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THE GROWTH OF *MONILIFORMIS DUBIUS* (ACANTHOCEPHALA) IN THE INTESTINE OF MALE RATS

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The helminth *Moniliformis dubius* forms a convenient source of material for studying the biochemistry and physiology of acanthocephalans because it grows to a relatively large size and its life-cycle is easily maintained in the laboratory. The parasite develops in the haemocoele of the cockroach *Periplaneta americana* and sexual maturity is achieved in the small intestine of rats. Biochemical work on helminths, however, will progress slowly until the worms in question have been cultured *in vitro*. One difficulty with culture work is the selection of a trustworthy means of assessing growth. Measurements of the lengths and wet weights are unreliable, and the aim of the work described below was to measure the protein nitrogen, the DNA and the RNA content of *M. dubius* during the course of its infection in rats. These results could then be used to monitor the growth of worms being kept *in vitro* and to form the basis for experimental studies of their growth *in vivo*.

MATERIALS AND METHODS

Moniliformis dubius of known age and sex were obtained from male albino rats (CFHB strain, Carworth, Europe) which weighed 150 ± 10 g on infection with 12 cystacanths. The worms, which were collected between 0900 and 0930 h, were quickly rinsed in Tyrode's saline before the measurements were made.

Extraction and analytical procedure

(1) Worms were minced in 2 ml of ice-cold 0·2N perchloric acid (PCA) and left for 20 min.

(2) The mince was centrifuged. All centrifugation was carried out for 7 min at 5000 rev/min.

(3) The supernatant fluid was discarded and the pellet was broken up in a further 2 ml of 0·2N PCA. During this period of extraction the sample was exposed to ultrasonication for 10 min (Soniprobe, Dawe Instruments Ltd). The tube holding the sample was supported in a bath of crushed ice.

(4) The sample was centrifuged and the supernatant fluid was discarded.

(5) Most of the lipid present in the pellet was removed by extraction for 30 min at 37 °C in 2 ml of a lipid solvent (1 part chloroform, 2 parts ethanol and 2 parts diethyl ether). The sample was centrifuged, the supernatant fluid was discarded and the extraction was repeated.

(6) The pellet was next extracted for 15 min at 70 °C with 0·8 ml 0·5N PCA. The

sample was centrifuged and the supernatant fluid was retained. The process was repeated, the supernatant fluids were combined and the volume was adjusted to 2·0 ml with 0·5N PCA.

(7) The pellet was sealed in an ampoule with a known volume of 30% KOH. The ampoule was heated for 2 h in boiling water and, after cooling, aliquots of the hydrolysate were analysed for nitrogen by a semi-micro method described by Umbreit, Burris & Stauffer (1949). The colour produced was measured at 490 mμ in a spectrophotometer (Beckman, DB) and the results were calculated from standard curves prepared against glycine and ammonium sulphate.

(8) The supernatant fluid from (6) provided two portions to enable DNA and RNA to be estimated. The first portion of 1·5 ml was analysed for deoxyribose by the diphenylamine method of Burton (1955, 1956). The colour produced during the incubation was measured at 600 mμ in a spectrophotometer (Unicam SP 500) and the results were calculated by calibration against weighed amounts of calf thymus DNA (Sigma Chemical Co.). A second portion of 0·25 ml of the supernatant fluid was analysed for ribose by the method described by Umbreit, Burris & Stauffer (1949). The orcinol used was recrystallized by refluxing with benzene in the presence of activated charcoal (Technicon, 1967). After incubation of the sample with orcinol, but before measuring the green colour at 660 mμ in a spectrophotometer (Unicam SP 500), the sample was clarified by centrifugation. The results were calculated from a standard curve prepared against ribose. Known samples of glycine and ribose were analysed to check the methods whenever nitrogen and RNA were measured. Blank samples, containing all reagents except the test materials, were prepared for use in the spectrophotometer on every occasion.

Measurements on young worms and on male worms had to be made on groups of worms varying from 26 to 2 worms per group, while measurements on older worms were made on individuals. The results were expressed as mg nitrogen, μg DNA and mg RNA per worm, and as μg DNA and mg RNA per mg nitrogen. The mean values for each substance were calculated from all the data obtained for worms of a given sex and age. The number of determinations made can be seen from Figs. 1–3, where each point represents a measurement from a group or an individual worm. The data shown in Table 1 are the computed weighted means and standard deviations for worms measured at weekly intervals; the weighting was necessary because of the unequal numbers of worms in the groups. In Fig. 1 many nitrogen values are plotted in addition to those obtained from the worms used for the DNA and RNA values.

Comments on the reliability and accuracy of the measurements

Trial determinations were made on known quantities of bovine serum albumin, calf thymus DNA and fresh rat liver to test the accuracy of the methods for nitrogen, DNA and RNA respectively. The results showed that approximately 91·5% of the albumin and 92·8% of the DNA had been detected, and the average value of 1·091 mg RNA per 100 mg of fresh rat liver was close to the figure tabulated by Kosterlitz (1961) in the *Biochemists' Handbook*.

Ultrasonication was introduced into the procedure to shatter the tough egg shells and to enable the nucleic acids to be extracted completely so that reproducible results could be obtained. A large sample of eggs of the same age was divided into several

equal portions and each portion was subjected to a given period of sonication before the DNA was measured. It was found that constant results were obtained after 10 min treatment; further sonication had no apparent effect. The need for sonication is illustrated by the fact that 2·13 μg of DNA were detected in eggs which had not been sonicated and 58·98 μg. were detected in an identical batch of eggs which had been sonicated for 10 min.

Lipid extraction proved to be necessary for two reasons. First, without lipid extraction, a green rather than a blue colour was produced in the diphenylamine reaction, and secondly, many small brown particles contaminated the sample after incubation with orcinol. A few of these particles persisted in spite of lipid extraction, and the samples were centrifuged before being examined in the spectrophotometer. It is not clear whether lipid was solely responsible for the brown particles. Some carbohydrates are known to interfere with the orcinol reaction (Fleck & Munro, 1962) and it is possible that traces of trehalose, an important sugar in *M. dubius* (Fairbairn, 1958), could have the same effect. Model experiments were carried out in which ribose was incubated with orcinol in the presence of either sucrose or trehalose. Brown particles appeared in both cases.

It must be emphasized that the orcinol method provides a very indirect means of measuring RNA, since only the ribose associated with the purine bases of the nucleic acid reacts with the orcinol. This ribose forms about 20 % of a typical RNA molecule. All values calculated for ribose from measurements made in the spectrophotometer were multiplied by five to give the final estimate of RNA. Since all the material has been subjected to a standardized procedure, the measurements presented in this paper permit reliable comparisons to be made between worms of known age. At present, it would be unwise to assume that the measurements of nitrogen, DNA and RNA reflect the exact amounts present in the worms.

RESULTS

The results for male and female worms are shown in graphical form in Figs. 1–3 and the weighted means and standard deviations are given in Table 1. The amounts of DNA and RNA per mg nitrogen are also listed in the table.

The amounts of nitrogen, which can be assumed to be associated with bound protein, are similar for both male and female worms for the first 3 weeks of the infection (Fig. 1). Copulation takes place at this time and it can be seen that the nitrogen content of the fertilized females increases at a greater rate than that of the males. By the time the female worms are 6 weeks old they have a mean nitrogen content of about 2·8 mg, compared with about 0·6 mg for the male worms. The increase in the amount of nitrogen in the females probably represents not only the early phase of egg production but also the growth of the worms' bodies to accommodate the developing eggs. The female worms eventually grow to achieve a mean nitrogen content of about 7 mg, while the male worms, which do not live as long as the females, reach a mean nitrogen content of about 1·2 mg.

A similar divergence between the amounts of DNA in the male and female worms can be seen after the fourth week of the infection (Fig. 2). There is less difference in DNA content, however, between some of the females and some of the males after the

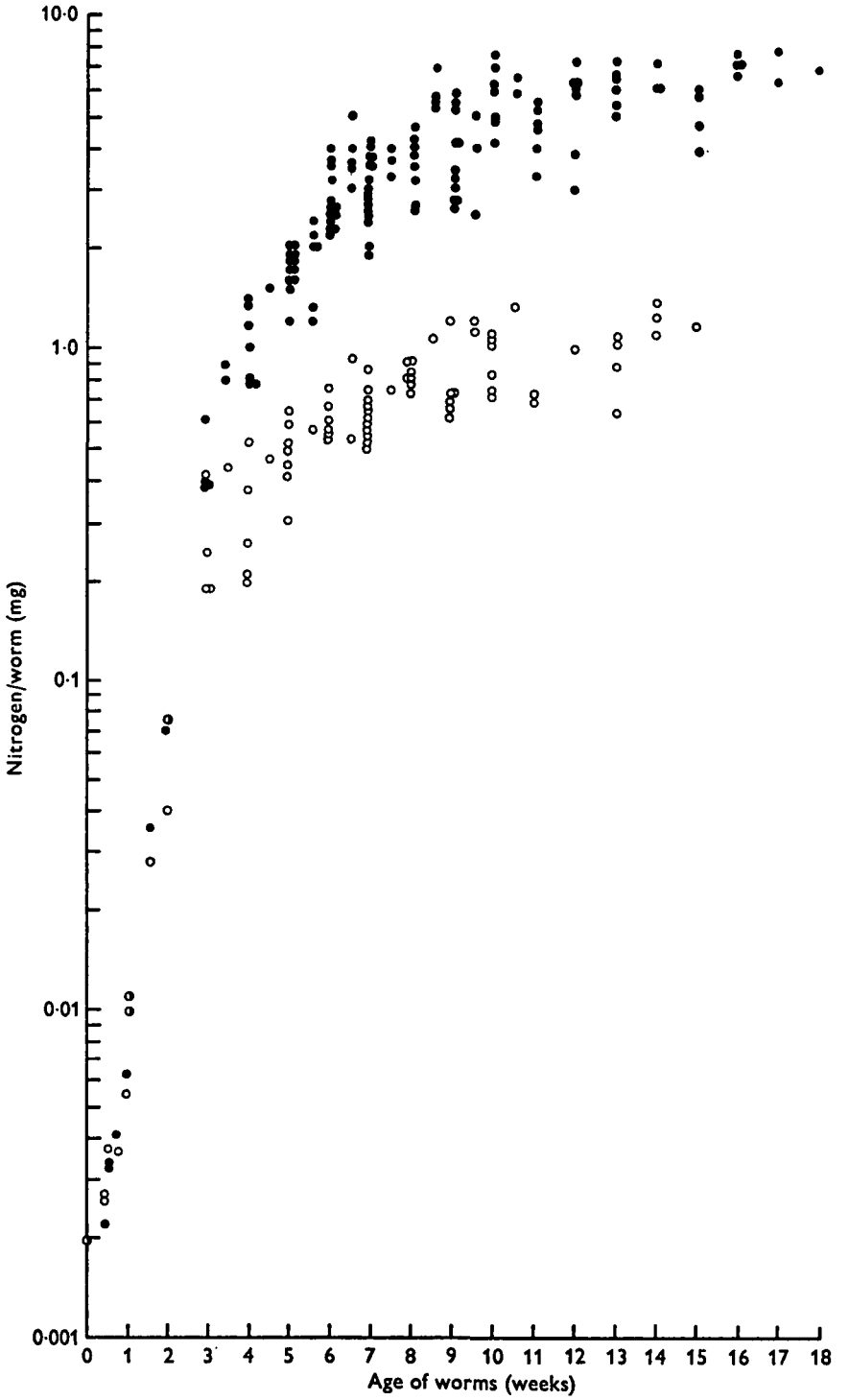

Fig. 1. Graph of the nitrogen content (mg) of *Moniliformis dubius* plotted against the age of the worms (weeks). ○, Male worms; ●, female worms; ◑, worms whose sex was not determined. Points on the ordinate represent values from cystacanths.

tenth week of the infection. This finding cannot be explained unless it results from measurements made on females which had just released a large number of eggs. The pattern for the amounts of RNA in the male and female worms (Fig. 3) is very similar to that for the nitrogen (Fig. 1), but the mean amounts of RNA in the females appear to vary more during the course of the infection than those of the males (Table 1).

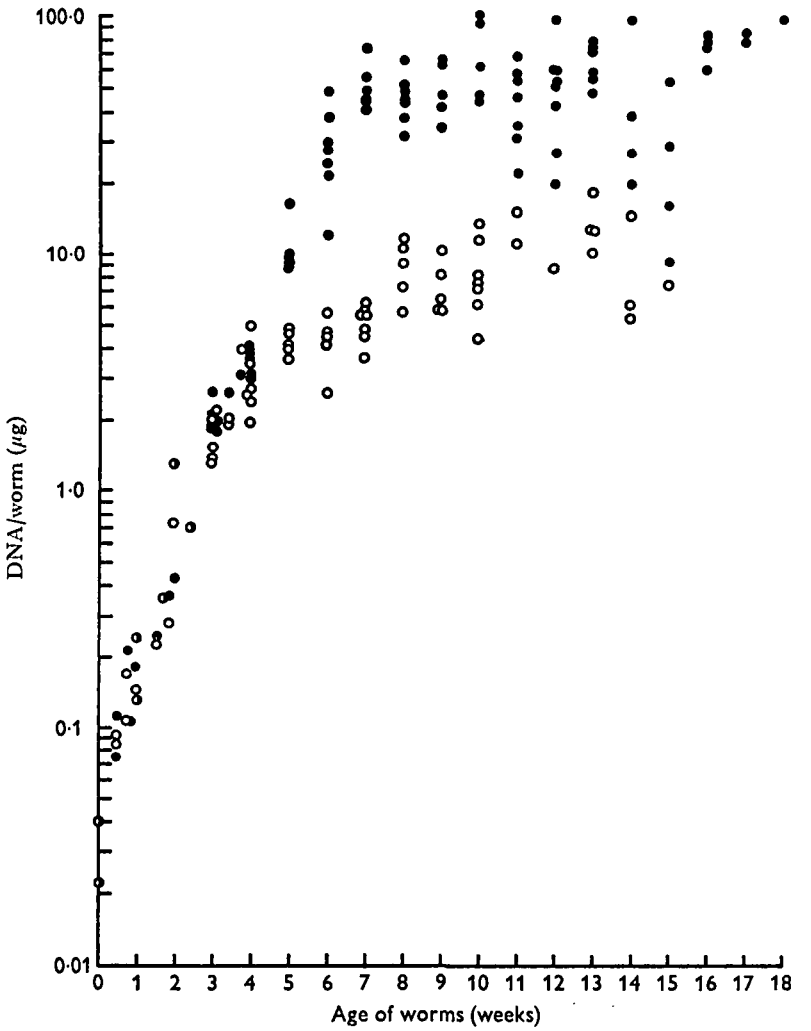

Fig. 2. Graph of the DNA content (μg) of *Moniliformis dubius* plotted against the age of the worms (weeks). ○, Male worms; ●, female worms; ◑, worms whose sex was not determined. Points on the ordinate represent values from cystacanths.

The fact that Figs. 1–3 are semi-log plots disguises the considerable individual variation in the amounts measured from the female worms. Much of this variation was assumed to be related to the release of eggs, although the time of mating could also influence the results. It was therefore decided to make preliminary measurements of the amounts of nitrogen, DNA and RNA in the eggs and in the body tissues of the worms which contained the eggs. The results, which are shown in Fig. 4 and Table 2,

are slightly high in the case of eggs, since the worm's efferent duct system was not separated from the eggs when these were collected. From the sixth week onwards until the end of the infection about 72% of the nitrogen is to be found in the body tissues and 28% in the eggs. Similarly, about 20% of the DNA and 62% of the RNA are contained in the bodies and 80% of the DNA and 38% of the RNA in the eggs.

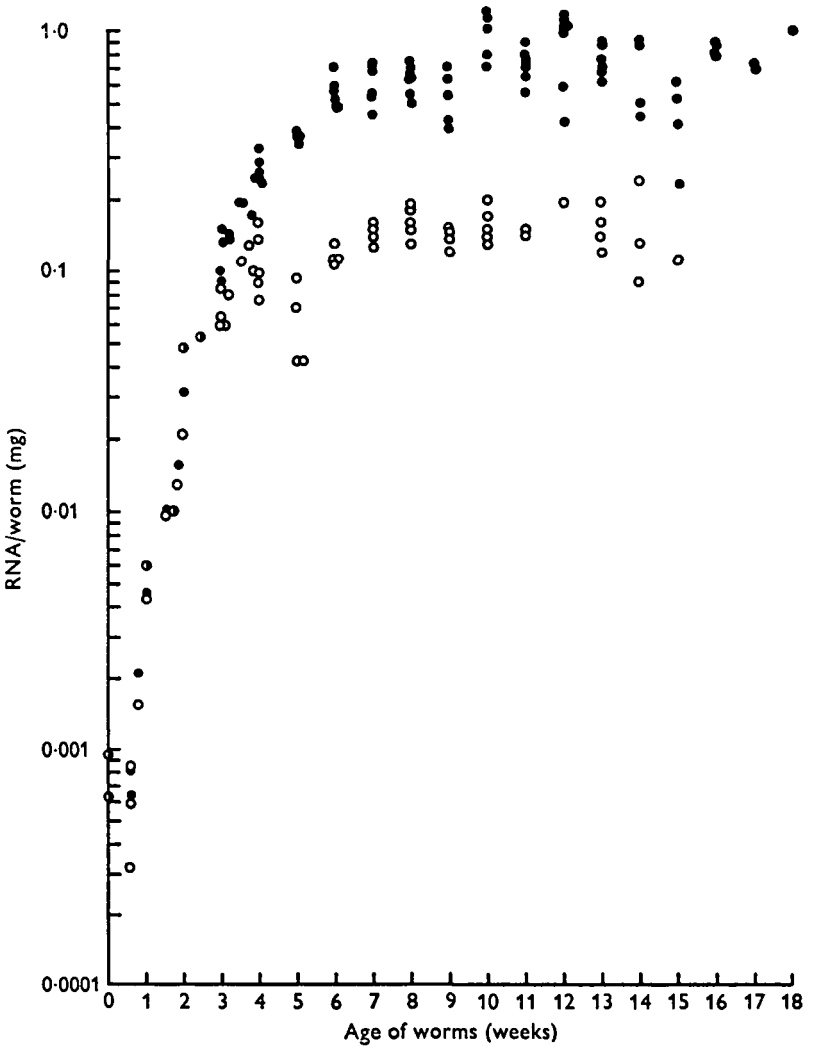

Fig. 3. Graph of the RNA content (mg) of *Moniliformis dubius* plotted against the age of the worms (weeks). O, Male worms; ●, female worms; ◑, worms whose sex was not determined. Points on the ordinate represent values from cystacanths.

Some estimate of the individual variation can be seen from the range of values for whole worms given in Table 2. The range of values for the nitrogen content of fertilized females cannot be explained in terms of egg release since the eggs account for only 28% of the nitrogen. The same argument applies to the range of RNA values, although egg release will have more effect. The amounts of DNA in the eggs could,

however, affect the range of values detected for this substance. The conclusion is that a natural large variation exists in the growth and size of fertilized worms.

Much circumstantial evidence could be cited to support the view that the values obtained from the diphenylamine reaction correspond to DNA. For example, the higher values are associated with the eggs in which relatively high concentrations of DNA would be expected (Table 2). The diphenylamine reaction, however, depends

Table 1. *The mean amounts of nitrogen, DNA and RNA in* Moniliformis dubius *growing in male rats*

		Males					Females				
Age (weeks)		N (mg)	DNA (μg)	RNA (mg)	μg DNA/mg N	μg RNA/mg N	N (mg)	DNA (μg)	RNA (mg)	μg DNA/mg N	μg RNA/mg N
1	Mean	0·0055	0·1470	0·0044	26·7300	0·8000 (22)	0·0062	0·1810	0·0045	29·1900	0·7257 (26)
	S.D.	0	0	0	0	0	0	0	0	0	0
2	Mean	0·0405	0·7200	0·0210	17·7700	0·5184 (20)	0·0690	0·4300	0·0310	6·2470	0·4493 (24)
	S.D.	0	0	0	0	0	0	0	0	0	0
3	Mean	0·2805	1·6010	0·0688	6·0877	0·2639 (30)	0·4373	2·0463	0·1154	4·8193	0·2740 (30)
	S.D.	0·0582	0·1742	0·0059	0·7257	0·0330	0·0519	0·1472	0·0135	0·6310	0·0223
4	Mean	0·3002	2·7622	0·1104	10·4739	0·3698 (23)	1·0327	3·7400	0·2614	3·7820	0·2575 (20)
	S.D.	0·0551	0·2452	0·0144	1·7573	0·0179	0·0974	0·2189	0·0132	0·4465	0·0161
5	Mean	0·5065	4·9173	0·0652	8·8188	0·1482 (15)	1·6934	10·3369	0·3589	6·2392	0·2139 (13)
	S.D.	0·0433	0·8323	0·0129	1·1194	0·0217	0·0635	1·2788	0·0051	0·9947	0·0065
6	Mean	0·6098	4·2975	0·1141	7·1063	0·1965 (16)	2·7829	26·2150	0·5277	10·0780	0·1959 (10)
	S.D.	0·0322	0·4731	0·0049	0·9641	0·0115	0·1357	4·1902	0·0278	1·6253	0·0079
7	Mean	0·6380	5·1867	0·1433	7·9233	0·1922 (27)	2·9757	49·7057	0·5884	16·5000	0·1806 (7)
	S.D.	0·0312	0·2732	0·0076	0·4472	0·0189	0·1523	5·0132	0·0459	1·1568	0·0084
8	Mean	0·8246	9·4400	0·1627	10·1092	0·1983 (14)	3·7292	42·6330	0·6219	10·6300	0·1501 (10)
	S.D.	0·0275	1·1692	0·0107	0·7214	0·0073	0·2382	3·8602	0·0309	1·3903	0·0141
9	Mean	0·8231	7·1376	0·1380	9·1500	0·1808 (17)	4·0300	49·8800	0·5334	15·4600	0·1660 (5)
	S.D.	0·1019	0·8596	0·0069	1·6983	0·0145	0·3576	6·0782	0·0591	1·1201	0·0100
10	Mean	1·0235	7·6960	0·1538	8·1680	0·1765 (20)	5·8788	66·0283	0·9283	10·6867	0·1507 (6)
	S.D.	0·0749	1·1176	0·0122	1·2115	0·0044	0·4437	12·2242	0·0877	1·6195	0·0074
11	Mean	0·7120	12·7100	0·1402	17·7800	0·1970 (5)	4·5417	44·3900	0·7200	9·3500	0·1540 (7)
	S.D.	0·0147	1·9351	0·0064	2·3025	0·0049	0·3288	6·0585	0·0424	1·7964	0·0088
12	Mean	1·0700	8·8900	0·1950	8·3080	0·1823 (2)	5·2922	47·1011	0·8623	8·8333	0·1604 (9)
	S.D.	0	0	0	0	0	0·5665	8·7187	0·1069	1·2690	0·0054
13	Mean	0·9168	13·5225	0·1520	14·8750	0·1685 (8)	6·0717	63·7833	0·7525	10·6150	0·1250 (6)
	S.D.	0·1018	1·7482	0·0164	1·2284	0·0136	0·3250	5·0783	0·0422	0·8631	0·0075
14	Mean	1·2499	9·6543	0·1619	7·7138	0·1350 (7)	6·3433	45·3150	0·6748	8·6500	0·1187 (4)
	S.D.	0·0879	3·2175	0·0446	3·1795	0·0459	0·3944	17·6152	0·1201	3·7630	0·0183
15	Mean	1·1800	7·4100	0·1090	6·2790	0·0924 (1)	5·0275	26·5300	0·4390	4·9125	0·0870 (4)
	S.D.	0	0	0	0	0	0·4863	9·5502	0·0791	1·3953	0·0067
16	Mean	—	—	—	—	—	6·9350	73·5275	0·8738	10·5500	0·1258 (4)
	S.D.	—	—	—	—	—	0·2394	4·9883	0·0497	0·5315	0·0040
17	Mean	—	—	—	—	—	7·0100	81·2000	0·8030	11·6500	0·1140 (2)
	S.D.	—	—	—	—	—	0·7200	3·6400	0·1170	1·8500	0·0050
18	Mean	—	—	—	—	—	6·8300	96·9600	1·0160	14·2100	0·1487 (1)
	S.D.	—	—	—	—	—	0	0	0	0	0

Figures in parentheses show the number of worms used in the determinations of DNA and RNA.

Table 2. *The amounts of nitrogen, DNA and RNA in the bodies and body contents (eggs) of fertilized female* Moniliformis dubius *growing in male rats*

Age of worms (weeks)	Nitrogen (mg)				DNA (μg)				RNA (mg)				No. of worms used
	Body	Eggs	Total	Range for whole worms*	Body	Eggs	Total	Range for whole worms*	Body	Eggs	Total	Range for whole worms*	
5	1·150	0·210	1·360	1·160–2·000	4·486	11·130	15·616	8·890– 16·290	0·223	0·078	0·301	0·343–0·375	3
6	1·836	0·622	2·458	2·200–4·050	5·120	20·470	25·590	12·000– 47·900	0·265	0·140	0·405	0·473–0·702	3
7	2·457	1·152	3·609	1·920–4·200	8·480	53·930	62·410	41·050– 74·340	0·370	0·270	0·640	0·439–0·718	4
8	3·450	1·427	4·877	2·600–4·650	8·480	58·980	67·460	31·510– 64·370	0·452	0·349	0·801	0·499–0·733	2
9	2·260	1·013	3·273	2·680–5·800	11·710	58·710	70·420	34·210– 64·640	0·304	0·234	0·538	0·390–0·702	2
10	3·005	1·427	4·432	4·180–7·660	11·980	41·740	53·720	43·900–104·500	0·378	0·218	0·596	0·710–1·141	2
11	2·995	1·653	4·648	3·330–5·530	12·905	52·450	65·355	21·820– 67·330	0·472	0·281	0·753	0·546–0·905	4
12	4·090	1·542	5·632	2·955–7·200	14·560	61·270	75·830	19·660– 96·960	0·398	0·339	0·737	0·421–1·141	2
13	4·025	1·260	5·285	4·980–7·130	14·140	57·100	71·240	47·130– 76·760	0·425	0·260	0·685	0·624–0·874	2
14	4·780	2·013	6·793	5·900–7·130	36·900	101·270	138·170	19·930– 96·960	0·601	0·398	0·999	0·437–0·889	1
15	4·780	2·013	6·793	3·860–6·000	17·780	79·990	97·770	9·160– 52·520	0·953	0·468	1·421	0·234–0·601	1

* From the results used to construct Figs. 1–3.

on the deoxyribose present and not on the characteristic bases of the molecule. Consequently, a chromatographic analysis was made of material extracted from the eggs to obtain direct evidence for the presence of DNA.

Eggs were collected from nine, 110-day-old, fertilized females and the extraction described above was completed as far as stage (4). The pellet was then digested overnight at 37 °C with N-NaOH to remove the RNA, and the DNA was precipitated with ice-cold 0·5N PCA. The precipitate was resuspended, washed with ice-cold 0·5N PCA and centrifuged. This procedure was carried out three times and the orcinol reaction was performed on a sample of the final supernatant fluid. No ribose was detected – a result which was assumed to indicate the removal of RNA.

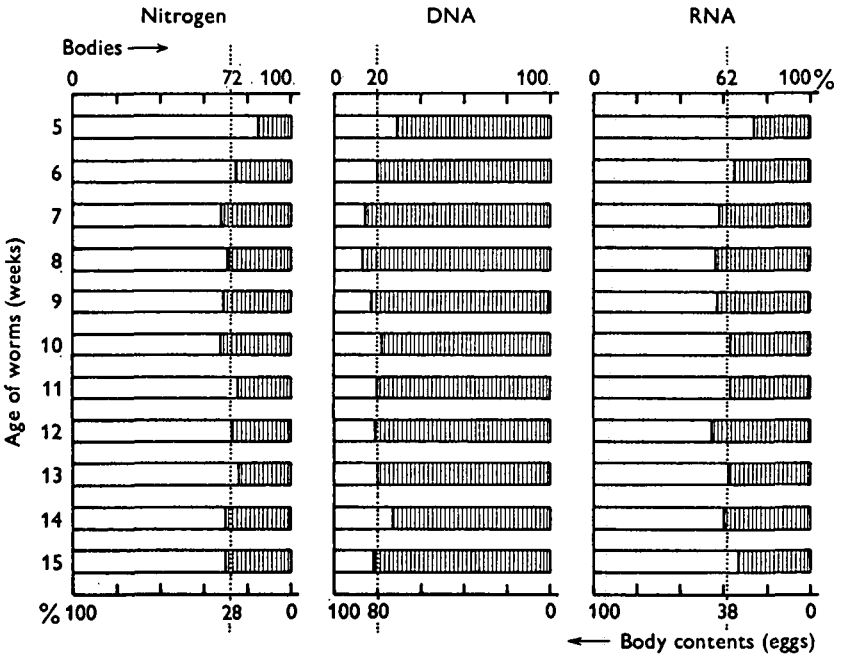

Fig. 4. Block diagrams showing the percentages of nitrogen, DNA and RNA in the bodies and body contents (eggs) of fertilized female *Moniliformis dubius*. Unshaded areas represent results from bodies and hatched areas represent results from eggs.

The material was sealed in an ampoule with N-HCl and heated for 2½ h in boiling water. A sample of calf thymus DNA was treated in the same manner. Details of the method and the amount of N-HCl required are given by Smith & Markham (1950). Aliquots of 5, 10 and 20 μl from the hydrolysates were spotted directly on to Whatman No. 1 paper together with samples of adenine and thymidine. Descending chromatograms were run at room temperature for 16 h in a solvent consisting of *n*-isopropanol, HCl (sp.gr. 1·18) and water (170:44:36) (Newton, 1957). After drying without heat the chromatograms were examined under u.v. light by the method of Markham & Smith (1949). A good separation of the bases from the calf thymus DNA had been achieved, but the egg material was contaminated with salt.

Samples of the egg material were eluted from the chromatograms with N-HCl and the eluates were concentrated by evaporation to give final volumes of 10 μl. These

samples were re-examined by chromatography and satisfactory separation was obtained, although the substances from the egg material tended to run slightly behind the marker substances. Four positions, where u.v. light was absorbed, were seen for calf thymus DNA and for the egg material. The R_F values for adenine and thymidine were 29·5 and 81·0 and the equivalent values from the calf thymus DNA and the egg material were 28·0 and 76·5, and 24·75 and 76·5, respectively. These results were considered to provide direct evidence for the presence of DNA in the eggs of fertilized female *M. dubius*. No attempt was made to obtain direct evidence for the presence of RNA in *M. dubius*.

DISCUSSION

Few data exist for comparison with these results since other workers studying aspects of the growth of *M. dubius* have used the worms' wet weights to measure growth. Large increases and decreases in the wet weights of female *M. dubius* were reported by Read & Rothman (1958), who were investigating aspects of the short-term effects of starvation and carbohydrate deprivation of the hosts on the parasites. The authors suggested that the striking decreases in wet weights, which they recorded, could have been caused by a massive loss of eggs. They also reported unpublished work which indicated that the decrease in the weight of the worms from starving hosts was not accompanied by any change in total nitrogen content. The results shown in Fig. 1 and Table 1 provide a foundation for a critical investigation of the events described by Read & Rothman.

Another interesting problem has been revealed by Graff & Allen (1963), who were primarily concerned with the glycogen content of *M. dubius*. On the basis of wet weights they reported that female *M. dubius* from female rats were much smaller than their counterparts from male rats. The same situation was encountered with the wet weights of male *M. dubius*, the heavier worms occurring in male rats. These observations of Graff & Allen need to be explained using a more reliable measure of growth than wet weight.

SUMMARY

1. The growth of male and female *Moniliformis dubius* has been defined by measuring the protein nitrogen, DNA and RNA content of the parasites during the course of the infection in male rats.

2. Details of the extraction and analytical procedure are described.

3. The growth of worms of both sexes is similar until after copulation, when female worms grow at a greater rate than males.

4. Large natural variations occur in the nitrogen, DNA and RNA content of individual fertilized female worms.

5. In fertilized female worms, the eggs contain about 28% of the nitrogen, 80% of the DNA and 38% of the RNA.

6. Evidence for the presence of DNA in the eggs was obtained by means of paper chromatography.

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