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Nutrient Composition of *Romanomermis culicivorax* in Relation to Egg Production and Metabolism[1]

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Abstract: The nutrient composition of postparasitic females (newly emerged juveniles, newly molted adults, and spent adults) and eggs of *Romanomermis culicivorax* was studied. Throughout postparasitic development, proteins increased and lipids decreased progressively as a proportion of the dry weight; the proportion of glycogen within the nematodes remained stable. The greatest decrease in the lipid moiety occurred during egg production. Eggs contained relatively low levels of lipids (12% dry weight), and ca. 20% of the dry weight of the eggs was unaccounted for by lipid, protein, and glycogen determinations. Chitin, mucoproteins, and peptides were present in the eggs. The fatty acid composition of nematodes remained constant during postparasitic development; eggs contained a similar profile of fatty acids as postparasites, with marginally higher content of unsaturated fatty acids. Radiotracer studies showed that the eggs could oxidize glucose and palmitic acid.

Key words: chitin, entomogenous nematode, fatty acid, glycogen, lipid, mermithid, mosquito parasite, mucoprotein, peptide, postparasite, protein, *Romanomermis culicivorax*.

The mermithid nematode *Romanomermis culicivorax* has considerable potential for the biocontrol of mosquitoes (20–22). Commercial deployment of the nematode in mosquito abatement programs will not be possible, however, until an in vitro method has been devised for mass producing the nematode's infective stage (6,21).

To establish an in vitro culture system, several studies have been done on the physiology of the parasitic juvenile stages that develop within the hemocoel of the larval culicid (11,12,14). The free-living portion of the life cycle is of longer duration than the parasitic phase and includes all of the processes involved in sexual reproduction. Under our rearing conditions, postparasitic juveniles emerge from *Aedes aegypti* larvae 7–11 days after initiation of infection. Female postparasites molt to adults (ca. 1 week postemergence), mate, and oviposit; oviposition is completed 5 weeks after emergence from the host. Postparasites are nonfeeding, relying for metabolic and developmental requirements upon storage products that are assimilated and incorporated by the parasitic juveniles into the nematode's trophosome.

The physiology of the free-living stages of *R. culicivorax* is incompletely understood. Before entering the pond substratum, postparasites oxidize lipids for energy metabolism (13). Within the pond sediment, metabolism is probably anaerobic (16). Despite the fact that a successful in vitro culture system must provide a milieu conducive to reproduction, the physiology of the adult female nematode has hitherto not been studied in relation to the process of egg development. Changes in the nutrient composition of parasitic juveniles, postparasitic juveniles, and adults were monitored up to a time that corresponded to the commencement of egg production (9,18), but no physiological studies were done on older nematodes or on the egg stage. The purpose of this study was to obtain qualitative and quantitative information on the utilization of nutrient reserves associated with egg development and oviposition and to examine the nature of the metabolism undertaken by the egg stage.

MATERIALS AND METHODS

A laboratory colony of *R. culicivorax* was maintained by propagating the mermithid through newly hatched *A. aegypti* larvae (2). Postparasites were obtained from experimentally infected mosquito larvae, sexed (18), and reared (27 C) in groups of 200 (sex ratio ca. 1 ♀:1 ♂) in petri dishes (9 cm

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d) containing ca. 45 ml autoclaved or millipore-filtered distilled water. Water was sterilized for mosquito and nematode rearing, because a bacterium, pathogenic to the postparasites, was found in the local water supply. The nutrient composition was determined for newly emerged (0–2 days), newly molted (6–8 days), and spent (5 weeks postemergence) females and for 0–3-day-old eggs.

The nutrient composition of the trophosome alone was compared with whole nematodes. Trophosomes were removed (12) from female postparasites, and their fatty acid composition was compared to that of whole nematodes of the same age and sex. Eggs (0–3 days old) were collected and consolidated into 40–100-µl (packed volume) sample sizes by repeated centrifugation. Analytical studies on postparasites and eggs were done using fresh material or samples that had been frozen (−20 C) for no longer than 48 hours. The percentage of oxygen saturation of the water in which the postparasites were reared was determined polarographically with a Clark-type O_2 electrode. Biochemicals used in this study were the purest available commercially and, except where otherwise stated, were purchased from Sigma Chemical Co., St. Louis, Missouri, and B. D. H. Chemicals, Dartmouth, Nova Scotia, Canada. Radioisotopes were obtained from Amersham Corp., Oakville, Ontario, Canada.

For protein determinations, nematodes (n = 20) of predetermined dry weight were homogenized in 2 ml distilled water with a motor-driven ground glass homogenizer, 0.2 ml of sodium deoxycholate (1.5 mg/ml) and 0.2 ml of 72% (w/v) trichloroacetic acid (TCA) were added, and then the homogenate colorimetrically was assayed (19) with a commercially available kit (No. P5656, Sigma Chemical Co.). To effect complete disruption of the egg shells, eggs (3–5 mg dry weight) were homogenized in 2 ml distilled water and sonicated (85 watts; 20 kHz; 30 seconds) with a Braun-Sonic 2000 sonicator (Canlab Laboratory Equipment, Dartmouth, Nova Scotia, Canada) prior to sodium deoxycholate-TCA treatment. Protein concentrations were expressed relative to a bovine serum albumin standard. To determine TCA-soluble peptides in eggs, 1 ml of the supernatant recovered from the TCA-treated homogenate was adjusted to pH 2.2 with 1.5 M lithium hydroxide and diluted with an equal volume of 0.3 M lithium citrate buffer (pH 2.2). Samples (50 µl) of the resultant extract were analyzed for free amino acids on a Beckman Model 121 MB amino acid analyzer; Benson D-X825 resin was used, and a single column three-buffer lithium method was followed (Beckman Bulletin 121 MB-TB-017, Spinco Division of Beckman Instruments, Palo Alto, California). To determine total amino acids, the remaining 1 ml of supernatant resulting from TCA-treatment was subjected to acid hydrolysis under vacuum (24 hours, 110 C) following the addition of 1 ml of 12 N HCl. Amino sugars were determined by adding 1 ml of 8 N HCl to the TCA supernatant and subjecting the solution to acid hydrolysis (8 hours, 100 C). The HCl was removed from amino acid and amino sugar hydrolysates under vacuum, and the dried samples were reconstituted with lithium citrate (total amino acids) or 0.2 M sodium citrate (amino sugars) buffer (pH 2.2) prior to analysis. Acid hydrolysis of TCA-soluble peptides resulted in levels of individual amino acids being increased above the free levels. Analysis of amino sugars was performed at 65 C on a modified Beckman Model 121 amino acid analyzer with a Beckman W2 resin bed (32 × 0.6 cm). The elution buffer was 0.4 M sodium citrate (pH 4.12), and the detection reagent was ninhydrin. Total lipids were quantified gravimetrically (18) after extraction (7) from homogenates (1 ml) of whole nematodes (n = 20) or sonicated eggs (2–5 mg dry weight). Glycogen was extracted from weighed samples of 20 nematodes or 40–50-µl (2–3 mg dry weight) aliquots of eggs by a modification of Steele's procedure (23). Samples were boiled for 20 minutes in 3 ml 30% (w/v) KOH, and glycogen was precipitated from 0.8-ml aliquots of digest with 50 µl saturated Na_2SO_4 and 20 ml 95% ethanol. The samples were vortexed and, after 15–20 minutes, centrifuged (1,000 *g*)

for 5 minutes. After decanting off the supernatant, the glycogen precipitate was colorimetrically assayed (5) by boiling for 10 minutes with 1 ml distilled water and 5 ml anthrone reagent. Absorbance values were related to those obtained from a glycogen standard. To assay eggs for glycoproteins, homogenates (2–4 mg egg dry weight/2 ml distilled water) were prepared and sonicated. Perchloric acid-insoluble glycoproteins and soluble mucoproteins were colorimetrically determined from 0.5-ml aliquots of homogenate using an orcinol reagent and expressing values relative (g equivalents/100 g dry weight) to a galactose–mannose standard (4). Chitin was extracted from 2–4 mg eggs using boiling KOH, then quantified colorimetrically after preparation of the 2′-,5-anhydrohexose derivatives (15). Chitin content was expressed relative to a commercially available chitin standard (from crab shells).

Egg and whole nematode homogenates were prepared for thin-layer chromatography (TLC) separation of carbohydrate fractions. Samples of 50 nematodes were homogenized in 1 ml half-strength Dulbecco's phosphate buffered saline (½ DPBS; Grand Island Biological Co., Grand Island, New York) using a tissue grinder; 80–100 µl eggs (packed volume) were sonicated for 10 minutes in 100 µl ½ DPBS. After centrifugation, 50-µl aliquots of supernatant were spotted on Silica Gel G-coated TLC plates that had been soaked in 0.1 M sodium bisulfite, air dried, and heat activated (30 minutes, 100 C). Chromatographs were developed with ethyl acetate : acetic acid : methanol : distilled water (60:15:15:10), dried, and sprayed with an ethanolic α-naphthol-sulfuric acid visualizing reagent; spots were identified by comparison with pure standards (8).

To prepare samples for separation of fatty acids by gas-liquid chromatography (GLC), 50 whole nematodes, 200 trophosomes, or 100 µl eggs (packed volume) were homogenized in 3 ml ½ DPBS, and lipids were extracted (7) with 1 ml chloroform : methanol (2:1) and 1 ml 9% sodium chloride. After the lower lipid-containing layer was transferred into a transmethylation tube, 0.65 ml chloroform was mixed with the original sample, lipids were re-extracted, and the combined chloroform extract was dried over nitrogen. The fatty acid methyl esters were prepared, and GLC analysis was conducted as described (12), except that hydroquinone was substituted for butylated hydroxytoluene as an antioxidant during the transesterification process.

The nature of the energy metabolism conducted by the eggs was investigated by measuring the $^{14}CO_2$ produced when eggs were incubated with ^{14}C-palmitic acid or ^{14}C-glucose. An egg suspension was prepared by vortexing ca. 40 µl eggs (packed volume) with 10 ml of 0.05 M Tris-HCl buffer (pH 7.0) containing 2,500 units of penicillin G and 2,500 µg streptomycin sulfate. Under aerobic conditions (atmospheric air), 6 µCi [^{14}C-U]glucose (2.8 mCi/mmol) or [^{14}C-U]palmitic acid (403 mCi/mmol) was added to 1 ml egg suspension in 100-ml serum bottles, the $^{14}CO_2$ produced during a 2-hour incubation period (27 C) was trapped in KOH, and associated radioactivity was measured by liquid scintillation counting (13). Controls (to test whether counts recorded were the result of egg metabolism or radioactive contamination) contained all ingredients except eggs and were assayed in parallel with the test bottles. Uptake of the isotope by the eggs was confirmed by centrifuging and washing them consecutively 12 times with 10 ml ½ DPBS. This procedure successfully removed any radioactivity adhering to the external surface of the eggs; 1 ml of the final wash had < 100 cpm above the background level. Washed eggs were placed in scintillation vials containing 0.5 ml NCS tissue solubilizer (Amersham Corp.) and incubated for ca. 1 hour until dissolved. Thirteen milliliters of Aquasol (New England Nuclear Co., Lachine, Quebec, Canada) liquid scintillation cocktail was added to each vial and acidified with 0.5 ml of 1 M HCl.

Results and Discussion

The protein concentration of the post-parasites increased proportionately and

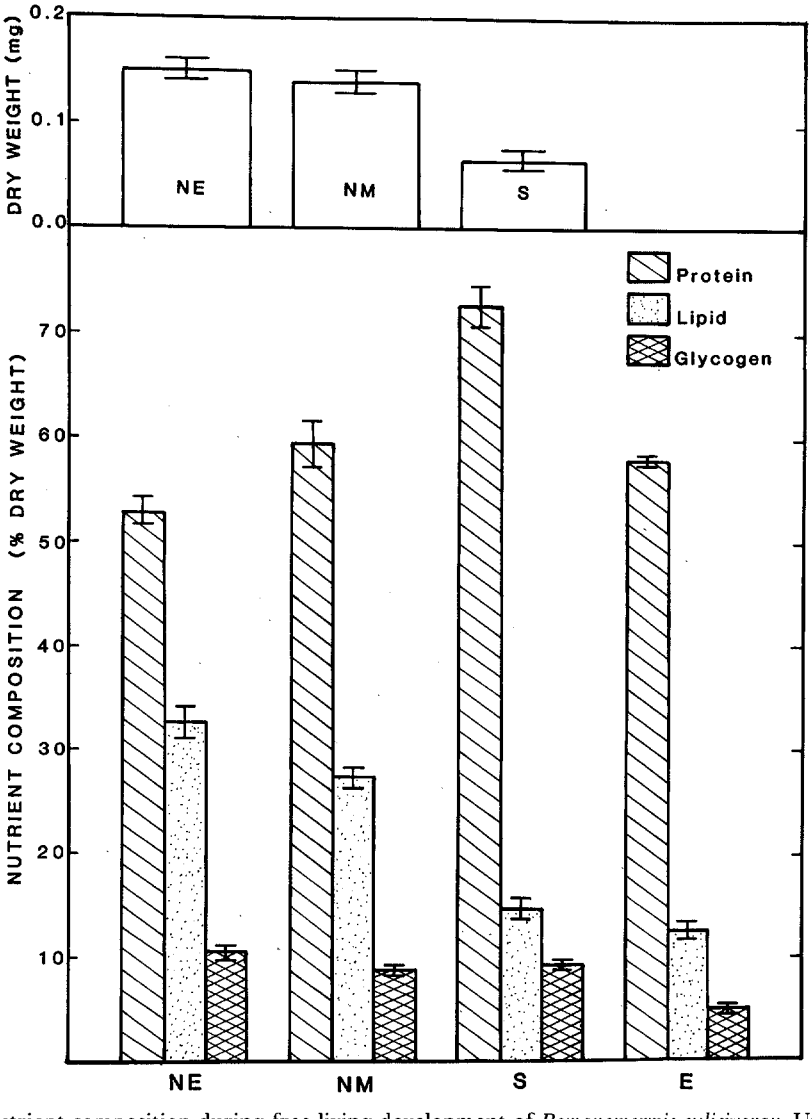

FIG. 1. Nutrient composition during free-living development of *Romanomermis culicivorax*. Upper section shows the dry weight of female nematodes (means ± SE of 7–10 replicates, each comprising 5–30 nematodes). Lower section shows the protein, lipid, and glycogen concentrations (means ± SE of 5–12 determinations) corresponding to each stage of postparasitic development as well as to the egg stage. NE = newly emerged. NM = newly molted. S = spent. E = eggs.

progressively throughout postparasitic development from 52.8 ± 1.3% in newly emerged postparasites to 72.6 ± 2.0% of the dry weight in spent females (Fig. 1). Concomitantly, lipids decreased from 32.5 ± 1.5% to 14.8 ± 1.0%, while the proportion of glycogen remained relatively stable, averaging 9.4% of the dry weight. Measurements of the percentage of oxygen saturation of the water in which the nematodes were reared indicated that aerobic conditions prevailed throughout the course of the study, as the saturation percentage remained stable at 90–95% fully saturated (7.2–7.6 ppm O_2). Thus, our data are at variance with those of Imbriani et al. (18), who found that, under similar oxygen tensions, glycogen increased proportionately at the expense of lipids, whereas protein remained constant during the initial 2 weeks after emergence. A proportionate increase in the protein fraction

is to be expected, because the nematodes develop without feeding and utilize lipid and glycogen reserves for energy metabolism. A net conversion from lipids to glycogen might also be predicted, however, on the basis that *R. culicivorax* postparasites possess the enzymes necessary for a glyoxylate pathway (10). Lipids were not metabolized to other carbohydrates, because the TLC separations showed glycogen to be the predominant form of carbohydrate at all stages of postparasitic development with only traces of trehalose and glucose present. It has been suggested (16–18) that lipid to carbohydrate conversion, following emergence from the host, is of adaptive significance because the nematode is a facultative anaerobe, relying upon anaerobic catabolism of glycogen via a fumarate reductase pathway. Because differences in postparasite rearing conditions (i.e., in parameters other than O_2 tensions) and (or) host species used by previous researchers occurred (18), we assume that the rate of gluconeogenesis by postparasites was counterbalanced in our study by an equivalent rate of glycogenolysis. During the process of egg production, stored lipids were preferentially utilized, as the lipid composition declined by ca. 12% from the newly molted proportion (27.8 ± 0.9%) of the dry weight; the lipid : glycogen ratio declined correspondingly from 3.1:1 to 1.6:1. From analyses of the eggs, it may be inferred that such a severe decline in lipid reserves resulted from utilization of lipids for energy metabolism and (or) conversion to carbohydrates, not from direct incorporation of lipids into the eggs. The proportion of the dry weight of the eggs accounted for by lipids was only 12.6 ± 0.9%, less than in any of the postparasitic juvenile or adult stages examined (Fig. 1). The glycogen content of the eggs (5.0 ± 0.3% of the dry weight) was also relatively low, and TLC separations of egg homogenates showed carbohydrates of lower orders of complexity, viz. trehalose and glucose, to be present in only trace amounts. A major portion, however, of the dry weight of the eggs is accounted for in the structural compo-

TABLE 1. Amino acid concentrations in eggs of *Romanomermis culicivorax*.

Amino acid or ninhydrin positive substance	Concentration (μmoles/g)	
	Free amino acid	Total amino acid
Cysteic acid/phosphoserine	3.28	9.92
Glycerophosphoethanolamine	0.00	0.00
Taurine	4.10	7.16
Phosphoethanolamine	2.10	2.76
Urea	0.00	0.00
Aspartic acid/asparagine	80.88	137.27
Hydroxyproline	0.00	1.09
Methionine sulfoxide	0.00	0.00
Threonine	25.58	58.66
Serine	43.26	97.12
Glutamic acid/glutamine	98.55	144.52
Sarcosine	0.00	0.00
α-Aminoadipic acid	7.72	2.97
Proline	17.78	58.06
Glycine	21.73	84.56
Alanine	46.51	92.20
Citruline	0.39	0.00
α-Amino-n-butyric acid	0.26	0.00
Valine	17.04	41.87
Cystine	0.41	5.13
Homocitruline	0.00	0.00
Methionine	12.60	2.46
Cystathionine	8.43	9.10
Isoleucine	13.00	27.03
Leucine	29.63	55.65
Tyrosine	9.87	19.24
Phenylalanine	17.09	25.65
β-Alanine	0.00	0.00
β-Aminoisobutyric acid	0.00	0.00
Homocystine	0.00	0.00
γ-Aminobutyric acid	2.61	1.94
Tryptophan	2.08	†
Ethanolamine	58.24	53.62
Ammonia	94.14	262.50
Hydroxylysine	0.00	0.44
Ornithine	3.18	8.54
Lysine	34.77	80.36
1-Methylhistidine	0.00	0.49
Histidine	17.45	29.22
3-Methylhistidine	0.34	0.54
Anserine	0.00	0.00
Carnosine	0.00	0.00
Arginine	24.29	41.10
Glutathione	0.00	0.00
Glucosamine	0.00	27.90
Galactosamine	0.00	21.57

All data are based on three replicates of egg homogenates (2–3 mg eggs/2 ml distilled water) and are expressed as μmoles/g dry weight of eggs of free amino acid or (total) amino acid after acid hydrolysis.

† This amino acid is degraded by acid hydrolysis.

TABLE 2. Fatty acid composition of free-living stages of *Romanomermis culicivorax*.

Fatty acid	Trophosomes		Whole nematodes		
	Newly molted	Newly emerged	Newly molted	Spent	Eggs
14:0	2.6	2.4	2.5	2.2	1.8
15:0	1.3	2.1	1.3	0.5	—
16:0	22.7	22.7	23.2	20.2	24.0
16:1	26.7	25.9	25.7	24.1	17.5
18:0	4.1	4.2	4.5	5.0	5.5
18:1	33.0	33.6	34.4	40.8	38.3
18:2	7.9	7.3	6.7	6.2	10.0
18:3	1.7	1.8	1.7	1.0	2.9
20:1	trace	trace	trace	trace	trace
Unsaturation† index	0.81	0.80	0.80	0.80	0.85

Expressed as mol %. Each value is the mean of four replicates of pooled trophosomes (200 trophosomes/replicate), whole nematodes (50 nematodes/replicate), or eggs (100 µl/replicate).

† [1.0 (mol % monoenes)/100] + [2.0 (mol % dienes)/100] + [3.0 (mol % trienes)/100]; from Sumner and Morgan (23).

nents of the egg shell and, possibly, secretions surrounding the eggs. Thus, the combined percentage of unconjugated proteins, lipids and glycogen totaled 95–97% of the dry weight of postparasitic juveniles and adult females, but only 76% of the egg dry weight. Structural carbohydrates included chitin, a polysaccharide component of the egg shells of several nematode species (1,3), which accounted for 3.6 ± 0.1% of the egg dry weight (n = 5). In addition, mucopolysaccharide–protein complexes accounted for 3.1 ± 0.6 g equivalent galactose–mannose/100 g egg dry weight (n = 5). Such conjugated heteropolysaccharides have been identified from the egg shells of a variety of helminths and, while their precise roles have not been established, they may function as lubricants or protectants (3). The characteristic agglutination of *R. culicivorax* eggs into clumps and their associated tendency to adhere to smooth surfaces (e.g., petri dishes) may be caused by the mucoproteins. A relatively high proportion (13.3 ± 1.1 g equivalents galactose–mannose/100 g egg dry weight; n = 5) of the proteins precipitated by TCA were determined to be glycoproteins. Thus, when structural components are considered, the overall carbohydrate content of the eggs is relatively high and the adult female may provide for such requirements by converting lipids to carbohydrates via the glyoxylate cycle (10) and effecting a net depletion of trophosomal lipid reserves. The eggs further contained peptides that were soluble in TCA and, thus, not assayed in the protein fraction. The concentration of all ninhydrin-positive substances, except methionine, citruline, α-aminoadipic acid, α-amino-n-butyric acid, and γ-aminobutyric acid, increased following acid hydrolysis (Table 1). The concentrations of most amino acids increased two to four times because of hydrolysis of TCA-soluble peptides. While further studies are needed to determine the number of peptides present, at least some of the amino acids released by acid hydrolysis are present in mucoprotein(s). Glucosamine and galactosamine, liberated by acid hydrolysis, are common constituents of glyco (and muco) proteins.

The fatty acid composition of trophosomes from newly molted females was the same as that of whole nematodes of the same developmental stage (Table 2). The trophosome almost completely fills the pseudocoelom and accounts for the bulk of the body mass, and the nutrient analyses of whole nematodes are indicative of the trophosomal composition. The fatty acid composition of the postparasites did not change before, during, or after egg pro-

TABLE 3. Uptake of [^{14}C-U]palmitate and [^{14}C-U]-glucose by eggs of *Romanomermis culicivorax* and oxidation to $^{14}CO_2$.

Exogenous source	Isotope absorbed	$^{14}CO_2$
^{14}C-glucose	8.8 ± 1.0	22.4 ± 3.0
Controls for $^{14}CO_2$ emission (glucose)		1.5 ± 0.1†
^{14}C-palmitate	53.3 ± 7.8	72.1 ± 6.8
Controls for $^{14}CO_2$ emission (palmitate)		0.5 ± 0.1†

All values are expressed as counts per minute × 10^3/mg nematode dry weight and are means ± SE of five determinations. Incubation time was 2 hours.

† Such a minimal level of contamination indicates that the counts recorded from the KOH were the result of CO_2 emission by the nematodes.

duction; an array of fatty acids was present with carbon chain-lengths ranging from C14 to C20. The values obtained agreed with those recorded for 0–7-day-old *R. culicivorax* postparasites that had emerged from *C. pipiens* (18), except that C16:1 accounted for a higher, and C18:3 a lower, proportion of the total lipids in postparasites that we reared. The fatty acid composition of the postparasites differed markedly from that recorded for the parasitic stages (12), which comprised substantially higher proportions of C14:0 and lower content of C18:1, indicating the nematode's capacity for fatty acid interconversions. The fatty acid composition of the eggs resembled that of the postparasitic juveniles and adults, except that the egg lipids contained higher proportions of polyunsaturated C18 (C18:2 and C18:3), so that the unsaturation index of the eggs was correspondingly greater. Because a shift toward unsaturated fatty acids in invertebrates is usually associated with cold temperature adaptation (12), the significance of such a marginally higher degree of unsaturation in *R. culicivorax* eggs (cf. postparasites) maintained at 27 C is unclear.

Radioisotope experiments showed that the eggs were capable of absorbing and oxidizing both carbohydrates (glucose) and lipids (palmitic acid) to yield $^{14}CO_2$ (Table 3). Thus, the eggs of *R. culicivorax* would seem to resemble those of *Ascaris lumbricoides* (25) as well as the postparasitic juvenile and adult stages (13) in possessing a functional β-oxidation pathway. Further enzyme analyses would be needed to confirm this point.

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