrated with hemoglobin as one band).

Previous studies into the composition of caecal contents of *C. marginatum* metacercariae (Osborn 1912 and Smallwood 1914) have not revealed proteinaceous material. This study demonstrates an accumulation and subsequent disgorgement of caecal proteins in *C. marginatum*. The latter action, if a

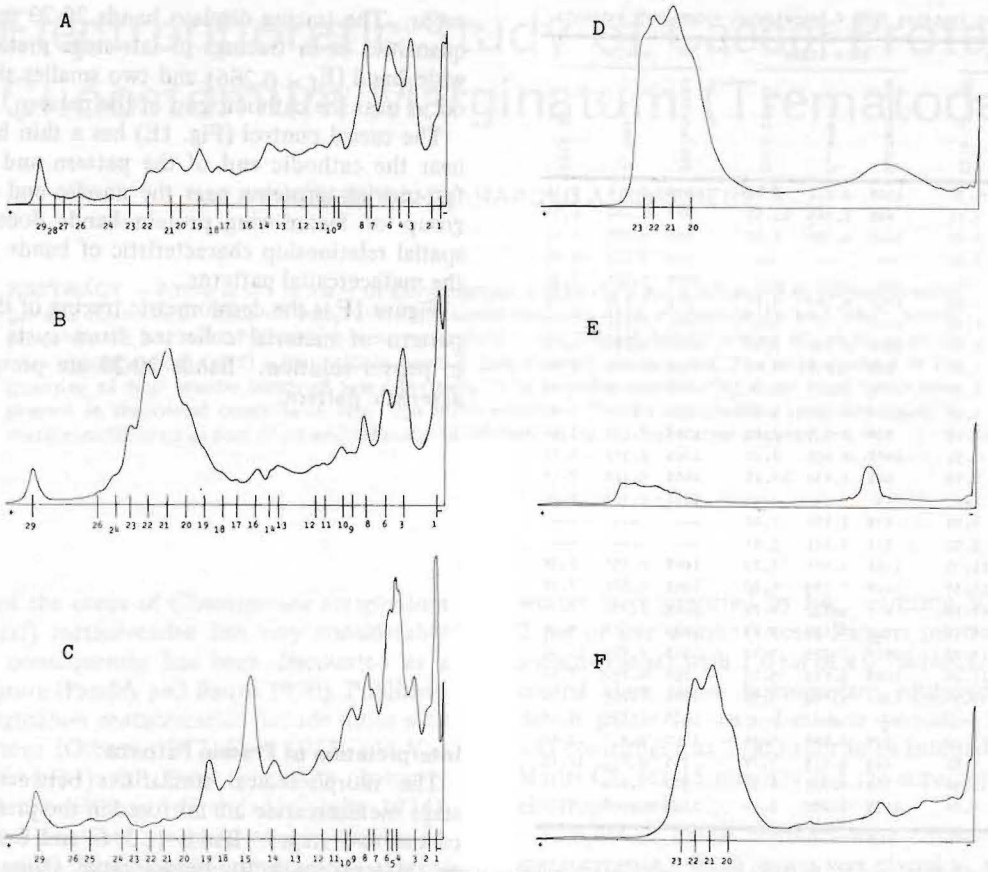

Figure 1. — Densiometric tracings of electrophoretic patterns of *C. marginatum* proteins. Whole-worm extracts of the early-stage metacercariae, late-stage metacercariae and adult (A-C, respectively); caecal material and the caecal control (D and E, respectively); contents of the vacated cyst (F). Protein bands with a percent occurrence of less than 50% are not represented.

natural phenomenon rather than a product of *in vitro* manipulations, may relate significantly to the possible function(s) of these proteins.

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