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In Vivo Growth of *Romanomermis culicivorax*: Biochemical Changes During Parasitism[1]

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Abstract: Biochemical analyses of total protein, lipid, carbohydrate, DNA, amino acid, and length, width, and dry weight measurements are reported for different stages of *Romanomermis culicivorax* cultured in the mosquito, *Culex pipiens.* The Bradford technique for assaying total protein was the most sensitive and reliable biochemical technique tested for assaying in vivo growth of *R. culicivorax.* Increases in total protein, lipid, carbohydrate, and dry weight during growth from preparasite to postparasite were greater than 6,900-fold for females and 2,300-fold for males. DNA increased 650-fold and 233-fold during development to female and male postparasites, respectively. The proportions of amino acids for preparasites were significantly different ($P \leq 0.01$) from female and male postparasites for all amino acids tested, except methionine and tyrosine. Female and male postparasites were similar in protein, lipid, carbohydrate, DNA, and most amino acid proportions, but were significantly different in relative concentrations of serine, glycine, and alanine ($P \leq 0.01$). Preliminary results suggest that the use of amino acid ratios from female postparasites improves the in vitro culture performance of *R. culicivorax.*

Key words: amino acids, carbohydrates, *Culex pipiens,* culture, DNA, lipid, mermithid nematode, physiology, protein, *Romanomermis culicivorax.*

The mermithid nematode *Romanomermis culicivorax* is an obligate endoparasite of the larvae of at least 17 species of mosquitoes (16). It has been studied extensively during the last decade because of its potential as a biological control agent of culicids, many of which are vectors for human disease (17). Attempts have been made to culture *R. culicivorax* apart from its host to decrease the cost of cultivation (4,8,18,22); none have been successful. These efforts are difficult to judge because greatest length and width measurements were used to assay parasite growth. While maximum length and width measurements are important for preliminary screening of parasite performance in test media, a more complete analysis of the average in vitro-produced parasite is needed for meaningful comparison and evaluation of experimental media. Gordon and Burford (11) reported using lipid, protein, and glycogen ratios to assay growth of *R. culicivorax* in vitro, but quantifications of these components were not reported.

We quantified total protein, lipid, carbohydrate, DNA, amino acid composition, length, width, and dry weight of different stages of *R. culicivorax* to establish how these factors change during normal in vivo development in *Culex pipiens.* These data will be used for future comparisons of parasite growth in vitro. Also, amino acid analysis was done on hydrolyzed postparasites of *R. culicivorax* to establish a reasonable amino acid ratio for in vitro culture media.

MATERIALS AND METHODS

Romanomermis culicivorax was maintained in *Culex pipiens* mosquito larvae as described by Platzer and Stirling (19). Preparasites were collected for biochemical studies as follows: 1) A storage pan was flooded for 1 hour with dechlorinated tap water. 2) The water with preparasites was poured through a 250-μm-pore screen to remove large debris. 3) Preparasites were concentrated on a 20-μm nitex filter (HD3-20, Tetko, Inc., Elmsford, New York) supported by a fritted glass funnel. 4) The nitex screen with trapped preparasites was placed in a plastic petri dish (100 × 15 mm) and covered with 2 or 3 ml of 1% low tem-

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perature gelling agarose (Type VII, Sigma Chemical Co., St. Louis, Missouri) at 27 C. 5) The agar was covered with a single layer of paper (Kimwipe, Kimberly-Clark Corp., Neenah, Wisconsin) and cooled at 4–5 C for 1 minute. 6) Agar (30 ml at 27 C) was poured over the filter. 7) Distilled water (3–4 ml) was poured on the surface of the congealed agar, and the plate was placed directly under a fluorescent light for 30 minutes. 8) Preparasites were collected with a plastic serological pipet and dispensed into a plastic millipore filter apparatus with a 3-μm nitex filter (HD3-5). 9) The preparasites were rinsed three times with a total of 250 ml distilled water to remove any agarobiose that might confound the carbohydrate microassay. 10) The clean preparasites were suspended in a measured volume, counted three times in diluted aliquots, dispensed into microfuge tubes in numbers of 2,000–5,000, quick-frozen on dry ice, and lyophilized.

R. culicivorax were dissected from *C. pipiens* larvae at 4 and 6 days postinfection (PI) in *Aedes* saline and placed in a drop of distilled water on a tared aluminum beaker (ca. 14 mg per beaker) on ice. Forty parasites were collected per beaker, frozen at −70 C, and lyophilized.

Postparasites were collected over a 24-hour period as they emerged from hosts starting at day 8 PI. Male and female postparasites were collected separately on day 10 PI, as described by Imbriani et al. (14), and placed in microfuge tubes for lyophilization (20 females or 30 males per tube).

Growth measurements: R. culicivorax for length and width measurements were gently killed over heat, processed into formalin-glycerol (24), drawn with a camera lucida, and measured with a Hewlett Packard 9111A graphics tablet.

Lyophilized samples were stored in a vacuum desiccator containing $CaSO_4$ and weighed in tared aluminum beakers with a Perkin-Elmer AD-2 microbalance. All aluminum beakers used in these experiments were rinsed five times in xylene and dried. An experiment was conducted to determine the error introduced by weighing samples in the open. The microbalance platform, implements, and samples were placed inside a large glove bag (IR^2) with an open container of $CaSO_4$ to equilibrate overnight while dried nitrogen was passed through the enclosure. Samples were weighed and then reweighed in the open room. Weight gains of about 10% were attributed to water gain during weighing in ambient air (average humidity ca. 20%).

Nematode samples for protein quantification were dried and weighed as above and digested in 125–250 μl of 1 N NaOH in a Pierce Reactivial at 60 C with constant stirring until completely digested (ca. 1 hour). The samples were neutralized with an equal volume of 1 M sodium acetate buffer (pH 5.0), and the final volume was measured with an Eppendorf pipetteman (5).

Samples for total carbohydrate, lipid, and DNA analysis were digested with 250–500 μl of a 200 μg/ml proteinase K solution (C. F. Boehringer, Mannheim, W. Germany), as described by Emmons et al. (7). The buffer of Brunk et al. (3) was used to avoid the fluorescence interference that occurs when SDS is present in the buffer. Digestions were run at 60 C for 1 hour.

Protein was estimated in duplicate samples (2) with crystalline bovine albumin fraction V (Sigma Chemical Co.) as the standard. One hundred microliters of sample was added to 900 μl reagent, vortexed, and allowed to stand for 5 minutes before measuring absorbance at 595 nm on a Gilford model 250 spectrophotometer.

Lipid was determined gravimetrically in duplicate samples of digested nematodes (1). Eighty microliters of sample was added to 100 μl chloroform and 200 μl methanol in a glass centrifuge tube with a Hamilton syringe and vortexed for ca. 1 minute. One hundred microliters of chloroform was added, the mixture vortexed, and 100 μl water added. The mixture was centrifuged at 1,000 for 2 minutes, the lower chloroform layer was marked for quantification, and a measured aliquot was taken up and slowly dispensed onto tared aluminum beakers on a heating pad at 60 C. Nitrogen

was blown onto the samples during this drying step. The samples were weighed on a microbalance as described previously.

Total carbohydrate was estimated spectrophotometrically in duplicate samples with the anthrone reagent (15) and a glucose standard series. Sixty microliters of sample was added to 940 μl distilled water, and 6 ml anthrone reagent was added forcefully into a 16 × 150-mm Pyrex tube. The reaction tube was incubated for 45 min at 80 C, and absorbance at 620 nm was determined.

Total DNA was determined with the 4′6-diamidino-2-phenylindole (DAPI) procedure of Brunk et al. (3). The fluorescence that resulted from the DAPI-DNA complex was read on a Farrand FOCI A-4 Florometer with a 7-60 excitation and a 3-73 emission filter. The fluorescence of Brunk et al. (3) buffer (1 ml) containing DAPI (1 μg per ml) was measured. Next, the slope from fluorescence readings of three sequential additions of 15-μl aliquots of proteinase K digested sample was compared with the slope from fluorescence resulting from the addition of three sequential 15-μl aliquots of a purified DNA standard. Total genomic DNA from *R. culicivorax* postparasites was isolated as described by Powers et al. (20) and used as the DNA standard. The concentration of DNA was measured spectrophotometrically at 260 and 280 nm. The 260/280 ratio equaled 1.83, indicating a clean DNA preparation. The DNA standard contained 5.5 μg DNA per ml.

Amino acid analysis: A nematode sample was dry weighed, added to a 10-ml hydrolysis tube containing one drop of liquid phenol (Sigma Chemical Co.) and 1 ml of constant boiling HCl (Pierce Chemical Co.), and sealed rapidly under a stream of nitrogen in an oxygen and gas flame. The hydrolysis was performed at 110 C in a Pierce Reactitherm constant temperature module for 24, 48, or 72 hours. Tubes were opened at the end of hydrolysis and maintained at 37 C for 1 hour while the hydrolysis mixture was blown dry with nitrogen under a fume hood. Dried samples were sent to the Protein Structure Laboratory, University of California, Davis, where they were taken up in a 0.2 M sodium citrate dilution buffer (pH 2.2) with a norleucine internal standard and run on a Durrum D-500 amino acid analyzer or taken up in a 0.2 M lithium citrate buffer (pH 2.2) with an internal standard of amino-B-guanidino proprionic acid and run on a Beckman 6300 amino acid analyzer. All glassware used during hydrolysis was carefully washed in a chromic acid cleaning solution and washed five times in distilled water. Quality control checks followed the above procedure without nematode samples.

Serine and threonine values were extrapolated to time zero with a linear regression equation generated from data from samples hydrolyzed for 24, 48, and 72 hours. Valine and isoleucine were determined from samples hydrolyzed for 72 hours. Cystine and methionine were estimated by performic acid oxidation of the sample prior to a 24-hour hydrolysis and quantified as cysteic acid and methionine sulfone on the amino acid analyzer (13). Tryptophan was not quantified in this study.

Results and Discussion

The biochemical changes and dry weights of in vivo cultured *R. culicivorax* are reported in Table 1. The assays for total lipid and carbohydrate were insensitive for samples of forty 4-day PI parasites and are not reported. Total protein analysis was the most sensitive, convenient, and reliable assay for growth of *R. culicivorax* in vivo. Confirmations of the Bradford protein quantifications were obtained using the sum of amino acids recovered from amino acid analysis of samples from preparasites and postparasites of *R. culicivorax.* The calculated values for total protein recovered from hydrolysis and amino acid analysis were 13.00 ± 3.57 (N = 5) ng protein per preparasite, 23.98 ± 1.83 (N = 6) μg protein per male postparasite (10 days PI) and 71.92 ± 17.39 (N = 6) μg protein per female postparasite (10 days PI). There was a 6,941-fold and 2,309-fold increase in to-

TABLE 1. Biochemical changes of *Romanomermis culicivorax* during in vivo growth in *Culex pipiens.*

Stage,* biochemical component (N)†	Mean ± SD weight per nematode (ng or μg)‡	% of theoretical dry weight§
Preparasite (J2)		
Protein (7)	10.41 ± 2.06	45.4
Lipid (8)	4.42 ± 2.63	19.3
Carbohydrate (6)	7.51 ± 1.12	32.7
DNA (12)	0.60 ± 0.22	2.6
Measured dry weight (9)	28.14 ± 4.38	
Day 4 PI (J3)		
Protein (9)	533.30 ± 196.30	—
Lipid	—	—
Carbohydrate	—	—
DNA (3)	18.53 ± 4.87	—
Measured dry weight (2)	543.33 ± 42.43	
Day 6 PI (J3)		
Protein (6)	13.10 ± 5.52	57.3
Lipid (5)	4.86 ± 2.62	21.3
Carbohydrate (6)	4.72 ± 2.09	20.7
DNA (6)	0.18 ± 0.04	0.7
Measured dry weight (16)	33.60 ± 6.27	
Day 10 PI female (J3)		
Protein (6)	72.26 ± 10.62	43.0
Lipid (4)	39.58 ± 2.87	23.5
Carbohydrate (6)	55.99 ± 18.47	33.3
DNA (6)	0.39 ± 0.07	0.2
Measured dry weight (12)	230.88 ± 43.69	
Day 10 PI male (J3)		
Protein (6)	24.04 ± 3.34	42.7
Lipid (5)	10.28 ± 3.95	18.3
Carbohydrate (6)	21.87 ± 5.81	38.8
DNA (6)	0.14 ± 0.04	0.2
Measured dry weight (12)	74.73 ± 10.08	

See text for description of biochemical analyses for each class of components.

* Preparasite: infective second-stage juveniles of *R. culicivorax* (> 2,000 preparasites per sample) day 0 postinfection (PI). Day 4 PI: third-stage juvenile parasites dissected from *Culex pipiens* day 4 PI (40 parasites per sample). Day 6 PI: third-stage juvenile parasites dissected from *C. pipiens* at day 6 PI (40 parasites per sample). Day 10 PI: female third-stage juvenile postparasites at day 10 PI (20 postparasites per sample). Day 10 PI: male third-stage juvenile postparasites at day 10 PI (30 postparasites per sample).

† (N) = number of samples processed.

‡ Preparasite and parasite (day 4 PI) weights are expressed as ng per nematode, remaining stages as μg per nematode.

§ Mean percentage of a particular biochemical component relative to the mean total recovered protein, lipid, carbohydrate, and DNA.

tal protein content during the growth from preparasite to female and male postparasites, respectively. Increases of the same magnitude were recorded for in vivo growth of *R. culicivorax* for lipid; 8,955 (female), 2,448 (male), carbohydrate; 7,455 (female), 2,912 (male), and dry weight; 8,205 (female), and 2,656 (male) (Table 1).

The differences between the theoretical dry weights (which represent the sum of the weights of total protein, lipid, carbohydrate, and DNA recovered) and the measured dry weights were 19.5% for preparasites, 31.8% for day 6 PI, 27.1% for day 10 PI female, and 24.5% for day 10 PI male *R. culicivorax*. Ten percent of these differences was due to water gained by the samples during the dry weight measurements (see Materials and Methods).

The proportion of protein for all stages

TABLE 2. Lengths and widths of *Romanomermis culicivorax* during in vivo growth in *Culex pipiens*.

Age*	N†	Length (mm) and SD‡	Width (μm)
0	17	1.15 ± 0.05	14.1 ± 5.1
2	17	0.87 ± 0.97	33.5 ± 7.0
4	23	1.61 ± 0.40	76.5 ± 15.3
6	18	8.22 ± 3.26	102.4 ± 50.1
10§	13	20.99 ± 4.84	158.6 ± 15.6
10‖	14	13.24 ± 3.60	124.6 ± 17.6

* Days postinfection.
† N = number of specimens examined.
‡ Standard deviation.
§ All female.
‖ All male.

of *R. culicivorax* was always greater than 40%. There appeared to be a trend for an increase in lipid and decreases in carbohydrate and protein through development, although it was much more variable than reported by Gordon and Burford (11). Imbriani et al. (14) reported that *R. culicivorax* postparasites contained 42% protein, 35% lipid, and 14% carbohydrate on a dry weight basis and that sex ratio did not effect these percentages. Our data (Table 1) support the contention that protein represents about 42% of the male or female postparasite. However, our lipid and carbohydrate quantifications diverged slightly from those reported by Imbriani et al. (14). These differences may be caused by loss of sensitivity in the scaling down of our assays.

Although the DNA relative to the theoretical dry weight of *R. culicivorax* declined from 2.6% to 0.2% through in vivo development (Table 1), the amount of DNA per parasite increased 650-fold for female and 233-fold for male day 10 PI postparasites. This dramatic increase in the total DNA per nematode is not surprising in light of Curran and Webster's (6) report of accretionary somatic cell proliferation during the postembryonic development of *R. culicivorax*. These authors reported a 30-fold increase in the number of somatic nuclei during the parasitic phase (preparasite through day 7 PI). Postembryonic gonadal cell proliferation in *R. culicivorax* was not examined. Thus, the increase in total nuclei during postembryonic development of *R. culicivorax* would be greater than reported by Curran and Webster (6) for somatic cell nuclei. A value of 0.5% DNA for *R. culicivorax* postparasites was obtained using the Burton diphenylamine technique with calf-thymus DNA as a standard (E. Balk, University of California, Riverside, pers. comm.).

In general, length and width measurements followed the same patterns as re-

TABLE 3. Amino acid compositions of *Romanomermis culicivorax* preparasites, male and female postparasites (10 days PI), *Culex pipiens* hemolymph, and Grace's insect tissue culture medium.

Amino acid	Preparasite	Postparasite		*C. pipiens* hemolymph	Grace's medium
		Male	Female		
Asparagine + aspartate	810 a	760 b	757 b	49	611
Hydroxyproline	250 a	84 b	65 b	—	—
Threonine*	407 b	566 a	525 a	240	170
Serine*	541 a	404 c	464 b	2,487	1,207
Glutamate + glutamate	796 b	931 a	960 a	274	947
Proline	586 a	418 b	398 b	648	350
Glycine	1,119 a	640 b	572 c	284	997
Alanine	736 a	489 b	455 c	704	291
Valine†	356 b	417 a	446 a	240	98
Methionine‡	166 a	199 a	180 a	127	39
Isoleucine†	288 b	331 a	338 a	157	44
Leucine	455 b	544 a	555 a	184	66
Tyrosine	168 b	206 b	211 a	748	32
Phenylalanine	196 b	267 a	275 a	119	105
Lysine	504 b	566 a	576 a	151	394
Histidine	123 b	182 a	195 a	1,041	1,857
Arginine	348 b	451 a	461 a	313	383
Cystine‡	173 b	159 a	150 a	—	10
Taurine	—	—	—	56	—

Amino acid composition of hemolymph calculated from Schmidt and Platzer (23), and Grace's medium from Grace (12). Unless otherwise designated N = five samples of preparasites (> 2,000 nematodes per sample) or N = six samples of postparasites (> 20 nematodes per sample) which were each hydrolyzed for 24 hours and analyzed (see text for description).

Data is presented as a mean (nanomoles of designated amino acid per milligram total amino acids). Means followed by different letters in a row were significantly different ($P \leq 0.01$). Data were compared with an ANOVA and means were separated with a Duncan's multiple-range test.

* Linear regression was performed on three samples each for 24-, 48-, and 72-hour hydrolysis and (T = 0) the y-intercept value was reported.

† N = three samples from 72 hour hydrolysis.

‡ N = three samples which were performic acid treated and hydrolyzed for 24 hours.

ported for in vivo growth of *R. culicivorax* (= *Reesimermis nielseni*) by Gordon et al. (10) and Curran and Webster (6) (Table 2). Length appeared to be sigmoidal with significant sex related differences at about day 8 PI. Length vs. time curve comparisons from these three studies (not shown) were shifted on the time axis indicating differences in the age, species, and (or) nutrition of the host larvae used in each case. All of these factors are known to effect the resulting size (10) and dry weight of *R. culicivorax* parasites (9). Width measurements appeared linear relative to time (Table 2) and were close to the values reported by Curran and Webster (6) for *R. culicivorax* reared in *Aedes aegypti.* The slope was much less steep, however, than it was in data for *R. culicivorax* harvested from *A. aegypti* (10).

No amino acids were recovered from the quality control experiment, indicating that the glassware and the chemicals used for hydrolysis were free from protein and (or) amino acid contaminants. The amino acid composition from hydrolysates of preparasites was significantly different ($P \leq 0.01$) from male and female postparasites for all amino acids except methionine and tyrosine (Table 3). Male and female postparasites were quite similar in amino acid composition, differing only in their relative concentrations of serine, glycine, and alanine. The relative amino acid compositions of hemolymph from fourth instars of *C. pipiens* (23) and Grace's insect tissue culture medium (12) are shown in Table 3. It is clear that the composition of both *C. pipiens* hemolymph and Grace's medium (which is based on the amino acid composition of the hemolymph of the silkworm, *Bombyx mori*) are quite different from the relative concentrations recovered from *R. culicivorax* life stages. In fact, more than 75% of the individual amino acid concentrations were divergent in each case. In vitro cultured *R. culicivorax* did not grow when the amino acid ratios from *C. pipiens* hemolymph were substituted for Grace's amino acid ratio (18). Recent work substituting the amino acid ratios for postparasitic females from this study for Grace's amino acid ratio has shown significant improvement of in vitro growth of *R. culicivorax* (Giblin and Platzer, unpubl. data). These findings support the contention that a reasonable starting point for the determination of the proper amino acid ratio for the in vitro culture of an organism is the amino acid ratio from the carcasses of organisms grown in vivo (21).

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