Research Note

Neutral lipids in cercariae, encysted metacercariae, and rediae of *Echinostoma caproni*

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Abstract

High performance thin-layer chromatography (HPTLC) was used to analyse the neutral lipids in the rediae, cercariae, and encysted metacercariae of *Echinostoma caproni* from *Biomphalaria glabrata* snails. Visual observations of the chromatograms showed that the most abundant lipid fraction in all stages was free sterol. Quantification of the free sterol revealed mean weights of 2.7 ± 0.64 ng per redia, 0.53 ± 0.023 ng per cercaria, and 0.081 ± 0.0098 ng per encysted metacercaria. Oil Red O staining of the larval stages confirmed the presence of lipids within the rediae and cercariae but did not show lipids in the encysted metacercariae. The dimunition in neutral lipids from the cercarial to the encysted metacercarial stage does not support a previous observation that fat increases in successive phases of the digenean life cycle.

Most studies of lipids in the intramolluscan stages of digeneans are based on qualitative histochemical tests. These studies showed variation in the distribution of Oil Red O- or Sudan Black-positive droplets, ranging from organisms (different species of cercariae, metacercariae, sporocysts, and rediae) with extensive lipid positive droplets to those with no or relatively few droplets (Ginetsinskaya, 1988). The function of lipids within the intramolluscan stages is speculative and suggested functions are buoyancy, energy reserves, metabolic waste products, and others.

Few studies have combined the method of high performance thin layer chromatography (HPTLC) with that of lipid histochemistry. The former method allows for distinction of neutral lipid classes with quantification of each class, whereas the latter method usually allows only for localization of lipids as a class.

Ginetsinskaya (1988) observed that the quantity of fat deposited in digenean tissues (specific examples of the organisms studied were not given) increased during successive phases of the life cycle and reached a maximum in the mature adult. Based on that statement, we were interested to see if the quantities of neutral lipids in the encysted metacercariae of an echinostome would be greater than that in the cercariae.

This study was undertaken to analyse by HPTLC the major neutral lipids in cercariae, encysted metacercariae, and rediae of *Echinostoma caproni*. We also used Oil Red O (ORO) histochemistry to examine the localization of lipids in these larval stages.

The life cycle of this echinostome is maintained in our laboratory using *Biomphalaria glabrata* snails and ICR mice (Fried & Huffman, 1996). All larval stages were obtained 8–10 weeks after the *B. glabrata* snails were infected with *E. caproni* miracidia. The rediae were obtained by dissecting them free of the digestive gland–gonad complex (DGG) of the snail. The encysted metacercariae were obtained by dissecting them free of the pericardial/kidney tissue. Cercariae were obtained by isolating snails individually for 2 h in dishes containing 10 ml of artificial spring water (ASW).

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https://doi.org/10.1017/S0022149X00701167 Published online by Cambridge University Press

Each larval stage was prepared separately, counted, and transferred in ASW to 1.5 ml microcentrifuge tubes. Samples were prepared as follows: for rediae, three samples were prepared consisting of 93, 92, and 94 rediae per tube; for metacercariae, three samples were prepared consisting of 226, 250, and 300 per tube, and for cercariae, three samples were prepared containing 71, 70, and 77 per tube. The samples were maintained in the tubes overnight at 4°C to inhibit mobility of the cercariae and allow the organisms to aggregate at the bottom of the tube.

To extract the lipids, the ASW above the larval pellet was removed with a Pasteur pipette and replaced with 1.0 ml of chloroform-methanol (2:1) in each tube. The sample was evaporated to dryness under nitrogen gas, and the lipid residue reconstituted with 25 μl of chloroform-methanol (2:1) measured with a 100 μl Drummond (Broomall, Pennsylvania, USA) digital microdispenser.

HPTLC analysis of neutral lipids was performed on Whatman (Clifton, New Jersey, USA) 10×20 cm LHPKDF silica gel plates containing 19 lanes and a preadsorbent zone. The plates were pre-cleaned by development to the top with dichloromethane-methanol (1:1) and air-dried under a fume hood. For qualitative and quantitative analysis, the standard used was neutral lipid standard 18-4A (Matreya, Pleasant Gap, Pennsylvania, USA), which was diluted in chloroform-methanol (2:1) to contain 0.200 $\mu g\,\mu l^{-1}$ each of cholesteryl oleate, methyl oleate, triolein, oleic acid, and cholesterol. Volumes of 1.0, 2.0, 4.0 and 8.0 μl of the standard and 20.0 μl of the reconstituted sample were spotted on a plate using a 25 μl Drummond digital microdispenser. The standard was also diluted 1:1 and 1:3, and a 1.0 μl aliquot of each of the dilutions was applied to the plates so that the lowest standard weight would bracket the lowest weight of the samples. Plates were developed in paper-lined Camag (Wilmington, North Carolina, USA) twin-trough HPTLC chambers with the Mangold solvent system (petroleum ether: diethyl ether: acetic acid 80:20:1) to a distance of 6.5 cm past the preadsorbent-silica gel interface. After development, the plate was air-dried under a fume hood, sprayed with 5% ethanolic phosphomolybdic acid (PMA), and heated at 110°C for 15 min. Neutral lipid zones appeared as dark blue spots against a yellow background.

Densitometry of the free sterol zones was performed using a CAMAG TLC Scanner II with a tungsten light source set at 700 nm, slit width 4, slit length 4, and scanning rate 4 mm sec^{-1}. The scanner was controlled by a CATS-3 software package, which produces a calibration curve relating the weights of the standard zones and their peak areas. The weights of free sterol in the sample zones were determined automatically from their areas by interpolation from the calibration curve. The weight of free sterol per larval stage was calculated as described in Muller *et al*. (1999).

Qualitative analysis of neutral lipids showed the major lipid fraction to be free sterol ($R_f = 0.21$) in all larval stages, with lesser amounts of triacylglycerols ($R_f = 0.32$) and steryl esters ($R_f = 0.90$) in only the redial samples. Quantitatively, the redia showed the greatest amount of free sterol (ng ± SE) with 2.7 ± 0.64 per redia, followed by 0.53 ± 0.023 per cercaria, and 0.081 ± 0.0098 per encysted metacercaria (n = 3).

Histochemical studies were done on at least ten whole organisms of each stage using Oil Red O (ORO). The material was fixed in cold 10% neutral buffered formalin as described in Fried *et al*. (1998) prior to ORO staining. The results showed the presence of ORO-positive droplets in the anterior region of the main excretory tubules of the cercariae. The droplets were found in close association with the excretory concretions. Encysted metacercariae did not stain with ORO. The rediae showed abundant ORO-positive zones in the subtegument, in the space between cercarial bodies, in the pharynx, and in the intestinal caecum. The HPTLC findings were in close accord with the histochemical findings in that the greatest amount of lipid was found in the rediae and least in the encysted metacercariae.

Muller *et al*. (1999) demonstrated that the cercariae of *Echinoparyphium* sp. had a mean weight of free sterol per cercaria of 0.022 μg, which is over 40 times the values reported for free sterol in the *E. caproni* cercaria. Low numbers of *E. trivolvis* cercariae in the Muller *et al*. (1999) study did not provide enough sample to quantify the weight of free sterol, and therefore those results could not be compared with this study.

Histochemical observations on isolated rediae of *E. caproni* have been reported (Beers *et al*. 1995) and are in accord with the present study. HPTLC analysis in this study is also consistent with that described in Beers *et al*. (1995), which showed free sterol as the most abundant lipid fraction, with trace amounts of steryl esters and triacylglycerols. Absolute weights of lipids were not reported in the Beers *et al*. (1995) study but in this study have been reported.

The function of neutral lipids in the larval stages of *E. caproni* is still unknown. The free sterols, especially cholesterol, probably play a role in cell and tissue structure in all stages. The cercaria showed 6.5 times more free sterol than the encysted metacercaria. The dimunition in neutral lipids from the cercarial to the encysted metacercarial stage does not support the observation of Ginetsinskaya (1988) that fat increases in successive phases of the digenean life cycle.

Major events that occur during the transformation of the cercaria to the encysted metacercaria are the loss of the cercarial tail and release of cystogenous material that will help form the cyst. Some free sterol may be lost following cercarial decaudation. Release of cystogenous material may also result in loss of free sterol or conversion of this lipid fraction into other substances. Clearly, further work is needed to explain the dramatic loss in free sterol content of the encysted metacercaria compared with the cercaria.

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https://doi.org/10.1017/S0022149X00701167 Published online by Cambridge University Press

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(Accepted 28 February 2000)