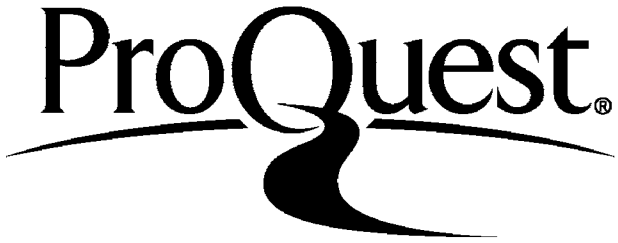
THE EFFECTS OF HOST AGE ON THE POULTRY CESTODE, RAILLIETINA CESTICILLUS (MOLIN, 1858) FUHRMANN, 1920.

J. S. GRAY.



ABSTRACT

The present study has shown that age resistance to *R. cesticillus*, manifested by worm destrobilization, occurred in male chickens. In females, age resistance developed more rapidly than in males, until the birds were about 84 days old and from this age onwards the manifestations of resistance declined. The roles of immune responses, mucus secretion and host hormone balance in the development of age resistance to *R. cesticillus* were subsequently investigated.

Host resistance to superinfection with *R. cesticillus* was demonstrated for the first time and this resistance was inhibited by the immunosuppressant dexamethasone. Further evidence for the immunogenicity of the cestode was provided by histological studies and by the demonstration of immunocytes and of ring precipitin and immunofluorescent antibodies. Experimental evidence suggested that these antibodies are not protective and it is probable the protective immune response is cellular.

An *in vitro* culture system, described by Schiller (1970), kept young adult cestodes in an active condition for more than eight days, although growth was poor. Chicken intestinal mucus extracts were shown to be lethal to *R.cesticillus in vitro* and mucus from old birds appeared to have a greater effect than 'young' mucus.

The effects of gonadal steroids on the course of infection of *R. cesticillus* were investigated by caponising male birds and implanting oestradiol or testosterone pellets.

This procedure reproduced to some degree the patterns of infection of R. cesticillus observed in intact, untreated, mature birds.

Scolex transplantation and the correlation of worm weights down the intestine with destrobilization strongly implicated intestinal environmental factors as being responsible for the phenomenon of worm destrobilization.

The possible mechanisms whereby the immune response, mucus secretions and host hormone balance affect worm viability are discussed.

ACKNOWLEDGEMENTS

I would like to express my thanks to Professor P.M. Butler for providing the facilities for this work, to Dr. J.W. Lewis for supervision, advice and criticism, to Dr. G.I. Twigg for temporary supervision and to Mr. M. Colthorpe, Mrs. R. Doran and many other members of the technical staff for their invaluable assistance. My best thanks are also due to Mr. E. Burt for looking after the chickens.

CONTENTS

SPECIES REFERRED TO IN THE MAIN TEXT

SPECIES	AUTHORITY	DATE
NEMATODA		
Ancylostoma caninum	Ercolani	1859
Ancylostoma duodenale	Dubrini	1843
Ascaridia galli	(Schrank,1788) Freeman	1923
Aspiculuris tetraptera	Nitzsh	1821
Bunostomum phlebotomum	Railliet	1900
Cooperia punctata	Linstow	1907
Dictyocaulus viviparus	Bloch	1782
Haemonchus contortus	Rudolphi	1803
Nematospiroides dubius	Baylis	1926
Nippostrongylus brasiliensis (muris)	Travassos (Yokogawa, 1920)	1914
Oesophagostomum columbianum	Curtice	1890
Ostertagia radiatum	Rudolphi	1803
Rhabditis briggsae	Dougherty and Nigon	1949
Trichostrongylus calcaratus	Molin	1860
Trichostrongylus colubriformis	Giles	1892
Trichostrongylus retortaeformis	Zeder	1800
Trichinella spiralis	Owen	1835
CESTODA		
Echinococcus granulosus	Batsch	1786
Hymenolepis citelli	Macleod	1933
Hymenolepis diminuta	Randolph	1819

SPECIES	AUTHORITY	DATE
CESTODA		
Hymenolepis microstoma	Dujardin	1845
Hymenolepis nana	Siebold	1853
Moniezia expansa	Rudolphi	1810
Raillietina fedjuschina	Skutar	1963
Taenia pisiformis	Bloch	1870
Taenia taeniaeformis (Cysticercus crassicollis)	Batsch	1876
TREMATODA		
Fasciola hepatica	Linnaeus	1758
PROTOZOA		
Eimeria maxima	Tyzzer	1929
Entamoeba histolytica	Schaudinn	1903
INSECTA		
Tribolium castaneum	Herbst	
Tribolium confusum	Duval	
AVES		
Colinus virgianus		
Gallus sonnerati		
Meleagridis galloparo domestica		
Phasianus gallus		

GENERAL INTRODUCTION

Host resistance to parasitic infections is usually divided into two main types, natural or innate, and acquired. Natural resistance is inherited by the host and bears no relation to any experience of parasitism. Most of the features of natural resistance are seen in the abnormal host and include decreased numbers and size, increased prepatent period, decreased patent period, decreased reproduction and abnormal distribution of the parasite. Many of the factors responsible for such features have been identified and these include nonspecific inflammation, physical resistance factors, nonspecific chemical resistance factors (bile salts, properdin, interferon), lack of developmental stimuli and nutrient factors.

Acquired resistance as the term suggests is brought about by physiological changes within the host in response to experience of parasitic infections. Acquired resistance is usually associated with a specific immune response, but other factors can undoubtedly contribute. For example, factors associated with natural resistance such as nonspecific inflammation and mucus production may be potentiated. Stahl (1966) demonstrated that the mouse pinworm Aspiculuris tetraptera evokes resistance to superinfection and suggested that intestinal mucus is a dominant factor in this resistance. The nonspecificity of the response was demonstrated by showing that concurrent infections with other helminth species also increased resistance to A. tetraptera.

It can be seen that the factors associated with natural resistance and those associated with acquired resistance are

not necessarily mutually exclusive and it is probably that a complex relationship between the various physiological factors of the two types of resistance exists.

Both natural and acquired resistance factors are undoubtedly involved in the phenomenon of age resistance to parasitic infections. It has long been recognised that the age of the host is related to its susceptibility to infection. The first experimental evidence was obtained by Loos (1911), who observed that the larvae of Ancylostoma duodenale developed to maturity in young dogs but not in old dogs, an abnormal host for this parasite. Since then age resistance has been demonstrated in many host-parasite systems, for example the nematodes Nippostrongylus muris (Africa,1931) in the rat, Ascaridia galli (Ackert, Edgar and Frick, 1939) in the chicken, Nematospiroides dubius (Dobson, 1962) in the mouse and the cestodes Hymenolepis nana (Larsh,1944) in the mouse and Raillietina fedjuschina (Skutar, 1963) and Raillietina cesticillus (Ackert and Reid, 1937) in the chicken.

There are some host-parasite relationships in which no age resistance develops, for example Ostertagia radiatum and Bunostomum phlebotomum (Mayhew, 1940) and Cooperia punctata (Bailey, 1949) in calves, Trichostrongylus calcaratus in rabbits (Sarles, 1932) and Trichostrongylus colubriformis in guinea pigs (Herlich, 1958). It was suggested by Sandground (1929) that when the parasite is closely adapted to its host, age resistance is almost or entirely absent and is most prevalent in poorly adapted host-parasite systems. If this is true, age resistance can be regarded as a form of intraspecific host specificity. Natural resistance factors obviously play an important part in the determination of host specificity.

Specific resistance factors associated with acquired resistance, such as serological and cellular immunity, probably play a minor role in grossly abnormal hosts, but are likely to be of significance in a situation where the parasite infects the host for sufficiently long to evoke an immune response. It would be expected that a situation such as this might occur in the development of age resistance to a parasite.

It is well known that neonatal and young animals have less immune potential than older animals and this would seem to add weight to the argument that age resistance is due primarily to the development of the immune system. It must be remembered, however, that extensive physiological changes occur during maturation and these are very likely to involve other resistance factors. Although there is much evidence to prove the existence of age resistance very little is known of the mechanisms involved. An investigation into this subject is overdue and will probably prove invaluable in the elucidation of the relationships between the resistance factors of natural and acquired immunity.

Most of the records of age resistance to parasites seem to apply to intestinal helminths. It is a well established fact that the intestinal physico-chemical environment is extremely complex and is in dynamic relation with the host tissues (Read,1950). Read discussed the physiology of the small intestine in relation to helminth parasitism and in this extensive review the importance of a wide range of physico-chemical factors was emphasised. These include host nutrients, digestive enzymes, endocrine secretions, mucin, bile, lipid secretion, oxygen tension, intestinal flora, oxidation reduct-

ion potential, pH and osmotic pressure. All of these factors could contribute to the development of age resistance to parasitism.

It is interesting to note that almost all examples of age resistance to parasitism involve parasites that are quite closely associated with host tissues at some time during their life cycle and are therefore exposed to a greater or lesser extent to the immune system of the host. A specific host response would obviously greatly complicate a study of the natural resistance component of age resistance. Raillietina cesticillus, the common poultry cestode, is, however, exceptional in that although age resistance in this system has been demonstrated (Ackert and Reid, 1937; Meinkoth,1947; Sinha and Srivastava,1958), the scolex does not penetrate beyond the base of the mucosa (Nath and Pande,1963) and several investigators failed to demonstrate acquired resistance (Luttermoser,1938; Sinha and Srivastava,1958; Meinkoth,1947). Meinkoth also failed to demonstrate serum antibodies or to induce resistance with injections of worm extract or by passive transfer of serum from infected birds.

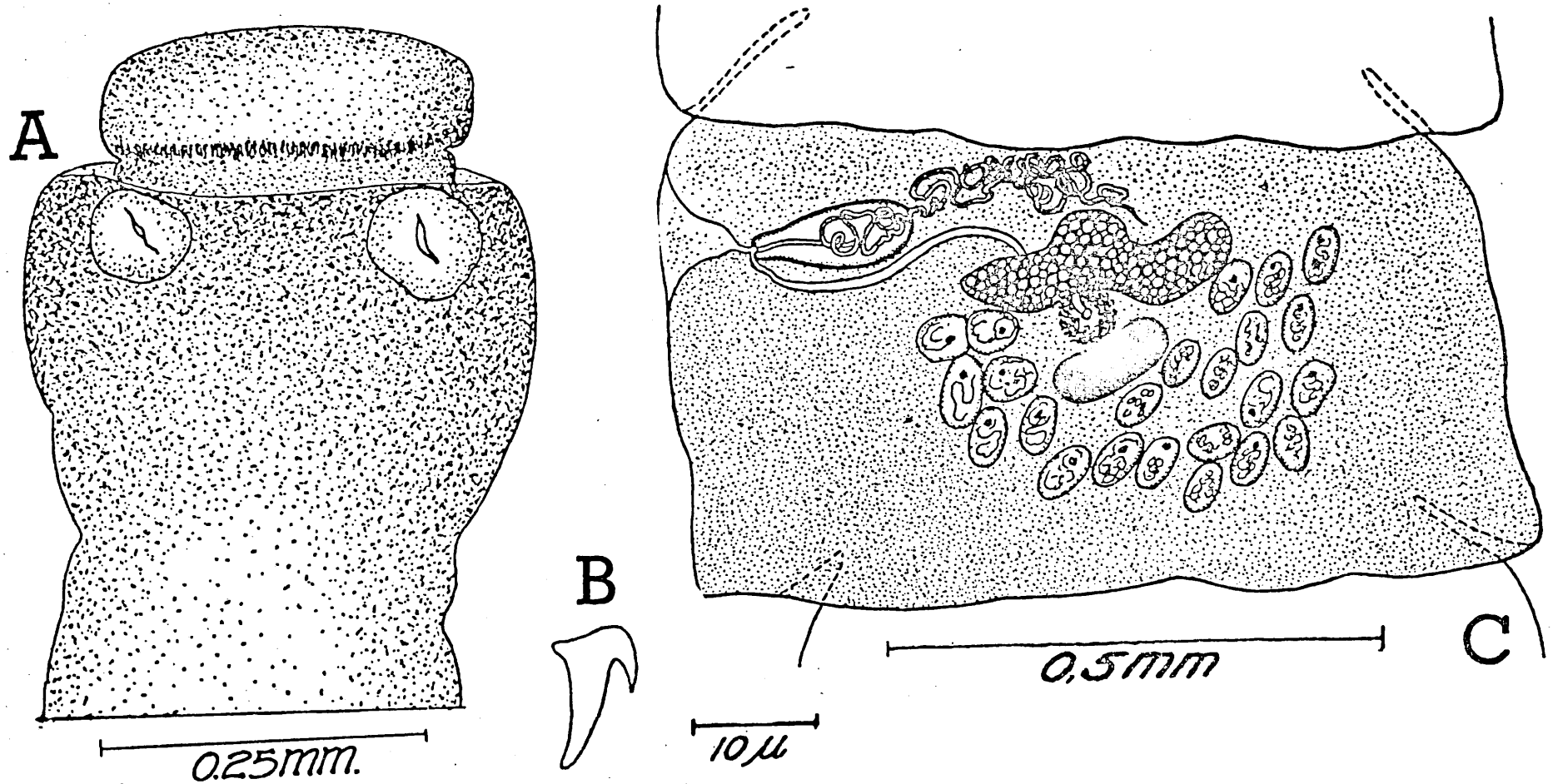
Since there appears to be no complicating immune response to Raillietina cesticillus in the chicken, this host-parasite system seems to be admirably suited to the study of the mechanisms of age resistance.

The adult Raillietina cesticillus was first described by Molin (1858) from Phasianus gallus. Molin placed it in the genus Taenia and the present day nomenclature dates from the classification of Fuhrmann in 1920 (after Reid, Ackert and Case, 1938).

It is cosmopolitan in distribution and is a very common parasite of the domestic fowl under natural conditions. It has also been reported from the grey jungle fowl, Gallus sonnerati (Southwell,1930), the domestic turkey, Meleagridis gallopavo domestica (Ransom,1909) and the bobwhite quail, Colinus virginianus (Stoddard,1931).

R. cesticillus is a large species and may reach a length of 13 cms, but is normally about half this length. Its natural habitat is the lumen of the upper third of the small intestine, but it may extend from the duodenum to Meckel's diverticulum. The large scolex (Fig.1) bears a broad flat rostellum with two rows of 300 - 500 hooks at the base. The suckers are unarmed. The genital pores are irregularly alternate and there are 16 to 30 testes per segment. The eggs occur singly in capsules and a gravid proglottid contains between 300 and 500 eggs. About 9 proglottids are produced by each worm per day in light infections and 4 proglottids per worm per day in heavy infections (Reid,1942). There is a distinct periodicity in the production of gravid proglottids. Reid, Ackert and Case (1938) correlated this periodicity with the digestive periods of the fowls and absorption and assimilation by the cestodes. Reid (1942) correlated glycogen levels in R.cesticillus with feeding activity of the fowls and suggested that this fluctuation in glycogen levels explains the daily periodicity of proglottid production.

The intermediate hosts are beetles and cysticercoids have been recorded from at least 20 carabid genera a number of scarabids and Tribolium and Tenebrio of the Tenebrionidae. The environmental temperature determines the rate of development of the cysticercoids, which are infective after 14 days at 30°C,



Raillietina cesticillus. A. Scolex. B. Rostellar hook. C. Mature Proglottid. (After Wehr, 1962.)

FIG. 1

but may require a month at lower temperatures. The cysticercoids develop to mature adults 11 - 18 days after being fed to chickens.

Individual cestodes cause little damage and only slight inflammation and flattening of the epithelium occurs at the site of attachment (Fig.2).

Ackert and Wisseman(1946) reported that 40 day old chicks tolerated up to 172 worms without adverse effect on growth, blood sugar and haemoglobin and blood cell counts. This contradicts a previous finding by Ackert and Case (1938), who reported that 4 - 25 cestodes caused weight retardation and reduced sugar and haemoglobin content of the blood of 3 - 4 month old White Leghorn chickens. This contradiction may have been caused by a difference in diet. Harwood and Luttermoser (1938) observed a marked retardation in manganese deficient birds and Luttermoser and Allen (1942) reported that a protein deficient diet plus R. cesticillus infection caused weight retardation.

However, Raillietina cesticillus is not regarded as a pathogenic species and clinical signs of infection are not easy to detect. From the evidence available it would appear that this is a well adapted host-parasite system.

It was decided to investigate the course of infection of the cestode in the susceptible bird in detail and then to determine the effect of host age on the course of infection. After these two basic studies attempts were made to identify and characterise the various host physiological factors that might contribute to age resistance to R. cesticillus.

Figure 2. Scolex *in situ* in the small intestine. Three week old infection in a chicken infected at three days of age. ×95

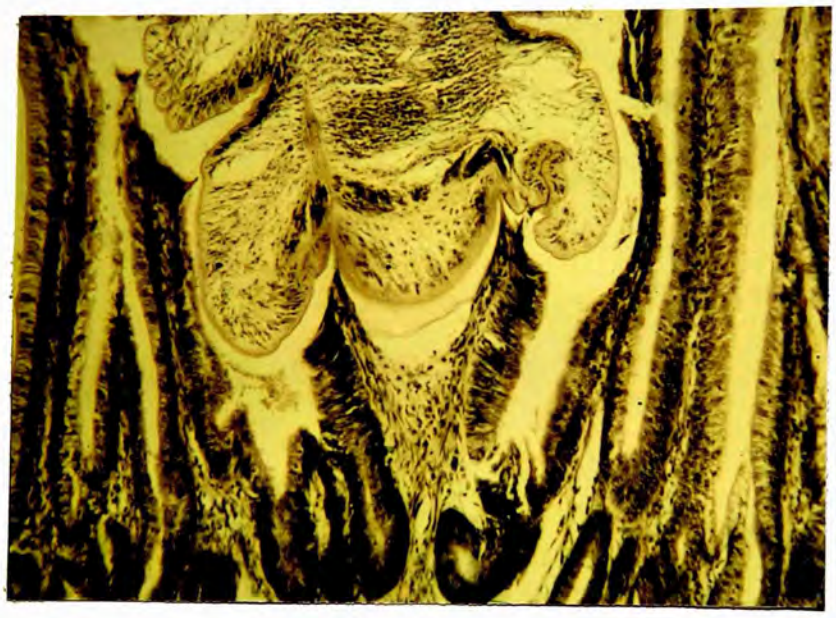


FIG. 2. Cestode Scolex *In Situ*

GENERAL MATERIALS AND METHODS

The cestode, Raillietina cesticillus (Molin,1858) was originally obtained from the University of Georgia and has been maintained in these laboratories for about four years.

The intermediate hosts were Tribolium castaneum and T. confusum. They were maintained in glass jars containing Scott's Porage Oats, white flour and yeast at 30°C and 60% R.H.

The chickens were a White Leghorn hybrid strain, known commercially as 'Apollo' and obtained as day olds from Sterlings Limited. They were maintained on wire at 22°C with a constant light intensity of 2 lumens, 12 hours on - off. Food (Spiller's Chick Crumbs) and water were given ad libitum.

Culture of R. Cesticillus

Isolation of Gravid Proglottids

Chicken faeces were homogenised in water and thoroughly washed in a 0.8 mm mesh sieve. The contents of the sieve were placed in a black dish and saturated sodium chloride was added. After mixing, the sediment was allowed to settle and proglottids were then pipetted from the surface. This technique gives good recovery and has no effect on the infectivity of the eggs.

Infection Procedure

The intermediate hosts were infected after a 24 - 48 hour starvation period. Twelve beetles were placed in each of several Petri dishes containing filter paper and three proglottids were placed on the filter paper for three successive days. Food was provided at the end of this period and the

beetles were kept at 30°C for 2 weeks, by which time the cysticercoids are infective. The beetles were then placed in specimen tubes and maintained at room temperature until required. This method yields approximately 10 - 20 cysticercoids per beetle and is designed to provide cysticercoids of uniform size.

For maintenance of the life cycle infected beetles were fed direct to chickens. For more exact infections cysticercoids were dissected out from beetles in 0.85% saline and administered by pipette. Food was withheld from the chickens for up to an hour before infection to ensure that their crops were empty.

In an attempt to reduce the variation in levels of infection in the chickens cysticercoids were selected with care and discoloured, deformed, very large and very small individuals were discarded. Care was also taken in the selection of chickens for infection. Each experimental group was matched as closely as possible before infection, usually by weight.

Autopsy Procedure

All chickens were killed by cervical fracture after weighing. When reflex activity had ceased the skin of the thorax and abdomen was snipped off. Using heavy scissors the clavicles, coracoids and ribs on both sides were cut and the sternum, with pectoral and abdominal muscles attached, removed. This procedure exposes thoracic and abdominal organs very rapidly and makes them easily accessible.

In most autopsies the organs removed for examination were the small intestine (Gizzard to Meckel's diverticulum), the

gonads and the adrenal glands. The spleen was also removed on occasion. The small intestine was examined for worms and the following values were usually obtained: total number of scoleces, number of destrobilated scoleces and distribution of scoleces. The process of destrobilisation is easily detectable and is most obvious when worms are reduced to about half their normal length of about 6 - 7 cms. Worms measuring less than 3 cms in length and lacking the characteristic terminal segment of the immature worm (Luttermoser, 1938) were therefore classified as destrobilated. The determination of the distribution of scoleces involved the measurement of the length of the small intestine, the position of the attached scoleces and the position of the bile/pancreatic ducts. It was important to measure the intestine in a uniformly relaxed condition. This was achieved by modifying a technique used by Brambell (1965). On removal from the chicken the intestine was placed in adrenalin-saline (0.85% NaCl, 1/10,000 adrenalin) at 40°C in a 3ft. long trough made from plastic guttering. The intestine was slightly stretched by hand and then allowed to relax. It was pinned out after straightening without stretching and slit open. The intestine was then divided into 50 equal sections and the numbers of scoleces occurring in each section recorded.

When dry weights of worms were determined each batch of worms was removed from the intestine and then washed in three changes of chilled (4°C) saline and placed in predried and preweighed silver foil boats. These were placed in an oven at 60°C and dried to constant weight. Only entire worms were used for these determinations. Worm weights were expressed in milligrams.

Wet weights of gonads and adrenals were determined in order to obtain a measure of physiological maturity and the degree of stress to which the birds were exposed. These organs were usually removed with considerable amounts of adjoining tissues attached as speed was essential.

Time had therefore to be spent in cleaning up these organs before weighing. It was not possible to do this in addition to the examination of the small intestine and these organs were therefore placed in a deep freeze as soon as possible after removal from the bird and were cleaned and weighed the following day.

Ideally the examination of the gut and collection of data would follow immediately after the removal of the relevant organs from the chicken. In practice this proved undesirable for two reasons. Firstly, the autopsy period would have to be extended over more than one day as considerable time would be wasted. Secondly, it was considered important to kill all chickens at the same time of day when dry weights were to be determined, since there is not only periodic release of proglottids to take into account, but also diurnal fluctuations in glycogen levels (Reid 1942).

The alternative procedure, killing all chickens at once, presents one major problem. This is the varying time lag between killing and removal of organs and the rest of the autopsy procedure. For each chicken the time taken for killing and removal of gut, gonads and adrenals averages five minutes, whereas it takes an average of thirty minutes to complete the autopsy. It was therefore necessary to maintain both gut tissue and parasites in a viable state for up to five

hours. It was not possible to do this by lowering the temperature as this causes the worms to release their hold on the gut mucosa. Incubating the gut in adrenalin-saline at 40°C, with a few drops of 1/1,000 thiomersal was eventually found to be the most satisfactory method. From a comparison of results from first and last autopsies of birds from the same experimental group it was concluded that this method works sufficiently well for the purposes described.

When samples of gut tissue were required for histological examination they were fixed in aqueous Bouin immediately after killing, embedded in paraffin wax and sectioned at 5u. It was found that sections in the longitudinal plane of the intestine gave the best results. For routine histopathological studies the sections were stained with haematoxyln and eosin. Other stains are mentioned where relevant.

Statistical variations are expressed as standard error.

CHAPTER 1

THE COURSE OF INFECTION OF R. CESTICILLUS IN THE SUSCEPTIBLE CHICKEN

Introduction

Before investigating age resistance to R.cesticillus it is necessary to characterise as far as possible the course of infection of the cestode in the young susceptible host.

The biology of R.cesticillus has been the subject of several studies, the most extensive of which are those of Reid, Ackert and Case (1938) and Dutt, Sinha and Mehra (1961). The morphology and development of the cestode from egg to adult is well described in both these papers, but little information is available on the course of infection of the adult worm in the definitive host. The only study on this subject is that of Foster and Daugherty (1959), who investigated the distribution and growth of the cestode in the prepatent phase of the infection. Their observations were, however, incidental to an investigation on cestode amino-acid metabolism and a more detailed study is necessary.

The present study provides more detailed information on the distribution and rate of growth of R. cesticillus in the susceptible chicken and also includes data on the dynamics of infection of the cestode.

Material and Methods

All birds were infected at 3 days of age with 100 cysticercoids each and autopsies were then carried out on ten birds (five male and five female) at each of the following times after infection: ½, 1, 4, 14, 28, 42, 56, 70 and 112 days.

From examination of the worms in the intestine the following values were determined: total number of scoleces, number of destrobilated scoleces, distribution of scoleces and dry weight of whole worms. The proglottid output from three birds of each sex was monitored throughout the infection by using the separation technique described on page 16 . The number of eggs in the gravid proglottids was determined as follows. Fifty or fewer proglottids were placed in graduated tubes and a digest solution of 0.2% pepsin and 1% HCl was added and made up to 10 mls or, if less than 25 proglottids were present, to 5 mls. This preparation was then incubated at 40°C for 4 hours and the proglottids well broken up by shaking. The suspended eggs were well mixed and 0.25 mls were then removed and placed in a counting chamber. Only eggs with a defined shell and embryo with hooks were counted. Two counts were made and a discrepancy of less than 10% was considered acceptable. If larger discrepancies were obtained further counts were made. Embryonated shelled eggs were observed to be unaffected after 8 hours incubation in the digest solution.

Results

	Table	Figure
Mean Number of Proglottids Per Bird	1	3
Mean Number of Eggs Per Proglottid	2	-
Mean Number of Scoleces	3	4
Mean Distribution of Scoleces	4	5
Mean Percent Number of Destrobilating Scoleces	5	6
Mean Worm Dry Weight	6	7

Where results for the two sexes do not differ all data is grouped together.

Table 1

Mean Number of Proglottids Per Bird

Days Post Infection	Male Host	Female Host	Male + Female
14	9.0	57.3	33.1
15	48.6	136.6	92.6
16	67.3	132.0	99.6
17	90.6	150.0	120.3
18	130.3	137.6	133.9
19	153.3	188.0	170.6
22	172.6	168.0	170.3
23	192.0	202.6	197.3
25	186.3	157.3	171.8
26	161.3	100.0	130.6
27	192.6	127.3	159.9
29	150.6	116.6	138.6
31	147.6	143.6	145.6
32	151.0	170.0	160.5
37	156.0	241.3	198.6
39	107.0	176.0	141.5
45	81.6	121.3	101.4
50	67.0	78.6	72.8
51	52.6	82.6	72.6
54	28.0	83.6	66.8
59	46.0	28.6	37.3
63	13.6	38.0	25.8
Mean Number of Scoleces at Autopsy	43.6 ± 11.25	50.6 ± 5.36	47.1 ± 5.79

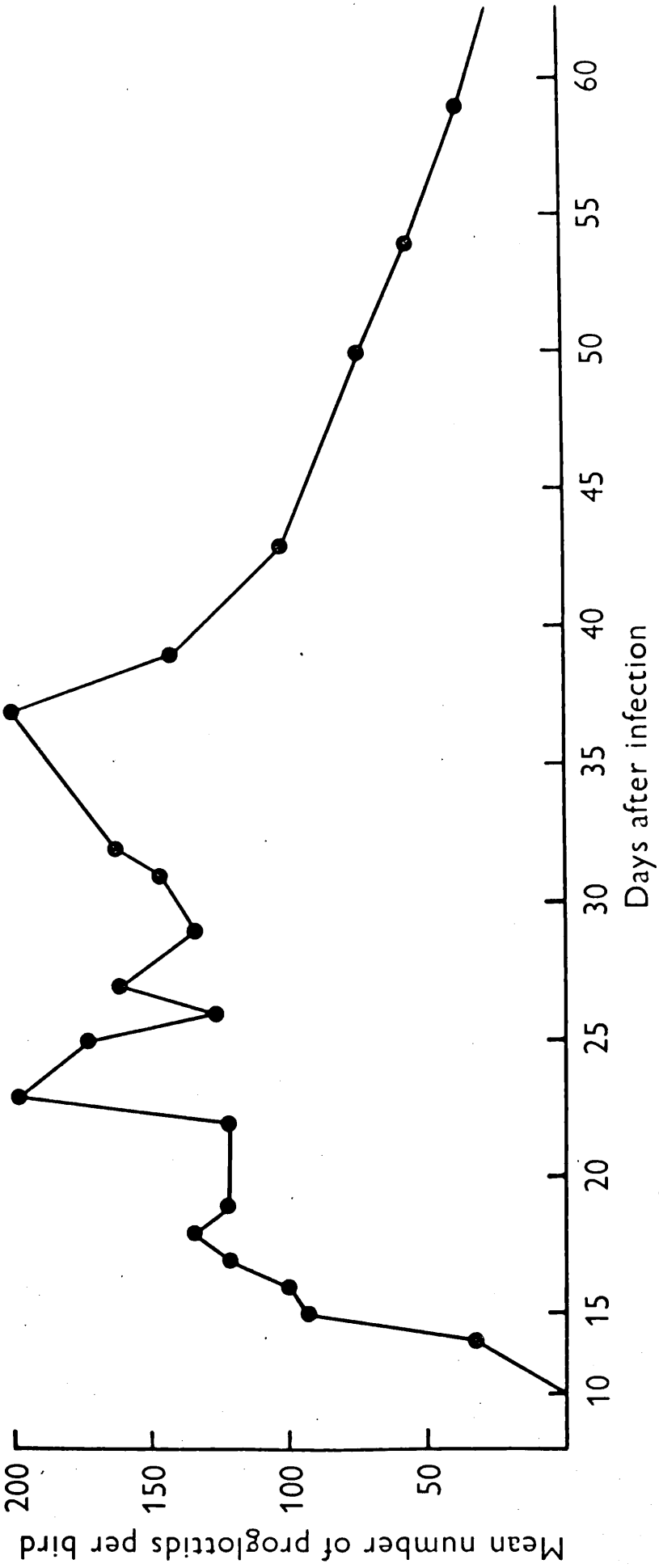

Mean number of proglottids per bird
200
150
100
50
10
15
20
25
30
35
40
45
50
55
60
Days after infection

FIG. 3

Table 2

Mean Number of Eggs Per Proglottid

Days Post Infection	Male Host	Female Host	Male + Female
14	197.0	289.5	243.25
15	245.8	278.3	262.05
16	354.5	345.7	350.1
17	373.0	388.0	380.5
18	349.0	336.0	342.5
19	329.5	324.5	327.0
22	376.0	346.0	361.0
23	383.5	342.5	363.0
25	263.0	247.9	255.45
26	327.5	242.5	285.0
27	316.5	295.0	305.75
29	334.3	357.5	345.9
31	274.0	264.4	269.2
32	276.4	298.5	287.45
37	311.0	288.5	299.75
39	301.8	270.0	285.9
43	350.1	341.0	345.55
50	300.7	376.0	338.35
51	271.3	352.5	311.9
54	265.7	281.0	273.4
59	314.2	373.0	343.6
63	259.0	278.5	268.75

Table 3

Mean Number of Scoleces

Days Post Infection	Male Host	Female Host	Male + Female
½	48.0 ± 4.04	60.0 ± 6.351	54.0 ± 4.30
1	29.2 ± 0.97	28.4 ± 1.12	28.8 ± 0.71
2	35.4 ± 1.60	39.2 ± 2.20	37.3 ± 1.31
4	34.0 ± 2.88	31.4 ± 2.13	32.7 ± 2.46
7	47.2 ± 3.65	46.8 ± 4.56	47.0 ± 2.76
14	48.2 ± 6.21	46.0 ± 4.28	47.1 ± 3.56
28	51.4 ± 3.49	56.2 ± 6.47	53.8 ± 3.55
42	51.2 ± 3.57	50.6 ± 3.03	50.9 ± 3.74
56	33.6 ± 6.21	23.2 ± 4.62	28.4 ± 4.04
70	45.0 ±12.45	24.6 ± 4.84	34.8 ± 9.64
112	2.0	1.0	1.5

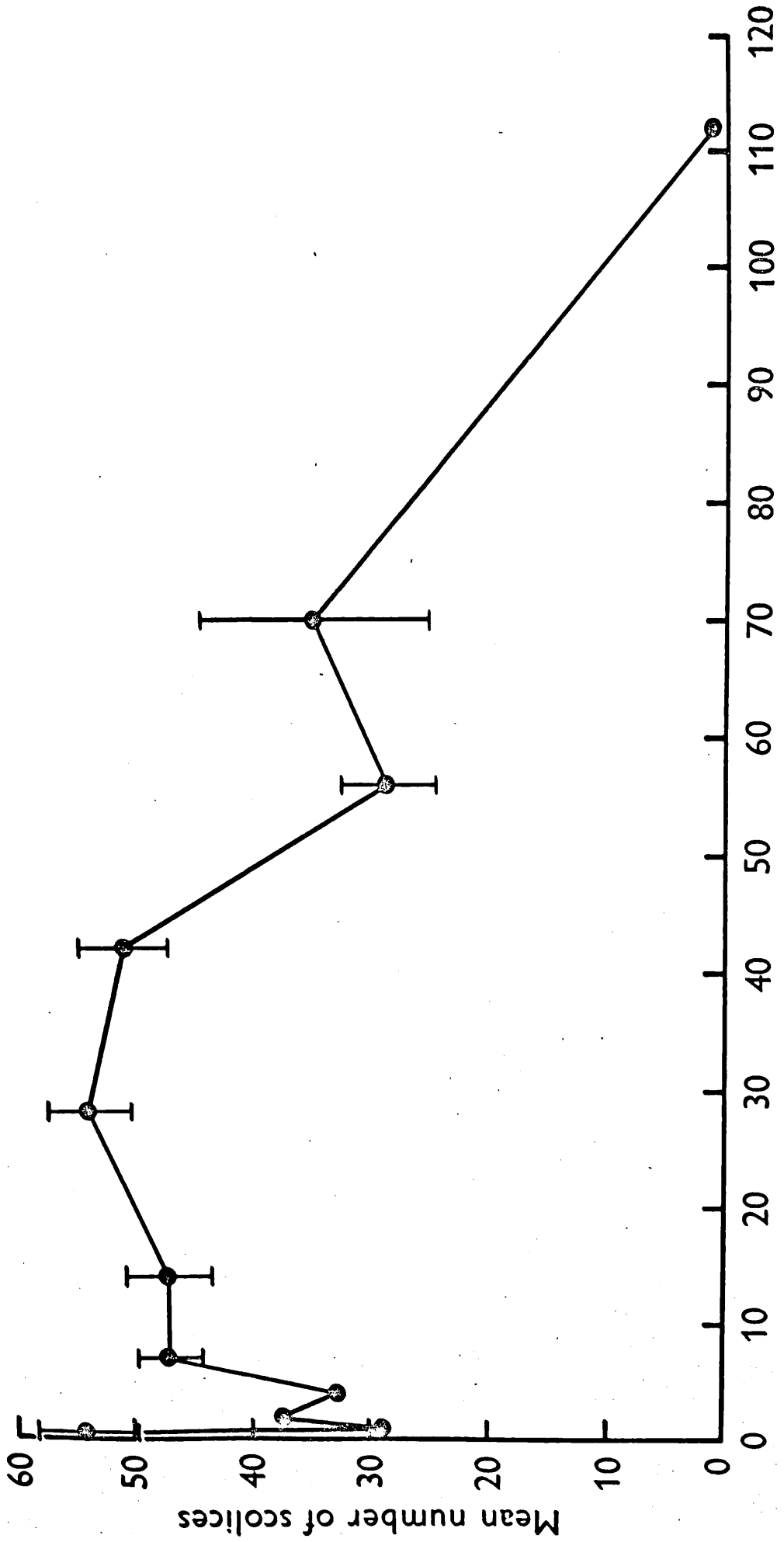

Mean number of scolices
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
120
Days after infection

FIG. 4

Table 4

Mean Distribution of Scoleces

Days Post Infection

½ Mean Number of Attached Scoleces Male - 46.3 Female - 58.9

Mean Position of Bile Ducts (Percent Length) Male - 32 Female - 30.0

Percent Length of Intestine -

2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40
♂ Percent No. Scoleces -																			
0.6	0	3.5	2.8	2.8	7.8	1.3	5.7	2.8	5.0	5.7	9.3	6.5	5.7	5.0	2.2	1.3	4.3	5.0	5.0
♀ Percent No. Scoleces -																			
3.4	2.2	2.7	3.4	2.7	6.8	5.1	2.7	4.4	8.5	7.8	7.2	3.4	3.9	6.8	3.4	2.7	5.1	1.7	10.0
Total Percent No. Scoleces -																			
2.0	1.1	3.1	3.1	2.7	7.3	3.2	4.2	3.6	6.7	7.7	8.7	4.9	4.8	5.9	2.8	2.0	4.7	3.3	7.5

Percent Length of Intestine -

42	44	46	48	50	52	54	56	58
♂ Percent No. Scoleces -								
1.3	4.3	3.5	2.8	2.2	2.2	0.6	0.6	0.6
♀ Percent No. Scoleces -								
3.4	3.9	1.7	0.5	5.1	0	0.5	0.5	
Total Percent No. Scoleces -								
2.3	4.1	2.6	1.6	3.6	1.1	0.5	0.5	0.3

Table 4 (Cont)

1 Mean Number of Attached Scoleces Male -28.8 Female - 28.4

Mean Position of Bile Ducts (Percent Length) Male -30 Female -32

Percent Length of Intestine -

4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40
♂ Percent No. Scoleces -																		
0.7	1.4	0	0	0	4.8mm	3.5	2.1	4.2	4.2	2.8	4.5	5.6	4.8	6.9	6.2	9.7	6.2	6.2
♀ Percent No. Scoleces -																		
		0.7	0.7	0	2.8	4.2	4.9	5.6	2.8	4.2	3.5	5.7	2.8	5.7	9.9	12.0	8.4	11.3
Total Percent No. Scoleces -																		
0.3	0.7	0.3	0.3	o	3.8	3.8	3.5	4.9	3.5	3.5	4.0	5.6	3.8	6.3	8.0	10.8	7.3	8.7

Percent Length of Intestine -

42	44	46	48	50	52	54	56
♂ Percent No. Scoleces -							
6.9	2.1	7.6	5.6	1.4	2.1	0.7	0.7
♀ Percent No. Scoleces -							
4.9	2.1	3.5	2.1	0.7	0.7	0.7	0
Total Percent No. Scoleces -							
5.9	2.1	5.5	3.8	1.0	1.4	0.7	0.3

Table 4 (Cont)

2 Mean Number of Attached Scoleces Male - 35.4 Female - 38.4

Mean Position of Bile Ducts (Percent Length) Male - 32 Female - 30

Percent Length of Intestine -

2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34
♂ Percent No. Scoleces -																
	1.1	0.6	4.5	2.3	5.6	2.8	1.1	2.3	2.3	2.8	6.2	6.2	6.8	10.7	7.3	7.3
♀ Percent No. Scoleces -																
0.5	1.7	0.5	0.5	2.3	1.7	4.0	2.8	4.6	2.8	4.6	5.1	5.7	8.0	4.6	8.6	9.2
Total Percent No. Scoleces -																
0.2	1.4	0.5	2.5	2.3	3.6	3.4	1.9	3.9	2.5	3.7	5.5	5.9	7.4	7.6	7.9	8.2

Percent Length of Intestine -

36	38	40	42	44	46	48	50	52	54	56	58	60
♂ Percent No. Scoleces -												
5.6	6.2	5.1	4.5	2.8	1.1	1.1	0	0.6	0.6	0	1.7	0.6
♀ Percent No. Scoleces -												
6.9	9.2	3.4	4.6	4.6	2.8	5.1	2.3	0.5	2.3	0	0.5	0
Total Percent No. Scoleces -												
6.2	7.7	4.2	4.5	3.7	1.9	3.1	1.1	0.5	1.4	0	1.1	0.3

Table 4(Cont)

4 Mean Number of Attached Scoleces Male - 34.0 Female - 31.4

Mean Position of Bile Ducts (Percent Length) Male - 30 Female - 30

Percent Length of Intestine -

6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
♂ Percent No. Scoleces -															
		0.6	0.6	0.6	2.4	2.9	1.2	0.6	1.8	0.6	1.8	2.4	6.5	6.5	15.3
♀ Percent No. Scoleces -															
0.6	0.6	2.5	1.3	1.3	3.8	5.1	1.9	2.5	5.1	3.8	3.8	5.7	9.6	5.1	8.3
Total Percent No. Scoleces -															
0.3	0.3	1.5	0.9	0.9	3.1	4.0	1.5	1.5	3.4	2.2	2.8	4.0	8.0	5.8	6.8

Percent Length of Intestine -

38	40	42	44	46	48	50	52	54	56	58	60
♂ Percent No. Scoleces -											
11.2	10.0	8.8	5.3	8.2	5.9	7.1	1.8	2.4	2.9	2.9	0.6
♀ Percent No. Scoleces -											
8.3	12.1	5.1	4.5	2.5	2.5	1.3	1.3	0.6	0.6	0	0.6
Total Percent No. Scoleces -											
9.7	11.0	5.9	4.9	5.3	4.2	4.2	1.5	1.5	1.7	1.4	0.6

Table 4 (Cont)

7 Mean Number of Attached Scoleces Male -47.8 Female - 45.8

Mean Position of Bile Ducts (Percent Length) Male -30 Female - 30

Percent Length of Intestine -

26	28	30	32	34	36	38	40	42	44	46	48	50	52	54	56
♂ Percent No. Scoleces -															
0.4	0.8	0.4	1.3	4.2	6.7	10.9	14.2	12.1	9.6	13.0	7.1	8.8	2.1	3.3	2.1
♀ Percent No. Scoleces -															
0.4	0.9	0	0	0.4	2.6	3.1	16.2	13.5	16.2	12.7	7.0	4.4	6.1	5.7	3.5
Total Percent No. Scoleces -															
0.4	0.8	0.2	0.6	2.3	4.6	6.5	15.2	12.8	12.9	12.8	7.0	6.6	4.1	4.5	2.8

Percent Length of Intestine -

58	60	62	64	66	68	70	72	74	76	78
♂ Percent No. Scoleces -										
0	0.8	0.4	0	0.4	0	04	0	0	0.4	0.4
♀ Percent No. Scoleces -										
2.2	1.7	1.7	0.9	0.4	0	0	0.4	0	0	0
Total Percent No. Scoleces -										
1.1	1.2	1.0	0.4	0.4	0	0.2	0.2	0	0.2	0.2

Table 4 (Cont)

14 Mean Number of Attached Scoleces Male - 40.8 Female 40.6

Mean Position of Bile Ducts (Percent Length) Male - 32 Female - 32

Percent Length of Intestine -

36	38	40	42	44	46	48	50	52	54	56	58	60	62	64	66
♂ Percent No. Scoleces -															
4.9	7.4	3.4	10.8	9.3	11.3	10.3	8.8	8.8	4.9	7.4	5.9	0.5	2.0	1.5	2.0
♀ Percent No. Scoleces -															
1.0	2.9	2.0	9.3	7.4	12.8	8.9	10.3	10.3	7.4	5.9	6.9	4.4	2.5	2.5	2.0
Total Percent No. Scoleces -															
2.9	5.1	2.7	10.1	8.3	12.1	9.6	9.5	9.5	6.1	6.7	6.4	2.4	2.3	2.0	2.0

Percent Length of Intestine -

68	70	72
♂ Percent No. Scoleces -		
0.5	0.5	0
♀ Percent No. Scoleces -		
1.5	0.5	1.5
Total Percent No. Scoleces -		
1.0	0.5	0.7

Table 4 (Cont)

28 Mean Number of Attached Scoleces Male - 50.0 Female - 56.2

Mean Position of Bile Ducts (Percent Length) Male -30 Female - 28.

Percent Length of Intestine -

32	34	36	38	40	42	44	46	48	50	52	54	56	58	60	62
♂ Percent No. Scoleces -															
2.0	1.2	0.8	5.2	6.8	5.2	8.4	6.8	4.8	7.6	8.0	6.0	10.4	6.0	6.0	4.8
♀ Percent No. Scoleces -															
0	0	3.6	3.9	6.0	5.3	7.5	4.3	6.0	7.1	5.0	7.5	5.3	5.7	6.0	6.0
Total Percent No. Scoleces -															
1.0	0.6	2.2	4.5	6.5	5.2	7.9	5.9	5.4	7.3	6.5	6.7	7.8	5.8	6.0	5.4

Percent Length of Intestine -

64	66	68	70	72	74	76	78	80	82	84
♂ Percent No. Scoleces -										
3.2	1.6	1.2	2.0	1.2	0.4	0	0	0	0	0
♀ Percent No. Scoleces -										
5.3	2.1	2.5	2.5	3.2	2.1	1.4	0.4	0.4	0.4	0.4
Total Percent No. Scoleces -										
4.2	1.8	1.8	2.2	2.2	1.2	0.7	0.2	0.2	0.2	0.2

Table 4 (Cont)

42 Mean Number of Attached Scoleces Male - 49.8 Female 49.6

Mean Position of Bile Ducts (Percent Length) Male -32 Female - 32

Percent Length of Intestine -

30	32	34	36	38	40	42	44	46	48	50	52	54	56	58	60
♂Percent No. Scoleces -															
0	2.0	2.8	1.6	2.8	7.6	7.2	5.6	10.4	7.6	9.6	9.2	8.4	5.2	2.4	4.4
♀Percent No. Scoleces -															
0.4	2.0	3.2	2.4	3.6	4.0	7.2	7.6	10.0	7.6	8.9	8.5	6.4	8.5	5.2	5.2
Total Percent No. Scoleces -															
0.2	2.0	3.0	2.0	3.2	5.8	7.2	6.6	10.2	7.6	9.2	8.8	7.4	6.8	3.8	4.8

Percent Length of Intestine -

62	64	66	68	70	72	74	76	78	80
♂Percent No. Scoleces -									
3.6	1.2	2.0	1.6	0.8	1.2	0.8	0.8	0.4	0
♀Percent No. Scoleces -									
2.0	2.8	0.4	1.2	0.8	0.4	0.4	0	0.4	0.4
Total Percent No. Scoleces -									
2.8	2.0	1.2	1.4	0.8	0.8	0.6	0.4	0.4	0.2

Table 4 (Cont)

56 Mean Number of Attached Scoleces (Male - 33.0 Female - 23.2

Mean Position of Bile Ducts (Percent Length) Male - 30 Female - 30

Percent Length of Intestine -

36	38	40	42	44	46	48	50	52	54	56	58	60	62
♂ Percent No. Scoleces -													
0.6	1.2	4.2	9.1	8.5	12.7	10.7	10.3	10.9	7.9	6.7	4.8	4.8	3.0
♀ Percent No. Scoleces -													
5.2	6.0	10.3	10.3	12.1	8.6	8.6	6.0	8.6	4.3	5.2	3.4	0.9	2.6
Total Percent No. Scoleces -													
2.9	3.6	7.2	9.7	10.3	10.6	9.6	8.1	9.7	6.1	5.9	4.1	2.8	2.8

Percent Length of Intestine -

64	66	68	70	72	74
♂ Percent No. Scoleces -					
1.2	1.2	1.2	0	0.6	0
♀ Percent No. Scoleces -					
3.4	1.7	0.9	0.9	0	0.9
Total Percent No. Scoleces -					
2.3	1.4	1.0	0.4	0.3	0.4

Table 4 (Cont)

70 Mean Number of Attached Scoleces Male - 44.8 Female - 24.1

Mean Position of Bile Ducts (Percent Length) Male - 30 Female - 28.

Percent Length of Intestine -

30	32	34	36	38	40	42	44	46	48	50	52	54	56
♂ Percent No. Scoleces -													
0.7	0.7	2.9	4.5	6.7	8.0	8.9	14.7	8.9	6.7	11.8	2.9	5.1	2.9
♀ Percent No. Scoleces -													
		1.2	0	8.3	13.7	9.5	16.6	10.8	2.5	5.4	6.6	8.3	1.2
Total Percent No. Scoleces -													
0.3	0.3	2.0	2.2	7.5	10.8	9.2	15.6	9.8	4.6	8.6	4.7	6.7	2.0

Percent Length of Intestine -

58	60	62	64	66	68
♂ Percent No. Scoleces -					
4.5	3.6	2.2	2.2	0.7	1.3
♀ Percent No. Scoleces -					
5.4	5.4	1.2	0	2.5	1.2
Total Percent No. Scoleces -					
4.9	4.5	1.7	1.1	1.6	1.2

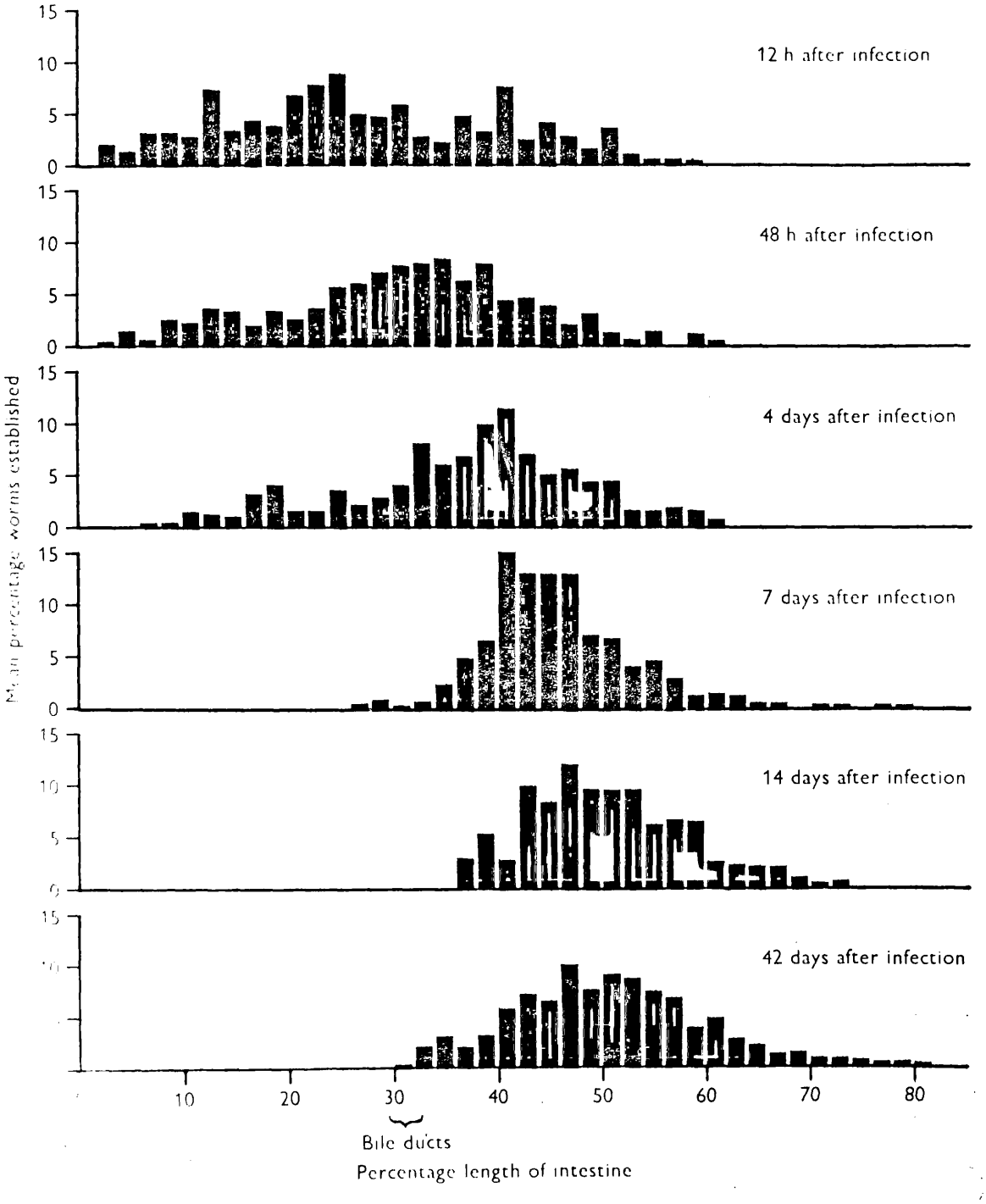

12 h after infection
48 h after infection
4 days after infection
7 days after infection
14 days after infection
42 days after infection
15
10
5
0
10
20
30
40
50
60
70
80
Bile ducts
Percentage length of intestine

FIG. 5

Table 5

Mean Percent Number of Destrobilating Scoleces

Days Post Infection	Male Host	Female Host
28	4.4	13.22 ± 7.98
42	10.79 ± 8.66	65.23 ± 12.13
56	27.57 ± 9.96	93.47 ± 2.35
70	93.53 ± 6.47	100.0 ± 0·0
112	100.0 ± 0·0	100.0 ± 0·0

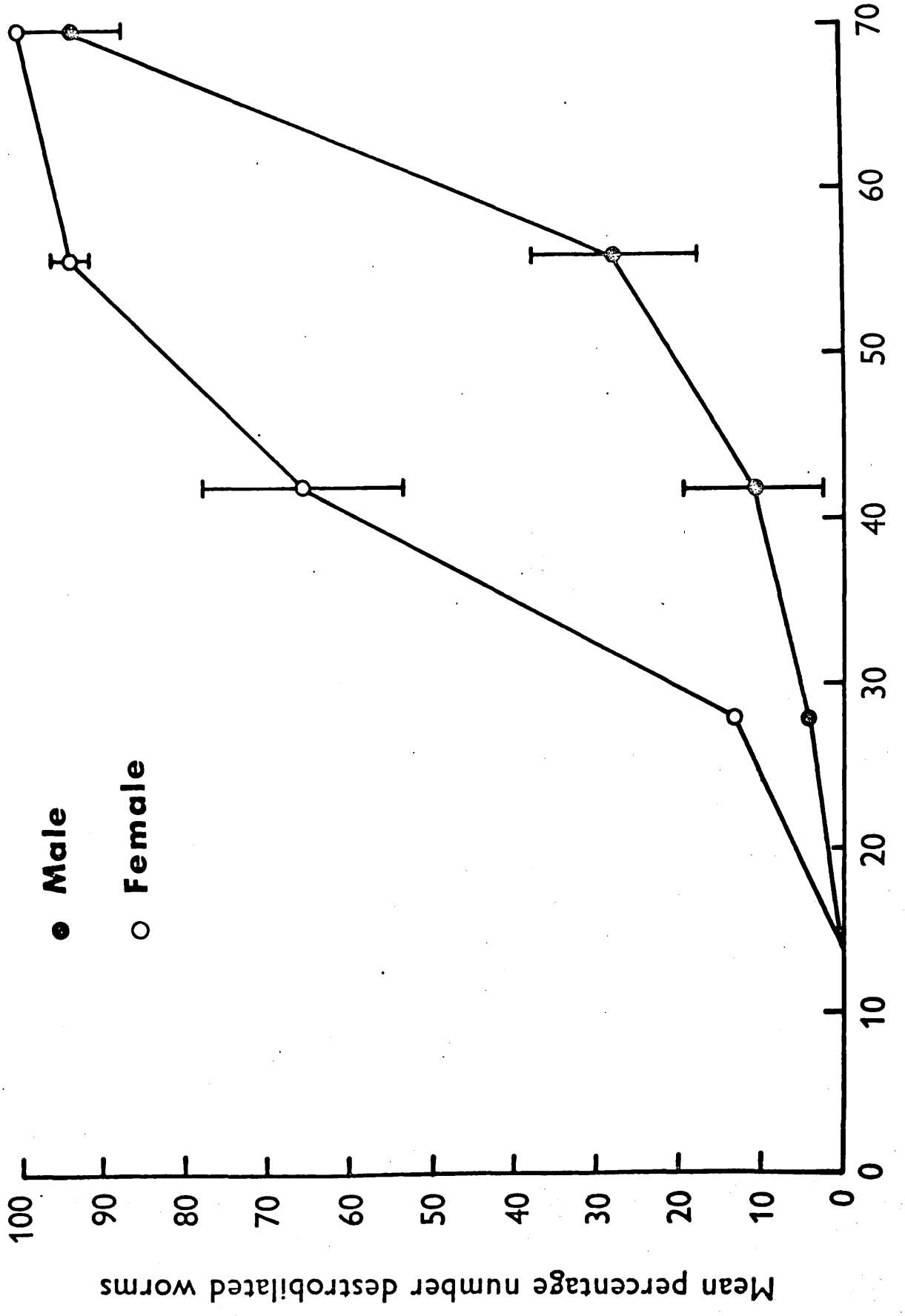

Mean percentage number destrobilated worms
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
Days after infection
● Male
○ Female

FIG. 6

Table 6

Mean Worm Dry Weight

Days Post Infection	Male Host	Female Host	Male + Female
4	0.034	0.034	0.034
7	1.95 ± 0.15	1.67 ± 0.14	1.81 ± 0.26
14	6.11 ± 1.03	4.69 ± 0.70	5.40 ± 0.73
28	5.91 ± 0.49	4.74 ± 0.47	5.32 ± 0.73
42	7.31 ± 0.41	7.07 ± 0.58	7.19 ± 0.86
56	8.74 ± 1.46	8.59	8.66 ± 0.94

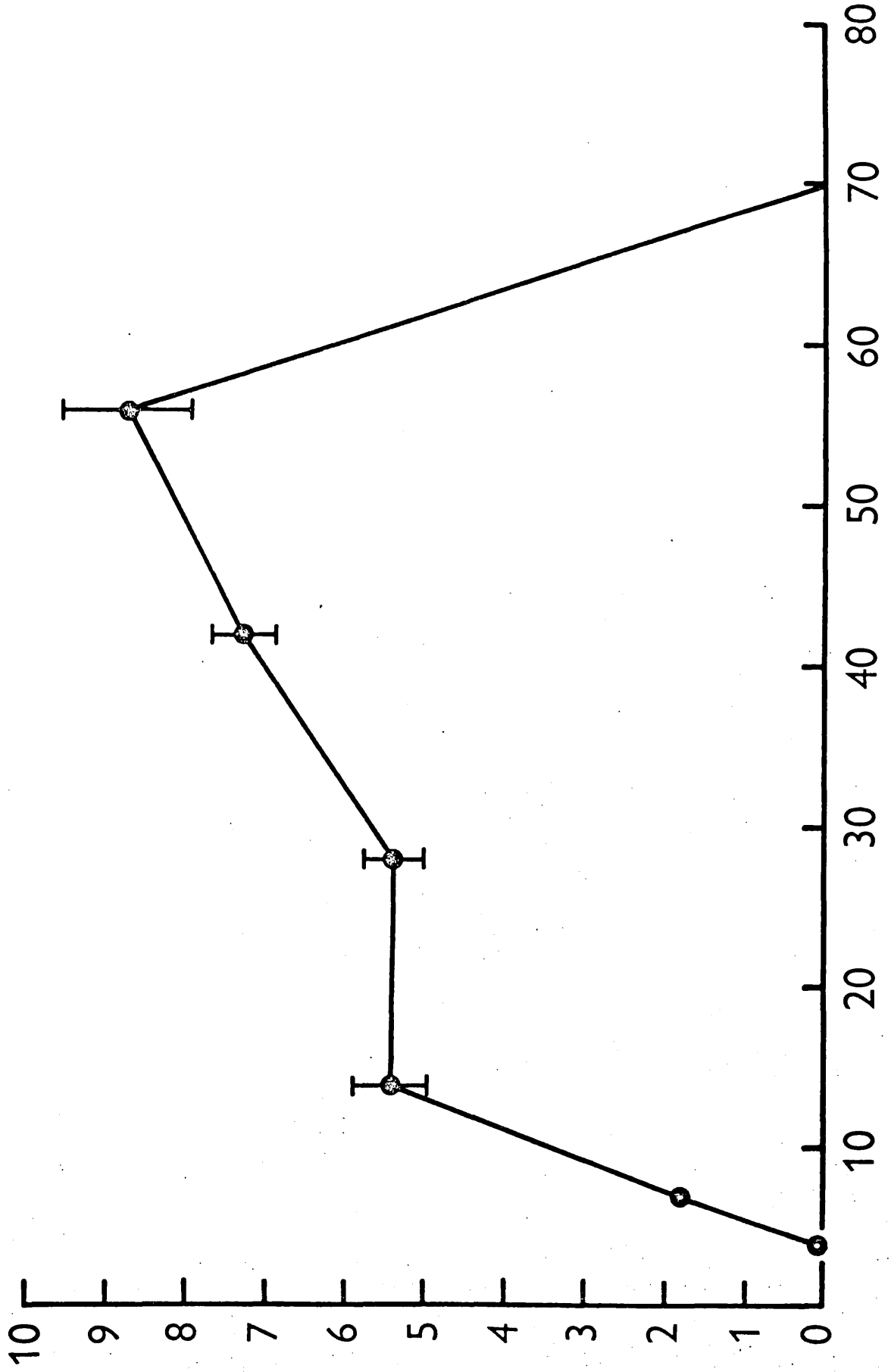

Mean dry weight (mg)
0
1
2
3
4
5
6
7
8
9
10
10
20
30
40
50
60
70
80
Days after infection

FIG. 7

Proglottid Output (Fig.3)

These results are expressed as proglottids per bird rather than per worm because variations in the size of the worm populations will influence the number of proglottids produced by each worm (Reid, 1942). This compensation should help to reduce the variation in numbers of proglottids produced by different sized populations.

A rapid rise in the rate of proglottid output occurs initially which levels out to an output of between 120 and 200 proglottids per bird per day. A slow decline in rate of output sets in 39 days after infection. The birds were autopsied 66 days after infection and it was found that 84.25% of the worms in the male birds and 83.83% in the female birds were partially or completely destrobilated.

Egg Production

The average number of eggs per proglottid varied between 197.0 and 388.0 over the infection. No particular pattern of production was detected.

Number and Distribution of Worms

There is a striking decrease in the number of worms recovered after the first 12 hours of infection (Fig.4). There follows a slight increase in numbers until another fall in recovery occurs at 56 days post infection. This fall continues until at 112 days post infection very few worms remain. The distribution of the worms is represented in Fig. 5, which shows that most worms attach themselves in a position anterior to the openings of the bile and pancreatic ducts in the first

12 hours. Consequent measurements indicate a gradual migration from this position and reattachment further down the intestine. The final position of attachment is reached by 14 days post infection and no further movement seems to occur.

This behaviour is probably responsible for the early fluctuations observed in worm numbers. After initial attachment the worms detach and move down the intestine during the next few days. It is likely that the decreased recoveries obtained at 1, 2 and 4 days post infection are due to some worms lying free in the lumen. They are microscopic at this stage and easily escape detection unless attached.

The incomplete recovery of numbers to ½-day post infection values at 7 days post infection suggests that a proportion of worms fail to reattach and are lost.

Numbers of Destrobilated Worms

It was observed that at 28 days post infection a small proportion of worms had lost part or all of the strobila. This process continues until at 70 days post infection almost all worms had destrobilated (Fig.6). It is apparent that destrobilization proceeds more rapidly in the female host than in the male, though by 70 days post infection the level of destrobilization is the same in both sexes.

There is no regeneration of strobilae and the scoleces are eventually discharged. Scoleces are lost from 56 days onwards and by 112 days post infection few are left, as already mentioned. However, the infection may be said to be effectively terminated when all worms have destrobilated at 70 days post infection.

Worm Growth

Dry weight measurements are presented in Fig.7 and show that there is a rapid period of growth until patency at 14 days post infection. There then follows a slower increase until complete destrobilization at 70 days post infection.

It should be noted that a crowding effect occurs in R. cesticillus infections (Reid, 1942) and this tends to obscure the true growth pattern when infections vary greatly in size. In the experiments described here, however, the sizes of adult worm infections were similar.

Discussion

In this study it has been shown that Raillietina cesticillus undergoes a posterior migration in the intestine of the fowl during the establishment phase. Initially the young cestodes attach themselves to the mucosa of the duodenum, but migration and reattachment occurs during the first 2 weeks of infection. By this time all surviving worms are attached in a position posterior to the openings of the bile and pancreatic ducts.

These results confirm those of Foster and Daugherty(1959), although in the present study the migration of the cestodes seems to commence slightly earlier than in their experiments. It is very likely that the time of day at infection is responsible for the timing of the posterior migration, as the peristaltic activity of the intestine is obviously related to the time of feeding. In the present study all birds were infected at 11a.m., 4 hours after the first feed. No mention

is made of the time of day at infection in Foster and Daugherty's paper.

Goodchild and Harrison (1961) reported a similar pattern of migration for Hymenolepis diminuta in the rat, with movement from the first to the second quarter of intestine in the first 5 days of infection. These observations contradict those of Chandler (1939) for the same host-parasite system.

It is not known what factors determine the final position of worm attachment. Foster and Daugherty suggest that this 'zone of viability' may be correlated with the glucose absorption area of the intestine and quote the work of Reynell and Spray (1957), who found that in the rat glucose is always absorbed in the upper half of the intestine. The proximity of the bile and pancreatic ducts to the 'zone of viability' suggests that bile and pancreas secretions may play an important part in the normal development of R. cesticillus. Bile constituents are known to affect helminth parasites in several ways (Smyth and Haslewood, 1963).

There is little information on the life span of R. cesticillus. Harwood (1938) reported that the cestode survives for 5 or 6 months, while Ackert (1921) found that chickens remained infected for up to 13.5 months, but in the present study the infection was effectively terminated by 70 days. This is a similar life span to that of Raillietina fedjuschina in the domestic fowl, described by Skutar (1963). This species survives for 44 - 61 days in young birds. It is markedly dissimilar to R. cesticillus in other respects however.

Read (1959) described three different patterns of cestode growth, ranging from Hymenolepis diminuta, which grows con-

tinuously for about 18 months and shows no senescence over this period, to Hymenolepis nana which has a life span of about 25 days with growth ceasing after 14 - 16 days. Hymenolepis citelli occupies an intermediate position between these two extremes. Early growth is rapid and then slows until 70 - 90 days after infection, when growth ceases and the worm becomes smaller and smaller as segments are shed. R. cesticillus shows a similar pattern of growth and onset of senescence as H. citelli.

Read shows that the different patterns of growth of the three Hymenolepid species can be correlated with sensitivity to shortage of carbohydrate and Reid (1942) shows that destrobilization of R. cesticillus can be induced by starvation of the avian host, though with eventual regeneration of strobilae. Starvation of the host results in a diminution of worm glycogen content and it is possible that senescence, characterised by destrobilization may be linked in some way with carbohydrate metabolism.

The question still remains, however, as to whether the destrobilization of R. cesticillus is a manifestation of inherent senescence, is caused by physiological interaction by the maturing avian host or is caused by other extraneous factors. Two such factors are the feeding behaviour of the chickens (in view of the observations of Reid (1942)) and the coccidiostats Pancoxin, Amprolium, Sulphaquinoxaline and Ethopabate contained in the feed. These factors were eliminated as being of any importance in destrobilization by checking the amount of feed consumed by the chickens and by the observation that coccidiostat-free feed did not prevent destrobilization.

Attention is therefore focussed on inherent senescence and host physiology. The marked sex difference in rates of destrobilization suggest that host physiology has at least some influence on the process.

CHAPTER 2

THE EFFECT OF HOST AGE ON THE COURSE OF INFECTION OF R. CESTICILLUS

Introduction

In the series of experiments described in the preceding chapter it was established that in the young susceptible host a progressive destrobilization of the worms occurs and the infection is effectively terminated 70 days after infection. A marked difference in the rate of destrobilization in the two host sexes was noted and this suggests that the course of infection of the cestode may well be affected by the process of host maturation.

To date, three studies on the effect of host age on R. cesticillus have been carried out. Ackert and Reid (1937) found that fewer worms established themselves in birds infected at 71 - 150 days old than in birds infected at 20 - 21 days old. Fewer proglottids were produced per worm in the former group than in the latter. It was concluded that age resistance comes in at 2½ - 5 months of age. Meinkoth (1947) and Sinha and Srivastava (1958) made similar observations for birds infected at 27 and 12 weeks respectively.

These studies demonstrate that as the host ages it becomes more resistant to R. cesticillus, a conclusion that is in agreement with most work on the effects of host age on parasitic infections. It is interesting to note, however, that Ackert and Case (1938) successfully infected adult (presumably female) chickens with R. cesticillus and they

point out that most records of tapeworm infestations of chickens are from examination of mature birds.

In the series of experiments described here the effects of host age on parasite take, destrobilization, distribution, growth, proglottid production and egg production were studied.

Materials and Methods

Each experimental group consisted of eight male and eight female chickens. They were infected with 100 cysticercoids each at the following ages:- 3, 14, 28, 56, 84, 112 and 224 days. Ten birds, five male and five female, of each group were autopsied 14 days after infection, with the onset of patency. This corresponds to the end of the second stage of the cestode's association with the host, as defined by Rogers (1962). The remaining three male and three female birds in the group were used to monitor proglottid and egg production during the infection.

Results

	Table	Figure
Mean Number of Proglottids Per Bird	7	8, 9
Mean Number of Eggs Per Proglottid	8	10, 11
Mean Number of Scoleces	9	12
Mean Distribution of Scoleces	10	13, 14
Mean Percent Number of Destrobilating Scoleces	11	15
Mean Worm Weight	12	16
Mean Body and Gonad Weights	13	17

Table 7

Mean Number of Proglottids Per Bird

Days Post Infection	3 day old		14 day old		28 day old		56 day old		84 day old		112 day old		224 day old	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
13			34.6	30.6			12.5	6.0						
14	9.0	57.3												
15	48.6	136.6	52.4	44.8	12.0	6.0	46.0	31.6	6.0	24.0				117.0
16	67.3	132.0												
17	90.6	150.0	85.3	103.3			33.5	41.0	15.0			102.0		
18	130.3	137.6			27.0	11.0	79.0	37.3	15.0			124.0		
19	153.3	188.0	150.0	187.3			60.5	38.0	23.5			123.0		
20									32.5			134.0		
21			130.0	163.3			83.0	55.0				127.0		
22	172.6	168.0			21.6	36.0			26.5			93.0		
23	192.0	202.6					115.0	76.0				18.0		
24							123.5	88.0						
25	186.3	157.3					83.0	56.5	46.5			22.0		
26	161.3	100.0	157.3	171.0	63.6	64.3			24.5					
27	192.6	127.3	171.3	145.3			112.0	10.0				28.0		

Table 7 (Cont)

Mean Number of Proglottids Per Bird

Days Post Infection	3 day old		14 day old		28 day old		56 day old		84 day old		112 day old		224 day old	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
28							77.0							151.0
29	150.6	116.6	137.0	205.3					4.0		23.0			
30					26.0	23.6	76.5		0.5					
31	147.6	143.6												
32	151.0	170.0					96.5							
33			113.3	128.6			72.0		12.0					
34					32.3	23.6	40.5				59.0			
35											109.0			
36			73.6	124.0										
37	156.0	241.3			6.3	9.3	74.5				145.0			
38							32.5				116.0			120.0
39	107.0	176.0					60. 0				151.0			
40			58.6	9.3			36.5							
41							34.5		8.0					
43	81.6	121.3					18.5							
44			12.0	83.0			19.5							
45							36.5							
46							14.0							

Table 7 (Cont)

Mean Number of Proglottids Per Bird

Days Post Infection	3 day old		14 day old		28 day old		56 day old		84 day old		112 day old		224 day old	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
47							9.0							
48			10.3	81.0			9.0							65.0
49														
50	67.0	78.6					8.0					173.0		
51			10.0	85.6										
52							10.0							
54	28.0	83.6												
55							19.0							
58														81.0
59	46.0	28.6												
60							18.5							
61												153.0		
63	13.6	38.0												
68														83.0
78														75.0
88												201.0		80.0

Table 7 (Cont)

Mean Number of Proglottids Per Bird

Days Post Infection	3 day old		14 day old		28 day old		56 day old		84 day old		112 day old		224 day old	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
98														44.0
108														36.0
118														109.0
128														74.0
148														150.0
Mean Number of Scoleces at autopsy	43.6± 11.25	50.6± 5.36	41.6± 12.54	39.3± 6.38	55.0± 12.41	54.3± 11.29	25.5± 3.5	36.6± 8.87	49.0± 3.0	58.5± 14.5	-	34.0	-	29.0

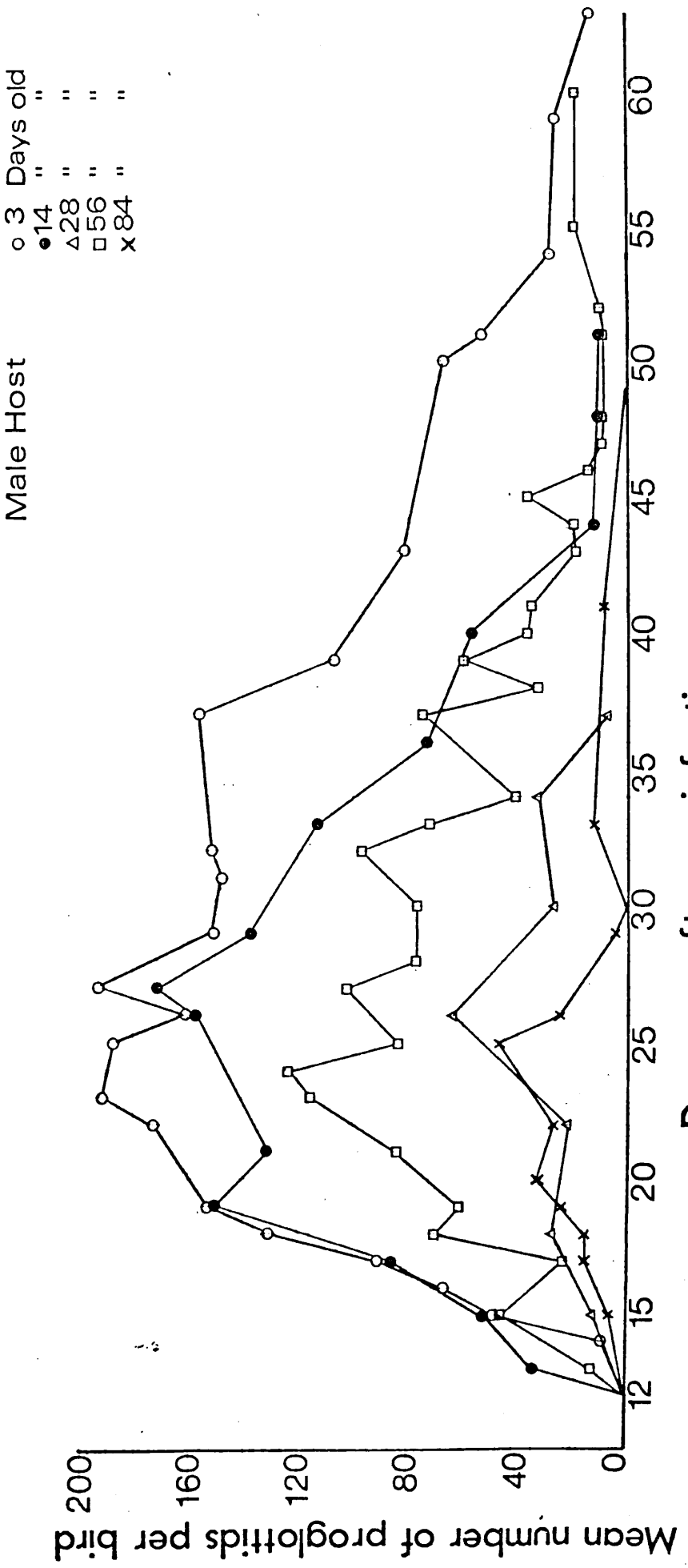

Male Host
○ 3 Days old
● 14 " " "
△ 28 " " "
□ 56 " " "
× 84 " " "
Mean number of proglottids per bird
0
40
80
120
160
200
12
15
20
25
30
35
40
45
50
55
60
Days after infection

FIG. 8

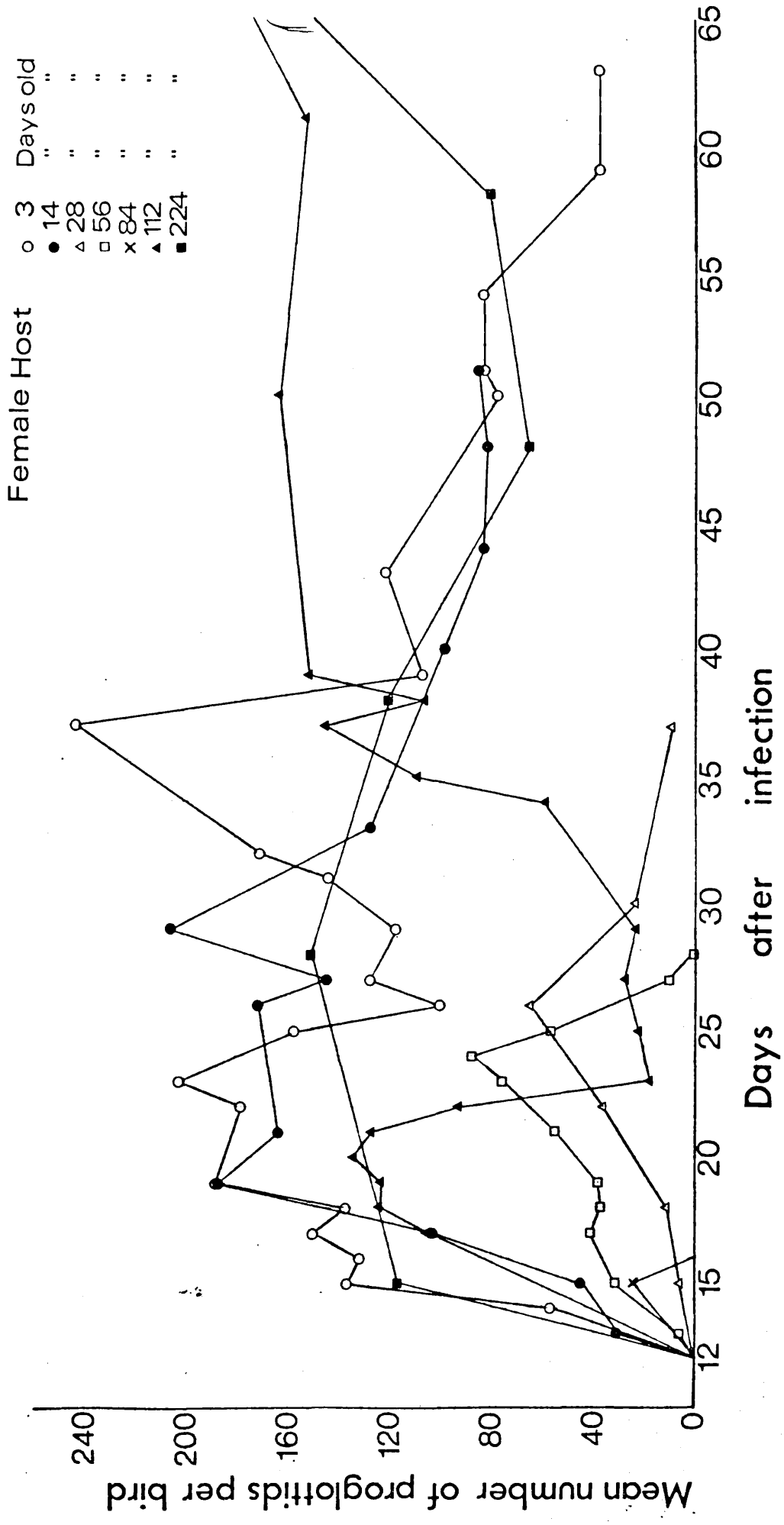

Female Host
3 Days old
14 " "
28 " "
56 " "
84 " "
112 " "
224 " "
Mean number of proglottids per bird
0
40
80
120
160
200
240
Days after infection
12
15
20
25
30
35
40
45
50
55
60
65

FIG. 9

Table 8

Mean Number of Eggs Per Proglottid

Days Post Infection	3 Day Old		14 Day old		28 Day old		56 Day old		184 Day old		112 Day old		224 Day old	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
13							280.0	143.2			—		—	
14	197.0	289.5	184.0	146.9										
15	245.8	278.3	248.7	276.0	282.0	364.5	333.7	330.0	366.2	110.4				217.1
16	354.5	345.7												
17	373.0	388.0	372.5	381.4			385.0	110.5	151.3			231.4		
18	349.0	336.0			326.0	280.0	397.9	312.0	298.1			291.3		
19	329.5	324.5	422.0	435.0			403.5	413.0	323.9			300.4		
20									250.0			271.2		
21			425.0	400.6			477.5	446.0				440.0		
22	376.0	346.0			284.0	286.3			240.8			397.4		
23	383.5	342.5					494.0	430.0				414.2		
24							292.0	352.0						
25	263.0	247.9					436.0	485.0	287.3			409.7		
26	327.5	242.5	405.0	386.5	275.5	347.5			326.3					

Table 8(Cont)

Mean Number of Eggs Per Proglottid

Days Post Infection	3 Day old		14 Day old		28 Day old		56 Day old		84 Day old		112 Day old		224 Day old	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
27	316.5	295.0	428.0	377.0			378.0	258.3				3845		
28							431.0						200.4	
29	334.3	357.5	417.5	260.0					236.2			420.8		
30					257.0	329.5	456.0							
31	274.0	264.4												
32	276.4	298.5					344.3							
33			474.0	327.0			400.5		198.9					
34					196.9	258.3	397.5							
35														
36			450.4	440.0										
37	311.0	288.5			224.0	114.2	402.5					217.4		
38							310.0						24.4	
39	301.8	270.0					296.5					287.9		
40			378.0	374.5			288.5							

Table 8 (Cont)

Mean Number of Eggs Per Proglottid

Days Post Infection	3 Day old		14 Day old		28 Day old		56 Day old		84 Day old		112 Day old		224 Day old	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
41					309.7				233.9					
43	350.1	341.0			316.7									
44			415.5	338.5	300.0									
45					370.3									
46					261.5									
47					243.5									
48			405.0	444.0	295.8									196.9
49														
50	300.7	376.0												
51	271.3	352.5	452.0	406.0	356.0									
52					355.5									
54	265.7	281.0												
55					184.8									
58														211.6
59	314.2	373.0												

Table 8 (cont)

Mean Number of Eggs Per Proglottid

Days Post Infection	3 Day old		14 Day old		28 Day old		56 Day old		84 Day old		112 Day old		224 Day old	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
60					190.9									
61												302.3		
63	259.0	278.5												
68														207.9
78														
88														
98														
108														
118														246.0
128														246.8
148														311.5

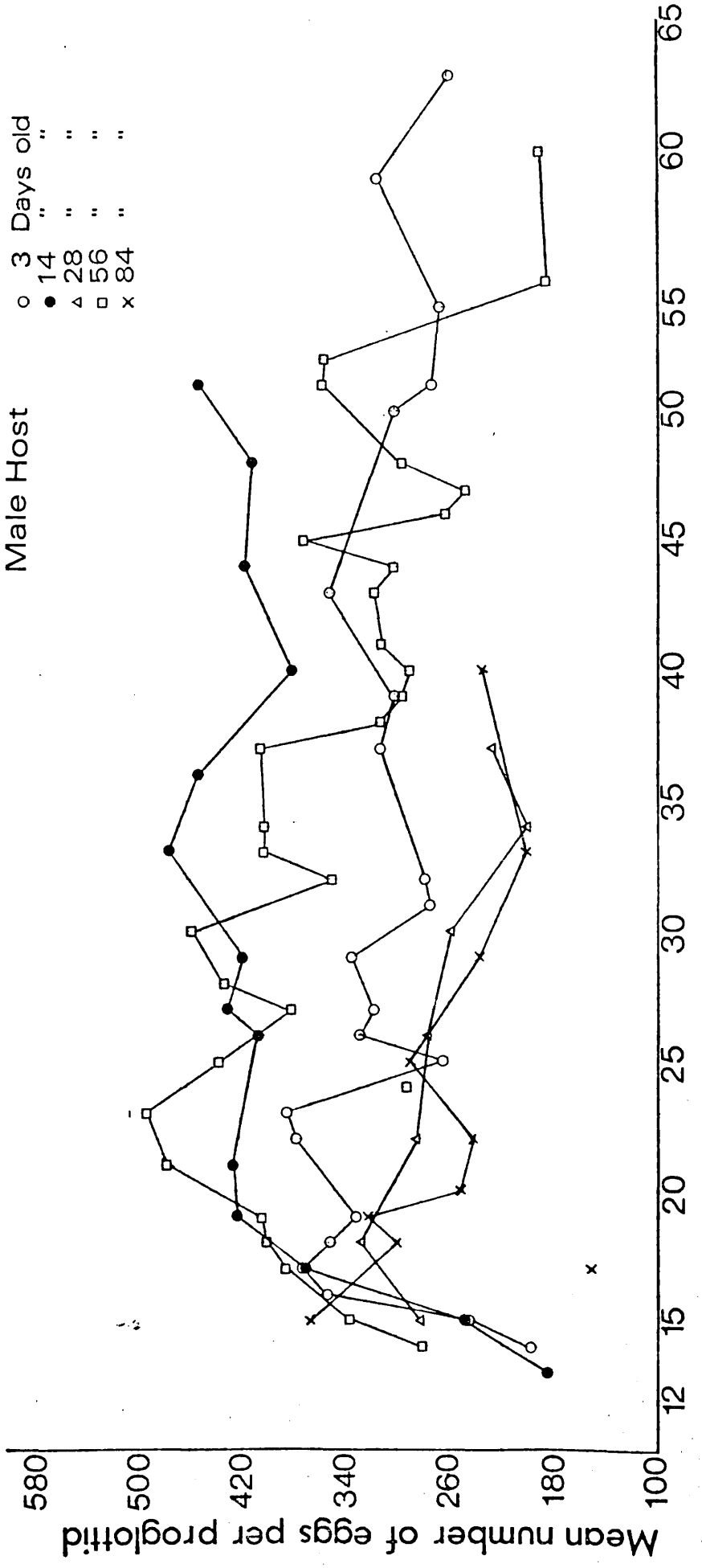

Male Host
○ 3 Days old
● 14 " "
△ 28 " "
□ 56 " "
× 84 " "
Mean number of eggs per proglottid
580
500
420
340
260
180
100
12
15
20
25
30
35
40
45
50
55
60
65
Days after infection

FIG. 10

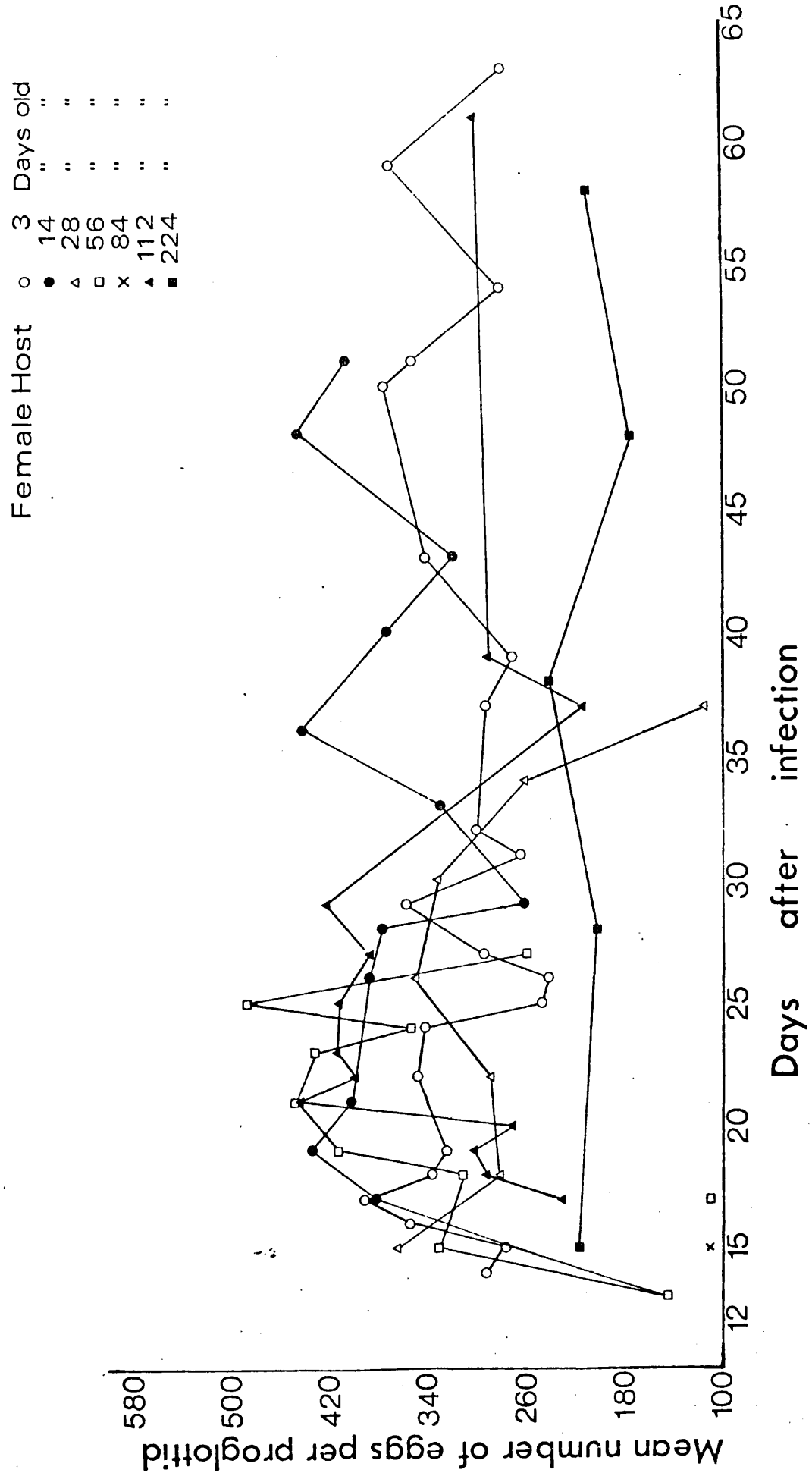

Female Host
3 Days old
14 " "
28 " "
56 " "
84 " "
112 " "
224 " "
Mean number of eggs per proglottid
580
500
420
340
260
180
100
12
15
20
25
30
35
40
45
50
55
60
65
Days after infection

FIG. 11

Table 9

Mean Number of Scoleces

Age of Chicken at Infection (Days)	Male Host	Female Host
3	48.2 ± 6.20	46.0 ± 4.30
14	51.4 ± 7.89	46.0 ± 1.95
28	52.4 ± 4.10	45.2 ± 5.64
56	52.5 ± 5.39	48.0 ± 4.75
84	48.2 ± 6.80	57.0 ± 5.13
112	45.8 ± 2.44	41.0 ± 5.20
224	23.8 ± 7.60	64.2 ± 6.57

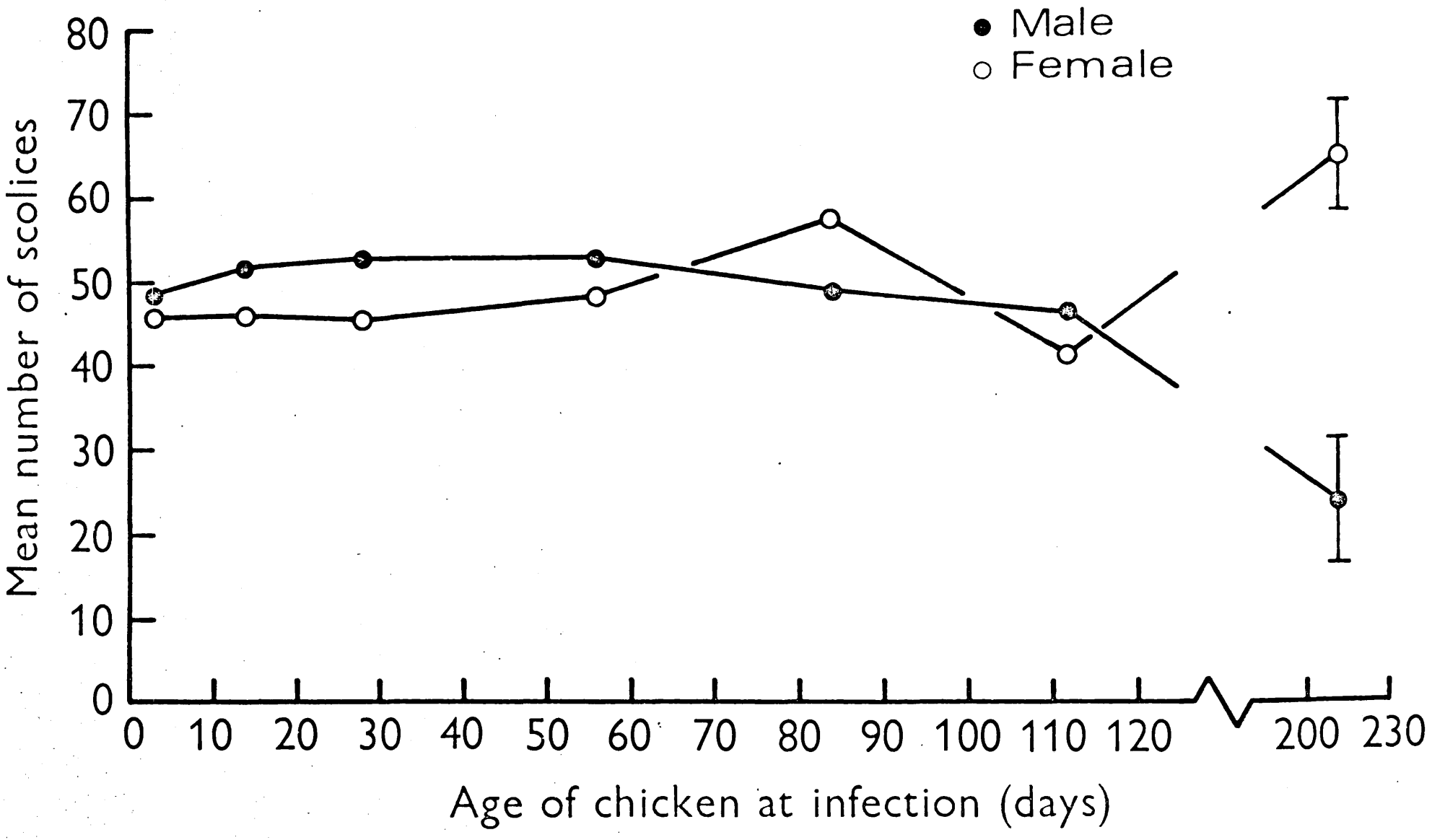

Male
Female
Mean number of scolices
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100 110 120 200 230
Age of chicken at infection (days)

FIG. 12

Table 10

Mean Distribution of Scoleces

Age of Chicken at Infection (Days)

3 Mean Number of Attached Scoleces Male - 40.8 Female - 40.6

Mean Position of Bile Ducts (Percent Length) Male - 32 Female - 32

Percent Length of Intestine -

36	38	40	42	44	46	48	50	52	54	56	58	60	62	64	66	68	70	72
♂ Percent No. Scoleces -																		
4.9	7.4	3.4	10.8	9.3	11.3	10.3	8.8	8.8	4.9	7.4	5.9	0.5	2.0	1.5	2.0	0.5	0.5	0
♀ Percent No. Scoleces -																		
1.0	2.9	2.0	9.3	7.4	12.8	8.9	10.3	10.3	7.4	5.9	6.9	4.4	2.5	2.5	2.0	1.5	0.5	1.5
Total Percent No. Scoleces -																		
2.9	5.1	2.7	10.1	8.3	12.1	9.6	9.5	9.5	6.1	6.7	6.4	2.4	2.3	2.0	2.0	1.0	0.5	0.7

14 Mean Number of Attached Scoleces Male - 42.2 Female - 39.0

Mean Position of Bile Ducts (Percent Length) Male - 32 Female -30

Percent Length of Intestine -

30	32	34	36	38	40	42	44	46	48	50	52	54	56	58	60	62	64	66	68	70	72	74	76	78	80	82
♂ Percent No.Scoleces																										
0	0	3.8	6.6	3.8	2.8	5.2	9.0	10.9	7.1	8.1	6.2	8.1	7.6	2.8	6.6	1.4	3.8	1.9	0.9	1.9	0.5	0	0.5	0	0	0.5

♀ Percent No. Scoleces -

0.5 0.5 2.6 4.6 4.1 9.7 7.2 6.2 10.8 5.1 8.7 8.2 4.1 5.6 3.6 4.6 3.1 1.0 3.1 1.5 2.6 0.5 1.0 1.0 0 0 0

Total Percent No.Scoleces -

0.2 0.2 3.2 5.6 3.9 6.2 6.2 7.6 10.8 6.1 8.4 7.2 6.1 6.6 3.2 5.6 2.2 2.4 2.0 1.2 2.2 0.5 0.5 0.7 o 0 0.2

28 Mean Number of Attached Scoleces Male -51.4 Female - 41.6

Mean Position of Bile Ducts (Percent Length) Male - 30 Female -30

Percent Length of Intestine -

28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76

♂ Percent No. of Scoleces -

1.6 2.3 4.7 9.3 10.1 8.2 5.1 6.6 7.0 9.8 7.8 5.8 3.9 3.9 2.7 2.3 2.3 1.2 1.6 0.8 1.2 1.2 0.4 0.4

♀ Percent No. Scoleces -

2.4 3.8 8.6 3.4 7.2 6.2 12.5 8.2 11.0 8.5 10.6 4.8 4.8 4.3 1.0 0.5 0.5 0.5 0.5 0 0 0.5 0 0 0

Total Percent No. Scoleces -

1.2 2.7 5.4 4.0 8.2 8.1 10.3 6.6 8.8 7.7 10.2 6.3 5.3 4.1 2.4 1.6 1.4 1.4 0.8 0.8 0.4 0.8 0.6 0.2 0.2

Table 10 (Cont)

56 Mean Number of Attached Scoleces Male - 50.5 Female - 47.0

Mean Position of Bile Ducts (Percent Length) Male -32 Female -30

Percent Length of Intestine -

28	30	32	34	36	38	40	42	44	46	48	50	52	54	56	58	60	62	64	66	68	70	72	74	76
♂ Percent No. Scoleces -																								
0.5	4.0	4.5	5.0	113	6.0	6.0	8.9	7.4	174	119	2.5	4.9	5.4	4.0	4.5	1.0	2.0	1.5	0	1.0	2.0	0.5	0.5	0.5
♀ Percent No. Scoleces -																								
0.9	0	1.3	3.8	1.7	3.4	3.0	7.0	8.5	3.8	102	8.0	7.7	8.9	8.5	4.3	6.0	5.5	3.0	04	1.3	2.1	0.4	0	0.4
Total Percent No. Scoleces -																								
0.7	2.0	2.9	4.4	6.5	4.7	4.5	7.9	7.9	5.6	110	5.2	6.3	7.1	6.2	4.4	3.5	3.7	2.2	02	1.1	2.0	0.4	0.2	0.4

Table 10 (Cont)

84 Mean Number of attached Scoleces Male - 47.6 Female - 56.6

Mean Position of Bile Ducts (Percent Length) Male - 30 Female - 28

Percent Length of Intestine -

14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48	50	52
♂ Percent No. Scoleces -																			
0	2.1	5.0	4.2	6.7	4.2	2.9	5.5	5.0	4.6	2.5	3.8	4.2	4.6	5.5	3.8	2.9	4.2	1.3	2.5
♀ Percent No. Scoleces -																			
0.7	1.1	1.4	1.8	7.1	4.6	5.7	3.2	2.8	4.6	5.3	3.9	8.5	3.9	8.1	8.1	2.5	0.4	0.7	4.2
Total Percent No. Scoleces -																			
0.3	1.6	3.2	3.0	6.9	4.4	4.3	4.3	3.9	4.6	3.9	3.8	6.3	4.2	6.8	5.8	2.7	2.3	1.0	3.3

Percent Length of Intestine -

54	56	58	60	62	64	66	68	70	72	74	76	78	80	82	84	86
♂ Percent No. Scoleces -																
2.1	2.1	2.9	1.7	2.1	2.1	2.1	1.3	1.7	1.3	0	1.7	0.8	1.3	0.4	0.4	0.4
♀ Percent No. Scoleces -																
3.5	3.2	0.7	2.8	2.5	1.4	2.1	2.1	1.4	1.4	0.4	0.7	0	0	0	0	0
Total Percent No. Scoleces -																
2.8	2.6	1.8	2.2	2.3	1.7	2.1	1.7	1.5	1.3	0.2	1.2	0.4	0.6	0.2	0.2	0.2

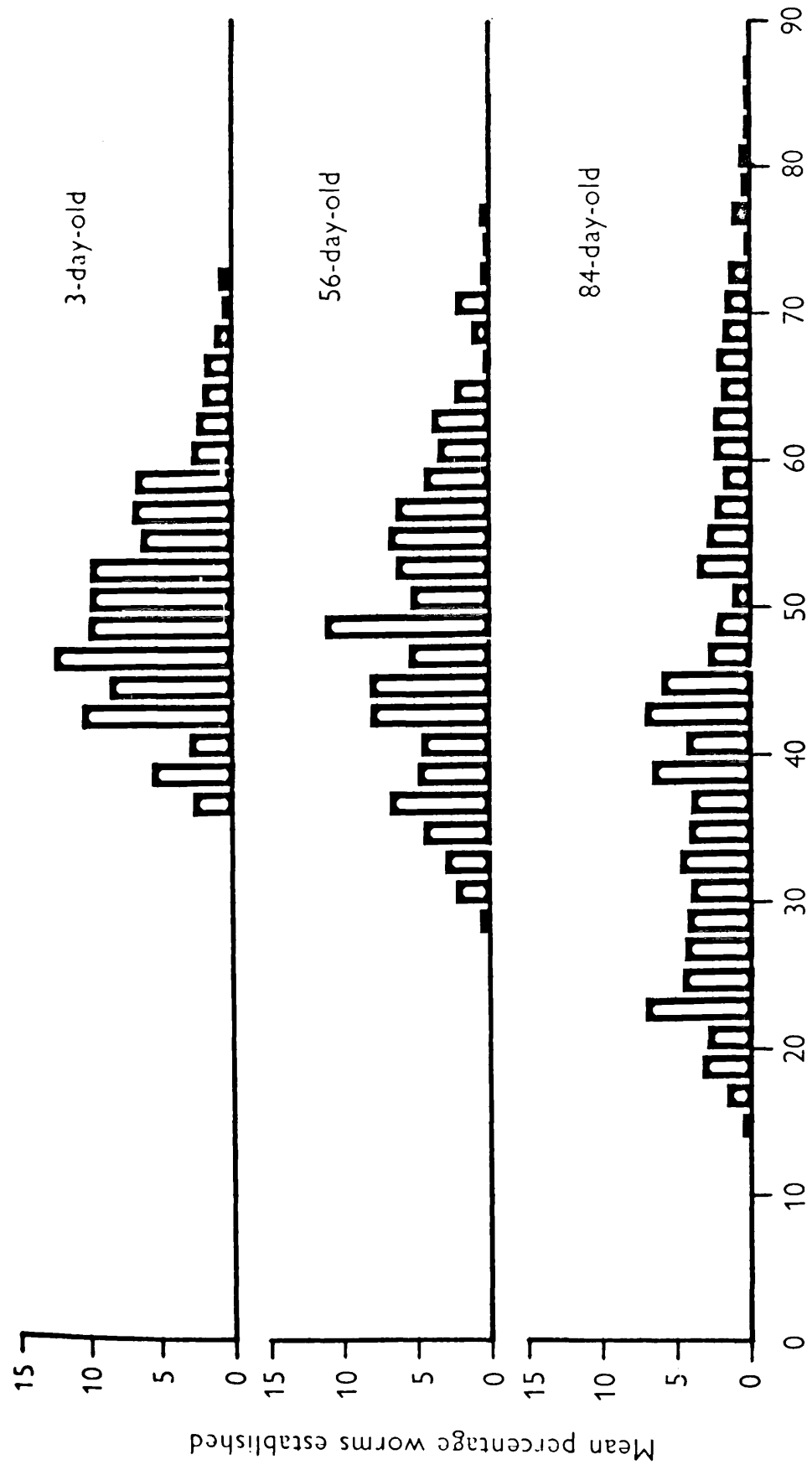

3-day-old
56-day-old
84-day-old
Mean percentage worms established
15
10
5
0
0
10
20
30
40
50
60
70
80
90
Bile ducts
Percentage length of intestine

FIG. 13

Table 10 (Cont)

112 Mean Number of Attached Scoleces Male -43.4 Female - 40.2

Mean Position of Bile Ducts (Percent Length) Male -30 Female -32

Percent Length of Intestine -

10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46
♂ Percent No. Scoleces -																		
0.5	1.8	0	0	4.2	1.4	5.1	3.2	3.2	6.0	6.5	3.2	0.9	1.8	3.2	4.2	4.2	6.0	4.6
♀ Percent No. Scoleces -																		
-	-	-	-	-	-	-	0.5	0	1.0	3.0	1.5	4.0	3.5	2.0	4.9	7.5	14.4	11.9

Percent Length of Intestine -

48	50	52	54	56	58	60	62	64	66	68	70	72	74	76	78
♂ Percent No. Scoleces -															
4.6	3.7	3.7	3.7	4.6	1.8	3.7	3.7	0.9	2.3	1.8	1.8	1.4	2.3	0.5	0
♀ Percent No. Scoleces -															
5.5	10.9	7.0	4.5	2.5	4.5	4.0	2.0	2.0	1.0	0.5	0.5	1.0	0.5	1.0	1.0

Table 10 (Cont)

224 Mean Number of Attached Scoleces Male - 23.8 Female - 64.2

Mean Position of Bile Ducts (Percent Length) Male - 32 Female -32

Percent Length of Intestine -

8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48	50	52	54
♂ Percent No. Scoleces -																							
0	0.8	0	0	1.7	0.8	0.8	0.8	1.7	0.8	1.7	2.6	6.0	2.6	o.8	2.6	0.8	4.3	3.3	7.7	2.6	4.3	5.1	5.1
♀ Percent No. Scoleces -																							
0.6	03	o.6	1.2	1.2	1.6	1.2	0.9	0.9	1.2	2.5	0.9	2.8	2.2	2.2	2.5	3.3	3.7	3.1	3.3	4.7	5.6	6.5	4.1

Percent Length of Intestine -

56	58	60	62	64	66	68	70	72	74	76	78	80	82	84	86	88	90	92	94	96	98	100
♂ Percent No. Scoleces -																						
5.1	5.1	9.4	9.4	2.6	0.8	0	4.3	0.8														
♀ Percent No. Scolec4s -																						
3.3	4.1	7.2	1.9	2.9	1.7	2.2	1.9	0.8	0.8	1.9	0.9	1.2	1.6	1.9	1.9	09	0.9	1.2	0.9	0.3	0.3	0.6

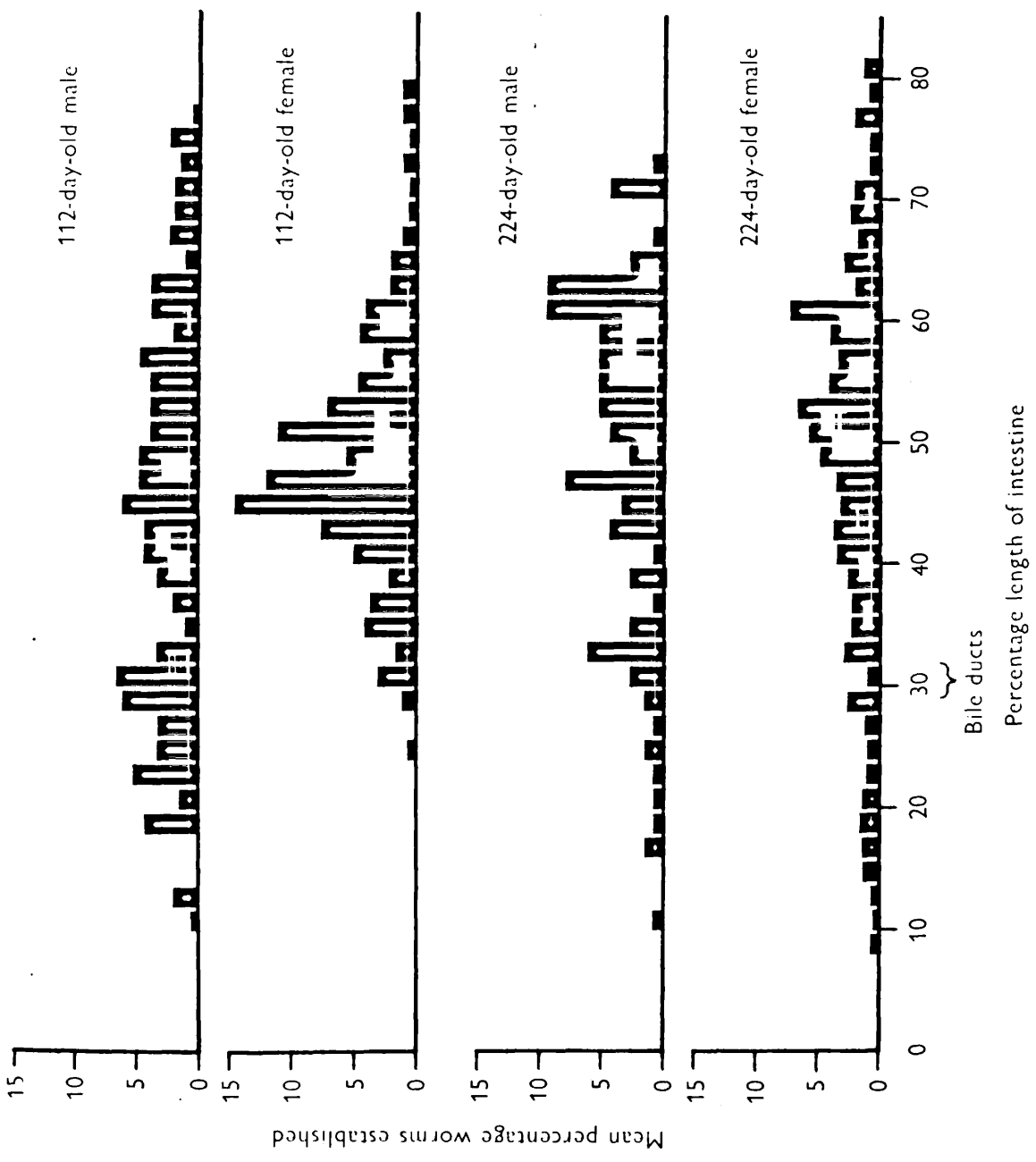

112-day-old male
112-day-old female
224-day-old male
224-day-old female
Mean percentage worms established
0
5
10
15
0
10
20
30
40
50
60
70
80
Bile ducts
Percentage length of intestine

FIG. 14

Table 11

Mean Percent Number of Destrobilating Scoleces

Age of Chicken at Infection (Days)	Male Host	Female Host
3	0.00	0.00
14	0.00	0.00
28	4.42 ± 2.03	7.52 ± 1.94
56	15.38 ± 5.16	58.38 ± 6.72
84	51.74 ± 8.30	56.26 ± 5.72
112	96.90 ± 3.08	60.71 ± 15.81
224	98.06 ± 1.19	55.38 ± 10.19

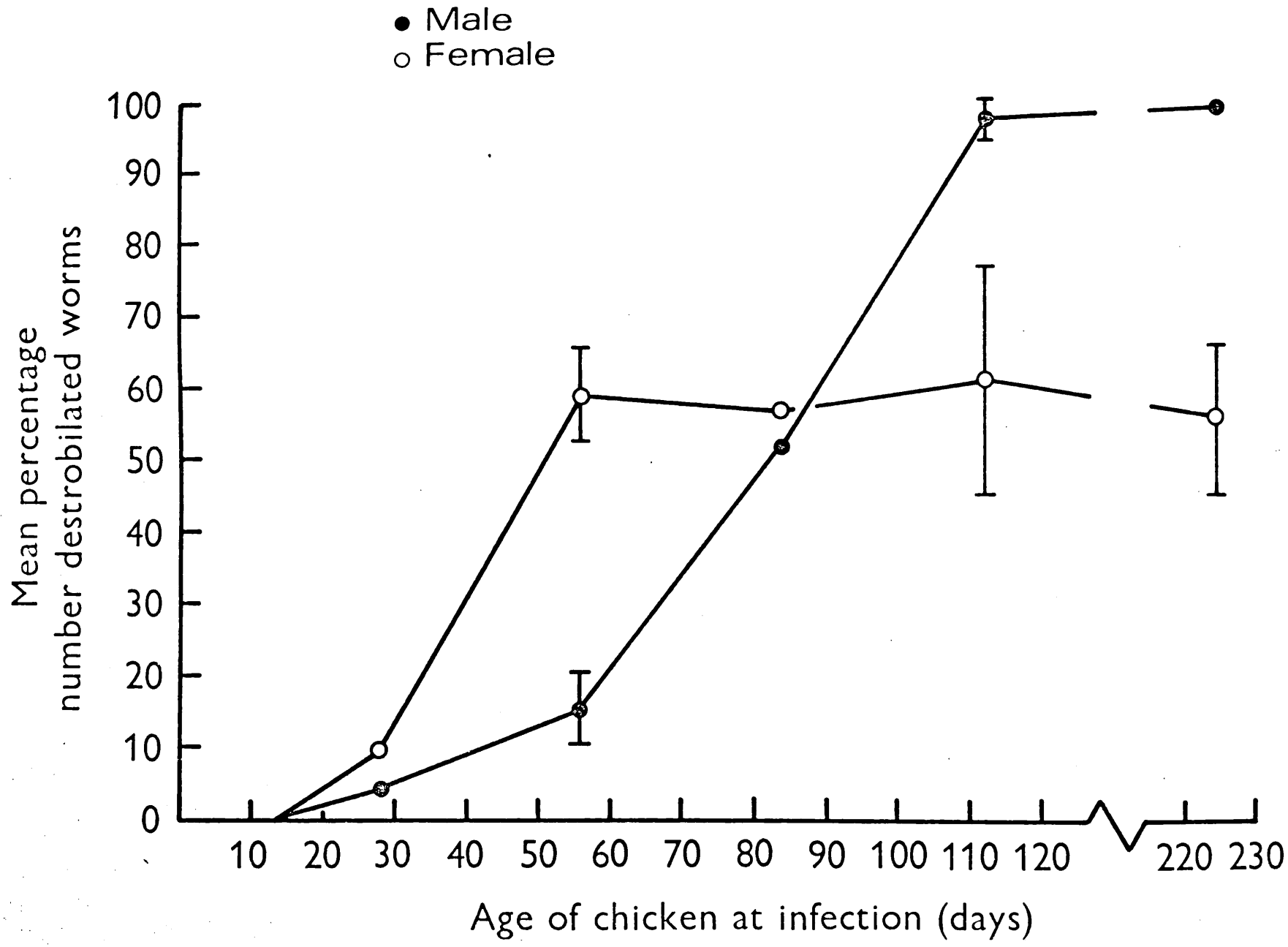

● Male
○ Female
Mean percentage number destrobilated worms
100
90
80
70
60
50
40
30
20
10
0
10 20 30 40 50 60 70 80 90 100 110 120 220 230
Age of chicken at infection (days)

FIG. 15

Table 12

Mean Worm Weight

Age of Chicken at Infection (Days)	Male Host	Female Host
3	6.11 ± 1.03	4.69 ± 0.70
14	7.72 ± 1.16	4.90 ± 2.39
28	5.66 ± 0.38	4.89 ± 0.32
56	6.00 ± 0.54	3.28 ± 0.49
84	3.92 ± 0.89	4.27 ± 0.42
112	[illegible]	6.22 ± 0.22
224	-	6.25 ± 0.19

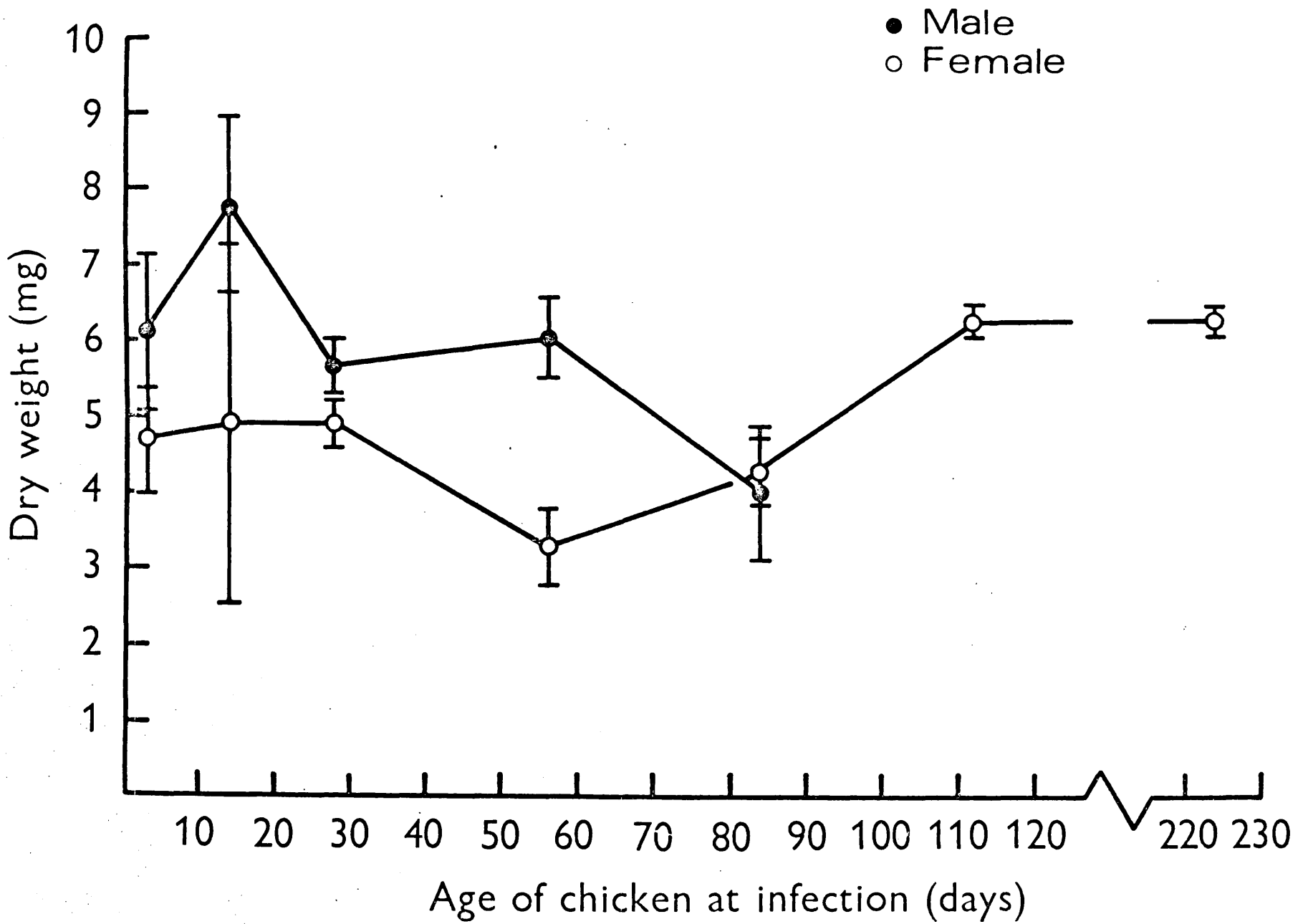

Male
Female
Dry weight (mg)
10
9
8
7
6
5
4
3
2
1
10 20 30 40 50 60 70 80 90 100 110 120 220 230
Age of chicken at infection (days)

FIG. 16

Table 13

Mean Body and Gonad Weights

Male Host

Age of Chicken at Infection Days	Body Weight (gm)	Gonad Weight (mg)	Gonad Weight Body Weight (%)
3	194.0	41.9	0.0216
14	375.0	81.3	0.0216
28	702.0	165.2	0.0236
56	1,287.5	4,470.1	0.347
84	1,776.0	14,466.7	0.814
112	2,020.0	17,659.2	0.874
224	2,680.0	37,614.8	1.402

Female Host

3	162.0	15.1	0.0094
14	312.0	80.8	0.0259
28	608.0	130.7	0.0214
56	960.0	260.1	0.0276
84	1,346.0	463.1	0.0344
112	1,580.0	1,285.8	0.0814
224	1,990.0	44,308.8	2.226

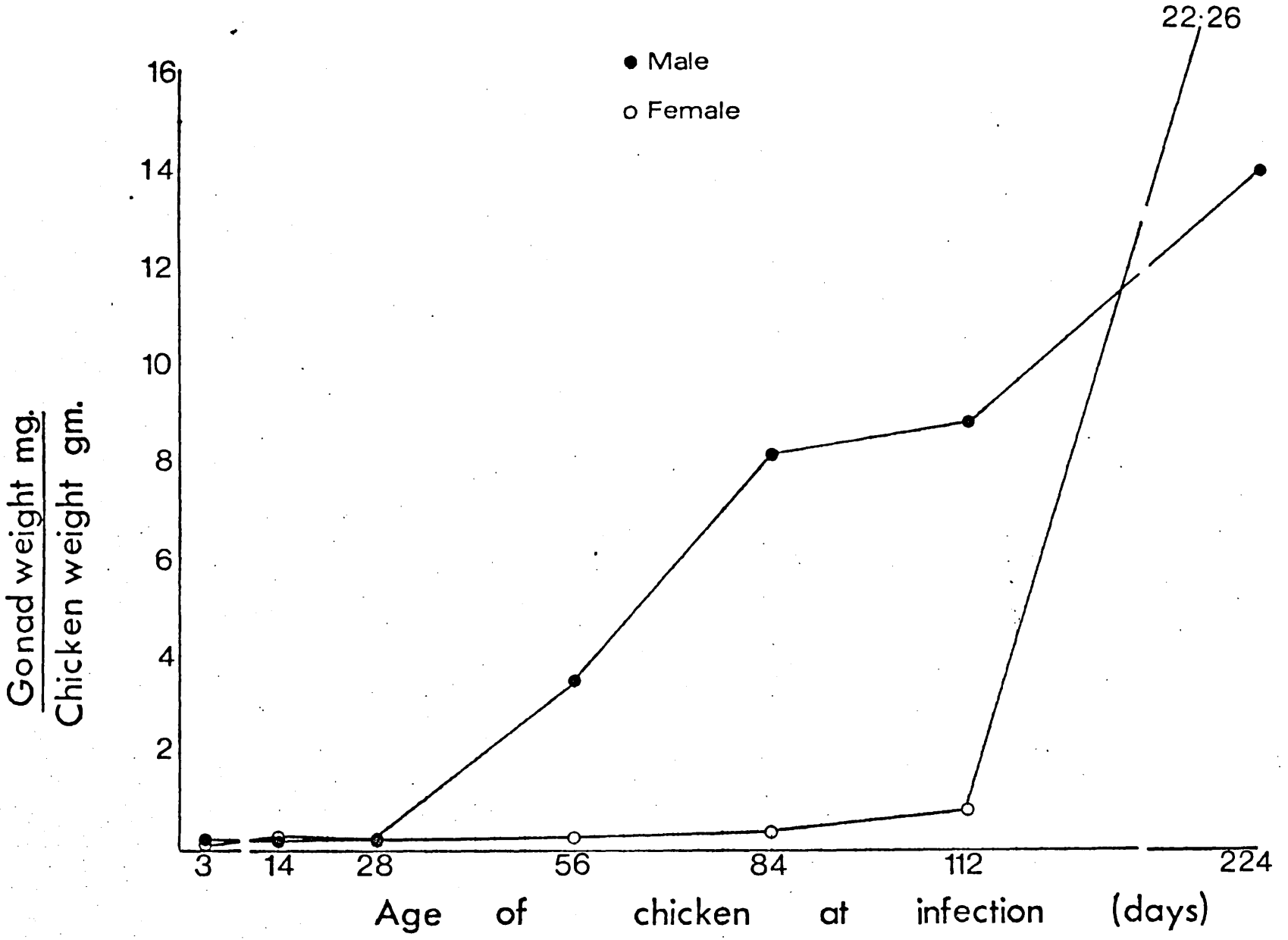

22:26
Male
Female
16
14
12
10
8
6
4
2
Gonad weight mg.
Chicken weight gm.
3
14
28
56
84
112
224
Age of chicken at infection (days)

FIG. 17

Proglottid Output (Figs. 8 and 9)

Both male and female birds infected at 3 and 14 days of age produced similar numbers of proglottids for about the same length of time, but a marked drop in numbers and a shortening of the patent period occurs for birds infected at 28 days of age. In the 56 day old infections the patent period is even shorter in the female birds, but in the males the patent period appears to be as prolonged as in the 3 day old infections and more proglottids were produced per day than in the 28 day old infections. This apparent recovery of susceptibility seems to be transitory, however, as in the 84 day old infections proglottid numbers are once more reduced and no proglottids were recovered from male birds infected at 112 and 224 days of age. In the females the depression of proglottid production and the shortening of the patent period is even more marked in the 84 day old infections and the cestodes only produce proglottids for three days. In the 112 and 224 day old infections a remarkable reversion had taken place and quite large numbers of proglottids were produced over long periods - three months in an individual infected at 112 days old and six months for a 224 day old bird.

Egg Production (Figs.10 and 11)

It is not easy to detect any effect of host age on egg production because of the considerable variation in values. It is noticeable, however, that where proglottid numbers were depressed the numbers of eggs per proglottid are often lower. This can be seen in the male 28 and 84 day old infections

and the female 84 day old infection. The increased susceptibility of male birds infected at 56 days old as indicated by increased proglottid production seems to be partially confirmed by increased egg production as well. The variation is such, however, that no firm conclusions can be drawn.

It is probable that the process of destrobilization is partly responsible for the fluctuations of both proglottid and egg production values. It is extremely likely that nongravid proglottids were counted on occasion and were also used in egg production determinations. In fact several proglottid production peaks can be correlated with sudden drops in the numbers of eggs per proglottid.

Number and Distribution of Worms

The number of established worms at patency varied little between the different age groups with the exception of the last - 224 days old (Fig.12). At this age the numbers of scoleces recovered from male birds decreased and the numbers from female birds increased from a value of approximately 50% take for the other age groups.

It is apparent that as the birds mature the cestodes become established over a greater length of intestine with a consequent flattening of the peak spanning the percentage lengths of 40-60 (Fig.13). The main spread of the infection is in an anterior direction and in the 84-day-old age group there is a concentration of worms in the percentage lengths 24-46.

In the 112-day-old age group there is a marked difference in worm distribution between the two sexes, with a reversion in the females to a pattern of distribution found in young birds (Fig.14).

In the 224-day-old age group there is an apparent contraction of the infected area in males compared with the preceding age group, whereas there is an extension of infected area in females, It should be pointed out, however, that in this particular age group a smaller number of scoleces was recovered from males and a higher number from females than from those of preceding age groups. The distributions of worms in birds of the 224-day-old age group are therefore not strictly comparable with those of the other age groups, even though values are expressed as a percentage of the total number of worms recovered.

Number of Destrobilated Worms

As the age at which chickens were infected increased, the proportion of destrobilated worms at the time of patency also increased (Fig.15). Initially destrobilization was further advanced in female than in male hosts, but in birds infected at 84 days of age values for the two sexes are similar. This is due in part to a sudden fall off of numbers of destrobilated worms in females and in part to a continuation of a steady increase of values in males. In birds infected at 112 days of age the trend in the male birds continues to a value of nearly 100%, whereas in the females the process of destrobilization is no further advanced than it was in the 84-day-old group.

Values for birds infected at 224 days of age are similar to those for the 112-day-old group. In the case of the males, however, the fact that scolex recovery is much reduced in this group suggests that there has in fact been a further advancement in the process of destrobilization followed by 'self-cure'. The mean value for the female hosts is slightly lower than that for the preceding age group and the standard error is smaller also. In both males and females therefore it can be argued that the trends observed in the 112-day-old group continue in the 224-day-old group.

Growth of Worms (Dry Weight)

There were considerable variations in values for the two youngest age groups, as shown by the large standard errors, but the variance decreases as the birds age (Fig.16). Mean values for worms from male hosts are higher than those from females in the first three age groups, but these differences are not significant. In the 56-day-old age group the mean value from male birds is significantly higher than that from females ($P = <0.001$). In the 84-day-old age group there is once again no significant difference as a result of a fall of male values and a rise of female values. In the 112-day-old age group the rise in values for worms from female hosts continues and levels out in the 224-day-old age group. No values were obtained for worms from males of the last two groups because of destrobilization.

Discussion

It has been shown that the physiology of the maturing host has a considerable effect on the course of infection of Raillietina cesticillus in the fowl. The general conclusion reached by Ackert and Reid (1937), Meinkoth (1947) and Sinha and Srivastava (1958) that ageing of the host results in increased resistance to the cestode is supported in the present study for the male host only. The situation is more complex in the female.

Increasing resistance in the maturing male host is manifested by a decreased percentage take when infected at 224 days old, an increased rate of destrobilization from a mean value of 4.42% at 28 days old to a mean value of 96.9% at 112 days old, a decreased rate of growth, as measured by dry weight determinations, at 84 days old and decreased proglottid production at 28 and 84 days old. This last parameter is probably related to destrobilization to a certain extent.

It is difficult to explain the recovery in proglottid output in the birds infected at 56 days old. The numbers of worms recovered from the two chickens involved was low compared with the other groups - a mean value of 25.5 as against 36.6 - 58.5. It is unlikely, however, that this fact alone is responsible for the increased rate of proglottid production, because the worms recovered at patency from the five other birds of this age group were quite large and were part of a population with a mean size of 52.5. It is also interesting to note that in birds of a related strain, 'White Link', a similar recovery of proglottid output occurred in birds infected at 56 days old.

Age resistance appears to develop more rapidly in the female than in the male host at first with a more rapid rate of destrobilization after earlier decreased worm growth rate. However, in birds infected after 56 days of age this trend is discontinued. Destrobilization values remain at the 50 - 60% level in the remaining three age groups, worm growth rate increases and in the last age group, 224 days old there is an increase in percentage take. In the birds used for proglottid and egg counts it was observed that patency was prolonged in the last two age groups - 3 months in a bird infected at 112 days old and 6 months in a bird infected at 224 days old. These observations are of particular interest in view of the successful infection of adult chickens with R. cesticillus by Ackert and Case (1938) (see Page 50.).

It is likely that there is a direct relationship between the various manifestations of resistance described here and the alteration of worm distribution in the intestine of maturing birds, particularly in view of the fact that a reversion to a worm distribution characteristic of young birds occurs in females infected at 112 days of age and this reversion is accompanied by a decline in the manifestations of resistance. An alteration of worm distribution in the intestine strongly suggests an alteration of physico-chemical and/or nutritional conditions and one might therefore expect an age resistance factor to have an effect on the intestinal environment.

The difference in the development of age resistance between the sexes implicates hormone balance as a possible factor and this view is strengthened by the fact that, at

the time of the decline of age resistance in the female, gonadal development is accelerating (Fig.17). The shape of the graph of testes weights as a percentage of body weight is remarkably similar to that for the percentage number of destrobilated worms in the male host. There is much information on the subject of hormones and parasites and this complex field is reviewed by Solomon (1969).

Another feature associated with maturation of chickens is the increase per unit area of goblet cells in the intestinal mucosa, reported by Ackert, Edgar and Frick (1939). They observed that this increase in number of goblet cells corresponds well with the development of age resistance to the nematode Ascaridia galli. When larval nematodes were cultured in the presence of mucus from resistant chickens, growth and development of the larvae was inhibited. Frick and Ackert (1948) provided evidence that mucus from resistant chickens contains an anthelmintic factor that is not affected by previous or current infections and is not an antibody, as it survives autoclaving at 15 p.s.i. for 20 min.

The third feature which might play a part in the development of age resistance is the increasing immune potential of the maturing bird. So far there is no evidence to suggest that R. cesticillus evokes an immune response. Luttermoser (1938), Meinkoth (1947) and Sinha and Srivastava (1958) failed to demonstrate resistance to superinfection and Meinkoth was also unable to detect circulating antibodies with ring precipitin tests. However, these investigators used numbers of established worms as their criterion of resistance and it has been shown in this series of experiments that worm number is a poor short-term measure

of resistance. The subject of acquired resistance to *R. cesticillus* requires further investigation, together with the use of other methods of antibody detection. In this latter context it is interesting to note that Wolfe and Dilks (1948) found that serological maturity of chickens was reached by the age of 5 weeks, as far as the production of precipitins is concerned, while Seto and Henderson (1968) found that haemagglutinin antibody titres increased most rapidly between the ages of 3 and 5 weeks with a levelling off of titres after 5 weeks. This age corresponds approximately to the age at which the manifestations of age resistance to *R. cesticillus* first appear.

It is not unlikely that all three of the factors discussed - hormone balance, mucus and an immune response - play a part in the effect of host age on *R. cesticillus*, acting independently or together. Hormone balance is known to influence immune responses (Weinstein, 1939; Nicol and Bilbey, 1958) and it is possible that goblet-cell activity constitutes part of such a response (Dobson, 1966a).

CHAPTER 3

HOST RESISTANCE TO SUPERINFECTION WITH R. CESTICILLUS

Introduction

It was suggested in the preceding chapter that immune mechanisms may be partly responsible for the development of age resistance to R. cesticillus. There is at present no experimental evidence to suggest that R. cesticillus evokes an immune response of any consequence and it has long been assumed that this parasite is immunologically inert, due mainly to its superficial attachment to the intestinal mucosa.

Luttermoser (1938), Meinkoth (1947) and Sinha and Srivastava (1958) all failed to demonstrate acquired resistance to R. cesticillus but, as already pointed out, these failures may be attributed to the use of numbers of scoleces as a criterion of resistance rather than numbers of destrobilated scoleces. The interpretation of their results was further complicated by the fact that the primary infections were not eliminated before the secondary infections were established, thus introducing the additional factor of worm competition. Furthermore, Sinha and Srivastava only used 2 - 8 cysticercoids in their primary infections, which, in view of the poor immunogenic contact between host and parasite, is unlikely to be sufficient to evoke an effective immune response.

In the two experiments described here attempts were made to demonstrate acquired resistance to the cestode with the numbers of destrobilated scoleces as the criterion of resistance. It was hoped that this, together with careful

experimental design, would increase the sensitivity of detection of any signs of host resistance.

Materials and Methods

Experiment 1.

Five male and five female 4 week old chickens were given a primary infection of 77.2 ± 6.81 cysticercoids each by beetle. Complementary birds were selected by weight as controls and maintained under similar conditions.

Before undertaking the secondary infections, which were timed to coincide with the destrobilization of the primary infections (6 weeks post infection), both experimental and control birds were dosed with 100 mg.hexachlorophene/Kg body weight in oil to remove all traces of the primary infections from the experimental birds. In preliminary trials this drug was found to be 100% efficient at this dose level. Kerr (1948) reported that 200mg/kg constituted a toxic dose. In the experiments described here no mortality was caused by 100 mg/kg. Control birds were also dosed because it is possible that hexachlorophene treatment may affect a following infection, although no effect was detected in a small pilot experiment.

Experimental and control birds were infected with 50 cysticercoids each by pipette two days after treatment with hexachlorophene. All birds were autopsied 14 days after this infection and observations were made on worm establishment, destrobilization, growth and distribution. Tissue samples of the worm *in situ* were taken from percent lengths of intestine 30 - 40 for histological examination (Haema-

toxylin and Eosin). Gonads and adrenals were removed for weighing.

Experiment 2.

This was undertaken in an attempt to confirm and amplify the findings of experiment 1. and the procedure followed was similar. Ten birds of each sex in the two groups (experimental and control) were used and the experimental birds were infected at 5 weeks of age with 65 ± 1.4 cysticercoids by beetle. This infection was eliminated with hexachlorophene at 4 weeks post infection and all birds were then infected with 50 cysticercoids. Autopsies were carried out 14 days post infection and the same observations were made as in experiment 1.

Results

Experiment 1

Table 14 (Mean Values)

Chicken Group	Chicken Weight	Number of Scoleces	Percent Number of Destrobilated Scoleces	Worm Weight	Adrenal Weight	Gonad Weight
Experimental	1675.0	21.0 ± 4.19	100.0	-	135.9	3526.7

Male	Percent Length of Intestine	24	26	28	30	32	34	36	38	40	42	44	46	
	Percent N_0. Scoleces	2.5	0	0	2.5	5.0	11.0	7.5	11.0	7.5	3.5	7.5	1.0	
	Percent Length of Intestine	48	50	52	54	56	58	60	62	64	66	68	70	
	Percent No. Scoleces	6.0	1.0	6.0	6.0	6.0	5.0	1.0	2.5	5.0	1.0	1.0	1.0	20.1

Control	1630.0	25.8 ± 1.89	53.42 ± 13.98	6.56 ± 0.61	129.2	4154.9								
Male	Percent Length of Intestine	28	30	32	34	36	38	40	42	44	46	48	50	
	Percent No. Scoleces	4.8	8.0	9.6	3.2	5.6	5.6	8.8	7.2	4.8	4.8	6.4	6.4	
	Percent Length of Intestine	52	54	56	58	60	62	64	66	68	70	72	74	
	Percent No. Scoleces	2.4	4.0	2.4	4.0	1.6	0.8	2.4	3.2	1.6	1.6	0	0.8	25.0

Experimental	1200.0	23.2 ± 2.89	100.0	-	77.4	332.0								
Female	Percent Length of Intestine	28	30	32	34	36	38	40	42	44	46	48	50	
	Percent No. Scoleces	2.6	2.6	8.6	6.0	6.0	7.8	6.9	5.2	5.2	5.2	1.8	2.6	

Percent Length of Intestine	52	54	56	58	60	62	64	66	68	70	72		
Percent No. Scoleces	3.4	4.3	5.2	1.7	3.4	2.6	2.6	4.3	5.2	0	0.9	23.2	

Control	1140.0	22.6 ± 2.18		83.85 ± 7.64		5.6			80.94		315.2		
Female	Percent Length of Intestine	24	26	28	30	32	34	36	38	40	42	44	
	Percent No. Soolecesz	0.9	0	3.2	3.2	4.5	6.3	7.2	13.5	13.5	9.0	10.8	
	Percent Length of Intestine	46	48	50	52	54	56	58	60	62	64	66	
	Percent No. Scoleces	7.2	3.2	1.8	4.5	3.2	0	3.2	0.9	0.9	0	0.9	22.2

T Values

Chicken Group	Percent Number of Destrobilated Scoleces	T Value	P
Experimental Male	100.0 ± 0.0	1.04	>0.05
Control Male	53.42 ± 13.98		
Experimental Female	100.0 ± 0.0	2.11	>0.05
Control Female	83.85 ± 7.64		

The results of experiment 1. are by no means clear cut, but they suggest that experience of a primary infection of R.cesticillus may increase resistance to a secondary infection. There were no differences between the four groups as far as number of scoleces are concerned, but 100% destrobilization occurred in both male and female experimental groups as against 53.42% and 83.45% in the male and female control groups respectively. These differences are, however, not statistically significant, due probably to the small number of birds in each group. In fact the results from only four birds in the experimental male group were considered, because the fifth bird had a very low gonad weight - 779.3mg. compared with a group mean of 2,577.3mg. It is of interest to note that the number of destrobilating worms in this particular bird was small - 27.6% - and it is therefore tempting to conclude that this aberration is due to the lack of gonadal growth.

The control birds were infected at 70 days of age and the number of destrobilating worms in the male birds was approximately 50%, as expected. In the female birds, however, this value was surprisingly high - 83.85%. The worm weights of the control male infections were slightly higher than expected. Only two values were obtained for the control females and these were also rather high. The distribution of the worms of the secondary infections did not appear to differ from that of the primary infections.

Experiment 2

Table 15 (Mean Values)

Chicken Group	Chicken Weight	Number of Scoleces	Percent Number of Destrobilated Scoleces	Worm Weight	Adrenal Weight	Gonad Weight
Experimental	1340.0	16.9 ± 2.44	94.63 ± 3.51	-	111.2	3108.4
Male Percent Length of Intestine	28	30	32	34	36	38	40	42	44	46	48	50	52	
Percent No. Scoleces	0.6	0.6	0	1.2	3.6	2.4	3.6	8.3	11.3	11.8	11.8	8.3	8.3	
Percent Length of Intestine	54	56	58	60	62	64	66	68	70	72	74	76		
Percent No. Scoleces	5.9	3.0	6.5	2.4	3.6	3.0	1.8	0.6	0	1.2	0	0.6		16.9
Control	1355.0	20.9 ± 2.5	67.31 ± 5.73	5.03 ± 0.73	113.4	3064.6
Male Percent Length of Intestine	28	30	32	34	36	38	40	42	44	46	48	50	52	
Percent No. Scoleces	1.0	1.0	2.5	2.5	5.4	4.5	7.4	15.8	10.4	9.4	6.4	10.4	5.9	
Percent Length of Intestine	54	56	58	60	62	64	66	68	70	72	74	76	78	
Percent No. Scoleces	4.0	2.5	3.0	1.5	0.4	2.5	0.4	1.5	1.0	0	0	0	0.4	20.2
Experimental	1030.0	9.8 ± 3.74	100.0 ± 0.0	-	91.0	297.6
Female Percent Length of Intestine	28	30	32	34	36	38	40	42	44	46	48	50		
Percent No. Scoleces	2.0	6.0	4.0	6.0	6.0	4.0	3.0	9.0	4.0	10.0	4.0	6.0		
Percent Length of Intestine	52	54	56	58	60	62	64	66	68	70	72			
Percent No. Scoleces	8.0	6.0	4.0	3.0	3.0	2.0	3.0	2.0	2.0	0	10.0			9.8

Control	1090. 0	24.3 ± 2.57			56.58 ± 8.47			5.1 ± 0.48		80.6		314.6			
Female	Percent Length of Intestine	32	34	36	38	40	42	44	46	48	50	52	54	56	
	Percent No. Scoleces	0.4	3.2	4.1	4.1	7.8	11.1	11.1	10.3	9.9	4.9	7.4	4.5	4.9	
	Percent Length of Intestine	58	60	62	64	66	68	70	72	74	76	78	80	82	
	Percent No. Scoleces	4.5	2.9	1.6	0.4	0.8	3.7	1.6	0	0	0	0	0	0.4	24.3

T Values

Chicken Group	Number of Scoleces	T	P	Percent Number of Destrobilated Scoleces	T	P
Experimental Male	16.9 ± 2.44	1.144	>0.05	94.63 ± 3.51	4.065	<0.001
Control Male	20.9 ± 2.50			67.31 ± 5.73		
Experimental Female	9.8 ± 3.70	3.625	>0.001	100.0 ± 0.0	5.127	<0.001
Control Female	24.3 ± 2.57			56.58 ± 8.47		

Figures 18-21. Scoleces *in situ* in the small intestine. Two week old primary (control) and secondary (experimental) infections in chickens infected at 70 days of age. X 95

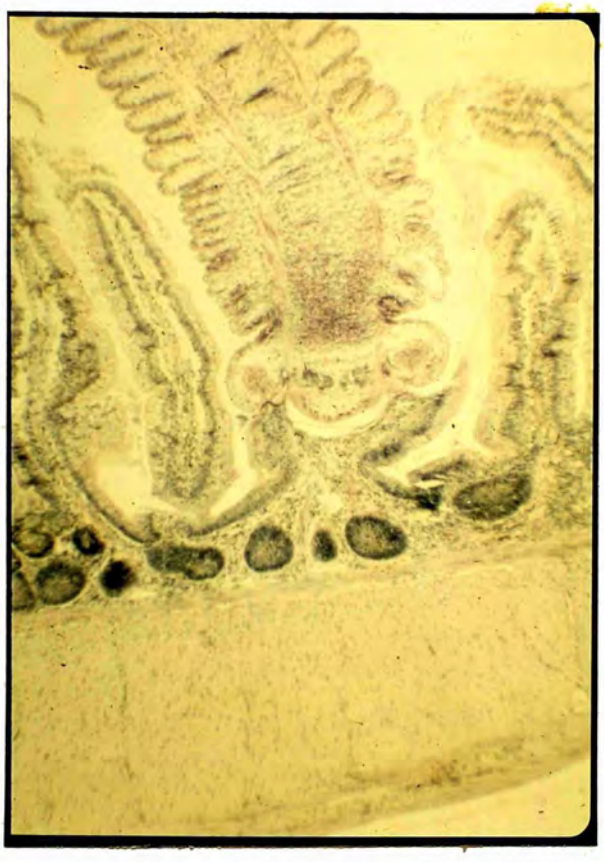


FIG. I8. Male Primary Infection

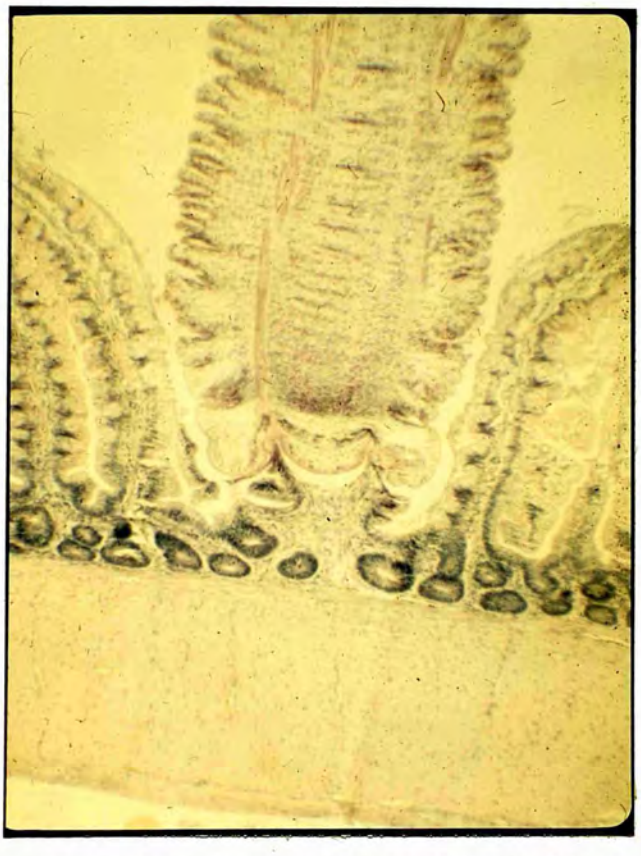


FIG. I9. Female Primary Infection

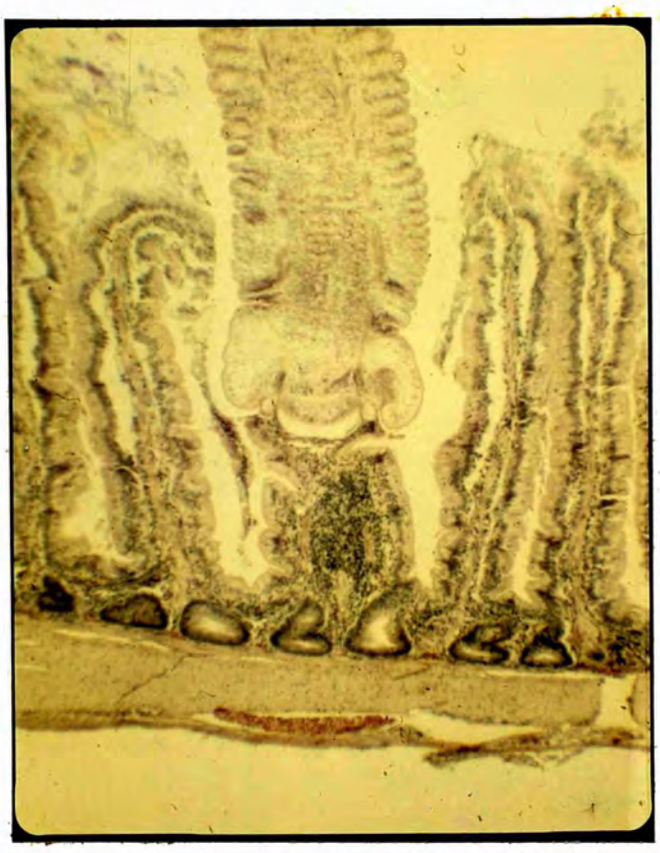


FIG. 20. Male Secondary Infection

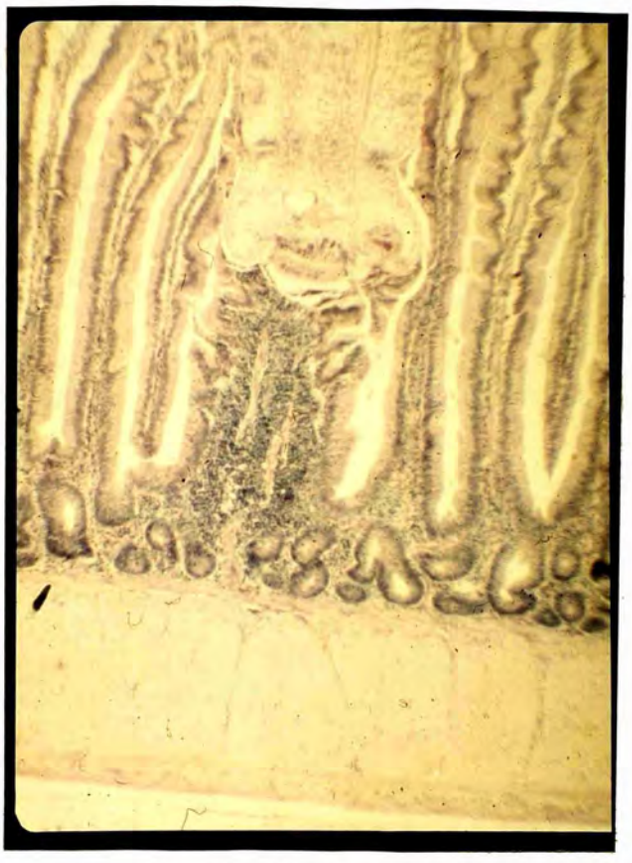


FIG. 2I. Female Secondary Infection

Twice as many birds were used in this experiment in an attempt to amplify the differences observed in experiment 1. and the birds were also a week older at the start of experiment 2. The results of this modified procedure provide strong evidence for an acquired resistance to R. cesticillus.

The numbers of destrobilating scoleces in the experimental birds of both sexes approached 100%, approximately twice as many as in the control birds. This difference was statistically very highly significant for both male and female hosts. The numbers of scoleces recovered from experimental and control males did not differ significantly, but in the case of the females there were significantly smaller numbers in the experimental group than in the control.

The distribution of secondary infection scoleces did not appear to differ from those of primary infections, as observed in experiment 1. The secondary infections of experiment 2. were carried out when the birds were about 63 days old and worms weights and numbers of destrobilating worms in the control birds were at their expected levels.

Gut tissue samples of worms in situ were obtained from birds of both experiments for histological examination. They were embedded in paraffin wax, sectioned at 5 u and stained with haematoxylin and eosin. The sections (Figs. 20 and 21) show an intense cellular reaction around the scoleces of secondary infection worms. This reaction consists predominantly of mononuclear cells and very few polymorphs are present. No cellular infiltration is present in the tissues obtained from the control birds

which had a primary infection of the cestode (Figs. 18. and 19).

Discussion

The results obtained from experiment 1. suggest that a primary infection of R.cesticillus might induce resistance to a secondary infection and the results obtained from experiment 2. prove this to be the case.

The phenomenon of acquired resistance to infection is usually due to a host immunological response, but this is not necessarily always the case. It is quite possible that the infectious agent, especially a helminth parasite, could alter the physico/chemical environment in the intestine by mechanical damage and irritation. Stahl (1966) suggested that acquired resistance to the mouse nematode, Aspiculuris tetraptera could well be due to the enhancement of natural resistance factors, such as mucus production.

In the experiments described here a local non-specific alteration of the intestinal environment by the primary infections cannot be ruled out. However, the fact that the distribution of the worms in the intestine was unaffected by primary infections argues against this possibility. The factor of direct competition between primary and secondary infection is not involved, as primary infections were eliminated before secondary infection.

The mononuclear infiltration at the site of scolex attachment, observed in tissue from the experimental birds, constitutes strong evidence to suggest that acquired resistance to R. cesticillus is immunological. It is possible that the primary infection gives rise to an accelerated inflam-

matory response to the secondary infection. Bailey (1951) reported that cysticercoids of Hymenolepis nana induce a typical inflammatory response in the mucosa, characterised by a predominance of neutrophils and eosinophils with a few lymphocytes. With secondary infections a few cysticercoids manage to penetrate the mucosa and induce a much accelerated inflammatory response. The reaction to R. cesticillus does not appear to be similar to that induced by H.nana as mononuclear cells predominate rather than polymorphs. It is possible, however, that the observed reaction may constitute the later stages of inflammation, in which polymorphs are largely replaced by mononuclear cells.

Hopkins, Subramanian and Stallard (1972a) reported that the destrobilation of Hymenolepis diminuta in mice in both primary and secondary infections is probably immunological and at first sight this system seems to behave in a very similar fashinn to R. cesticillus in the chicken. The big difference between the two systems is that the mouse is an abnormal host for H. diminuta, whereas the chicken is the normal host for R. cesticillus.

In order to obtain further evidence that a true immune response to R. cesticillus has been demonstrated it was decided to attempt to suppress the resistance to a secondary infection of R. cesticillus with chemical immunosuppressants (Chapter 4).

CHAPTER 4

THE CHEMICAL SUPPRESSION OF ACQUIRED RESISTANCE TO R. CESTICILLUS

Introduction

The design of the experiments that demonstrated acquired resistance to R. cesticillus suggested that this resistance was of an immunological nature. Additional confirmation that a true immune response is involved is required and in the following experiments attempts were made to suppress acquired resistance to R. cesticillus with chemical immunosuppressants.

Experiment 1. Dexamethasone Therapy

Perhaps the best known chemical immunosuppressants are the adrenal steroids. They are potent inhibitors of inflammatory activity and have long been used in this role in medicine. They inhibit immune responses in a variety of ways and no doubt their range of activities in this field is larger than is at present known. They inhibit proliferative responses, are powerful lympholytic agents, stabilise lysosomal membranes (thus possibly affecting the processing of antigenic information), and there have also been reports of anti-complementary activity.

Lerner, Bianchi, Turkheimer, Singer and Borman (1964) tested the activity of thirteen different adrenal steroid derivatives. They investigated effects on inflammation (induced by cotton implants), thymus weights and liver glycogen deposition in adrenalectomised rats. For the

purposes of the experiment under consideration it was necessary to select a steroid with a high anti-inflammatory activity and a low glycogen deposition activity. The best derivative in this respect, according to Lerner et al, was paramethasone followed by dexamethasone and then betamethasone. Dexamethasone was finally selected as it is more easily available than paramethasone.

Dexamethasone is approximately 200 times more potent than cortisone in its anti-inflammatory activity and was used in the following experiment to suppress resistance to a secondary infection of R. cesticillus. Observations were made on worm establishment and destrobilization, the wet weights of spleens, gonads and adrenals and on the histopathology.

A secondary experiment was run in order to investigate the effect of the glycogen deposition activity of dexamethasone on the cestode. There is already circumstantial evidence to suggest that the phenomenon of destrobilization may be linked to the nutritional condition of the cestode (Page 48.). Dexamethasone-induced glycogen deposition and lipogenesis may therefore obscure any effects of immunosuppression. In order to check on this possibility the effect of dexamethasone on the biochemical composition of a primary infection of R.cesticillus was investigated.

Materials and Methods

Fourteen male and fourteen female 4 week old chickens were infected with 50 cysticercoids each by pipette. The remains of these primary infections were eliminated with 100mg Hexachlorophene /Kg body weight 32 days after infection. After another 7 days all birds were reinfected with 50 cysticercoids each.

The immunosuppressant therapy was started 2 days after the secondary infections so that the establishment of the cestodes would not be disrupted. The dexamethasone was obtained from Sigma Chemical Company and was injected into the pectoral muscles of 7 male and 7 female birds as a suspension in physiological saline at the rate of 0.5mg/Kg body weight/day. The 14 control birds, which were matched with the experimental birds by weight, were injected with physiological saline alone.

Great care had to be taken to ensure that mortality due to decreased resistance to infection was kept to a minimum. Daily spraying with a 2% solution of the amholytic biocide 'Tego' together with strict isolation of the experimental room were successful in this respect and all birds remained free of disease.

In order to determine the effect of dexamethasone on the biochemical composition of a primary infection two male and two female birds were infected with 100 cysticercoids each. One male and one female were treated with 0.5mg dexamethasone/Kg every day for two weeks and the controls were injected with saline.

The birds were autopsied 14 days post infection and the worms were counted and weighed and then analysed for protein lipid and polysaccharide content as follows (after Graff, 1970):-

1. The samples were extracted in 10mls. 70% ethyl alcohol overnight.

2. The supernatant was decanted and the sample homogenised in 70% ethyl alcohol and centrifuged at 3,000 r.p.m. for 5 minutes.

3. Four volumes of 3:1 v/v ether: 70% EtOH was added to the precipitate which was well shaken and then centrifuged. The supernatant was saved for analysis of lipids. This was repeated once.

4. Ten mls. of 10% trichloroacetic acid was added to the precipitate, mixed and heated to 80°C. After centrifugation the supernatant was saved for step 6.

5. Ten mls. cold 10% trichloroacetic acid was added to the precipitate, mixed and then centrifuged. The precipitate is mainly protein. The supernatant was saved for step 6.

6. The supernatant from steps 4 and 5 were mixed with KOH to give a 15% solution of potassium hydroxide, heated to 100°C for 15 minutes, cooled and added to 1.2 volumes of 95% EtOH. This was then heated to boiling, cooled and centrifuged. The precipitate contains polysaccharide.

The lipid, protein and polysaccharide samples were dried under vacuum in a dessicator containing silica gel and finally weighed.

Results

Table 16 (Mean Values)

Effect of Dexamethasone on Secondary Infections

Chicken Group	Chicken Weight	Number of Scoleces	Percent Number of Destrobilating Scoleces	Adrenal Weight	Gonad Weight	Spleen Weight
Experimental Male	1142.8	20.2 ± 2.40	50.3 ± 5.63	91.4	826.3	767.4
Control Male	1442.8	12.0 ± 1.71	100.0 ± 0.0	129.7	2335.4	2774.9
Experimental Female	835.7	19.4 ± 2.25	49.9 ± 4.60	54.0	206.5	361.7
Control Female	1042.8	14.1 ± 2.36	100.0 ± 0.0	79.3	314.5	1968.6

T Values

Chicken Group	Number of Scoleces	T	P	Percent Number of Destrobilating Scoleces	T	P
Experimental Male	20.2 ± 2.40	2.783	>0.01	50.3 ± 5.63	8.401	<0.001
Control Male	12.0 ± 1.71			100.0 ± 0.0		
Experimental Female	19.4 ± 2.25	1.625	>0.1	49.9 ± 4.60	11.109	<0.001
Control	14.1 ± 2.36			100.0 ± 0.0		

Table 17

Effect of Dexamethasone on Primary Infections

	Experimental Male	Control Male	Experimental Female	Control Female
Chicken Weight	800	1000	700	850
Number of Scoleces	29	55	30	66
Percent Number of Destrobilating Scoleces	27.6	63.6	26.6	34.9
Total Dry Weight (mg)	48.1	30.1	49.6	35.8
Protein %	45.0	43.2	45.2	47.7
Polysaccharide %	36.8	33.2	33.3	32.7
Lipid %	13.1	23.6	21.6	18.7

Figures 22 and 23. Scoleces *in situ* in the small intestine. Secondary infections of female chickens infected at 67 days of age and treated with the immunosuppressant dexamethasone (experimental) and saline (control). X95.

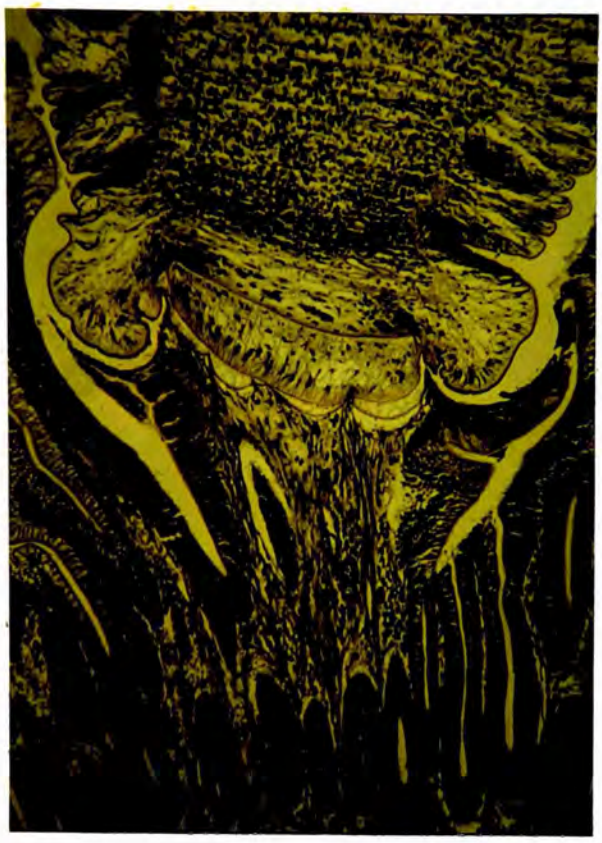


FIG. 22. Female Experimental

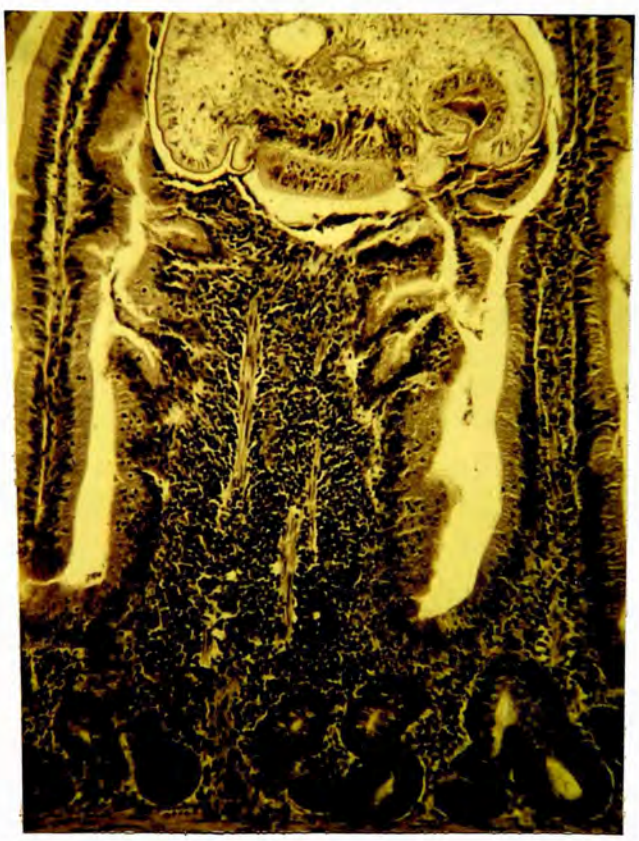


FIG. 23. Female Control

Discussion

The dexamethasone therapy can be seen to have suppressed acquired resistance to R.cesticillus extremely effectively. In both male and female experimental groups destrobilization only reached a value of about 50 percent as against complete destrobilization in the control groups. These differences are very highly significant ($P < 0.001$). In addition to this there has been a greater loss of scoleces from the control males than from the experimental males ($P > 0.01$). The difference between control and experimental female loss of scoleces is not so marked.

Histological examination showed a complete lack of cellular host reaction to the scoleces of the experimental groups (Fig.22). In contrast to this a marked reaction to the scoleces can be seen in the control groups (Fig.23). This reaction has already been described (Chapter 3).

The spleen weights of the experimental groups were very much less than those of the controls, again indicating effective immunosuppression. As one might expect, adrenal weights in the experimental birds were lowered due to negative feedback to the pituitary. Gonad growth was also inhibited and it is probable that this is due to negative feedback as well. Body weights were lowered by dexamethasone therapy and this was almost certainly caused by the side effects associated with adrenal steroid therapy, which can be severe. There was obvious sodium retention in the treated birds, as they were always thirsty, and glycogen deposition occurred in the liver.

It was possible that such side effects might have influenced the course of infection of R. cesticillus and obscured the effects of immunosuppression. This possibility was investigated by treating birds with a primary infection of the cestode with dexamethasone. From the results (Table 17) it can be seen that fewer worms were recovered from the treated birds than from the controls, but the total weight of each population was much the same. The lower worm take in treated birds may be due to the fact that therapy was started only one day after infection and may have interfered with the establishment of the cestode. There was no discernible difference between the groups as regards worm protein, polysaccharide and lipid content. This leads one to the tentative conclusion that the reduction in numbers of destrobilated secondary infection worms in the treated birds is due to an inhibited immune response and not to improved nutritional conditions due to side effects. This conclusion is supported by the fact that the percent destrobilization values of the experimental groups are similar to those of birds of the same age that had not received a previous infection (controls of the acquired resistance experiments).

It is interesting to note however that about half the numbers of established worms destrobilated despite suppression of immune mechanisms. This suggests that destrobilization is caused by a non-specific factor the effects of which immune mechanisms simply amplify. Such a factor could be nutritional conditions, which has already been implicated in previous experiments.

Experiment 2. Cyclophosphamide Therapy I.

Dexamethasone effectively suppressed a protective immune response to a secondary infection of R. cesticillus, but the side effects were considerable and although steps were taken to show that these effects do not affect the cestode it was considered desirable to attempt other means of suppressing immunity.

There are now several non-steroid chemical immunosuppressants, which can be grouped into five general classes 1) Alkylating agents, 2) Antimetabolites, 3) Antibiotics, 4) Folic acid antagonists, 5) Plant alkaloids. These compounds interfere with nucleic acid metabolism, denature D.N.A. or inhibit mitosis. They are cytotoxic and prevent cell proliferation, sometimes killing them. Many of them are difficult to use invivo because of their toxicity, but none of them cause complicated side effects as do adrenal steroids.

Cyclophosphamide, an alkylating agent and a derivative of mustard gas, was finally selected because of its comparative non-toxicity, its success as an immunosuppressant in many experiments, its availability and the fact that it has already been used in chickens (Floersheim and Seiler, 1967; Lerman and Weidanz, 1970; Glick, 1971).

In these experiments the effect of cyclophosphamide on secondary infections of R. cesticillus was investigated. The design of these experiments was broadly similar to that of the dexamethasone experiment.

Materials and Methods

Fourteen male and fourteen female 4 week old chickens were infected with 50 cysticercoids each and this infection was eliminated with 100 mg hexachlorophene/Kg 4 weeks later. Two male birds died as a result of this treatment. All remaining birds were reinfected with 50 cysticercoids each 7 days after the anthelmintic treatment.

The cyclophosphamide monohydrate was supplied by Koch-Light Ltd and the dose level used was arrived at after preliminary toxicity studies together with white blood cell counts. It was finally decided to treat half the infected birds with a single intraperitoneal injection each of 300mg cyclophosphamide monohydrate/Kg in saline. The injections were carried out on the day of infection. All birds were autopsied 14 days after infection.

As in the dexamethasone experiment a spray of 2% Tego solution was used to reduce the risk of disease. No mortality or disease occurred.

White blood cell counts were made from samples obtained 3,6, 9 and 13 days after infection from one bird of each group. Differential counts were made from the blood samples of the 13th day after infection. The diluting fluid used for the total white cell counts consisted of physiological saline containing 4% formalin and 0.001% Gentian violet. The differential count stain was Leishman's and a battlement counting technique was used.

Table 18 (Mean Values)

Chicken Group	Chicken Weight	Number of Scoleces	Percent Numbers of Destrobilising Scoleces	Spleen Weight
Experimental male	1316.6	0,0	0.0	1615.8 ± 137.2
Control Male	1566.6	22.0	95.3	2816.2 ± 123.5
Experimental Female	914.2	0.0	0.0	1268.5 ± 95.6
Control Female	1114.2	18.1	100.0	2108.5 ± 118.8

Effect of Cyclophosphamide on White Blood Cell Counts /cu.mm.

Days after Infection	Experimental Male	Control Male	Experimental Female	Control Female
3	5,600	42,200	9,000	32,700
6	4,200	40,100	2,100	37,400
9	11,900	38,600	16,800	31,400
13	14,100	41,500	13,900	39,700

Differential Counts %

Cell Type	Experimental Male	Control Male
Monocytes	11	4
Large Lymphocytes	18	19
Small Lymphocytes	27	58
Leucocytes	47	19

Discussion

The immunosuppressant therapy in this experiment successfully depressed the white blood cell counts to 13 - 28% normal values by 3 days after the injections. By day 13 d.p.i. the counts had recovered slightly to 34 - 35% normal values - still less than half the number of white blood cells in the control birds. The spleen weights of the treated birds are lower than those of the controls and these differences are statistically significant. The body weights of treated birds were also lower than those of control birds.

It would appear that this particular regimen of cyclophosphamide monohydrate has had some effect on the immune system, but no conclusions can be drawn as far as the effect of depressed immunity on worm survival is concerned since no worms were recovered from any of the treated birds. The situation in the controls was normal. Cyclophosphamide monohydrate is a cytotoxic agent and it is known that it does not have its cytotoxic effect until it has been processed by the liver. It is likely that the bile contained considerable quantities of the processed form during the time the cyclophosphamide was being absorbed from the peritoneal cavity and processed in the liver. In accordance with other work on this subject the drug was administered at about the same time as the antigen (cysticercoids) and it is probable that the establishing young cestodes were killed by the products of cyclophosphamide degradation in the bile.

Experiment 3. Cyclophosphamide Therapy II.

It was decided to repeat the previous experiment with a different regimen of cyclophosphamide treatment. It was desirable to eliminate the cytotoxic action of the drug during the secondary infections while ensuring that the immune system is still inhibited. The only way to accomplish this was to increase the initial dose of cyclophosphamide and to increase the period between treatment and secondary infection.

Materials and Methods

Six male and six female 4 week old chickens were infected with 100 cysticercoids each and this infection was eliminated with 100mg hexachlorophene/Kg. 9 weeks later. Two days later the experimental birds, three male and three female, were injected intraperitoneally with 400mg cyclophosphamide monohydrate/Kg. in saline. The controls received saline alone. The secondary infections of 100 cysticercoids each were carried out 7 days after drug treatment.

White blood cell counts were made at infection and at autopsy, which was carried out 14 days post infection.

Results

Table 19 (Mean Values)

Chicken Group	Chicken Weight	Number of Scoleces	Percent Number of Destrobilating Scoleces	Spleen Weight
Experimental male	1786.6	21.3 ± 7.22	99.3	3150.61
Control Male	1740.0	13.3 ± 2.02	100.0	3263.1
Experimental Female	1216.6	11.0 ± 1.73	100.0	1958.3
Control Female	1366.6	3.6 ± 1.76	100.0	2156.6

White Blood Cell Counts /cu.mm.

Days after Secondary Infection	Experimental Male	Control Male	Experimental Female	Control Female
0	4,300	35,400	2,200	37,700
14	25,500	43,400	19,200	41,200

Discussion

In contrast to experiment 2. destrobilated worms were present in the treated birds as well as in the controls birds. This suggests that the interval between drug therapy and secondary infection was sufficient for clearance of the cytotoxic agents in the bile.

The fact that the worms were destrobilating in the treated birds suggests that the drug failed to prevent the immune response to secondary infection. This was borne out by: a) the rapid recovery of white blood cell counts from 6 - 12% normal at infection to 47 - 59% at autopsy; b) the normal spleen weights and c) the lack of inhibition of mononuclear infiltration in treated birds.

The immunosuppressive action of cyclophosphamide appears to be quite selective. The drug depresses white blood cell numbers and has its maximum effect on small lymphocytes. Glick (1971) observed that after cyclophosphamide therapy spleen and thymus lymphoid tissue regenerated whereas the bursal follicles did not. It has long been established that the thymus gives rise to the components of cellular immunity and the bursal follicles to humoral immunity. Floersheim and Seiler (1967) reported that cyclophosphamide was one of a group of immunosuppressants that inhibited delayed cutaneous reactions in chickens in the early stages, but that this inhibition did not last long. Lerman and Weidanz (1970) observed that when chicks were treated with cyclophosphamide in the first three days of life their antibody formation abilities were depressed, but cellular immunity was apparently unimpaired.

This comparative lack of effect of cyclophosphamide on cellular immunity is of interest in view of the failure of treatment with this drug to suppress the secondary response to R.cesticillus infection. It is possible that cellular immunity is of prime importance in acquired resistance to R. cesticillus.

Most successful suppressions of immunity with cyclophosphamide in chickens have been accomplished by treating the birds very early in life. In this way the stem cells of humoral and cellular immunity, in the bursal follicles and in the thymus respectively, can be exposed to the drug. This approach may well prove useful in the elucidation of the nature of the immune response to R.cesticillus infection. The use of other cytotoxic immunosuppressants may also prove helpful in this respect. However, cyclophosphamide has been used in the experiments described here simply in an attempt to confirm the existence of an immune response to R.cesticillus. It is apparent that the use of cytotoxic immunosuppressants is attended by many complex problems and it was therefore decided that further experimentation with such drugs was not justified. It would also appear that the use of other immunosuppressants such as antilymphocytic serum and X-irradiation in an attempt to confirm the immunogenicity of R.cesticillus is too complex to be worthwhile.

It can be argued that sufficient evidence for an immune response to R. cesticillus has already been demonstrated. Further evidence for the immunogenicity of the cestode would be the demonstration of antibodies and/or cellular immunity

by specific tests. Such tests, if positive, though not necessarily demonstrating functional immunity would, together with the dexamethasone therapy experiment, constitute very strong evidence for immunological involvement in acquired resistance to *R. cesticillus*.

CHAPTER 5

IMMUNOLOGICAL TESTS

Introduction

Considerable evidence was presented in the last two chapters to support the suggestion that R.cesticillus evokes an immune response. However, some of this evidence, particularly that of the immunosuppressant experiments, is questionable and it was felt that additional confirmation was required. It was therefore decided to carry out specific immunological tests in an attempt to provide this additional confirmation and also to characterise the immune response to some degree.

Attempts were made to detect specific antibody in both serum and mucus. It is unlikely that serum antibody is protective against R.cesticillus, but its detection would provide positive evidence for an immune response. Intestinal mucus antibody is more likely to be protective and considerable attention has been focused on the problem of local immunity and secretory immunoglobulins in recent years (Tomasi and Bienenstock, 1969). Gamma-A seems to be the predominant immunoglobulin in mucus secretions.

Tests were also made for cellular immunity and counts were made of cellular components normally involved in immune responses, such as mast cells and eosinophils.

GENERAL MATERIALS AND METHODS

Serum (after Campbell, Garvey, Cremer and Sussdorf 1964)

Blood was obtained by cardiac puncture using a 2 inch, 19 gauge serum needle and a 20ml. syringe. It was possible to take samples of up to 20ml. from birds weighing one kilogram or more without apparent ill effect. Bleeding was always carried out early in the morning and not later than one hour after the first feed, in order to reduce the amount of lipid in the blood. The blood was placed in 90 x 25mm tubes in 10ml. aliquots and allowed to clot at room temperature for 2 hours. The clot was then freed from the sides of the tube with a clean spatula and centrifuged at 1000xg for 30 minutes. The serum was then removed, placed in 10ml. tapered centrifuge tubes and centrifuged again at 1000xg for 30 minutes. A high proportion of the lipid remaining in the serum was removed by centrifuging at 25,000xg for 30 minutes at 0°C. The lipid formed a layer on the surface and the serum was removed by passing a precooled 15 inch, 20 gauge needle through the lipid layer and withdrawing into a 5 ml. syringe.

Serum samples were stored at -20°C with no preservative and before use were centrifuged at 60,000xg for 30 minutes at 4°C to clarify. Samples were not used after being deep frozen more than once.

Mucus Extracts

The preparation of intestinal mucus extracts was adapted from the method described by Dobson (1966a). The

intestine was slit open and pinned out in warm saline; the contents were removed and the mucosal surface washed with saline. The intestine between percentage lengths 30 and 80 was then cut up into 2cm. sections, placed in 25 ml. of chilled saline at 4°C and refrigerated overnight. This procedure causes the goblet cells in the mucosal epithelium of the intestine to contract and expel their contents into the saline.

After vigorous stirring the pieces of intestine were removed and the mucus extract thoroughly mixed and allowed to extract for a further 4 hours at 4°C before centrifuging at 25,000xg for 30 minutes. The supernatant was freeze dried or concentrated osmotically with carbowax. Extracts were used at a concentration of 100mg protein/ml.

Antigen

Living adult R.cesticillus were washed three times and thoroughly homogenised in phosphate buffered saline (pH 7.2). The homogenate, which was allowed to extract overnight at 4°C, was centrifuged at 1,000xg for 30 minutes and the precipitate discarded. Further centrifugation at 25,000xg was sometimes carried out when clear antigen solutions were required.

Lipid was removed from antigen by extraction with an equal volume of 3:1v/v diethyl ether: petroleum ether. The extraction was carried out by mixing the antigen solution thoroughly with the solvent mixture in 150 x 15mm tubes, allowing the two phases to separate and then removing the solvent by aspiration. This procedure was carried out three

times and the antigen was then dried and all traces of solvent removed by placing it under vacuum over silica gel. The antigen was usually stored at 4°C, but made up solutions of antigen could be preserved for a few days by the addition of 1:10,000 units of thiomersal.

Protein Determinations

Accurate protein determinations were made with the Folin Phenol reagent, as described by Lowry, Rosebrough, Lewis and Randall (1951). A useful rapid method utilising spectrophotometric absorbance at 280 and 260u, described by Warburg and Christian (1941)(after Colowick and Kaplan, 1957), was also frequently used.

A. THE EFFECT OF INFECTION ON MAST CELL, EOSINOPHIL AND GLOBULE LEUCOCYTE NUMBERS

I. Mast Cell Counts

Mast cells, which secrete the vasodilator histamine, are involved in the inflammatory process and an increase in number or degranulation of these cells is often associated with helminth infections. Such changes suggest that the host is capable of responding immunologically to the parasite and this was investigated in chickens infected with *R. cesticillus.*

Materials and Methods

Four groups of ten week old male birds, of two birds per group, were used:-

1) Uninfected
2) Primary infection of 14 days

3) Primary infection of 50 days

4) Secondary infection of 14 days.

Gut tissue was taken from percentage length 40, fixed in Bouin's fluid, embedded in paraffin and sectioned at 5u. The sections were stained in a 0.1% solution of toluidine blue for 5 minutes. Counts were made of mast cells in 200u long lengths of lamina propria from the base of the villus. Ten counts were made in each of five sections for each bird, making one hundred counts for each group.

Results

Table 20

Group	Mean Number of Mast Cells per Count
1	0.44 ± 0.074
2	0.78 ± 0.092
3	2.14 ± 0.153
4	6.93 ± 0.257

Groups	d (=)	P
1:2	2.807	0.01
2:3	8.324	0.001
1:3	10.018	0.001
3:4	13.444	0.001
2:4	22.610	0.001
1:4	24.289	0.001

These results show that infection with R.cesticillus results in an increase in the numbers of mast cells present in the lamina propria of the small intestine of the chicken.

Figure 24. The effect of R.cesticillus infections on intestinal mast cell numbers.

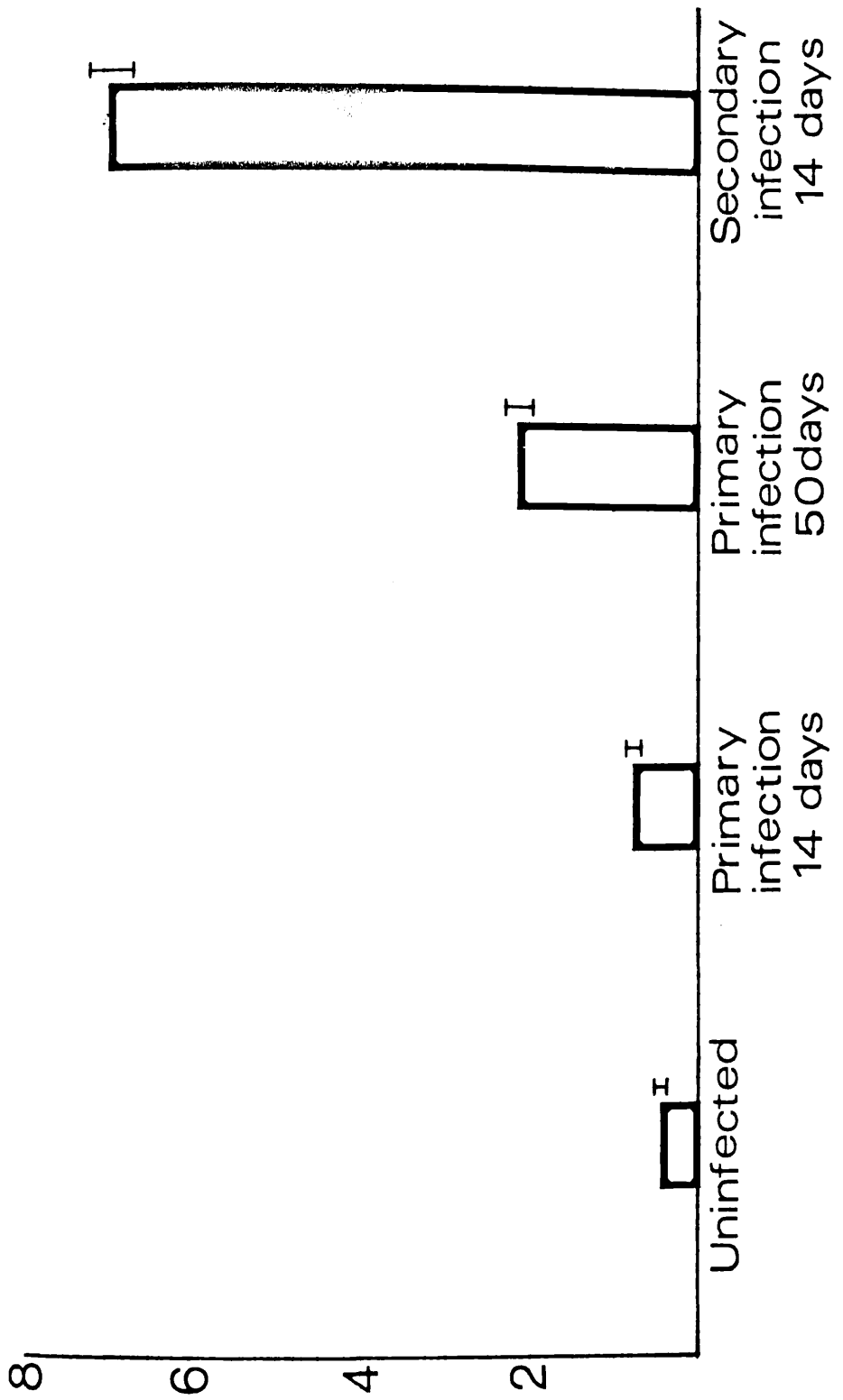

Mean number of mast cells
per unit area
8
6
4
2
Uninfected
Primary infection 14 days
Primary infection 50days
Secondary infection 14 days
Chicken Group

FIG. 24

The degree of this increase seems to be related to the length of infection (Fig.24). Differences in numbers of mast cells between the experimental groups are all statistically significant. In addition to an increase of mast cells in infected birds, degranulation of mast cells seems to be further advanced in birds with secondary infections than in the other groups.

II Eosinophil Counts

Eosinophilia has long been associated with helminth infections, particularly those involving histotropic helminths. Fernex and Fernex (1962) suggested that eosinophilia is a secondary phenomenon following mast cell hyperactivity and, since R. cesticillus infections cause a multiplication of mastcells in the lamina propria of the intestine, it was decided to investigate the effect of infection on blood eosinophilia and on eosinophil infiltration in the intestine.

Materials and Methods

Blood was obtained from male birds with different aged primary infections (7, 28, 49 and 70 days) and from birds with a 14 day old secondary infection and the number of eosinophils present were determined from counts in an improved Neubauer haemocytometer with a diluting fluid of 50% propylene glycol and 1% eosin in physiological saline. Gut tissue sections were obtained in the usual way and stained with haematoxylin and eosin or with Leishman's and

then with Giemsa. For both blood counts and tissue sections samples were obtained from uninfected control birds of the same age.

Results

The figures obtained for the eosinophil counts varied considerably for different aged infections (2,700 - 11,400/ cu.mm), but this is undoubtedly a reflection on the technique, as these variations are not consistent and there was no difference in counts between the experimental and control birds of any one group. It must be concluded that R.cesticillus does not cause a blood eosinophilia.

Eosinophils were never detected in the intestines of uninfected birds or in those with primary infections - even when longstanding. They only seem to occur, and then in small numbers, in the intestines of birds with secondary infections.

These results are not consistent with the suggestion of Fernex and Fernex (1962) that eosinophilia is linked to mast cell hyperactivity, but apart from this they are not surprising in view of the fact that R. cesticillus does not penetrate the mucosa and causes little tissue damage.

III 'Globule Leucocyte' Counts

Considerable numbers of cells, that resemble the globule leococyte of mammals, occur amongst the epithelial cells of chicken small-intestine mucosa. Mammalian globule leucocytes increase in numbers, as do mast cells, in infect-

ions of N. brasiliensis the rat nematode, but as yet their function is unknown. It has been suggested that they may be modified plasma cells or mast cells (after Ogilvie and Jones, 1971).

Counts of the chicken 'globule leucocyte' were carried out in an attempt to determine whether or not this cell plays a part in the immune response to R. cesticillus.

Materials and Methods

Gut tissue from percentage length 40 was obtained from the same birds that were used for the mast cell counts and was fixed in Bouin's fluid, embedded in paraffin wax, sectioned at 5u and stained with haematoxylin and eosin. Counts were made from 50u square areas of mucosal epithelium taken at random. Ten counts were made in each of five sections for each bird, making one hundred counts for each group.

Results

Table 21

Group	Mean Number of 'globule leucocytes' per count
Uninfected	0.88 ± 0.098
14 day Primary	0.96 ± 0.107
50 day Primary	1.02 ± 0.092
14 day Secondary	0.87 ± 0.092

There is no significant difference between the values of any of the groups and it must therefore be concluded that the chicken 'globule leucocyte' is not affected by R.cesticillus infections.

B. IMMUNOCYTO-ADHERENCE

This test is extremely useful for the detection of an immune response, as it does not just depend on antibody being present, but can detect cells that may not be secreting antibody, but are immunologically committed (Zaalberg, van der Meul and van Twisk, 1968). Such a test would appear to be an admirable method of confirming the immunogenicity of R. cesticillus.

The test consists of exposing the suspected immunocytes to red blood cells coated with antigen. A positive result is indicated by the red cells adhering to the immunocytes forming clusters that may be several red cells deep.

The test described here was adapted from the procedure of Duffus and Allan (1970), who investigated the immune responsiveness of chicken circulating leucocytes.

Materials and Methods

Blood was collected in Alsever's solution from a chicken with a secondary infection, a chicken that had received a 150mg protein/ml intravenous antigen injection with Freund's adjuvant 10 days before and an uninfected chicken. The blood was dispensed into eight 5 x 100mm tubes

and centrifuged at 900xg for 25 minutes. The buffy coats were carefully removed with fine pipettes and washed three times in 199 tissue culture medium with 10% inactivated rabbit serum. The cells were then resuspended in about 2 ml of 199 TC/10% serum.

Sheep red blood cells in Alsever's solution were washed three times in saline and a 2.5% suspension of cells was exposed to a 0.001% solution of tannic acid to increase their sensitivity to antigen. The cells were coated with 7-10ug protein/ml antigen at room temperature during a 30 minute incubation period and after being thoroughly washed were suspended in 199TC/10% serum. Uncoated control cells were also prepared.

The chicken leucocytes were diluted to give a count of 25,000/cu mm and were mixed with an equal volume of sheep red cells, with a count of 5 x 105/cu mm, in 6 x 20mm tubes to give a redcell:leucocyte ratio of20:1. The preparations were incubated at 37°C and observations of samples in haemocytometers were made after 30, 60, 90 and 120 minutes of incubation.

Results

Initially, nonspecific clumping of red cells occurred, probably as a result of the tanning process, but after changing the batch of both red cells and tannic acid this problem was overcome and positive results were obtained in the incubations of coated cells with the experimental and the positive control leucocytes. No adherence was observed in the incubations containing uncoated cells or in the

Figures 25 and 26. Immunocytoadherence. Sheep red blood cells around chicken white blood cells. X 900.

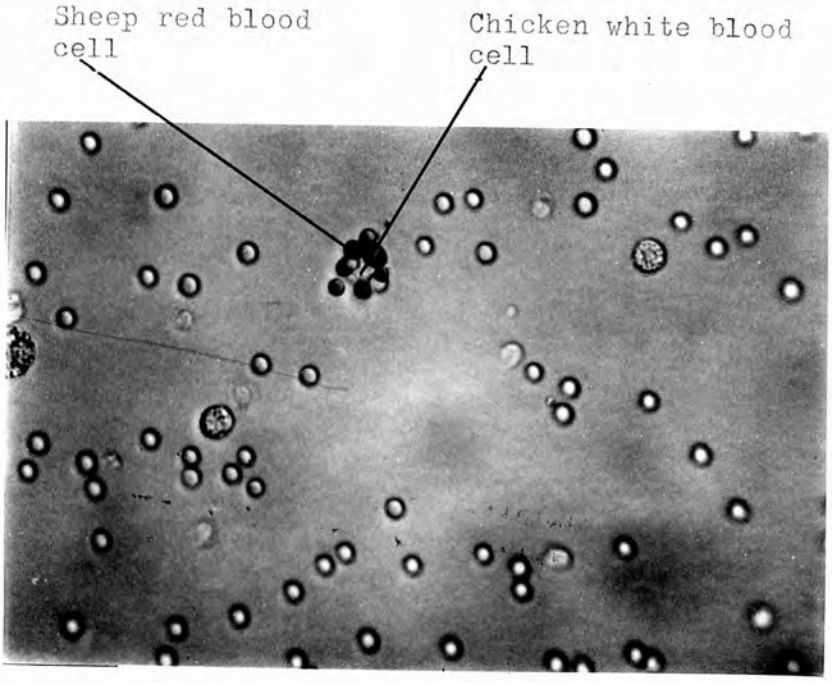


FIG. 25

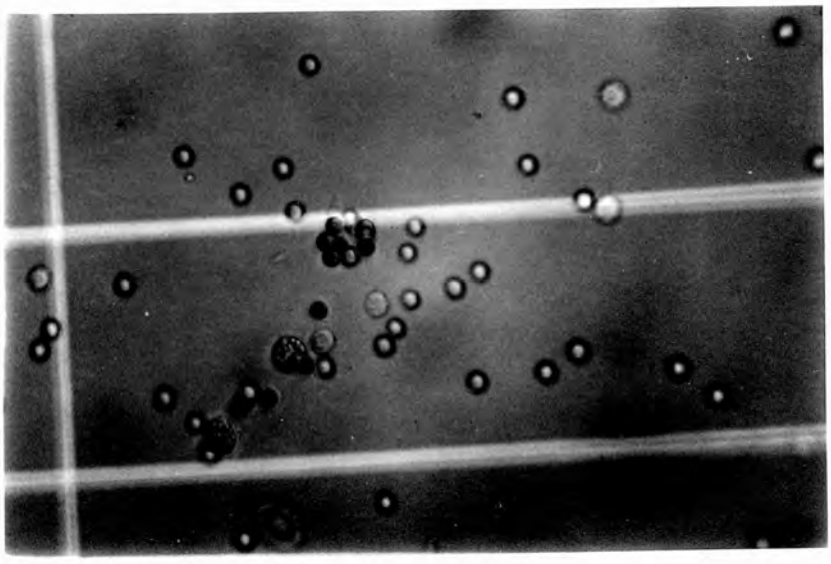


FIG. 26

incubations with coated cells and leucocytes from an uninfected chicken.

Selected red cell clumps were photographed to show the central leucocyte (Figs.25,26). Large clumps of red cells and leucocytes were occasionally observed, but many-cell layered rosettes were never seen possibly because the detected immunocytes were producing no or very small amounts of antibody. However, it is difficult to see how this could apply to positive control leucocytes.

Red cell clumps first appeared in both experimental and positive control preparations after only 30 minutes incubation and were numerous after 60 minutes. In one experiment an antiserum from a primary infection was included and the fact that no clumps were detected until 60 minutes of incubation suggests that the immune status of these leucocytes was lower than that of leucocytes from secondary infections, as one might expect.

These tests provide good supporting evidence for an immune response to R. cesticillus infections.

C. THE INTERFACIAL RING PRECIPITIN TEST

This test is a useful qualitative method for the detection of precipitin antibodies and involves the overlaying of the antiserum with a solution of the antigen so that a sharply defined interface is formed. It is possible to detect as little as 1.0ug. of protein with this method.

Meinkoth (1947) attempted to demonstrate serum antibodies to R. cesticillus in serum from infected birds, but was not successful. Positives were obtained with serum

from birds that had received injections of worm homogenate.

In the tests described here attempts were made to demonstrate antibodies to R.cesticillus homogenate in both serum and mucus from birds with various stages of infection.

1). Serum

Materials and Methods

The serum was clarified by centrifugation at 60,000xg for 30 minutes at 4°C and introduced into 6 x 50mm tubes with a pasteur pipette to a height of 5 mm from the base of the lucite rack. A crude extract of R. cesticillus was centrifuged at 60,000xg for 30 minutes at 4°C and the resulting clear solution was diluted with phosphate buffered saline to a protein concentration of 50mg protein/ml. Serial dilutions of this antigen were made with a twice normal strength of phosphate buffered saline and the prepared antiserum was carefully overlayed with these solutions. The antigen was diluted with a twice normal strength of phosphate buffered saline, as Goodman, Wolfe and Norton (1951) had shown that increased precipitation occurred in chicken serum with increasing concentrations of NaCl. Maximum precipitation occurred at a salt concentration of 8%. The twice normal strength of phosphate buffered saline used in these tests corresponds approximately to the 1.8% saline used by Wolfe and Dilks (1948) as the antigen diluent in ring tests with chicken serum.

The negative controls consisted of the antiserum plus phosphate buffered saline and normal serum plus antigen solution. The positive control consisted of serum from a

bird injected with R. cesticillus homogenate plus antigen solution.

Readings were made after two hours incubation at room temperature.

Results

Initially it proved very difficult to obtain unequivocal results and false positives occurred frequently. After several tests it was found that the most consistent results were obtained with serum that had been frozen for a short time. In addition to this, clear and lipid free serum and antigen solutions were essential.

Positive ring precipitin tests were eventually demonstrated (Fig. 27.); tubes 1 - 4 contain antiserum overlayed with 10 fold serial dilutions of antigen (5mg protein/ml), tube 5 contains antiserum overlayed with phosphate buffered saline and tube 6 contains normal serum overlayed with 1/10 antigen. Positive reactions can be seen in tubes 1, 2 and 3. No reaction has occurred in tube 4 or the two negative controls. The reaction in the tubes containing concentrated antigen is usually visible after a few minutes and is sharply defined at this stage. Dispersal of the precipitate occurs after a few hours and this can be seen to be occurring in tube 1 after an incubation of two hours.

It is by no means certain that the observed precipitation is caused by antibody-antigen reaction. However, it was noted that in birds with primary infections titres reached 1:4,000 (12.5ug/ml) and in birds of the same age titres reached 1:10,000 (5.0ug/ml). A single intravenous

Figure 27. Ring precipitin test. Serum from chickens infected with R.cesticillus against antigen of diluted worm homogenate. X2.

Tube I 2 3 4 5 6

FIG. 28.

injection of 40mg protein antigen/ml resulted in a titre of 1:10,000 one week later. These results suggest that the titres bear some relation to the course of the cestode infection and thus to an immunological response. It was decided to investigate the change in titres throughout the infection in more detail.

Serum was collected from three male and three female 5 week old chickens which were then infected with 100 cysticercoids of R.cesticillus each. Serum was collected 2, 5, 10, 15, 20, 25, 30, 35, 40, 60, 63, 68, 75 and 82 days post infection and the samples from the individual birds pooled. The primary infection was eliminated 60 days after infection and a secondary infection from a dose of 100 cysticercoids was established 68 days after the primary infection.

The samples were tested 5 days after collection against diluents of a 50mg protein/ml antigen solution.

Table 22.

Titres

Days after primary infection	Male		Female	
	Dilution	Protein ug/ml	Dilution	Protein ug/ml
2	1:200	250.0	1:100	500.0
5	1:400	125.0	1:200	250.0
10	1:800	62.4	1:200	250.0
15	1:1000	50.0	1:800	62.0
20	1:2000	25.0	1:1000	50.0
25	1:4000	12.5	1:2000	25.0
30	1:4000	12.5	1:4000	12.5

Table 22(Cont)

Days after primary infection	Titres			
	Male		Female	
	Dilution	Protein ug/ml	Dilution	Protein ug/ml
35	1:8000	6.2	1:10,000	5.0
40	1:10,000	5.0	1:10,000	5.0
60	1:10,000	5.0	1:10,000	5.0
Infection eliminated				
63	1:10,000	5.0	1:10,000	5.0
68	1:8,000	6.2	1:8,000	6.2
Secondary reinfection				
75	1:10,000	5.0	1:10,000	5.0
82	1:20,000	2.5	1:20,000	2.5

These results show a pattern typical of a primary and secondary antibody response (Fig. 28.). Titres rose slowly to a plateau of 1:10,000 and fell to 1:8000 after the primary infection was eliminated with the anthelmintic hexachlorophene. This is probably not a significant fall in titres, but it is probable that the dramatic increase to 1:20,000 after secondary infection represents a genuine anamestic response.

The ring test is not regarded as a quantitative test, as it is not dependent on critical initial ratios of antibody and antigen, but on diffusion between the two components and the establishment of a ratio optimal for precipitation.

However, providing as many factors as possible are kept constant it can be used qualitatively to compare relative antibody levels. Wolfe and Dilks (1948) used this method when they correlated the precipitin production

Figure 28. Ring precipitin test titres of serum taken from chickens with primary and secondary infections of R.cesticillus.

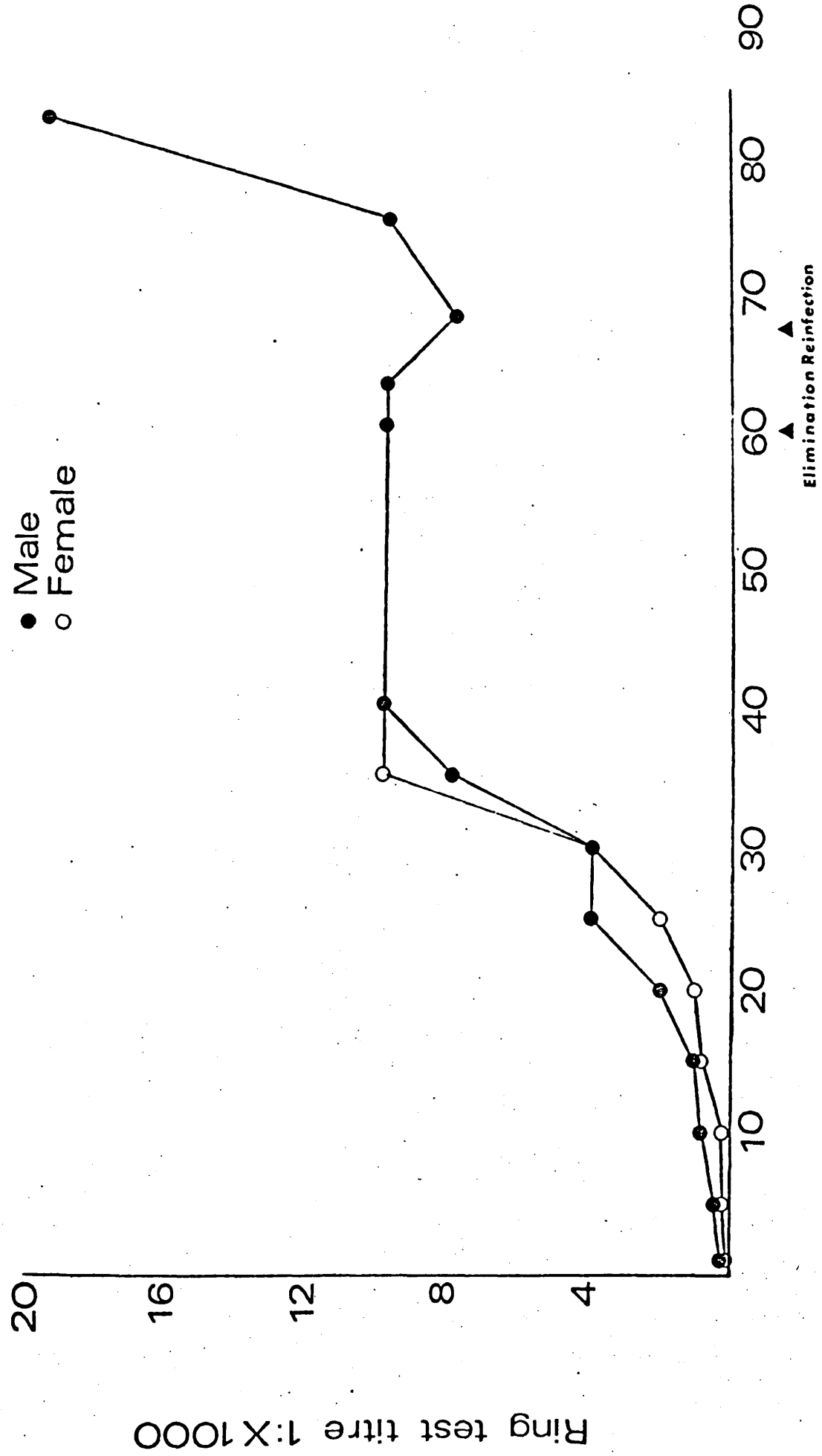

Ring test titre 1:X 1000
20
16
12
8
4
10
20
30
40
50
60
70
80
90
Days after infection
Elimination Reinfection
● Male
○ Female

FIG. 28

of chickens with age.

The titres obtained in the test described here correlate well with host resistance to R. cesticillus as determined by the number of destrobilating scoleces and this experiment therefore lends further support to the hypothesis that R. cesticillus evokes an immunological response. It is not suggested that the detected antibodies are active against R. cesticillus, but merely that they reflect the activity of the immune system in infected birds.

It is difficult to explain the fact that Meinkoth (1947) failed to obtain positive sera from infected birds using the ring test. He gives very few details about the preparations of antigen and antiserum. It is perhaps significant that the birds that supplied the antiserum had rather small infections compared with those in the birds used in the tests described here.

It was considered desirable to attempt to confirm the presence of serum antibodies to R. cesticillus with other tests and if possible to monitor the production of antibody during an infection more accurately.

2). Mucus

Many attempts to demonstrate antibodies in clarified mucus extracts of 100mg protein/ml were made, but no positive results were obtained. Mucus from chickens injected with antigen could not be considered as a positive control as it is not likely that an intravenous injection of antigen would stimulate antibody production in the intestine.

D. IMMUNODIFFUSION AND IMMUNOELECTROPHORESIS

Immunodiffusion

Immunodiffusion techniques are similar in principle to the ring precipitin test in that diffusion of antigen and/or antibody occurs until a ratio optimal for precipitation is established. The difference between the two techniques is that diffusion occurs directly between the antigen and antibody components in the ring test and a positive reaction is usually visible after a matter of minutes, whereas in immunodiffusion a third component is present in which the antigen and antibody diffuse and precipitate. This third component is usually agar gel, but can take other forms such as cellulose acetate membranes. Positive reactions usually take longer to develop in immunodiffusion tests than in the ring test. The fact that immunodiffusion and the ring test are fundamentally similar and that they both detect precipitins means that immunodiffusion should be an ideal technique for the confirmation of the positive results obtained with the ring test.

Immunodiffusion techniques can be characterised in several ways. When a concentration gradient is established for only one of the reactants the technique is termed simple diffusion. When concentration gradients are established for both reactants the technique is termed double diffusion. In both simple and double diffusion the reactants may diffuse in one, two or three dimensions.

Attempts were made to demonstrate antibodies using simple one dimensional and double two dimensional diffusion.

Simple One Dimensional Diffusion

This technique is similar to the ring test, but the antiserum is dispersed in agar gel, so that when antigen is overlayed precipitin lines take longer to develop and move down the agar column as time progresses.

The tests were carried out in 6 x 50mm tubes with 0.85% Ion Agar No.2, which contained 0.01% thiomersal, 0.01% trypan blue and 25 or 10% serum. The sera used were obtained from birds with secondary infections of R. cesticillus (experimental), from birds that had received injections of worm homogenate with Freund's adjuvant (positive control) and from uninfected birds (negative control).

Ten fold dilutions of an antigen stock of 50mg protein/ml in phosphate buffered saline were layered on top of the solidified agar and the tubes were placed in a humidity chamber for up to two weeks with daily observations.

The results obtained were very equivocal. Precipitin lines were consistently demonstrated with positive control sera, but experimental preparations were often negative while positive readings were sometimes obtained with the negative controls. The precipitin lines obtained rarely moved very far from the interface of the agar gel and the antigen solution and their significance is questionable.

Double Two Dimensional Gel Diffusion

This technique was developed by Ouchterlony (1949) and its chief advantage is that it can be used to demonstrate more than one antigen-antibody system. In the tests described, ten fold serial dilutions of antigen were tested against antisera and against mucus extracts in 0.85% Ion Agar No 2, containing 0.01% thiomersal and trypan blue, in petri dishes.

The hot agar was pipetted into the petri dishes in 8ml aliquots and allowed to cool and solidify. After solidification another 8 ml of hot agar was pipetted into each dish. One central well and six surrounding wells were cut with a penicylinder and the test samples added. The serum or mucus extract was placed in the central well and the antigen solutions in the surrounding wells. The same sera and antigen were used as described for single one dimensional diffusion (Page147). Mucus extracts were obtained from uninfected birds and birds with secondary infections of R. cesticillus.

A piece of filter paper was attached to the lid of each petri dish to prevent the accumulation of condensate on the surface of the agar and the petri dishes were then placed in a humidity chamber at room temperature for up to two weeks. Several tests were carried out, but no precipitin lines were detected in the experimental or negative control preparations. Positive control preparations consistently showed precipitin lines at antigen concentrations of 50, 5 and 0.5 mg protein/ml.

Immunoelectrophoresis

This technique combines the methods of electrophoresis and immunodiffusion. The antigen or antibody is first separated electrophoretically and the antibody or antigen is then applied to a parallel trough from which it diffusss out to react with the electrophoretically separated components. It was hoped that this technique might separate the antigenically active component of the worm homogenate resulting perhaps in greater sensitivity.

There are many factors of importance to be considered when applying this technique such as the distance between the point of origin and the trough, the concentration ratios of antigen and antibody, the pH and ionic strength of the buffer, the applied electrical field, the temperature, the time allowed for electrophoretic separation and the time allowed for diffusion. With so many factors affecting the system optimal conditions for reaction must be determined by trial and error.

The basic procedure carried out for each test was as follows. Microscope slides were coated with a thin film of 1.5% Agarose which was allowed to harden and 2 mls of 0.85% Agarose was pipetted onto the slides. After solidification the troughs and wells were cut with a Shandon cutter. Antigen or antibody was placed in the wells and the slides placed between the two buffer vessels with the wells nearest the cathode. The buffer wicks were applied and the current applied at 5 - 6v/cm constant voltage and 20m/A amperage for up to 4 hours. Bromophenol, an albumin

dye was occasionally added to the wells in order to follow the run. Antiserum or antigen was then placed in the troughs and allowed to diffuse for 48 hours in a humid atmosphere. The slides were fixed in 1%CH_3COOH in 50% EtOH and stained in nigrosine (nigrosine - 1g, 12% CH_3COOH - 500ml, 1.6% sodium acetate - 500 ml) for 30 minutes. Differentiation took place in 2% CH_3COOH.

This method was used several times with various modifications such as length of electrophoretic run, diffusion time, different buffering systems (barbitone acetate, pH8.6; barbital, pH8.2; phosphate, pH7.2), different concentrations of antigen and different concentrations of agar. Despite these modifications precipitin lines were only detected once with experimental serum. On this occasion the agar gel concentration was 1.5%, the buffer being barbitone acetate with an ionic strength of 0.1m; the electrophoretic run took four hours and the antiserum was allowed to diffuse out from the trough for 48 hours. Possible precipitin lines were detected on both replicates of the experimental serum preparation.

Attempts to demonstrate precipitin lines with positive control serum were never successful and it was concluded therefore that the failure of these tests was probably due to the inadequacies of the electrophoretic technique used. Several other electrophoretic tests, such as crossed over electrophoresis and C.A.M. electrophoresis, were tried, but unequivocal results were never obtained.

E. SERUM PROTEIN SEPARATION

It is possible to separate the antibody-containing components of antiserum electrophoretically. By this means it should be possible to detect specific antibodies to R. cesticillus. Serum containing antibodies to R. cesticillus is likely to have a greater proportion of globulins, probably gamma globulins, than serum from uninfected birds. This difference could be detected by a quantitative comparison between the serum protein profiles of the 'infected' and 'uninfected' sera. An alternative method of detection of these antibodies, which is both simpler and more satisfactory, is to incubate the sera with R. cesticillus antigen. The sera with specific antibodies to the cestode will have altered protein profiles, as a proportion of the antibody will have reacted with the antigen and precipitated.

Quantitative comparison of the protein profiles is unnecessary as any antibody-antigen reaction will alter the shape of the profile and should be obvious. This method is unlikely to be very sensitive and cannot justifiably be used as the sole means of demonstration of antibodies, but it is an adequate confirmatory method, providing sufficient controls are included.

In the experiments described here this technique was applied to both serum and mucus extracts. Dobson (1966) states that serum proteins are present in the mucus of sheep, but are in different proportions, with less albumin and more globulin.

Materials and Methods

The sera and mucus extracts were incubated with the same volume of 5mg/ml of clarified R.cesticillus antigen in 6 x 50mm tubes at 37°C for 1 hour. The tubes were centrifuged for 10 minutes at 3000 x g before samples were taken for electrophoresis.

All separations were done on cellulose acetate membrane strips with a current of 6m amps at a voltage of 100-200v/cm for 4 hours. The samples were applied as a streak 2.5 cms from the end of the strip and bromophenol blue was used as a tracer dye. Strips were impregnated with buffer before the run; several buffers were tried the most successful being Tris EDTA boric acid. After the run, strips were fixed in trichloroacetic acid for 10 minutes, washed in 5% acetic acid and stained in 0.02% nigrosine overnight. They were differentiated in 5% acetic acid for about 3 hours and then air dried.

This procedure was carried out many times before good separation of both experimental and control preparations was achieved. For photometric evaluation of the strips a Locarte Densitometer was used. This instrument was adjusted to give the maximum separation of the protein profiles. It was sometimes necessary to read the strips from the reverse side to obtain the best results.

Results

Serum Separation

The experimental antiserum was obtained from 10 week old male birds with secondary infections of R.cesticillus

and was then pooled. The negative control serum was obtained from a single 10 week old male bird and the positive control serum was obtained from a bird that had received an intravenous injection of 40mg protein/ml antigen seven days before. Each serum was incubated with antigen and with phosphate buffered saline. Up to three replicates were prepared for each incubation and after separation each C.A.M. strip was processed by the Densitometer.

Results were very variable, but the clearest examples can be seen in Fig.29. . The most noticeable difference between the normal serum preparations and the experimental and positive control serum preparations is the greater proportion of globulin relative to the albumin in the latter two preparations. McGhee (1971) states that it is generally accepted that in birds, as in mammals, an increase in gamma-globulin levels in the blood usually means an increase in specific antibody content. The profiles of the experimental and positive control serum/antigen incubations suggest that a proportion of the globulin fraction, especially gamma-globulin, has been eliminated. The suggestion is that antigen has reacted with specific antibodies in the gamma-globulin fraction and the resulting complexes have been precipitated. No such effect is seen in the normal serum/antigen incubation.

These results lend further support to the suggestion that R. cesticillus evokes a humoral immune response.

Figure 29. Protein separation of sera from chickens infected with R.oesticillus, from uninfected chickens and from chickens injected with worm homogenate. Incubated with antigen and with saline.

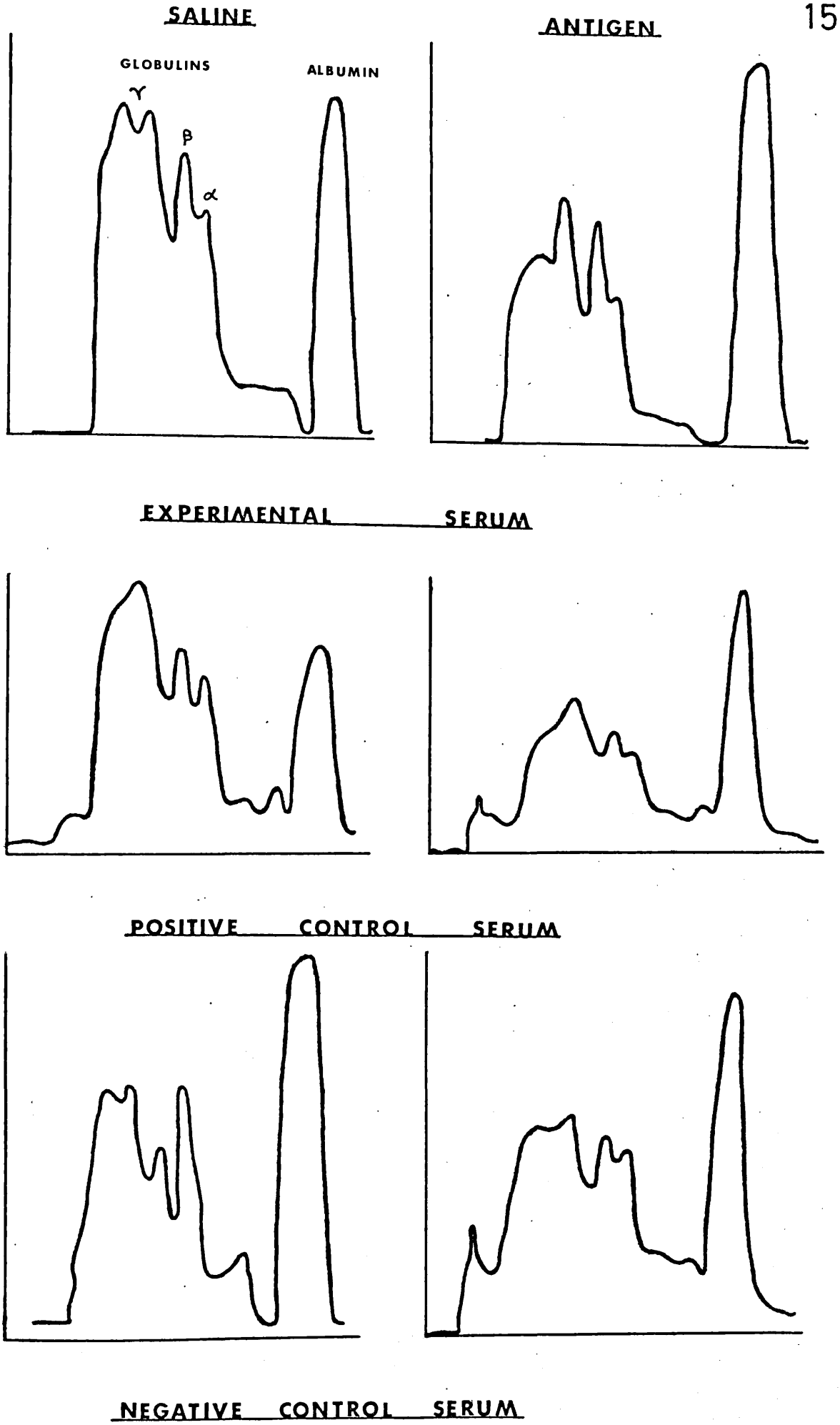
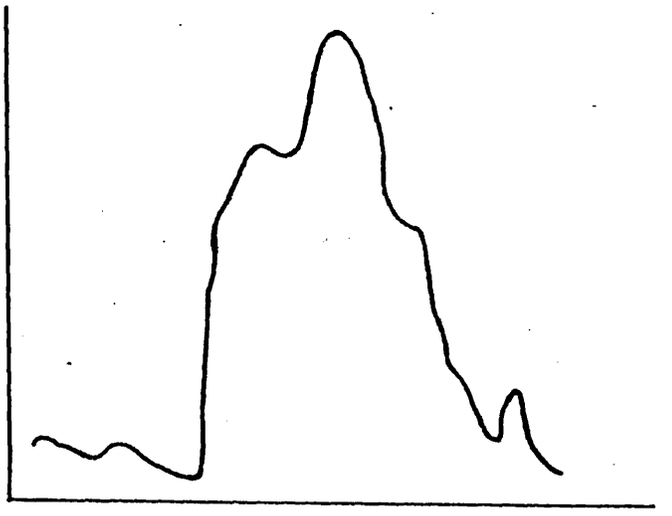
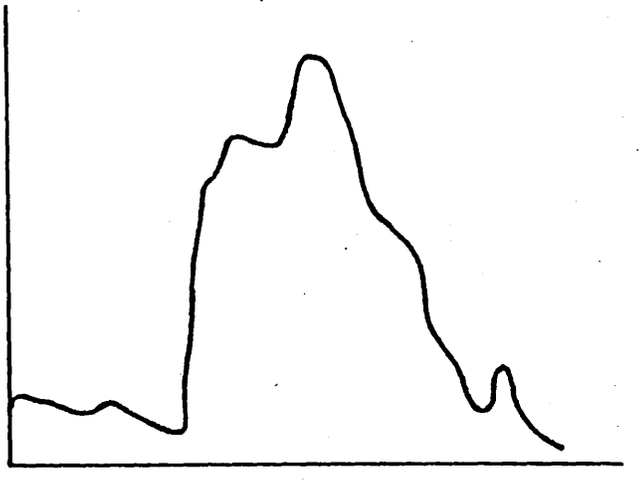

SALINE
ANTIGEN
GLOBULINS
ALBUMIN
γ
β
α
EXPERIMENTAL SERUM
POSITIVE CONTROL SERUM
NEGATIVE CONTROL SERUM

FIG. 29

Figure 30. Protein separation of mucus extracts from chickens infected with R.cesticillus. Incubated with antigen and with saline.



IMMUNE MUCUS / SALINE



IMMUNE MUCUS / ANTIGEN

FIG. 30

Mucus Separation

Mucus extracts obtained from an uninfected bird and a bird with a secondary infection of R. cesticillus were concentrated osmotically with carbowax and used at a protein concentration of 100mg/ml. These extracts were incubated with antigen or phosphate buffered saline, as described for the serum separations. Good separations of the mucus extracts were difficult to obtain, but it is evident from the 'immune' mucus/antigen and the 'immune' mucus/saline profiles (Fig.30.) that no antibody-antigen reaction has occurred.

F. PASSIVE HAEMAGGLUTINATION

This technique utilises the red blood cell, with its complex surface membrane, as an antigen carrier particle and is sensitive enough to detect 0.003-0.006ug of antibody nitrogen.

It was hoped that this technique would detect agglutinating antibodies to R. cesticillus and perhaps provide a sensitive means of monitoring antibody production during an infection.

Materials and Methods

The basic procedure followed was as set out by Campbell, Garvey, Sussdorf and Cremer (1964).

The red blood cells were well washed with physiological saline and a 3 ml suspension in phosphate buffered saline (pH 7.2) was tanned with an equal volume of 0.001-0.005% tannic acid at 37°C for 10 minutes. After thorough

washing with phosphate buffered saline the cells were exposed to 0.8 - 15.0ug protein antigen in phosphate buffered saline pH 6.4 at room temperature for up to 60 minutes. Control cells were treated similarly with saline instead of antigen. The cells were washed in phosphate buffered saline pH 7.2 and finally suspended as a 2.5% suspension in 1% normal serum in saline.

Titrations were carried out against 0.5ml doubling dilutions of antiserum or mucus extracts with 0.05ml of tanned coated red blood cells.

The sera were always inactivated (56°C for 30 minutes) and were sometimes absorbed with their own volume of packed red cells to remove interspecific precipitins.

A lot of difficulty was experienced with this technique and thirty-five separate tests were carried out in attempts to obtain unequivocal results, during which the following modifications and variations of the described procedure were made.

1) Both chicken red blood cells (fresh) and sheep red blood cells (in Alsever's Solution and formalinised) were used.

2) When sheep red blood cells were used the sera were absorbed with their own volume of sheep cells for 1 hour at room temperature. On some occasions when chicken cells were used the sera were absorbed with chicken cells.

3) The cells were tanned with concentrations of tannic acid of 0.001, 0.0025 and 0.005%. In some tests the cells were untanned.

4) Cells were exposed to antigen concentrations ranging from 0.8 - 15ug/ml.

5) The length of coating time ranged from 10 minutes to 1 hour and took place at 37°C and at room temperature, at pH 6.4 and 7.2.

6) A variety of different wells and tubes were used and one test using a microhaemagglutinating kit was carried out.

The main problem was panagglutination and it proved very difficult to obtain good negative readings for the controls. Despite many precautions such as using fresh sera, washing cells well after tanning and coating, reducing the tannic acid concentration, the use of citrate saline as a normal serum diluent and the washing of tanned cells in 1% bovine serum albumin, nonspecific panagglutination continued to be a problem and was never completely resolved.

Attempts were made to detect agglutinating antibodies to R. cesticillus in both sera and mucus extracts.

14) SERA

Antiserum, with a ring test titre of 1/10,000, was obtained from birds with secondary infections and the normal serum from uninfected birds of the same age. The positive control serum was obtained from birds that had received 40mg protein/ml antigen intravenously ten days before bleeding and had a ring test titre of >1/10,000.

Negative readings were consistently obtained with untanned cells in both antiserum and positive control serum.

Cells tanned with 0.001% tannic acid usually gave negative results in antiserum and the negative controls and positive results in the positive control serum. Cells tanned with 0.0025% tannic acid gave very mixed results and cells tanned in 0.005% tannic acid, the recommended concentration, invariably panagglutinated.

The least equivocal results were obtained in the following two tests.

1) Materials and Methods

Chicken red blood cells were collected by cardiac puncture and delivered into an equal volume of Alsever's Solution. They were tanned with 0.0025% tannic acid and were washed with 0.5% bovine serum albumin after the tanning process. They were then exposed to 2.5ug protein/ml antigen at pH 6.4 for fifteen minutes at room temperature. The test was carried out in 12 x 75mm tubes and the results were scored +++, ++, +, -, according to the extent of agglutination.

Table 23.

Test

Volumes in mls.

Tube Number	1	2	3	4
N.S.diluent	0.5	0.5	0.5	0.5
Ag. R.B.C.	0.1	0.1	-	0.1
Control R.B.C.	-	-	0.1	-
Antiserum	0.5	-	0.5	-
Normalserum	-	-	-	0.5

Result

Tube Number	1	2	3	4
Experimental serum	-	-	-	-
Positive control serum	++	-	-	-
		Negative Controls		

This result suggests that R. cesticillus infections do not evoke an agglutinating antibody response.

2) Materials and Methods

Chicken red cells were again used and were tanned with 0.001% tannic acid. The cells were exposed to 5.0mg protein/ml antigen for 30 minutes and the test was carried out in 12 x 75 mm tubes.

Table 24.

Test

Tube Number	1	2	3	4	5	6	7
N.S.diluent	-	0.5	0.5	-	0.5	-	0.5
Ag.R.B.C.	0.05	0.05	0.05	-	-	0.05	0.05
Control R.B.C.	-	-	-	0.05	0.05	-	-
Antiserum	0.5	0.5	-	0.5	0.5	-	-
Normalserum	-	-	-	-	-	0.5	0.5

Result

Tube Number	1	2	3	4	5	6	7
Experimental serum	-	-	-	-	-	-	-
Positive control serum	++	+	-	-	-	-	-
			Negative Controls				

The second test also suggests that no agglutinating antibodies are produced in response to R. cesticillus infection.

Tannic acid increases the instability of red cells and also causes them to take up protein antigens. Cells have been shown to absorb protein antigens and become agglutinable without having been tanned, but the sensitivity of such preparations is low (after Weir 1967).

One would perhaps expect the positive control serum to agglutinate untanned cells, as the antigen injection is likely to have contained immunogenic polysaccharides as well as proteins. Agglutination of untanned cells did occur, but not consistently enough to be regarded as significant. The frequent panagglutination encountered is almost certainly due to the tanning process, but it would seem that other factors are involved as well.

Although the results obtained suggest that there are no agglutinating antibodies in serum from infected chickens very few of the tests were entirely satisfactory and it must be concluded that the absence of serum agglutinating antibodies to R. cesticillus has not been proved.

2) Mucus Extracts

Mucus extracts were obtained from the same birds that contributed the serum and were used at a concentration of 200mg protein/ml.

Lysis of red cells occurred when mixed with the mucus extracts and inactivation of the mucus extracts at 56°C for 30 minutes resulted in the precipitation of most of the

mucus protein. The supernatants were concentrated with carbowax to about 100mg protein/ml and tested with chicken red blood cells that had been tanned with 0.001% tannic acid and exposed to 7ug protein/ml antigen for 30 minutes. No haemagglutinating activity was detected in the mucus extracts and no panagglutination occurred. Once again there was some lysis of red cells, but it had been much reduced by inactivation.

Positive results were not obtained with the 'positive control' mucus extract as expected. The failure of this test once again does not constitute evidence for the absence of agglutinating antibody. There is plenty of room for improvement of this test using mucus extracts especially in preparatory procedures.

G. IMMUNOFLUORESCENCE

The labelling of antibody or antigen with a substance that fluoresces in ultra-violet or blue light has many applications and is a much more direct way of observing antibody - antigen reactions than agglutination, precipitation or anaphylactic techniques. Whole cells or organisms may be used as antigen and the interference of contaminant antigens with specific reactions can be eliminated.

Another advantage of immunofluorescence is the opportunity to localise antigens in the internal structures of parasites. This may be of significance as far as *R. cesticillus* is concerned because it is not unlikely that the functional *in vivo* antigen is not present in the whole

worm extracts used as antigen in the precipitation and agglutination techniques. This might perhaps explain the lack of success with immunodiffusion and passive haemagglutination, but does not explain the positive results obtained with the ring test.

It was decided to use immunofluorescence as a possible means of demonstrating serum and mucus antibodies to R. cesticillus and in an attempt to localise functional antigen in the cestode.

Immunofluorescent techniques are not regarded as having the sensitivity of passive haemagglutination, but are probably more sensitive than complement fixation. Two different methods of immunofluorescence are used to detect antibodies. The direct method involves the fluorescent labelling of the globulin fraction of the antiserum, or sometimes the labelling of whole antiserum, and then incubation with the antigen. The indirect method involves the incubation of unlabelled antiserum with the antigen and then incubation of the antigen with a labelled antiserum prepared against the first antiserum. The indirect method is more sensitive than the direct method and an added advantage is that labelled anti-species serum is available commercially. The indirect method was selected for the tests described here.

Evaginated cysticercoids were used as antigen, anti-chicken rabbit globulin labelled with fluorescein isothiocyanate (RACh/FITC) was obtained from Nordic Diagnostics and this combination was used to detect antibodies in serum and mucus.

Materials and Methods

Cysticercoids were dissected out from beetles and washed in phosphate buffered saline. They were pipetted into saline containing 1% pepsin and 0.5% HCl and incubated for ten minutes at 37°C. They were then transferred to warm Hank's balanced saline containing 0.5% sodium glycotaurocholate. Evagination of most cysticercoids occurred after about 30 minutes incubation at 37°C. The evaginated cysticercoids were thoroughly washed in phosphate buffered saline and then incubated for 2-3 hours at 37°C in 6 x 20mm tube containing the following preparations:

1. Phosphate buffered saline
2. 1:10 normal serum
3. 1:100 normal serum
4. 1:10 positive control serum
5. 1:100 positive control serum
6. 1:10 experimental serum
7. 1:100 experimental serum
8. Normal mucus extract
9. Experimental mucus extract.

The normal serum was obtained from an uninfected 10 week old male bird, the positive control serum was obtained from a 10 week old male chicken that was injected with 150mg protein/ml antigen with Freund's adjuvant 10 days before bleeding and the experimental serums were obtained from birds that had both primary and secondary infections. Mucus extracts were obtained from an uninfected bird and from a bird with a secondary infection. They were used at protein concentrations of 100mg/ml.

The cysticercoids were washed in phosphate buffered saline after incubation and were fixed in acetone for 15 minutes. They were washed again in phosphate buffered saline and incubated in RACh/FITC for 15 minutes. After further washing in phosphate buffered saline the cysticercoids were mounted in glycerine with 10% phosphate buffered saline and observed under blue light. The best results were achieved by leaving the cysticercoids in phosphate buffered saline at 4°C overnight. About 30% fluorescence is lost after this time and this procedure eliminates much of the nonspecific background fluorescence. The cysticercoids were photographed under blue light using Kodachrome II with an exposure time of 8 minutes.

Results

Specific fluorescence was observed in evaginated cysticercoids that had been incubated in positive serum and in experimental serum. No fluorescence was observed in cysticercoids that had been incubated in saline, normal serum or mucus extracts.

This test was repeated three times with consistent results with both 1/10 and 1/100 serum dilutions and clearly demonstrates the presence of antibodies to R. cesticillus in serum from chickens with both primary and secondary infections (Figs.31-35.).

It is possibly significant that the distribution of specific fluorescence in the cysticercoids of the positive control serum incubation differs from those of the experimental serum incubation. The fluorescence is concentrated

Figures 31-35. Immunofluorescence. Cysticercoids incubated in sera from chickens with primary and secondary infections of R.cesticillus from uninfected chickens and from chickens injected with worm homogenate. Labelled with fluorescein iso-thiocyanate (conjugated with rabbit anti-chicken globulin) and photographed under blue light. X 200.

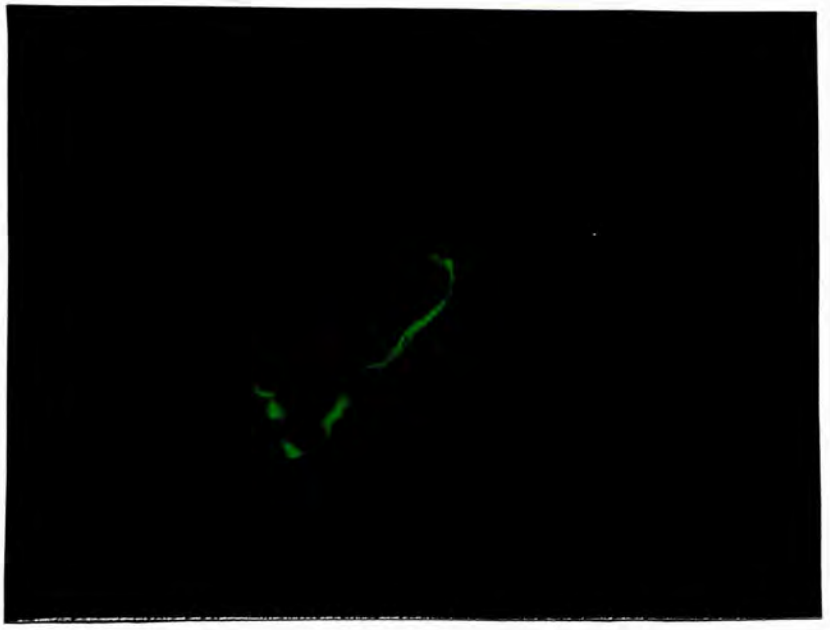


FIG. 31. Primary Infection Serum

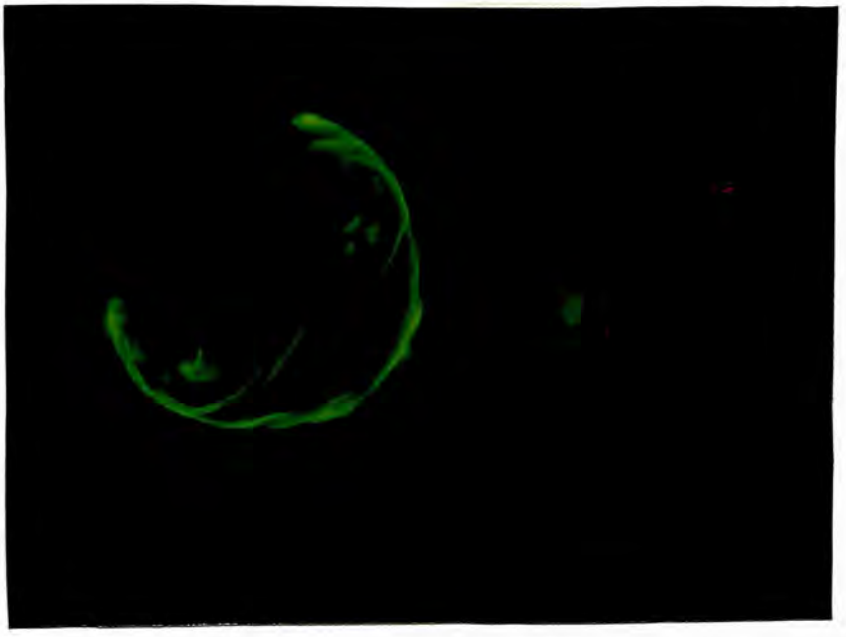


FIG. 32. Secondary Infection Serum

FIG. 33. Negative Control Serum

FIG. 34. Negative Control (Saline)

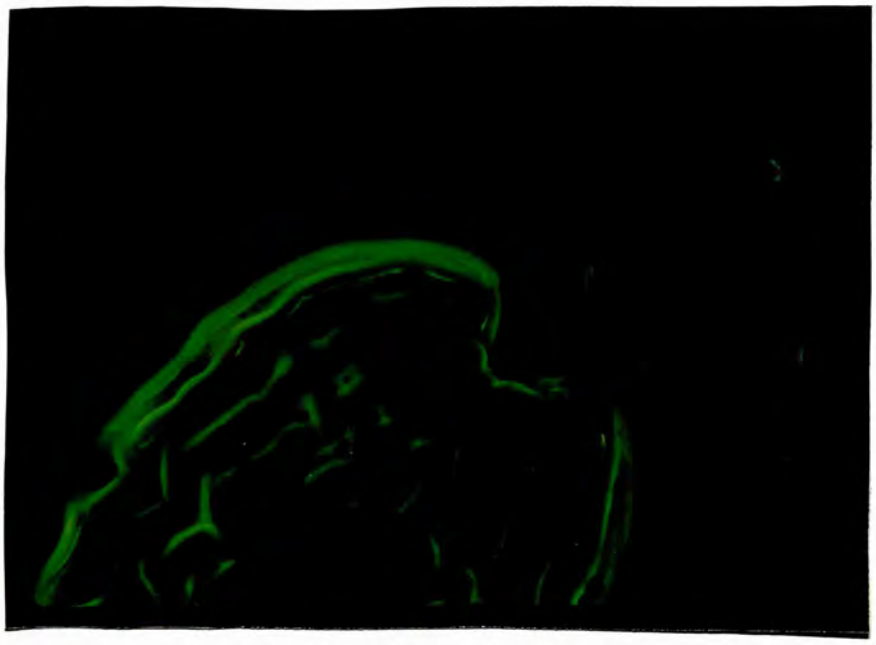


FIG. 35. Positive Control Serum

in the body wall of the positive control serum cysticercoids and is less prevalent in the scoleces. This was to be expected as the whole worm extract injected into the positive control chicken consisted predominantly of strobilae. In the cysticercoids of the experimental serum incubation, on the other hand, the specific fluorescence seems to be restricted to the scoleces. This again is to be expected, as it is the scolex of the cestode that is in intimate contact with the host tissues and it is likely that it is at this point that antigenic information is made available to the host.

If the functional *in vivo* antigen is located in the scolex the failure of some tests with whole worm extract antigen can be explained, but the validity of the results obtained with the ring precipitin test becomes doubtful. Unfortunately, it was not possible to monitor the level of antibodies produced in an infection with the immunofluorescent test due to lack of time and materials. However, this test has conclusively demonstrated the presence of antibodies to *R. cesticillus* in the sera, but not the intestinal mucus, of infected chickens.

H. HYPERSENSITIVITY REACTIONS

Passive Cutaneous Anaphylaxis

The increase in mast cell numbers with infection suggests that *R. cesticillus* may well evoke an anaphylactic reaction involving reaginic antibody. It has been suggested that anaphylactic reactions play a part in the elimination of several helminth parasites, including *Nippostrongylus*

brasiliensis, Dictyocaulus viviparus, Trichinella spiralis (Bloch, 1967), Haemonchus contortus, Trichostrongylus colubriformis, Trichostrongylus retortaeformis (Mulligan, 1968). Several helminth infections, including schistosomiasis, trichinosis, paragonomiasis, ascariasis, filariasis and echinococcosis can be detected by anaphylactic skin reactions (Bloch,1967).

Most studies on anaphylaxis have involved mammals, but anaphylactic reactions also occur in birds. Celada and Ramos (1961) demonstrated passive cutaneous anaphylaxis in chickens for the first time with bovine serum albumin as the antigen. Conway, van Alten and Hirata (1968)and Kubo and Benedict (1968) also demonstrated P.C.A. in chickens.

It was decided to attempt to demonstrate P.C.A., with R. cesticillus homogenate as antigen, in chickens and rats.

The belly hair of two 2 month old rats was removed with the depilatory 'Nair' 48 hours before the test and the breast feathers of two 1 month old chickens were plucked, also 48 hours in advance. The skin areas were divided into four with a marker and intradermal injections of experimental 'immune' serum, positive control serum, negative control serum and phosphate buffered saline were given in 0.05ml. aliquots. After five hours 1 ml of an antigen - Evan's blue preparation, consisting of 10mg/ml of each constituent, was injected into the femoral vein (rats) and the wing medial vein (chickens), observations being made over the next 30 minutes.

This basic technique was used on three occasions with more concentrated antigen (50mg/ml), more dilute antisera

(50%) and older chickens (7 weeks), but no positive results were obtained.

It is possible that this failure is due to the crudity of the antigen. Unfortunately, no attempt was made to test the technique with an antigen such as bovine serum albumin. Systemic anaphylaxis was never observed despite repeated injections of worm homogenate for the preparation of antisera.

It seems unlikely, therefore, that R. cesticillus evokes a reaginic antibody response and this conclusion is supported by the fact that the pattern of elimination of the cestode is not characteristic of anaphylaxis and also that reaginic responses do not seem to be associated with adult cestode infections.

Delayed Hypersensitivity

Delayed hypersensitivity differs from other allergic reactions in that it cannot be transferred with serum and is therefore not antibody dependent. It can, however, be transferred with lymphoid cells and is thought to be a form of cellular immunity. In skin tests it is characterised by erythema and induration which first appear about 5 hours after injection and take about 12 hours to reach a maximum. A massive infiltration of mononuclear cells takes place and polymorphs are few in number.

The histology of delayed hypersensitivity seems to fit in quite well with the cellular reaction evoked by R. cesticillus (Chapter 3). Larsh (1967) described experiments with 5 species of helminths (Trichinella spiralis, Tricho-

strongylus colubriformis, Ancylostoma caninum, Fasciola hepatica and Hymenolepis nana), in which lymphoid cells from immune hosts conferred some degree of immunity to these parasites in susceptible hosts. These experiments suggest that cellular immunity to helminth parasites may be of great importance, particularly in view of the fact that protective circulating antibodies are difficult to find in helminth infections (Sinclair, 1970).

It was not possible to transfer immunity to R.cesticillus with lymphoid cells, because no sufficiently in-bred strain of chicken was available. Attempts were made, therefore, to demonstrate delayed hypersensitivity with skin tests. These tests involved chickens with both primary and secondary infections and also some 'hyperimmune' birds, which had received repeated infections. Antigen preparations of varying strengths up to 100mg protein/ml were injected intradermally with and without Freund's adjuvant. In no case was delayed hypersensitivity demonstrated.

This failure might well be due to the crudity of the antigen or possibly to the fact that whole worm homogenates may not bear any relationship to the functional antigenicity of living worms (Immunofluorescence P.172.). The chicken is certainly capable of exhibiting delayed hypersensitivity type skin reactions, as demonstrated by Floersheim and Seiler (1967).

Discussion

Considerable evidence has now been presented to support the hypothesis that R. cesticillus evokes a protective immunological response. In Chapters 3 and 4 it was shown

that resistance to superinfection could be induced and that this resistance could be suppressed with the cortisone derivative, dexamethasone. In the present chapter the immunogenicity of the cestode has been demonstrated by immunocytoadherence, ring precipitin tests and immunofluorescence and further supported by the demonstration of mast cell hyperactivity, by eosinophil infiltration in secondary infections and by the protein separation studies.

It is difficult to reconcile the positive results obtained with ring precipitin tests and the failure to demonstrate precipitins by immunodiffusion and immunoelectrophoresis, or agglutinins by passive haemagglutination. As already mentioned (P.172.), these failures may be due to the fact that the functional antigens of the cestode may be localised in the scolex and consequently whole worm homogenates, used as antigen in the tests, would contain very small amounts of functional antigen. If this is the case, the validity of the ring precipitin tests is questionable. However, the immunofluorescent studies prove conclusively that R. cesticillus evokes a humoral immune response.

Although the immunogenicity of R. cesticillus has been established, it has not been proved that the immune response evoked is truly protective, though this is highly likely in view of the results of the superinfection experiments.

It has already been suggested that a delayed hypersensitivity type of reaction may be of importance, despite

the failure to demonstrate delayed hypersensitivity with skin tests. As already mentioned it was not possible to confer immunity passively by the transfer of lymphoid cells due to the lack of an inbred host strain.

Despite the demonstration of antibody in sera, antibody was not demonstrated in mucus extracts, though one might expect protective antibody to be present in intestinal mucus rather than in serum. It was decided to investigate the effects of sera and mucus extracts on R. cesticillus in vivo and in vitro and these experiments are described in Chapter 6.

CHAPTER 6

THE EFFECT OF SERA AND MUCUS EXTRACTS FROM INFECTED CHICKENS ON R. CESTICILLUS IN VIVO AND IN VITRO

Despite some equivocal results serum antibodies to *R. cesticillus* have been successfully demonstrated. So far no antibodies have been detected in the intestinal mucus of resistant birds, but this does not mean that they are not present, as a wholly satisfactory test has not yet been developed. In fact one might expect a protective antibody response to *R. cesticillus*, if it exists, to have its effect in the mucus rather than the serum and it is possible that antibody levels in the serum of resistant birds are a reflection of the protective levels in the intestinal mucus.

The protective qualities of sera and mucus extracts from infected birds against *R. cesticillus* were investigated by passive transfer of sera and mucus extracts and by *in vitro* incubation of worms in sera and mucus extracts.

A. Passive Transfer Experiments

Materials and Methods

Sera and mucus extracts were obtained from 10 week old birds with 2 week old secondary infections. According to the ring precipitin test, the highest serum antibody titres occur at this stage and the 100% destrobilization values, which are usually obtained suggest that protective mucus antibody, if any, is also likely to be high.

Two male and two female 3 week old chickens received daily intravenous injections of 0.5ml sera throughout the prepatent period of a primary infection, which was initiated one day after the start of the injections with 100 cysticercoids each. Two male and two female controls received similar treatment, but were injected with saline instead of serum.

A second experimental group of two male and two female 3 week old birds received 0.5ml of a 200mg protein/ml mucus extract intraperitoneally every prepatent day starting one day before infection, which consisted of a dose of 100 cysticercoids each. A control group received saline instead of mucus extract.

Autopsies of the serum treated group and their controls were carried out 14 days after infection. The mucus treated group and controls were autopsied 16 days after infection. Observations were made on the total numbers of worms, the numbers of destrobilating worms and worm growth.

Results

The values obtained for the two host sexes were not sufficiently different to warrant separate treatment and are therefore grouped together.

Table25(Mean Values)

Chicken Group	Number of Scoleces	Percent Number of Destrobilating Scoleces	Worm Weight
Serum Transfer			
Experimental	59.9 ± 4.96	17.2 ± 2.37	3.12 ± 0.72
Control	51.2 ± 6.77	23.8 ± 6.39	3.24 ± 0.49
Mucus Transfer			
Experimental	33.2 ± 4.90	8.4 ± 4.05	4.82 ± 0.73
Control	47.2 ± 6.41	9.4 ± 3.17	3.98 ± 0.56

The results suggest that neither sera nor mucus extracts confer immunity to R. cesticillus when passively transferred from resistant to susceptible birds. However, these experiments are by no means conclusive and were only carried out to see whether this approach was worth pursuing further. They would need to be repeated several times with large numbers of birds in each group before one could state that no protective antibodies were present in the sera or mucus extracts. Rose (1971) points out that the many failures to demonstrate protection to Eimeria spp with passively transferred serum is quite probably due to the difficulty of ensuring high titred serum and the fact that resistance to infection is not necessarily correlated with serum antibody levels. Rose finally managed to confer passive immunity to Eimeria maxima with serum obtained from birds with active infections rather than from birds that were refractory. Campbell (1938) reported that serum

from rats infected with Cysticercus crassicollis conferred protection when given before and during the infective process, but not when given 10 days after infection, which suggests that stage specific immunity occurs in this system and this constitutes a further obstacle to the successful demonstration of passive immunity. Weinmann (1966) found that passive protection against Hymenolepis nana was "obtained in five of eleven experiments, but the remainder inexplicably failed to demonstrate a protective effect".

It would appear that the problems associated with the passive transfer of immunity are complex and it was therefore decided that further experiments of this nature were not justified unless the in vitro studies suggest the presence of serum and/or mucus antibodies.

B. In Vitro Studies

Several workers have incubated living cestode material in 'immune' sera and have observed such reactions as formation of precipitates, formation of enclosing membranes, immobilisation, lysis and death (Chen, 1950; Silverman,1955; Mueller, 1961). These experiments involved the use of sera from animals infected with larval cestodes, which are known to be markedly immunogenic. Adult cestodes living in the intestinal lumen of their vertebrate hosts are widely regarded as being poorly immunogenic and it is not surprising therefore that few in vitro experiments involving sera from hosts infected with lumenal cestodes have previously been carried out. However, immune canine serum has been

shown to contain antibody to adult Echinococcus granulosus and in such serum a vigorous reaction to live worms occurs (Smyth 1969). E. granulosus evokes little cellular reaction in the intestine, but the scolex enters and dilates the crypts of Lieberkühn and sometimes penetrates the lamina propria. Heyneman and Welsh (1959) investigated the action of antiserum produced by antigen injections in rabbits on various stages of Hymenolepis nana and observed that after 48 hours at 37°C eggs showed a retraction of the central hydrophilic membrane, granular depositions in the central peripheral band, clumping and reduced infectivity. Cysticercoids were incubated for 24 hours and eversion of the rostellum, immobilisation of suckers, bubbles in the cuticle of the tail and reduced infectivity were observed. Young adult worms showed increased motility causing ruptures and precipitation layers around the scolex, eventually extending to the whole worm, after 48 hours incubation.

In marked contrast to these findings Weinmann (1966) reported that antiserum from resistant mice, that had been immunised by infection with eggs of H.nana, had no detectable effect on eggs, cysticercoids or young adult worms when compared with incubations in serum from uninfected mice. Although immunofluorescent studies have shown that antibodies combine with adult worms implanted intraperitoneally (Coleman, 1971; Coleman and Fotorny, 1962) Weinmann's experiments suggest that these antibodies are not responsible for the resistance of immunised mice. However, in another experiment Weinmann (1966) showed that mucosal

extracts from immune mice had a marked effect on immature worms compared with extracts from normal mice. Worms in both immune and normal extracts were adversely affected. They became dark and granular in appearance and inactive, but after 3 hours incubation at room temperature 80 percent of the worms in the normal extract were still active and undistorted, whereas in the immune extract 30 percent of the worms were dead after 3 hours and all worms were almost inactive; by 4 hours incubation a distinct membrane could be seen around some of the still viable worms. These observations suggest that protective antibodies may well be present in the intestinal mucosa of immune mice.

Immunofluorescent studies with R. cesticillus have shown that serum antibodies react directly with immature cestodes. The same technique failed to detect specific antibodies in mucus extracts, but this is possibly due to the way the mucus extracts were prepared and used. It was therefore decided to investigate the protective properties of both sera and mucus extracts from resistant birds.

Four separate experiments were carried out; one with cysticercoids and three with immature adult cestodes.

The Effect of Immune Serum on Cysticercoids

Materials and Methods

Immune serum was obtained from a group of 10 week old birds with 14 day old secondary infections. Normal serum was obtained from a 10 week old uninfected bird. Both sera

were sterilised by high speed centrifugation and then by filtration through oxoid filters using positive pressure. Two ml. of each sera were pipetted into three autoclaved solid watch glasses. Cysticercoids were dissected out from beetles and well washed in sterile Hanks balanced saline. One hundred cysticercoids were placed in each watch glass, covered and incubated at 40°C. Observations were made after 6, 18, 24 and 48 hours of incubation.

After 48 hours incubation 20 unevaginated cysticercoids from each well were fed by pipette to six 4 week old males in order to test the effect of the antiserum on cysticercoid infectivity. The birds were autopsied 7 days after infection and the number of established worms counted.

Results

No morphological changes were observed in any of the cysticercoids apart from evagination. The evaginated young cestodes were very active at first, but all detectable movement had ceased after about an hour in both immune and normal serum.

Table 26.

Incubation Time (Hours)	Percent Evagination					
	Immune Serum			Normal Serum		
	1	2	3	1	2	3
6	3	7	4	9	1	3
18	4	11	7	12	6	5
24	12	18	15	17	12	13
48	27	35	21	40	19	17

Table26(Cont)

	Immune Serum			Normal Serum		
Infectivity Percent Number of Established Worms	1	2	3	1	2	3
	55	35	45	45	35	50

There appears to be no difference between the two groups as far as evagination of cysticercoids or their infectivity are concerned.

It must be concluded that cysticercoids are unaffected by the antibodies contained in serum from resistant chickens.

The Effect of Immune Serum and Mucus Extracts on Young Adult R. cesticillus

Three separate experiments were carried out.

Experiment 1

Materials and Methods

Immune sera and mucus extracts were obtained from birds with 14 day old secondary infections and normal serum and mucus extract from uninfected birds. The mucus extracts were used at a concentration of 100mg protein/ml. Both sera and mucus extracts were sterilised by high speed centrifugation and filtration as before and then 2 ml of each preparation were placed in two watch glasses containing 2 ml sterilised Hanks balanced saline.

Young cestodes were obtained from a 4 day old infection and were well washed in six changes of Hanks balanced saline containing 100 i.u. penicillin/ml. Five worms were placed in each watch glass which were covered and incubated at

40°C. Observations were made at 3, 12, 24, 36 and 48 hours incubation.

Activity of the worms was arbitrarily assessed on a four point scale as +++, ++, + or - and the number of surviving worms was also recorded.

Results

Table 27

	Hours after Incubation	3		12		24		36		48	
		Activity	Survival	Activity	Survival	Activity	Survival	Activity	Survival	Activity	Survival
Immune	1	+++	5	+++	5	+++	5	++	3	+	1
Serum	2	+++	5	+++	5	++	5	-	0	-	0
Normal	1	+++	5	+++	5	++	5	+	3	-	0
Serum	2	+++	5	++	4	++	4	+	3	+	2
Immune	1	++	4	+	2	-	0	-	0	-	0
Mucus	2	+	2	+	2	+	1	-	0	-	0
Normal	1	++	3	+	2	-	0	-	0	-	0
Mucus	2	+	3	+	1	-	0	-	0	-	0

No precipitation occurred on any of the worms, but blistering of the cuticle occurred in every case and was first observed after only 3 hours incubation in the sera. Blistering was present in all groups after 12 hours incubation. It was less marked in the worms in the mucus extracts, which were much less active than those in sera. The mucus extract worms began to fragment after only 12 hours and were dead after 24 hours. No significant differences between the immune and normal serum groups or between the immune and normal mucus extract groups were observed.

The poor survival in normal serum and mucus could be due to non-specific antibodies. It is thought that the rostellar precipitate of adult Echinococcus in normal serum or in hydatid fluid may be due to reaction with natural antibodies (Smyth 1969). A non-specific anti-helminth factor has been demonstrated in chicken mucus by Frick and Ackert (1948). They found that the nematode, Ascaridia galli, was inhibited in growth in vitro by mucus and concluded that the factor involved was not an antibody as it survived autoclaving at 15 p.s.i. for 20 minutes. This factor may well be responsible for the poor survival of R. cesticillus in normal mucus extract.

This experiment has failed to demonstrate specific protective qualities in immune serum and mucus, but this failure may be due to the obscuring of any such qualities by non-specific factors. The use of more dilute preparations and a longer incubation period may overcome this problem.

Experiment 2

In view of the fact that most worms in the previous experiment were dead after 48 hours incubation, including those in normal serum and mucus extract, it was decided to attempt a more prolonged culture of the cestodes and to use more dilute serum and mucus preparations.

The *in vitro* culture of *R. cesticillus* does not seem to have been attempted since Smith (1954) reported that adult worms survived for 60 hours in a liquid culture medium consisting of Ringer's-glucose-corn starch with added vitamins and chicken serum in low concentrations. Attempts were made to improve on this work and then to investigate the protective properties of immune serum and mucus on the worms in culture.

Materials and Methods

The *in vitro* system used was based on that described by Schiller (1965) for *Hymenolepis diminuta*. The medium consisted of 10 ml oxoid nutrient agar containing 3% inactivated normal chicken serum, which was overlaid with 10 ml Hanks balanced saline containing 2.5mg glucose/ml and 100 i.u.penicillin/ml. This medium was incubated in 50 ml Erlenmayer flasks at 40°C, shaken at 20 cycles/ minute and renewed daily.

Worms were obtained at 7 days old and after washing in six changes of Hanks balanced saline with 100 i.u. penicillin/ml, up to three worms were placed in each flask. This system maintained worms in an active condition for 144 hours and no blistering occurred. However, the worms

started to lose proglottids after 48 hours of incubation so it would appear that the medium is not nutritionally adequate. Increased fragmentation was observed in media that contained no inactivated serum in the agar or contained lipid-poor inactivated serum. Blistering was observed when the media contained serum that had not been inactivated.

These observations suggest that fragmentation of the worms is associated with the nutritional conditions in the media, that blistering is dependent on the presence of complement in the serum and is therefore probably caused by non-specific antibodies as previously suggested.

For the immune serum and mucus extract incubation experiment, the agar contained inactivated serum, which had been obtained from a laying hen 2 hours after feeding and was therefore rich in lipids.

The immune and normal sera and mucus extracts were obtained from the same sources as for experiment 1. The mucus extracts were used at a protein concentration of 100mg/ml. Both sera and mucus extracts were sterilised by high speed centrifugation and by filtration and dispensed in 1.0 ml (sera) and 0.5ml (mucus extracts) aliquots into the prepared media to give a concentration of HBS:sera or mucus extracts of 5:1 (sera) and 10:1 (mucus extracts).

Worms were obtained from 7 day old infections and, after washing, three worms were placed in each medium, incubated at 40°C and shaken at a rate of 20 cycles/minute for 15 hours. The sera and mucus extracts were then added to two flasks for each preparation and observations were made 28, 40 and 55 hours after their addition.

Results

Table

	Hours after addition of sera and mucus extracts	28		40		55	
	Flask	Activity	Survival	Activity	Survival	Activity	Survival
Immune Serum	1	+++	3	++	3	+	3
	2	+++	3	++	3	+	3
Normal Serum	1	+++	3	++	3	+	3
	2	+++	3	++	3	+	3
Immune Mucus	1	+	3	+	2	-	0
	2	+	3	+	3	-	0
Normal Mucus	1	+	3	+	1	-	0
	2	+	3	+	3	-	0

Survival in this system was not appreciably better than in experiment 1 despite the use of less concentrated serum and mucus extract. No difference in worm activity or survival in the immune and normal sera and mucus extracts was detected. Slight cuticular blistering and scolex distortion occurred in the worms incubated in sera.

Blistering was much accentuated in the worms incubated in mucus extract and none of these worms survived.

The results of this experiment are very similar to those obtained in experiment 1 and they suggest that specific protective antibodies to R. cesticillus are not present in serum or mucus extracts from infected birds. As in experiment 1 the mucus extracts seem to have a non-specific anthelmintic effect on the cestodes.

It was decided that the in vitro system would probably be improved by the provision of an artificial atmosphere of 5% CO_2 and 95% N_2 as used by Schiller (1970). This was carried out in experiment 3.

Experiment 3

In this experiment Schiller's artificial atmosphere was used with the medium described in the previous experiment in a Gilson respirometry unit. The culture vessels contained half the amounts of media as previously described. The gas mixture was passed through the flasks at a rate of 30ml/minute.

In a preliminary experiment 3 week old adult worms were maintained in good condition for six days. Three to

four gravid proglottids were produced each day by each worm and the eggs proved to be infective to beetles, but at a reduced level. The worms were still very active when the culture had to be terminated.

In another experiment 7 day old worms were cultured for 8 days and were active throughout. However, growth was poor, as may be seen from Fig.36. , in which a worm from culture is compared with a worm of the same age (15 days) obtained from an infected chicken.

This culture system was used to investigate the effect of immune and normal mucus extracts on R. cesticillus.

Materials and Methods

Mucus extract was obtained from immune and normal birds of the same age and was used at a concentration of 100mg protein/ml after sterilisation by centrifugation and filtration.

Seven day old worms were well washed in HBS with 100 i.u. penicillin/ml and three were inoculated into each of three culture vessels. One of these culture vessels received 0.2 ml of immune mucus, the second received 0.2 ml of normal mucus and the third received no mucus. The media, including mucus extracts, were renewed daily. Photographs were taken at each change of media.

Results

After 24 hours incubation, the worms in both mucus extracts showed considerably reduced activity compared with the control worms. After 42 hours incubation, the worms in

Figure 36. Comparison of *in vivo* and *in vitro* growth of 15 day old *R.cesticillus*. *In vitro* worm cultured for 8 days.X5.

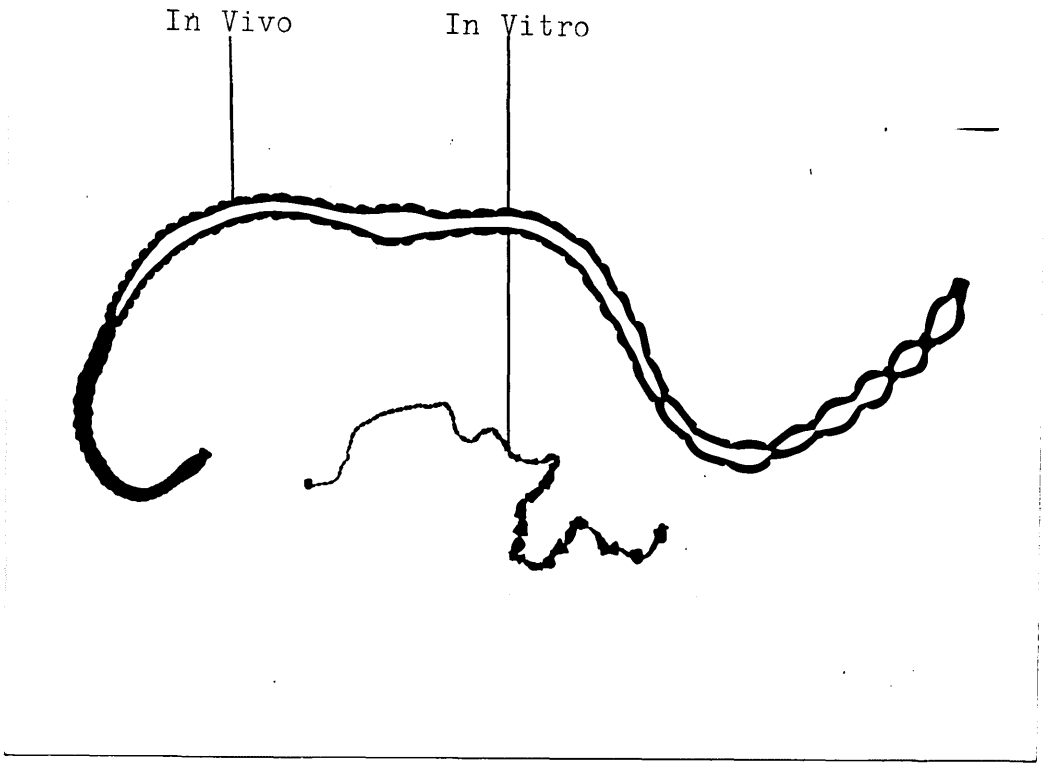


FIG. 36.

Figures 37-39. The effect of mucus extracts from infected and uninfected birds on R.cesticillus after 42 hours of in vitro incubation.X5.

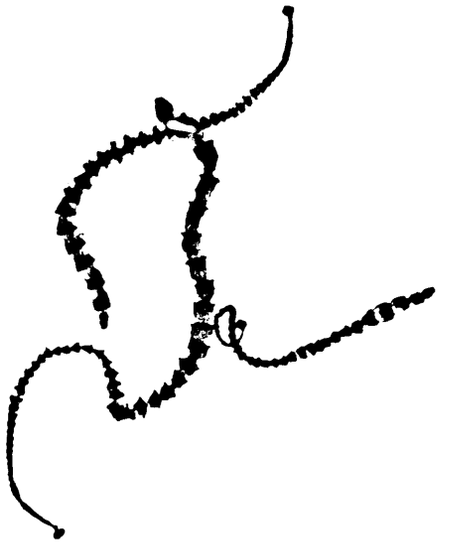


FIG. 37. 'Infected' Mucus

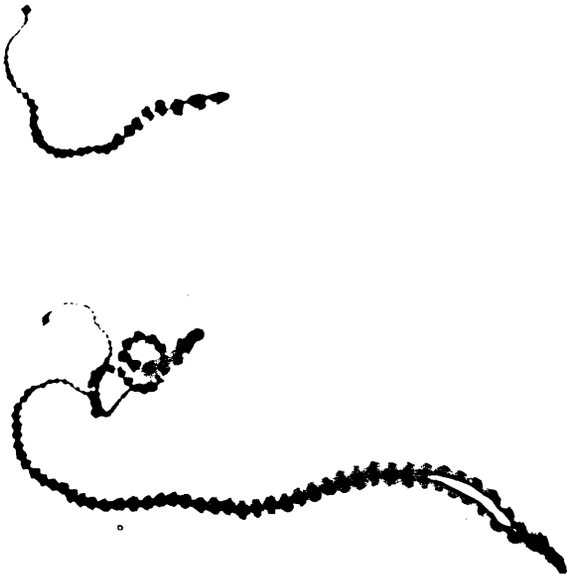


FIG. 38. 'Uninfected' Mucus

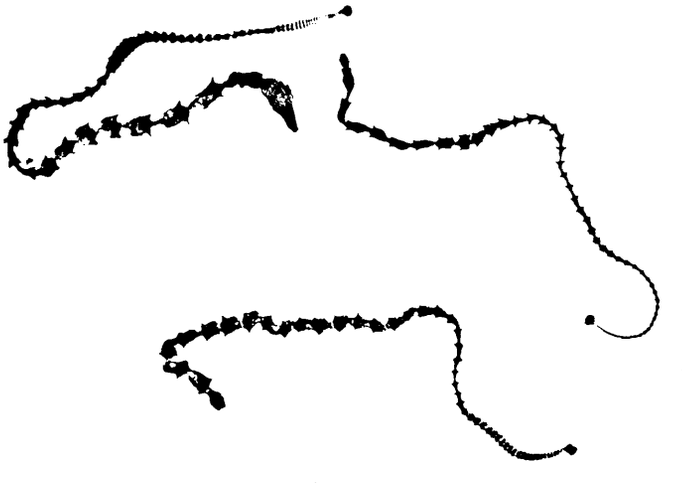


FIG. 39. Control

the mucus media were almost completely motionless and some degree of cuticular blistering was present (Figs.37,38). The control worms continued to be very active and showed no signs of blistering (Fig.39.). After 66 hours of culture the worms in both immune and normal mucus media were very obviously dead while the control worms were still very active.

No differences in the antiworm properties of immune and normal mucus were detected.

Discussion

This series of experiments has provided no evidence to suggest that sera or mucus extracts contain specific protective antibodies to R. cesticillus. It is possible that specific protective properties were obscured by non-specific effects, especially as far as mucus extracts are concerned where one might perhaps expect to find antibodies directed against the cestode. It is probable that intestinal antibody production to R. cesticillus, if any, is very localised and therefore very difficult to demonstrate in crude extracts. However, it was suggested earlier that serum antibodies to R.cesticillus may be a reflection of protective levels in intestinal mucus. Coleman and Fotorny (1962) reported that antibody binding sites on Hymenolepis nana, detected by immunofluorescence, were similar in worms implanted intraperitoneally, and therefore exposed to humoral antibody, and in worms obtained from the ileum. This suggests that the serum antibodies are similar to those produced in the intestine. If this is the case in chickens then the lack of protective serum antibody

to R. cesticillus suggests that intestinal antibodies are not protective either. This is, of course, highly speculative.

Non-specific antibody is probably present in serum and appears to attack R. cesticillus in culture, causing cuticular blistering. Inactivation of serum by heating to 56°C for 30 minutes eliminates this property, which suggests that complement is involved. The non-specific action of mucus extract has already been mentioned, but it is not known whether this is due to antibody or not. This problem was investigated during studies on the role of mucus production in age resistance.

CHAPTER 7

THE ROLE OF INTESTINAL MUCUS IN AGE RESISTANCE TO R. CESTICILLUS

Introduction

Intestinal mucus is one of the factors mentioned that might play a part in the development of age resistance to R. cesticillus.

Ackert, Edgar and Frick (1939) correlated increasing numbers of goblet cells in intestinal epithelia of both chickens and rats with increasing age and also with the development of resistance to Ascaridia galli. Goblet cell numbers reach their maximum at about 3 - 4 months of age in the chicken and this is also the age at which resistance to R. cesticillus manifests itself.

Frick and Ackert (1948) managed to demonstrate that mucus from old birds contained a factor which inhibited the growth of larval Ascaridia galli in vitro. They concluded that this factor is soluble in 0.832% saline, is not an antibody, as autoclaving at 15 p.s.i. for 20 minutes has no effect on its inhibitory properties, and its effects are nutritional and temporary. Evidence has already been provided to suggest that intestinal mucus contains a nonspecific factor that is lethal to R. cesticillus in vitro (Chapter 6).

It was decided to investigate the role of intestinal mucus in age resistance to R.cesticillus by carrying out goblet cell counts and by in vitro studies.

The Effect of Primary and Secondary Infections of R. cesticillus on Numbers of Intestinal Goblet Cells.

It has already been established by Ackert et al (1937) that the numbers of goblet cells in chicken intestinal mucosa can be correlated with age resistance to Ascaridia galli and also to R. cesticillus. It was felt that further evidence was required to substantiate the hypothesis that goblet cell numbers are directly related to resistance to helminth parasites and it was therefore decided to investigate the effect of a primary infection on goblet cell numbers. It has already been shown that primary infections of R. cesticillus induce resistance to secondary infections. Stahl (1966) suggested that intestinal mucus is a contributing factor in the natural resistance of mice to Aspiculuris tetraptera and that it becomes the dominant factor in the increased resistance of mice to superinfection. There is no doubt that helminth parasites affect the amounts of mucus in an infected intestine. Wells (1963) reported that Nippostrongylus brasiliensis causes hypertrophy of mucin producing cells in rats and Dobson (1966b) has shown an increase in numbers of goblet cells in sheep infected with Oesophagostamum columbianum.

Materials and Methods

Two 10 week old birds of each sex with primary and secondary infections and one uninfected 10 week old bird of each sex were selected. The intestines were removed and stretched in adrenalin saline at 40°C. Mucosal tissue was taken from percent lengths 12, 24, 36, 48, 60, 72, 84 and 96

and were fixed in aqueous Bouin's fluid overnight. The tissue was embedded in paraffin, sectioned at 5u and stained in Erlich's haematoxylin for ten minutes and in mucicarmine, at a concentration of 1 in 10 with distilled water, for 30 minutes.

Counts were made at a magnification of 400x of goblet cells in strips of villus mucosal epithelium 205u long. Goblet cells are about 10u in diameter and counts were made from every third section to ensure that the same goblet cells were not counted twice. With sections of 5u thick a single layer of goblet cells in each section should result. Five counts were made from five sections from each gut section, resulting in 25 counts per gut section.

Results (Mean Values)

Table 29.

Percent Lengths	Male			Female		
	Uninfected Control	Primary Infection	Secondary Infection	Uninfected Control	Primary Infection	Secondary Infection
12	8.92 ± 0.419	8.1 ± 0.787	8.4 ± 0.374	8.04 ± 0.275	7.4 ± 0.346	8.7 ± 0.787
24	9.6 ± 0.572	7.8 ± 0.346	9.3 ± 0.635	8.6 ± 0.414	8.2 ± 0.451	8.3 ± 0.363
36	9.1 ± 0.400	8.0 ± 0.379	7.6 ± 0.424	8.0 ± 0.433	9.2 ± 0.464	9.5 ± 0.648
48	9.2 ± 0.424	8.4 ± 0.395	7.9 ± 0.489	7.9 ± 0.303	10.1 ± 0.473	10.6 ± 0.613
60	10.4 ± 0.481	9.9 ± 0.547	9.4 ± 0.316	10.5 ± 0.346	10.2 ± 0.517	11.1 ± 0.442
72	10.6 ± 0.334	9.9 ± 0.469	9.0 ± 0.485	11.7 ± 0.346	11.2 ± 0.447	10.4 ± 0.802
84	13.6 ± 0.485	10.4 ± 0.456	9.4 ± 0.509	12.6 ± 0.456	11.9 ± 0.506	16.6 ± 0.710
96	15.0 ± 0.275	12.9 ± 0.748	11.7 ± 0.536	13.5 ± 0.494	16.3 ± 0.460	15.4 ± 0.812

Figures 40-42. Numbers of goblet cells in the small intestine mucosal epithelium of chickens. Comparison between the host sexes and the effect of primary and secondary infections of R.cesticillus.

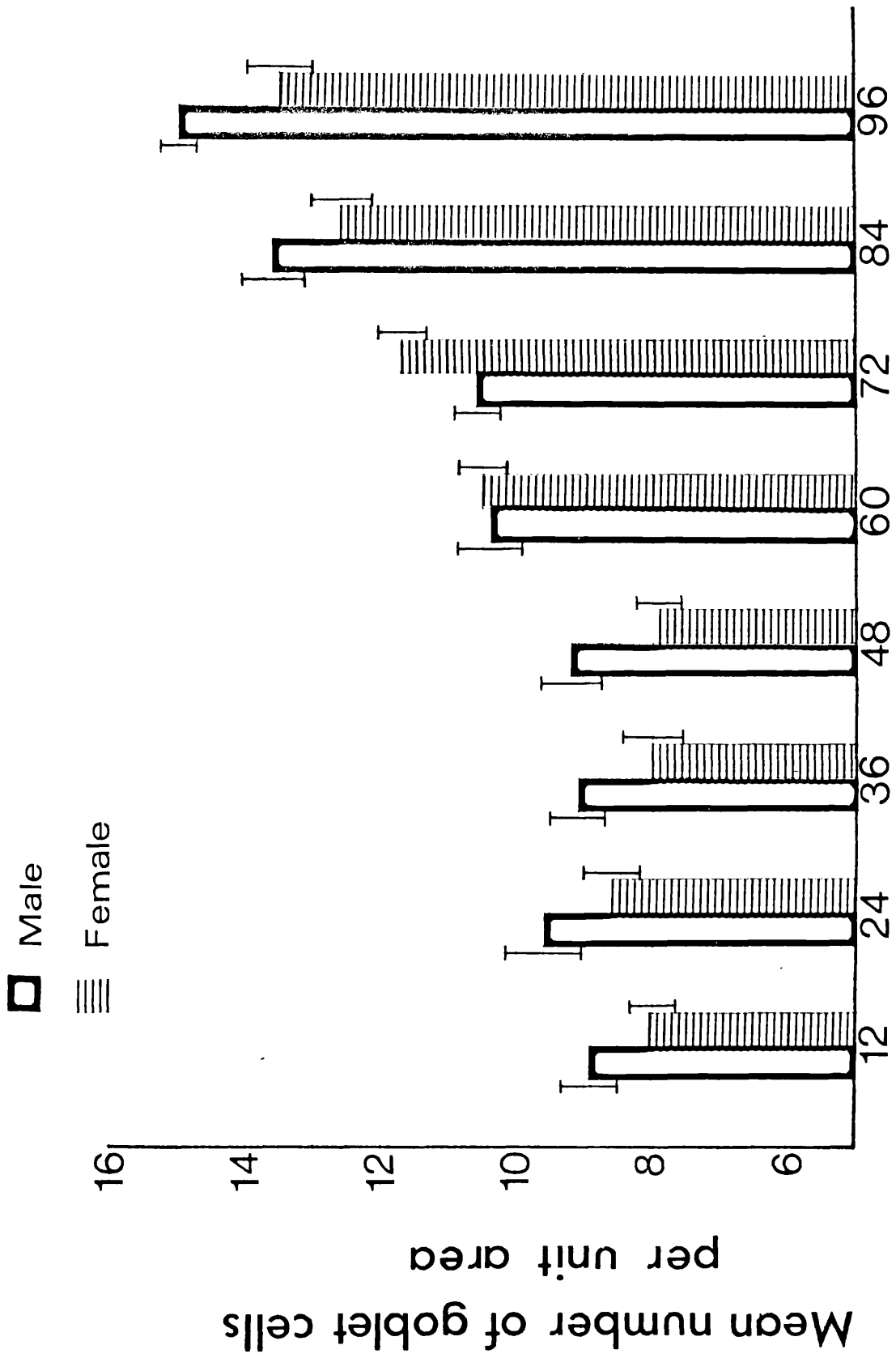
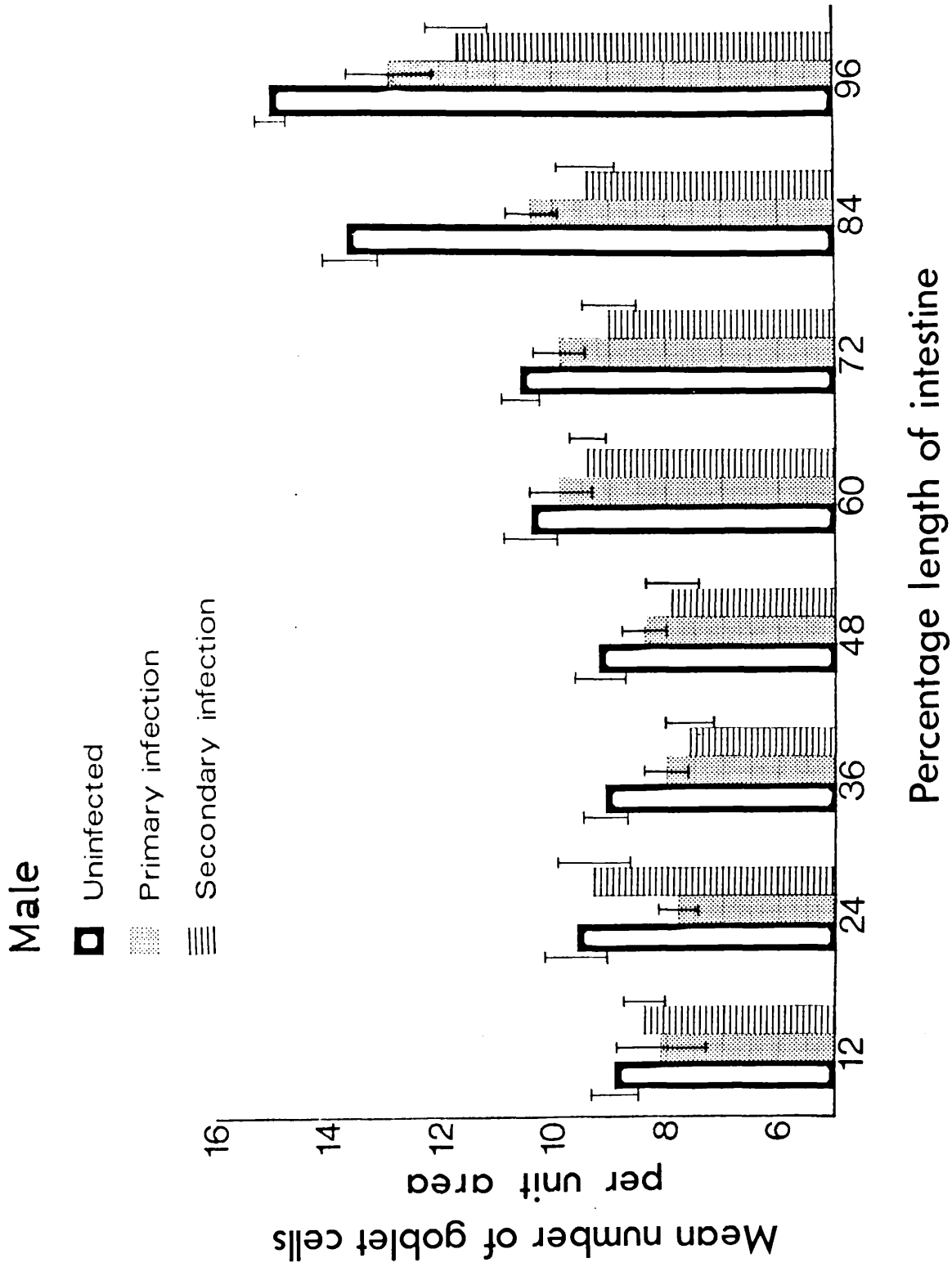

Male
Female
Mean number of goblet cells per unit area
16
14
12
10
8
6
12
24
36
48
60
72
84
96
Percentage length of intestine

FIG. 40

FIG. 41


Male
Uninfected
Primary infection
Secondary infection
Mean number of goblet cells per unit area
16
14
12
10
8
6
12
24
36
48
60
72
84
96
Percentage length of intestine

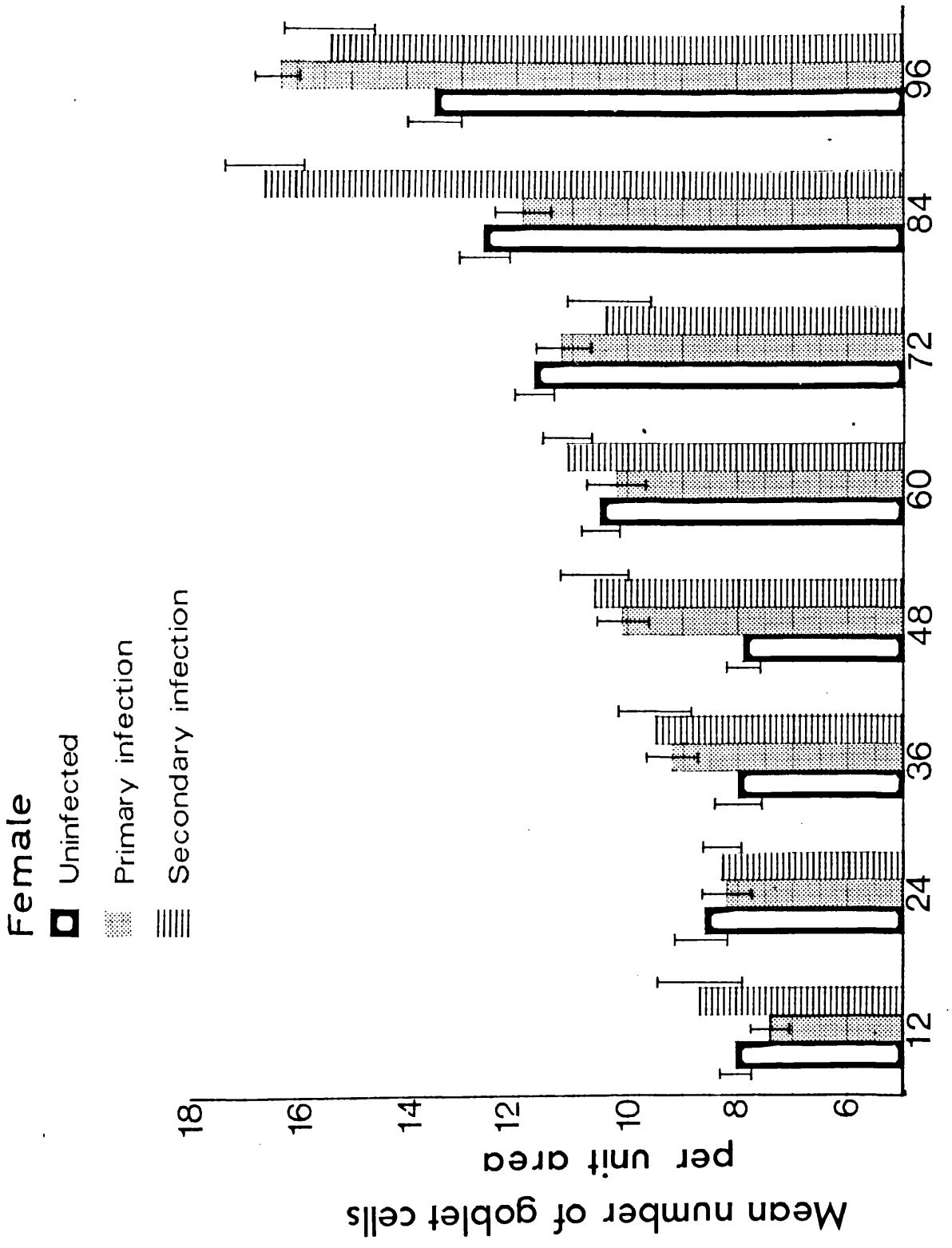

Female
Uninfected
Primary infection
Secondary infection
Mean number of goblet cells per unit area
18
16
14
12
10
8
6
12
24
36
48
60
72
84
96
Percentage length of intestine

FIG. 42.

Discussion

The only consistent feature of the results is the increasing number of goblet cells towards the distal end of the intestine. There appears to be no correlation between numbers of goblet cells and infection with R. cesticillus. It is possible that samples from a greater number of birds would have revealed some sort of correlation, but this is unlikely and greater variation may well have resulted. The number of goblet cells is not the only parameter by which increased mucus production might be measured. The size of individual goblet cells is also likely to be of significance, but the practical difficulties involved in such measurements are considerable and were therefore not attempted.

The results of this experiment lead one to the conclusion that resistance to superinfection with R. cesticillus is not directly related to the numbers of goblet cells in the intestinal mucosa. This conclusion does not invalidate the hypothesis that age resistance is related to goblet cell numbers, but it suggests that the mechanisms of resistance operating against a superinfection may not be the same as those of age resistance.

The Effect of Mucus from Different Aged Birds on R. cesticillus in Vitro.

Mucus extracts have already been found to have a deleterious effect on R. cesticillus in vitro and it is possible that this is due to the anthelmintic factor in mucus described by Frick and Ackert (1948). They demonstrated that mucus

from old birds has a greater inhibitory effect on the growth of A. galli in vitro than mucus from young birds and they suggested that this was due to an increasing concentration of inhibitory factor in 'old' mucus. It is likely that the larger amount of mucus in older chickens, as determined by goblet cell counts, plays a secondary role in age resistance to A.galli and that the increasing concentration of the inhibitory factor is of greater importance. This conclusion would perhaps explain the failure to relate goblet cell numbers with resistance to superinfection with R. cesticillus in the last experiment.

In the present experiment an attempt was made to repeat the work of Frick and Ackert using R. cesticillus instead of A. galli.

Materials and Methods

The in vitro system used was based on that described by Schiller (1970) for Hymenolepis diminuta and consisted of a nutrient agar-lipid-rich-serum medium overlayed with Hanks balanced saline containing glucose and pencillin, with an artificial atmosphere of 95% N2 and 5%C02. This medium was contained in Gilson respirometry flasks, incubated at 40°C and renewed daily. (See Chapter6. Page192.)

Mucus extracts were obtained from 14, 70 and 175 day old male chickens and were used at concentrations of 50 and 100mg protein/ml. The selection of the chicken ages was based on experimental infections; chickens aged 14 days were fully susceptible to infection, those aged 70 days were partially susceptible, in that 50 percent of the established worms were destrobilizing at patency, and those aged 175 days

were fully refractory.

Two separate experiments were carried out.

Experiment 1. Seven day old worms were removed from a chicken intestine and washed in six changes of sterilised HBS containing 100 i.u.penicillin/ml. Three worms were then inoculated into each of four culture vessels and incubated for 12 hours. Sterilised mucus extracts (14, 70 and 175 day old) were added in 0.2ml aliquots from 100mg protein/ml saline solutions to give a liquid medium concentration of 4.0mg protein/ml. No mucus extract was added to the fourth culture vessel which served as a control. The culture was run for 90 hours, the media being changed daily and the worms photographed at each change.

Experiment 2. Worms were obtained from a 10 day old infection, washed and incubated for 12 hours. Sterilised mucus extracts were added in 0.2ml aliquots from solutions of 50mg protein/ml to give a liquid medium concentration of 2.0mg protein/ml. One worm was inoculated into the culture vessels containing 14 and 70 day old mucus and into the control vessel. Two worms were inoculated into the 175 day old mucus vessel and two worms were inoculated into a vessel that contained autoclaved 175 day old mucus (15 p.s.i. for 20 minutes). Autoclaving of the mucus caused precipitation of about half the protein content and should have eliminated the anthelmintic property of the mucus if the inhibitory factor is a non-specific antibody.

The culture was run for 96 hours and the media were changed every 24 hours with photographs being taken at each change.

Results

In both experiments there appeared to be little difference between the mucus extracts from different aged birds in their inhibitory action on R. cesticillus. After only 24 hours incubation the worms in mucus extracts were markedly inactive and in a contracted state. There were signs of cuticular blistering in some worms. The worms in the autoclaved mucus extract were the exception in that they appeared to be perfectly healthy and were fully as active as the control worms.

After 42 hours incubation worms in unautoclaved mucus were totally inactive and cuticular blistering was present. This can be seen in the worms of experiment 1 (Figs.43-45.). It also appears that the worms of the 175 day old mucus extract incubation are slightly more affected than those of the 14 and 70 day old mucus extract incubations. Cuticular blistering is conspicuous, especially in the 175 day old mucus worms. This constitutes the only evidence to suggest that the inhibitory factor is present in greater concentrations in 'old' than in 'young' mucus as reported by Frick and Ackert (1948). In contrast to the findings of these workers, however, autoclaving of mucus completely eliminated its inhibitory properties. After 72 hours of incubation all worms in unautoclaved mucus were dead, but the worms in autoclaved mucus were fully active at this stage and were still in good condition after 96 hours when the experiment was terminated. Figures 47. and 48. show the worms of the 175 day old mucus extract incubation and of the autoclaved 175 day old mucus extract incubation respectively after 72 hours incubation. The worms in the unautoclaved mucus are

Figures 43-46. The effect of mucus extracts from chickens of 14, 70 and 175 days of age on R.cesticillus after 42 hours *in vitro* incubation. X5.

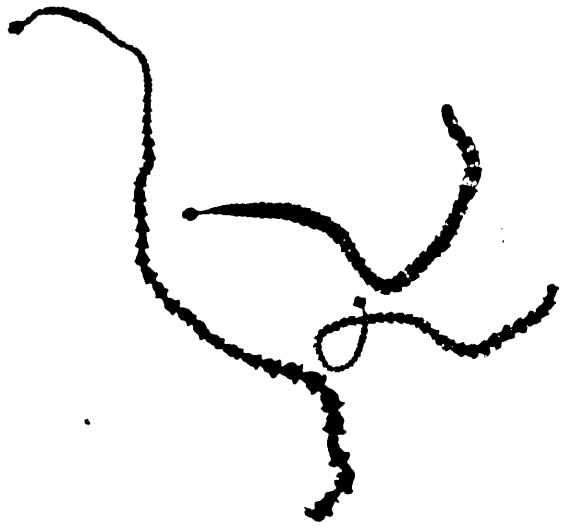


FIG. 43. 'I4 Day Old' Mucus

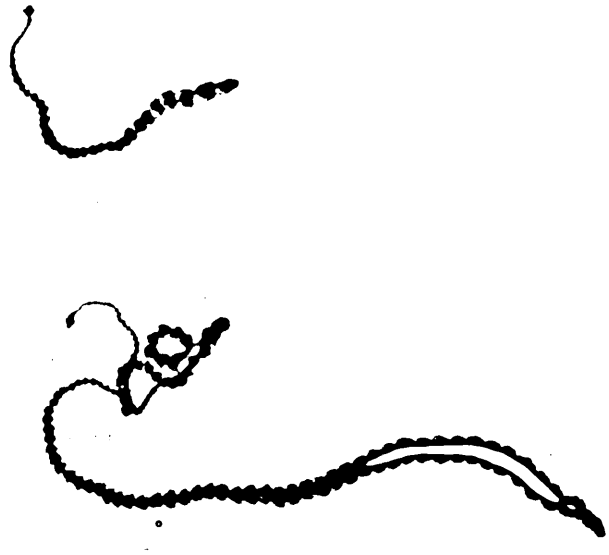


FIG. 44. '70 Day Old' Mucus

FIG. 45. 'I75 Day Old' Mucus

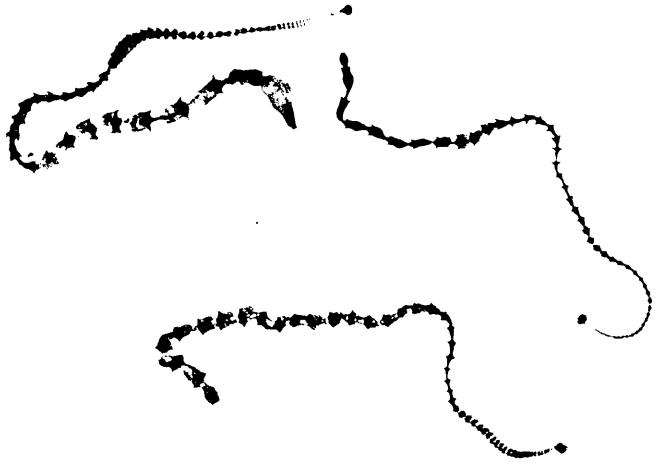


FIG. 46. Control

Figures 47-48. The effect of autoclaved and unautoclaved mucus extracts from 175 day old chickens on R.cesticillus after 72 hours *in vitro* incubation. X5.

FIG. 47. Un-autoclaved Mucus

FIG. 48. Autoclaved Mucus

contracted and stunted and there are signs that one of them is fragmenting, whereas the worms in the autoclaved mucus are obviously in good condition and were very active. This suggests that the inhibitory factor demonstrated against R. cesticillus may well be an antibody.

Frick and Ackert (1948) showed that the action of the inhibitory factor on A. galli was temporary as worms recovered their normal growth rate when removed from the mucus medium and placed in a mucus free medium. An attempt was made to reproduce this result with R. cesticillus and one worm from each medium of experiment 1 was placed in mucus free medium after 42 hours incubation. However, no recovery occurred and it is likely that damage to the worms was too far advanced at this stage.

Discussion

There can be little doubt that the intestinal mucus of chickens contains a factor that is active against R. cesticillus. This fact alone could explain the development of age resistance to the cestode as it had already been established by Ackert et al (1939) that older chickens have more goblet cells per unit area in the intestinal epithelium than younger birds. If more mucus was present the cestode would be exposed to more of the inhibitory factor.

However, Frick and Ackert (1948) were unable to correlate the increase in numbers of goblet cells with the increase in efficiency as a growth inhibitor of mucus from old chickens.and in the experiments described here resistance to secondary infections was not associated with an increase

in goblet cell numbers.

Frick and Ackert (1948) provided evidence that the inhibitory factor increased in concentration in mucus from chickens as they age and this conclusion is supported by one of the experiments described here, in which mucus from a 175 day old bird appeared to have a more rapid lethal effect on R. cesticillus than mucus from a 14 or a 70 day old bird.

The failure of worms to recover after being placed in mucus-free medium is probably due to the limitations of the in vitro system, in addition to the rather concentrated mucus extract used and therefore cannot be considered as evidence that mucus has a permanent irreversible inhibitory effect on R. cesticillus.

These experiments can only be regarded as pilot studies and suggest that the in vitro system used for R. cesticillus was probably inadequate as it did not give good worm growth. In order to substantiate the findings of Frick and Ackert for the R. cesticillus system it is necessary to use more dilute mucus extracts, to reduce the degree of inhibition, and to obtain better growth of the worms, so that a more exact measure of growth inhibition may be made. It is hoped to continue with further studies at a later date.

CHAPTER 8

THE EFFECT OF GONADAL STEROIDS ON THE COURSE OF INFECTION OF R. CESTICILLUS

Introduction

In the series of experiments on the effects of host age on the course of infection of R. cesticillus (Chapter 2) it was found that male birds become increasingly resistant to the cestode as they mature and are refractory by the age of 224 days. The manifestations of this resistance are a progressive destrobilization of the worms followed by loss of scoleces and a decreasing rate of growth of the worm. It was also noted that the worms became more diffusely distributed in older birds, mainly in a proximal direction.

These manifestations develop more rapidly in female birds initially, but at 84 days of age appear to decline and at 224 days of age female birds maintain quite heavy infections for several months. Furthermore, the diffuse distribution characteristic of mature male birds reverts back to a distribution characteristic of young birds at the age of 112 days.

The difference between the sexes in the development of age resistance implicates hormone balance as a possible factor and this view is strengthened by the fact that, at the time of the decline of age resistance in the female, gonadal development is accelerating. In these experiments attempts were made to reproduce the phenomena observed above by treatment with homologous gonadal steroids.

The influence of gonadal steroids on parasitic infections has been the subject of many studies. It is very difficult to make any generalisations in this complex field as there are many contradictory reports, a considerable lack of standardisation of experiments and a fair degree of laxity in the control of experimental conditions. One of the probable factors responsible for the confusion is the use of dose levels of hormones that bear little or no relationship to normal levels. This is a particularly intractable problem as protracted preliminary studies would be necessary to achieve a regimen that would simulate normal conditions. The problems associated with under - or overdosing are exemplified by the observations of Sadun (1948), who reported that heavy doses of testosterone propionate decrease the resistance of chickens to Ascaridia galli, but moderate doses increase resistance.

Perhaps one of the more reliable methods of ensuring that abnormal dose levels are not used is to correlate dose levels with the reproductive behaviour of the experimental animal. This is particularly applicable to the chicken, whose stereotyped behaviour patterns have frequently been analysed by the injection of hormones. Noble and Zitrin (1942) attempted to produce complete copulatory behaviour in single-comb White Leghorn chicks with homologous hormone injections. Some success was achieved with 15 day old chicks which received 0.5mg testosterone propionate/day or 0.17 mg oestradiol benzoate/day. Davis and Domm (1941) obtained crowing, copulation behaviour and waltzing (courtship dance) with capons that received 2.5 and 3.75 mg

testosterone propionate/day. Another convenient method of assessing the effectiveness of hormone treatment is the measurement of comb growth. Comb growth has a direct relationship with dose levels of testosterone.

In a preliminary experiment dose levels of 1.0mg testosterone and 0.4 mg oestradiol/Kg body weight/day were selected and were injected in arachis oil into the pectoral muscles of 14 day old male and female chickens. The chickens were infected a week later with 50 cysticercoids each and autopsied two weeks after infection. Hormone therapy was continued throughout the infection and the doses adjusted according to the weight of the birds every three days. At this particular age the naturally produced hormone levels of the chickens are very low and the first manifestations of age resistance are appearing.

Unfortunately, the evidence suggests that the hormone levels used were too low. There was comb growth in the testosterone treated birds initially and these birds became much more aggressive than their controls. Growing was also noted in the second week of treatment. However, the controls caught up rapidly and at autopsy no great difference was in evidence as regards size of combs or weights of testes. Slower growth of testes was expected in testosterone treated birds due to negative feedback. No sexual behaviour whatever was observed in female birds treated with oestradiol and organ and body weights were also similar.

It is not surprising, in view of these observations, that no differences in the course of infection with R. cesticillus betwenn the experimental and control groups was observed, which is undoubtedly due in part to the low

hormone dose levels. Another possible reason for the lack of effect of hormone therapy on R. cesticillus is the fact that cestodes in chickens of this particular age are strobilated and healthy. The experiments on the effects of age on the course of infection (Chapter 2) suggest that oestrogens inhibit the manifestations of resistance rather than potentiate them and it would be difficult to detect any such effect in healthy cestode infections.

It was decided therefore to create hormonally neutral adult birds, as far as gonadal steroids are concerned, by castrating young male birds and then treat them with testosterone and oestradiol pellets for long term continuous gonadal steroid therapy. In this way adult birds were made artificially male or female. The effect of such a system on the course of infection of R.cesticillus was then studied.

Materials and Methods

Twentyfour male 7 week old chickens were surgically caponised after anaesthetisation with intravenous Nembutal (50 mg/Kg). The testes were removed through lateral incisions between the last two ribs. The birds were kept in quiet surroundings for a day after the operation and subsequently maintained in outdoor fold units on a wooden slatted floor. Regeneration of testicular tissue, as evidenced by comb growth, occurred in seven birds and these were discarded.

Three months after caponization Organon hormone pellets were implanted under the skin at the back of the neck. Six

birds were implanted with 25mg pellets of Testosterone, six birds with 10mg pellets of Oestradiol and five were left untreated as controls.

The testosterone treated birds rapidly developed male characteristics, but the oestradiol treated birds did not show any signs of female behaviour, such as squatting, two months after implantation. A second oestradiol pellet was therefore implanted into each of the 'female' birds. The testosterone pellets have a life of only 60 days, as against 200 for the oestradiol pellets, and it was therefore necessary to reimplant the 'male' birds as well.

The 'female' birds had still shown no signs of squatting 4 months after the first implantation, but squatting was eventually induced by oestradiol injections of 0.5 mg/Kg/2 days in arachis oil. Five days after the start of this treatment all birds were seen to squat. One bird was also seen to attempt copulation on many occasions. Guhl (1950) reported that oestrogen may be used to elicit male copulatory behaviour, but in this particular case the size of the comb suggested that slight regeneration of the testicular tissue had occurred. On autopsy this proved to be the case. This bird was removed from the experimental groups.

All birds were infected at 280 days old with 100 cysticercoids each 7 days after the start of the oestradiol injections of the 'female' birds, which was continued throughout the prepatent period of the infections. All birds were autopsied 14 days after infection and observations were made on numbers of worms, numbers of destrobilated

worms, the distribution of worms and worm growth. Tissue samples of the worm *in situ* were also taken for histological examination.

Results (Mean Values)

Table 30.

Chicken Group	Chicken Weight	Number of Scoleces	Percent Number of Destrobilating Scoleces	Worm Weight	Adrenal Weight	Spleen Weight
Testosterone	2375.0	22.3 ± 6.23	100.0 ± 0.0	-	184.6	2434.0
treated Percent Length	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46
Percent No. Scoleces	0	0	0	1.5	0	3.0	1.0	1.5	3.0	3.0	5.0	3.0	10.5	10.5	11.5	5.5
Percent Length	48	50	52	54	56	58	60	62	64	66	68	70	72	74	76	78
Percent No. Scoleces	6.5	5.5	5.0	5.0	3.0	5.5	0	5.0	0	1.5	2.5	0	1.0	0	0	1.0
Oestradiol	2510.0	8.6 ± 2.64	60.0 ± 7.10	4.52 ± 0.21	230.6	2176.0
treated Percent Length	42	44	46	48	50	52	54	56	58	60	62	64	66	68	70	72
Percent No. Scoleces	7.0	9.3	9.3	16.2	4.6	11.6	11.6	4.6	7.0	2.3	4.6	2.3	7.0	0	0	2.3
Controls	2608.0	32.4 ± 5.42	93.7 ± 6.32	4.4	205.0	2709.8
Percent Length	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38
Percent No. Scoleces	0.6	0.6	0	0	1.2	0	0	0.6	1.2	4.3	1.9	5.7	1.9	1.9	5.2	5.2	5.2	3.2
Percent Length	40	42	44	46	48	50	52	54	56	58	60	62	64	66	68	70	72	74
Percent No. Scoleces	2.6	6.5	7.8	5.2	6.5	5.7	5.2	5.7	5.7	2.6	1.2	4.3	0.6	0.6	1.2	0	0.6	1.2

Percent Number of Destrobilating Scoleces - T Values

Chicken Group	T	P
Testosterone treated		
	0.997	>0.10
Control		
Oestradiol treated		
	3.546	<0.01
Control		
Testosterone treated		
	5.633	<0.001
Oestradiol treated		

The results of this experiment show that some success was achieved in the attempt to reproduce by hormone therapy the course of infection characteristics of normal mature male and female birds. Mature male birds have been shown to be resistant to infection with R. cesticillus as judged by numbers of destrobilating worms, whereas mature female birds were susceptible. This situation has been reproduced in this experiment by treatment with gonadal steroids. Testosterone treated birds contained 100% destrobilated worms, but in the oestradiol treated birds this value was only 60.0%. This is a statistically significant difference ($P = < 0.001$).

However, the numbers of worms recovered from the oestradiol treated birds were abnormally low. It is very likely that this is due to overdosage with oestradiol, despite the precautions taken. No worms were found prior to percent length 42 and it is probable that high levels of oestradiol and oestradiol breakdown products in the bile prevented establishment in the area of the gut just posterior to the bile duct opening. This supposition implies that high levels of oestradiol are toxic to the cestodes. Rosen, Dougherty and Bern (1951) reported that 40ug of α - oestradiol/ml depressed egg production by the nematode Rhabditis briggsae. This concentration of oestradiol is certainly higher than normal physiological values. O'Grady (1968) reported that concentrations of oestradiol in chicken plasma ranged from 8.3 - 21.4 ng/ml

It is interesting to note that no worms have established anterior to the bile duct opening in the oestradiol-treated birds. This distribution is found in young birds

and also in mature females, which revert to the juvenile pattern at 16 weeks of age, shortly before coming into lay. This distribution may be a reflection of the small numbers of worms present in the oestradiol-treated birds or it may be caused by the same factors that are operating in mature females and that cause a 'juvenile' distribution. If the latter hypothesis is the case it would appear that the factors responsible are linked with the presence of oestrogens.

In the testosterone treated birds both the numbers of scoleces recovered and their diffuse distribution are similar to the situation found in the normal mature male.

The situation in the untreated caponised birds seems to be intermediate in some respects between that found in the testosterone- and oestradiol-treated birds. Substantial numbers of worm scoleces were recovered and in four of the birds destrobilization was less far advanced than in the testosterone-treated birds although all worms had started to destrobilate. In the fifth bird the number of destrobilating worms was only 68.4%. Judging by the parameter of destrobilization it would appear that untreated capons are very slightly less resistant than testosterone-treated birds.

Discussion

These results suggest that in this particular system androgens potentiate host resistance to the parasite whilst oestrogens reduce it. This conclusion seems to be in conflict with the generalisation that male hosts are usually

more susceptible to parasitic infections than female hosts (Solomon 1969, Gray 1969). However, as previously mentioned, there are many contradictory reports. It is important to bear in mind the fact that helminth host-parasite systems differ in many ways and different parasites are likely to be exposed to different hormonal effects.

Several mechanisms whereby gonadal steroids might affect helminth parasites have been postulated. Solomon (1966) suggested that androgens affect integumentary structures allowing easier penetration by helminth larvae such as Nippostrongylus brasiliensis.

Androgens have been implicated in helminth nutrition by Addis (1946) and Beck (1952), who established that the utilisation of an hydrolysed yeast extract by Hymenolepis diminuta in vivo was enhanced by the presence of testosterone. Biochemical evidence for an anabolic effect of androgens on helminths was provided by Aldrich, Chandler and Daugherty (1954), who observed that castration of the host resulted in decreased protein synthesis by H. diminuta and Daugherty (1956) reported that host castration also resulted in a decreased rate of glycogenesis by this cestode.

There is some evidence to suggest that oestrogens enhance immune mechanisms (Nicol and Bilbey, 1958; Sprunt, McDearman and Raper, 1937; Von Haam and Rosen, 1942) and Dobson (1961) postulated that this explained the higher resistance of female mice to the mematode Nematospiroides dubius.

These suggested mechanisms provide evidence for the widely held view that androgens reduce resistance and oestrogens potentiate it. They do not, however, explain the results obtained with R. cesticillus in this experiment.

The cestode does not penetrate host tissues, so the action of androgens on connective tissue is irrelevant. The enhancement of immunity by oestrogens is a possibility in this particular system, but is unlikely in view of the experimental results. Histological studies of scoleces in situ from the two experimental and the one control group provided no evidence for enhanced immunity. However, this mechanism may well explain the differences observed between male and female responses to secondary infections in 70 day old chickens (see Chapter 3).

In the experiment described here oestradiol appears to reduce resistance to R. cesticillus. Gibbs (1967) claimed that diethylstilboestrol increased the fecundity of trichostrongyle nematodes in sheep and Dunsmore (1971) found that an oestrogen implant increased the numbers of Trichostrongylus retortaeformis naturally acquired by grazing rabbits of both sexes. It is quite possible that oestrogens exert these effects by creating a favourable nutritional environment in the intestine, either directly or indirectly. Several workers have provided evidence that high cholesterol levels facilitate ulceration and liver invasion by Entamoeba histolytica and cholesterol has been shown to be a nutritional factor in the culture of E. histolytica (after Solomon, 1966). Oestrogens

undoubtedly enter the intestine in the bile and there is also evidence that oestrogens can pass from the blood through the gut wall into the lumen (Pearlmann, Rakoff, Paschis, Cantarow and Walking, 1948). Oestrogens can therefore have a direct effect on parasites in the intestine. An indirect effect is perhaps more likely in view of the wide range of action that oestrogens have on vertebrate tissues. In the chicken oestrogens cause a rapid increase in lipogenesis and in yolk protein synthesis in the liver. The bile is therefore likely to be considerably enriched in lipids and other nutrients in the mature female.

The effect of oestradiol on lipogenesis in the birds of this experiment was very obvious at autopsy. A lot of fat deposition had occurred subcutaneously and in the mesentery of the intestines. The bile also appeared to be very rich in lipids. In the birds that received testosterone implants no fat deposition had occurred and there was no evidence for increased amounts of lipid in the bile. This is as one would expect. In the untreated caponised control birds lipogenesis seemed to be intermediate between the two experimental groups. Androgens increase the basal metabolic rate and protein anabolism in vertebrates and it is therefore predictable that castration causes increased deposition of fat. The anabolic action of androgens on H. diminuta reported by Aldrich, Chandler and Daugherty (1954) does not appear to have had any effect in this particular experiment, but it is interesting to note that worms recovered 14 days after infection from 56 day old male birds were significantly heavier than those from

female birds of the same age.(Page 84).

There appears to be some correlation between the amount of lipid in the intestine and the degree of resistance to R. cesticillus, as judged by the numbers of destrobilating worms. This conclusion adds more weight to the hypothesis that the primary factor involved in the phenomenon of destrobilization is that of worm nutrition.

CHAPTER 9

THE ROLE OF WORM NUTRITION IN THE PROCESS OF DESTROBILIZATION.

INTRODUCTION

Considerable evidence has accumulated from previous experiments to suggest that the nutritional status of *R. cesticillus* is related to the process of destrobilization. In a discussion on the possible cause of destrobilization (Page48.) it was stated that Read (1959) had correlated senescence of *Hymenolepis diminuta*, *H.nana* and *H. citelli*, characterised by destrobilization, with their sensitivity to shortage of carbohydrate and Reid (1942) demonstrated that destrobilization of *R. cesticillus* could be induced by starvation of the host. It was suggested that destrobilization may be linked in some way with carbohydrate metabolism.

More evidence for the hypothesis that nutritional conditions influence destrobilization was obtained from the experiments on the effect of host age on the course of infection of *R. cesticillus* (Chapter 2). As the age at which birds were infected increased, the numbers of destrobilating worms 14 days after infection also increased and the distribution of the worms in the intestine became more diffuse. Female chickens became more susceptible to *R. cesticillus* on reaching maturity and the distribution of the worms became more compact, reverting to the same pattern of distribution as found in younger more susceptible birds.

These observations establish a relationship between destrobilization and distribution. It was pointed out (Page 86.) that the distribution of the cestodes in the intestine is likely to be dependent on the nature of the physico/chemical and nutritional environment. If this is the case it is reasonable to suggest that a relationship between destrobilization and the intestinal environment has been established.

This hypothesis suggests that destrobilization of the worms is caused by an unfavourable alteration of the environment and restrobilization should occur in a favourable environment.

A. SCOLEX TRANSPLANT

In this experiment destrobilated scoleces of both primary and secondary infections were transplanted into the small intestines of uninfected young chickens in an attempt to determine whether worm destrobilization is caused by damage to the worm or by environmental changes.

Materials and Methods

Donor Birds

Destrobilated scoleces were obtained from seven donor birds.

1) Female; infected at 3 days old; 100 cysticercoids; 54 day old primary infection.

2) Female; infected at 28 days old; 100 cysticercoids; 31 day old primary infection.

3) Male; infected at 105 days old; 200 cysticercoids; 28 day old secondary infection.

4) Female; infected at 105 days old; 200 cysticercoids; 28 day old secondary infection.

5) Female; infected at 147 days old; 200 cysticercoids; 28 day old secondary infection.

6) Male; infected at 3 days old, 100 cysticercoids; 12 day old primary infection. Control.

7) Male; infected at 3 days old; 100 cysticercoids; 12 day old primary infection. Control.

Removal of Scoleces

Donor birds were autopsied individually and the small intestines removed and slit open in cold physiological saline. Chilling of the scoleces makes them relax their hold and they are easily removed without damage. Fully destrobilated scoleces were selected from donor birds 1) - 5). The worms from donors 6) and 7) were strobilated and the strobilae were removed at the neck. The scoleces were placed in Tyrode's saline at 40°C and kept at this temperature in preparation for transplanting.

Operative Procedure

The recipient birds were 7 day old male Apollo and they were anaesthetised with an intraperitoneal injection of 50 mg Nembutal/Kg body weight. The ileum was exposed by cutting between the last two ribs on the right side and held in position with wax-coated forceps. A small incision was made in the intestinal wall and the scoleces were introduced into the intestine with a fine pipette. The body-wall incision was sutured with silk thread and the birds allowed to recover. All were fully active three hours after the operation.

Autopsies were carried out 12 days after the transplantations and the numbers of established strobilated worms recorded.

Results

Table 31.

Number of Donor and recipient chicken	Number of Implanted Scoleces	Number of established worms	Percent take
1	23	2	8.7
2	10	0	0.0
3	47	16	34.0
4	42	6	14.3
5	44	20	45.4
6	20	7	35.0
7	20	15	75.0

Scoleces restrobilated in all recipient birds with the exception of number 2), which only received 10 scoleces.

This experiment proves the hypothesis that damage to destrobilated scoleces is temporary and regeneration of strobilae occurs in a favourable environment. This result provides further evidence for the suggestion that destrobilization is caused by an adverse alteration of the cestode's environment, rather than by actual damage to the worm.

It is noticeable that the percentage takes of transplanted primary infection scoleces are much lower than the percentage takes of secondary infection scoleces. This may be due to the fact that fewer primary infection scoleces were transplanted, but it is also possible that scoleces that have been destrobilated for some time (as is the case

forscoleces of donor bird 1) lose the ability to regenerate strobilae once their energy reserves fall below a certain level. An alternative explanation is that the destrobilization of primary infections is caused by different factors than those responsible for the destrobilization of secondary infections. In the absence of more data these explanations are highly speculative.

It has been suggested that the immunological response evoked by R. cesticillus has its effect by altering the environment of the cestode and this hypothesis is supported by the results of this experiment. Further evidence is provided by Hopkins, Subramanian and Stallard (1972a), who reported that destrobilated Hymenolepis diminuta restrobilate when transplanted into susceptible rats and mice. As already mentioned (Chapter 3) these workersconcluded that immunological factors are responsible for the destrobilization of H. diminuta in mice. It seems very likely that destrobilization of secondary infections of R. cesticillus is caused in part by an immune response.

It was mentioned previously (Chapter 4) that a nonspecific factor also seems to contribute to destrobilization of secondary infections and it is probable that this factor plays a part in the destrobilization of primary infections. Such a factor may be the anthelmintic factor demonstrated by Frick and Ackert (1948) in chicken intestinal mucus (Chapter 7). This factor exerts a temporary inhibitory effect on the growth of Ascaridia galli larvae and Frick and Ackert suggested that this inhibition is probably nutritional. Attempts to demonstrate a similar effect on

R. cesticillus have not been successful, but this was probably due to the limitations of the design of the experiment.

B. CORRELATION OF DESTROBILIZATION WITH WORM POPULATION SIZE

Crowding is well known to cause decreased growth rates of the larger cestode species and Reid (1942) demonstrated that this phenomenon applies to *R. cesticillus*. Chandler (1939) concluded that as far as *Hymenolepis diminuta* is concerned this effect is due to competition between the worms, probably nutritional. In view of the suggestion that nutritional factors are possibly involved in worm destrobilization it was decided to investigate the effect of the size of worm populations on percentage destrobilization.

It was not considered necessary to conduct a specific experiment on this subject as plenty of data is available from other experiments. Within some of the groups considered there are sufficient differences between sizes of worm populations for a reasonable assessment to be made.

Results

Table32.

	Numbers of worms recovered at autopsy	Percent Number of Destrobilating worms
Group (1) Chickens infected at 3 days old. 56 day old infections.		
Male 1	21	71.4
2	26	32.5
3	31	9.7
4	33	33.3
5	57	31.6

Results (Cont)

Table 32 (Cont)

	Numbers of worms recovered at autopsy	Percent Number of Destrobilating Worms
Group (1) Chickens infected at 3 days old. 56 day old infections.		
Female 1	14	100.0
2	16	87.5
3	19	89.5
4	28	92.9
5	39	97.4
Group (2) Chickens infected at 56 days old. 14 day old infections.		
Male 1	40	22.5
2	47	19.1
3	61	19.7
4	62	0.0
Female 1	32	66.7
2	43	60.4
3	51	37.2
4	57	77.2
5	57	52.6
Group (3) Chickens infected at 84 days old. 14 day old infections.		
Male 1	32	37.5
2	32	46.9
3	54	33.3
4	59	62.7
5	64	78.1

Table 32(Cont)

	Numbers of worms recovered at autopsy	Percent Number of Destrobilating Worms
Group (3) Cont. Chickens infected at 84 days old. 14 day old infections.		
Female 1	45	77.8
2	47	46.8
3	58	56.9
4	62	53.2
5	73	46.6
Group (4) Chickens infected at 70 days old. 14 day old infections.		
Male 1	21	33.3
2	22	50.0
3	27	14.8
4	28	78.6
5	31	90.4
Female 1	14	64.2
2	23	87.0
3	25	68.0
4	25	100.0
5	26	100.0
Group (5) Chickens infected at 70 days old. 14 day old infections.		
Male 1	4	100.0
2	14	64.2
3	19	36.8
4	20	85.0
5	20	50.0
6	21	71.4
7	25	68.0
8	26	80.8

Table 32(Cont).

	Numbers of worms recovered at autopsy	Percent Number of Destrobilating Worms
Group (5) (Cont)	Chickens infected at 70 days old. 14 day old infections.	
Male 9	27	59.3
10	33	57.6
Female 1	16	43.8
2	18	44.4
3	18	100.0
4	18	55.5
5	18	77.7
6	23	30.4
7	29	100.0
8	30	26.6
9	34	41.2
10	39	46.2

From these figures it is immediately apparent that there is no consistent relationship between the size of a worm population and the extent of its destrobilization. The only conclusion to be drawn here is that the crowding effect is not of prime importance in the phenomenon of worm destrobilization. It would be surprising if the size of a worm burden did not exert some influence on destrobilization because other factors in addition to competition for nutrients (such as antigenicity, inhibitory worm excretory products and actual physical crowding) probably play a part in the crowding effect (Reid, 1942; Roberts, 1961). It is possible that a detailed investigation of this subject would establish some relationship between worm burden and destrobilization, but it would appear that variations in the physiology of individual birds is of far greater significance.

These results do not eliminate worm nutritional status as being of importance in destrobilization.

C. CORRELATION OF DESTROBILIZATION WITH WORM WEIGHT

The factors that cause destrobilization have already been linked with those that influence the distribution of the cestode in the gut (Page 234). The evidence for this relationship is further supported by the fact that in previous experiments destrobilization has been observed to be further advanced at the anterior and posterior limits of the infection zone. This is not very surprising as physico/chemical and nutrient conditions vary considerably along the intestine and the zone for optimal viability of the worm is therefore likely to be restricted. The implication is that destrobilization is directly related to the viability of the worm.

Reid (1942) states that R. cesticillus collected from anterior locations in the gut weighed appreciably more than those from posterior locations. This seems to be slightly at odds with the suggestion that destrobilization is associated with low worm viability. One would expect worms at the anterior and posterior limits of the infection to weigh less than those occupying the optimum viability zone. It was therefore decided to determine the weights of worms obtained from different parts of the intestine and to attempt to correlate these weights with destrobilization.

Materials and Methods

The worms were obtained from the male birds of the first gonadal hormone experiment (Chapter 8), in which it was found that low-dose testosterone treatment had no detect-

able effect on the course of infection of the cestode. The birds were infected at 3 weeks of age with 50 cysticercoids each by pipette and autopsied 17 days after infection, by which time the birds were 5 weeks old, the age at which the factors responsible for destrobilization start to operate.

The intestines were removed and worms from each percent length from each bird were pooled and after thorough washing in saline were dried to constant weight in an oven at 60°C.

Destrobilization values were obtained from the ten male control birds of the second acquired resistance experiment (Chapter 3). These values have been expressed as the number of strobilated worms as a percentage of the total number of worms in each percent length.

Results

Table 32.

Percentage Length -	30	32	34	36	38	40	42	44	46	48	50	52	54	56	58	60	62	64	66	68	70	72	74	76	78
Total Worm Number -	2	7	8	8	15	22	31	20	22	33	17	25	10	8	11	10	8	4	2	6	1	0	0	3	
Mean Worm Weight -	11.4	6.9	6.8	8.0	8.6	7.8	8.6	8.2	9.7	9.6	12.2	9.6	10.9	8.8	9.6	7.9	8.0	8.4	6.8	6.1	3.8	0	0	4.5	
Percentage Strobilated Worms -	0	0	0	0	0	0	25	28	63	54	71	83	50	40	33	33	0	0	0	0	0	0	0	0	0

Figure 49. Correlation of destrobilization of R.cesticillus down the intestine with worm weights.

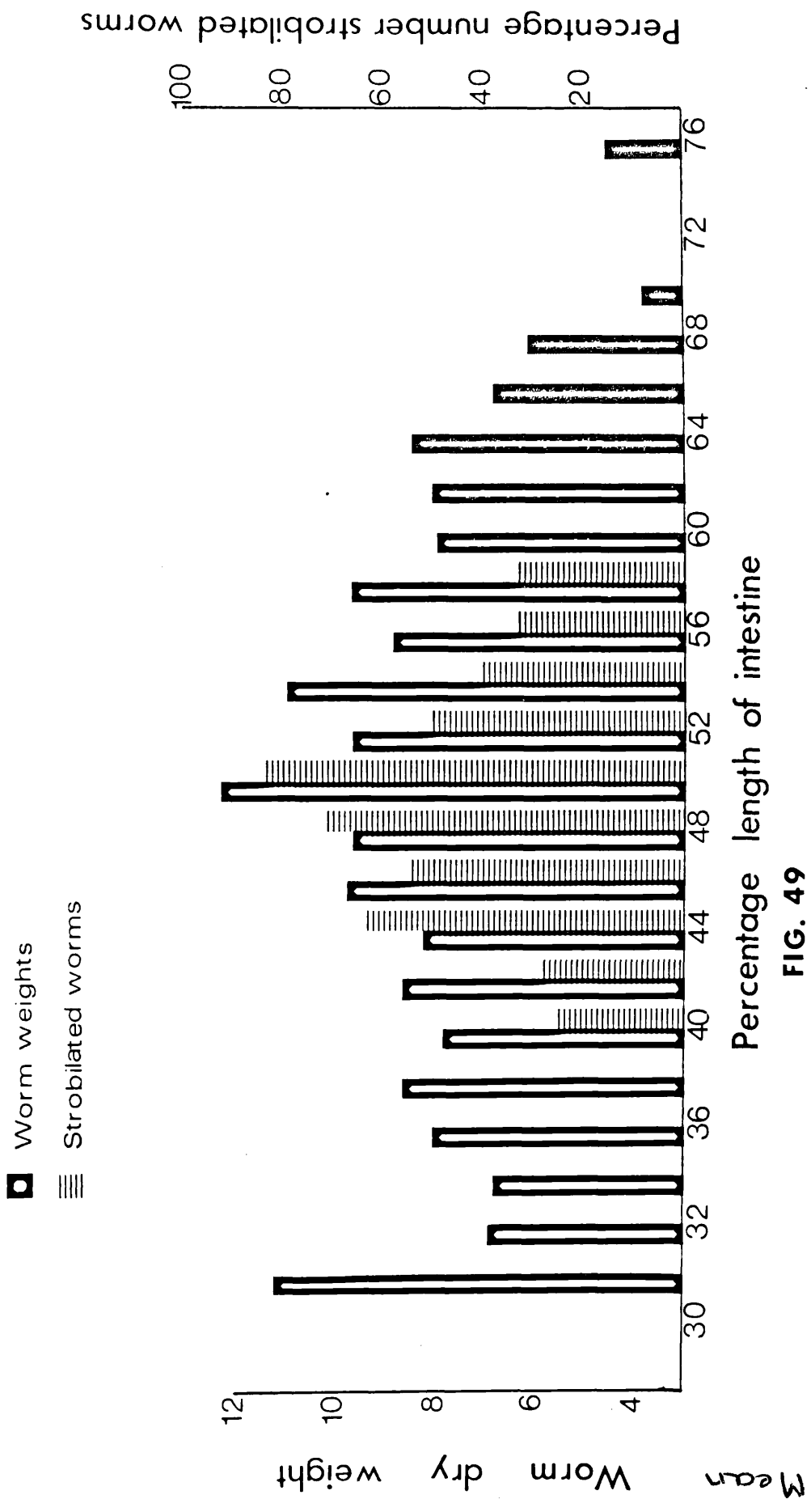

Worm weights
Strobilated worms
Percentage number strobilated worms
100
80
60
40
20
Mean Worm dry weight
12
10
8
6
4
30
32
36
40
44
48
52
56
60
64
68
72
76
Percentage length of intestine

FIG. 49

These results show that the weights of worms depend on their location in the intestine. The largest worms occur towards the centre of the infected zone and the smallest worms are found at the anterior and posterior limits. This is not in agreement with the observations of Reid (1942), but corresponds well with rates of destrobilization down the intestine (Fig.49.). Worm weight therefore seems to be directly related to destrobilization and the healthiest and largest worms appear to destrobilate last. This strongly implicates worm nutritional status as being of prime importance in the process of destrobilization.

It is interesting to note that the optimum viability zone for the cestode (percent lengths 40-60) contains both the largest and the most worms. This constitutes further evidence for the suggestion that nutritional competition between worms is of little significance in destrobilization.

It is difficult to explain the large worms found in percent length 32, but it is possible that extra nutrients are obtained from the bile, which enters the intestine in this area.

D BIOCHEMICAL ANALYSIS OF DESTROBILATING WORMS

The involvement of worm nutrition in the process of destrobilization is further supported by the results obtained in the gonadal hormone experiment (Chapter 8), in which destrobilization was correlated with host lipogenesis. It was also observed that worms cultured *in vitro* in lipid-poor serum tended to fragment rather easily and that medium containing serum rich in lipid seemed to prevent fragment-

ation. This suggests that host lipid levels have a direct relationship with the nutritional status of R. cesticillus. Botero and Reid (1969) reported that the lipids of R. cesticillus reflect the fatty acid make up of the environment.

In the following experiment an attempt was made to detect some change in the amount of a major chemical constituent, such as protein, lipid or carbohydrate (polysaccharide), associated with the process of destrobilization. It was expected that any such change would probably be sufficiently large to detect by the measurement of total protein, lipid and carbohydrate and that detailed analysis would be unnecessary. Reid (1942) managed to correlate depleted total glycogen in R. cesticillus with host starvation and it was anticipated that a similar situation might be found in worms approaching destrobilization.

Materials and Methods

Five male and five female 4 week old chickens were infected by pipette with 100 cysticercoids and were autopsied at three different periods after infection in an attempt to obtain a gradation of worm destrobilization. One male and one female were autopsied 14 days after infection, two males and two females 28 days after infection and the remaining two males and two females 56 days after infection. At each autopsy whole worms were removed from percent lengths 40-80, washed three times in phosphate buffered saline and deep frozen at -20°C with 1:10,000 thiomersal.

The worms from each bird were separated into their major chemical fractions by the method of Graff (1970), described in Chapter 4, dried under vacuum in a dessicator containing silica gel and finally weighed.

Results

Table 33.

Chicken	Number of worms	% Destrobilizing worms	Dry weight	% Protein + Ash	% Polysaccharide Carbohydrate	% Lipid
1 ♂	15	0.0	237.6	41.2	55.0	3.7
2 ♂	60	0.0	334.8	38.4	59.0	2.6
3 ♀	49	32.0	103.3	57.9	37.4	4.7
4 ♀	40	37.5	109.5	46.3	36.2	17.4
5 ♂	38	44.5	242.9	68.0	24.9	7.2
6 ♀	35	68.6	67.1	38.4	52.9	8.6
7 ♂	27	74.0	143.9	62.2	35.3	2.5
8 ♀	49	77.5	51.4	31.3	41.4	27.2
9 ♂	35	88.4	65.5	40.1	45.5	14.3
10 ♀	35	91.5	102.7	63.7	29.7	6.6

From these results it can be seen that there is no apparent correlation between the numbers of destrobilating worms and the relative amounts of protein, lipid and polysaccharide (Fig.50.). This may be due to one of several reasons.

Firstly, the number of birds involved was small, as was the number of worms. When destrobilization values reached 80 and 90 percent only one or two worms were available for analysis, as only whole worms were used. It is possible that larger samples would have reduced the variation in values.

Figure 50. Biochemical analysis of destrobilating R.cesticillus.

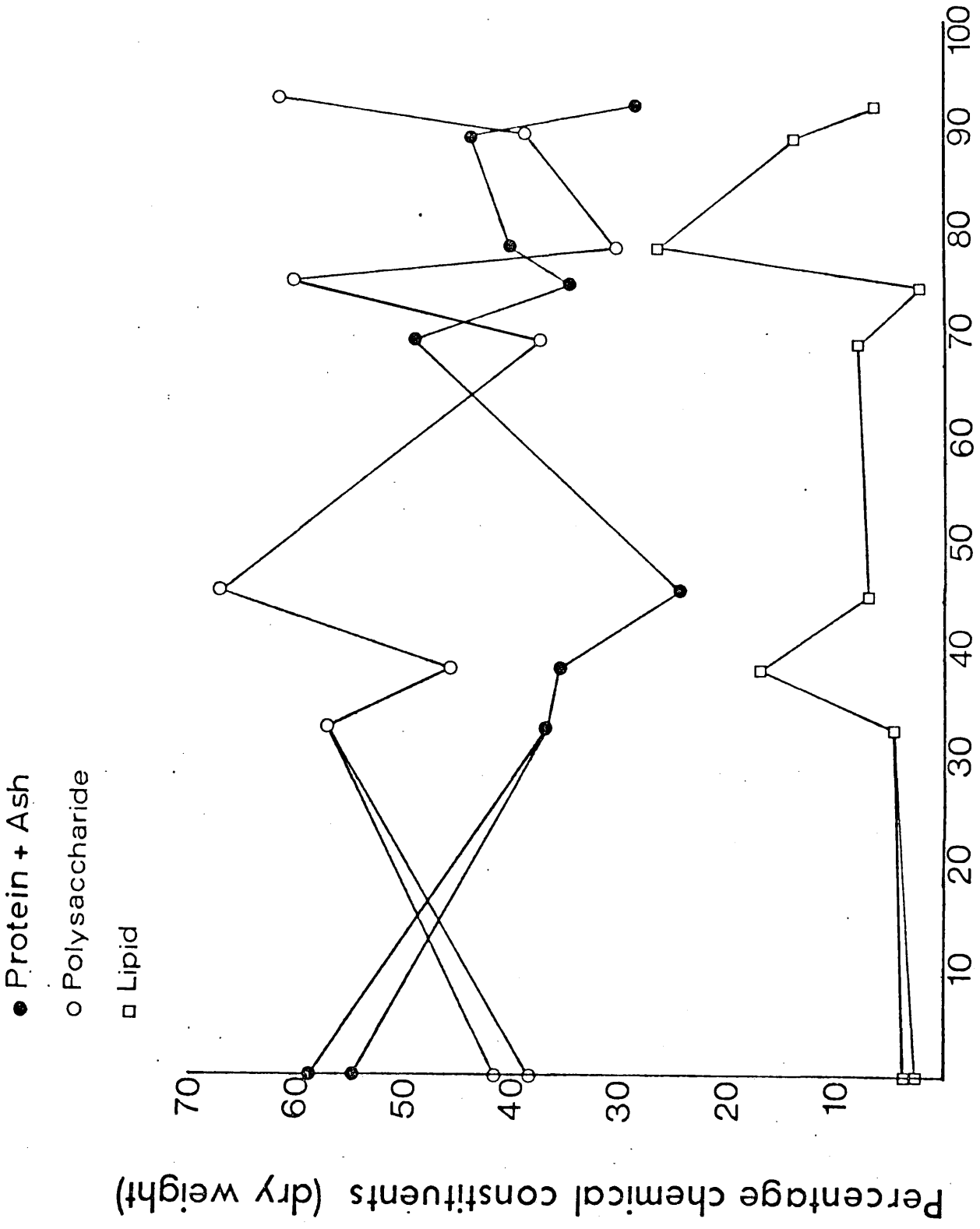

● Protein + Ash
o Polysaccharide
□ Lipid
Percentage chemical constituents (dry weight)
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
90
100
Percentage number destrobilated worms

FIG. 50

Secondly, it is possible that the changes in chemical composition that may initiate the process of destrobilization are too small to be detected by the methods used. Such changes may occur within the three classes of chemical constituents considered and more detailed analysis would be required in this case.

Thirdly, it is by no means certain that the whole worms removed for analysis at each autopsy were themselves approaching destrobilization even though most of the rest of the worm population may have destrobilated.

The results of this experiment are, therefore, somewhat inconclusive and it can only be suggested that destrobilization is probably not caused by a large change in the relative amounts of worm protein, lipid and polysaccharide.

Discussion

In this series of experiments it has been established that alterations in the intestinal environment cause worm destrobilization and that the factors responsible are probably related to the nutritional requirements of the worms as the largest worms, which occur in the optimum viability zone (percent lengths 40-60), destrobilate last.

However, there is apparently no correlation between destrobilization and worm burdens, so that it seems that nutritional competition has no effect on destrobilization, and there are apparently no changes in levels of protein, lipid and polysaccharide in worms approaching destrobilization. This compares well with the findings of Reid (1942), who showed that dwarfing of worms due to the 'crowding effect' was not accompanied by depletion of glycogen levels.

GENERAL DISCUSSION

Progressive destrobilization of R. cesticillus was observed in chickens infected at 3 days of age with complete destrobilization occurring 70 days after infection. The marked difference in the rate of destrobilization in the two host sexes suggested that this phenomenon is probably caused by host interaction and is not inherent to the worm. Studies on the effects of age on the course of infection of the cestode confirmed this theory. It was found that a distinct age resistance occurred in male birds, with complete destrobilization occurring before patency in birds infected at an age of more than 112 days. In the females, however, resistance, as measured by destrobilization, decreased once the birds reached maturity. As a result of these studies the possible influence on R. cesticillus of three factors associated with age resistance to helminths (immune potential, mucus secretion and hormone balance) was investigated.

Until recently there has been little evidence to suggest that lumenal cestodes, with the exception of Hymenolepis nana, evoke a protective immune response. However, evidence for resistance to cestode superinfections has been provided by Seddon (1931) for Moniezia expansa, Boughton (1932) for Cittotaenia (after Chandler, 1939) and Heyneman (1962) for the lumenal stage of H. nana. More positive evidence for a protective immune response to lumenal cestodes was presented by Hopkins, Subramanian and Stallard (1972a), who demonstrated resistance to superinfection with Hymenolepis diminuta in the mouse, an abnormal host, and inhibited this resistance with the

immunosuppressants cortisone acetate, methotrexate and anti-lymphocytic serum (Hopkins, Subramanian and Stallard, 1972 b).

In Chapter 3 experiments were described which demonstrated resistance to superinfection with R. cesticillus for the first time. This resistance was suppressed by the immunosuppressant dexamethasone (Chapter 4) and in Chapter 5 immunocytes and antibodies were demonstrated.

There can be little doubt that R. cesticillus evokes a protective immune response, but it is not yet known how this response attacks the cestode. Serum antibodies do not seem to be protective in vivo or in vitro (Chapter 6) and mucus extracts from immune birds do not seem to have any specific effect on the cestode either. So far no antibodies to R. cesticillus have been detected in mucus extracts, but, as previously suggested, this is probably due to the limitations of the preparatory procedures.

Mast cell numbers appear to increase in R. cesticillus infections, as they do in most other helminth infections, but there is no evidence to suggest that anaphylaxis occurs. Anaphylaxis is thought to play a part in the elimination of several nematode infections, including Nippostrongylus brasiliensis and in this species the 'self-cure' is thought to take place in two steps. In Step 1) the worms are damaged immunologically and in Step 2) they are eliminated by an anaphylactic reaction (Ogilvie and Jones, 1971). Although Step 2) does not seem to operate in R. cesticillus infections, there is no reason why Step 1) should not.

It has been suggested that Step 1) is dependent on sensitised small lymphocytes rather than on antibodies (after Ogilvie and Jones, 1971) and this may explain the failure to immunise chickens against R. cesticillus passively with serum. The possible role of a cellular immune response is considered later.

The protective immune response to R. cesticillus must have its effect at the intestinal mucosa-worm interface, since there are no tissue stages of the parasite, and there is now considerable evidence to suggest that the mucosal immune barrier to invading parasites is of great importance. In the case of H. nana the experiments of Bailey (1951) and Weinmann (1966) suggest that the mucosal barrier is more important than tissue immunity. Leonard and Leonard (1941) came to the same conclusion when working with the cysticerci of Taenia pisiformis in rabbits.

Many of the mononuclear cells involved in the cellular response to R. cesticillus have abundant cytoplasm, which suggests that they may be producing antibody locally. A local antibody response might explain the failure to demonstrate antibodies in mucus. An alternative explanation is that the protective immune response is purely cellular. Both local antibody production and cellular immunity have been advanced to account for the characteristics of delayed hypersensitivity and there is considerable evidence to suggest that delayed hypersensitivity-type reactions are of importance in protective immune responses to helminths.

Weinmann (1964) induced resistance to Hymenolepis nana with infections of the lumen-dwelling bile-duct cestode

H. microstoma, which normally evokes a non-protective antibody response (Moss, 1971). Tan and Jones (1967) successfully immunised mice with heavily X-irradiated H.microstoma, which were soon eliminated. The resistance induced to a secondary infection took 20 days to develop. Tan and Jones (1968) also showed that this resistance lasts for about 150 days.

The cross immunity, indicating non-specificity, demonstrated by Weinmann and the slow induction of immunity demonstrated by Tan and Jones are characteristic of delayed hypersensitivity. Larsh (1967) demonstrated that delayed hypersensitivity is probably partly responsible for immunity to several helminths, including the cestode, Hymenolepis nana. He transferred peritoneal exudate from infected mice to uninfected mice, which then received a dose of H.nana cysticercoids. Recovery was reduced and this was attributed to local cellular injury, resulting in an alteration of the cestode's environment. The importance of cellular responses in immunity to helminths is further supported by the fact that protective antibodies have rarely been demonstrated (Sinclair, 1970) and Kagan (1970) states that the probable mechanisms involved in immunity to helminths are immediate and especially delayed hypersensitivity.

It has already been suggested (Page 174.) that the cellular response to R. cesticillus is characteristic of delayed hypersensitivity. Some circumstantial evidence for the role of delayed hypersensitivity is also provided by the experiments involving the immunosuppressant cyclophosphamide (Chapter 4). In the second experiment the

cellular reaction to R. cesticillus was not inhibited and neither was the destrobilization of the cestodes. Floersheim and Seiler (1967) reported that cyclophosphamide did not inhibit the early stages of delayed hypersensitivity in chickens; Lerman and Weidanz (1970) reported that, while the antibody formation abilities of chicks, treated in their first three days, were depressed, cellular immunity was unimpaired, and Glick (1971) observed that after cyclophosphamide therapy regeneration of spleen and thymus tissue (cellular immunity) occurred, but that the bursal follicles (humoral immunity) did not regenerate.

There seems, therefore, to be considerable circumstantial evidence for the hypothesis that immunity to R. cesticillus is affected by a delayed hypersensitivity-type response. It is not easy to see how sufficient antigenic information can cross the mucosa in order to induce an immune response when the cestode causes so little tissue damage. It may be that longstanding primary infections, such as those used in the experiments described in Chapters 3 and 4, are necessary. An alternative explanation may be that a local immune response occurs in the mucosa, which is well supplied with diffuse lymphoid tissue, and antigen passage across the intestinal wall may not be necessary.

The observations of Ackert, Edgar and Frick (1939) and Frick and Ackert (1948) on goblet cells and a mucus anthelmintic factor (Page 200.) suggested that intestinal mucus might be a factor involved in age resistance. It was shown in Chapter 7 that mucus extracts are lethal to R. cesticillus in vitro and that mucus extracts from old birds have a more rapid effect on the cestodes than mucus extracts

from young birds. These findings are in agreement with those of Frick and Ackert (1948) for Ascaridia galli, but their experiments suggested that the factor responsible for the anthelmintic effect is not an antibody, as it is thermolabile. It was found that autoclaving mucus destroyed the lethal factor for R. cesticillus. It was suggested in Chapter 6 (Page 199) that chicken serum contains a non-specific antibody that attacks R. cesticillus in vitro and it is quite possible that the anthelmintic factor demonstrated in mucus extracts is a non-specific antibody.

This anthelmintic factor could well be responsible for age resistance to R. cesticillus, because not only is there some evidence to suggest that its concentration increases with age, but also Ackert et al (1939) have shown that older chickens have more goblet cells per unit area in the intestinal epithelium than younger birds.

In Chapter 7 an attempt was made to correlate goblet cell numbers with the resistance to R. cesticillus induced by superinfection, but no such correlation was found despite the reports of Wells (1963) and Dobson (1966) that helminth infections increase the amount of intestinal mucus produced. No hypertrophy of goblet cells was noted either and it was therefore concluded that the resistance to superinfection with R. cesticillus is not directly related to the anthelmintic property of mucus.

It was shown in Chapter 1 that the rate of destrobilization depended to some extent on the sex of the host, which suggests that gonadal hormones probably influence the process of destrobilization in some way. This was confirmed

in the experiment described in Chapter 8, in which it was shown that oestradiol treatment inhibited destrobilization. There was also a suggestion that testosterone treatment might enhance destrobilization of the worms, since destrobilization values in untreated caponised birds were slightly lower than those of the testosterone-treated birds. Thus, oestrogens seem to decrease resistance to R. cesticillus and androgens may increase resistance. This is in keeping with the situation found in untreated intact mature birds (Chapter 2). In most host-parasite systems oestrogens increase host resistance, probably through their enhancement of immune responses. R. cesticillus seems to evoke a more vigorous immune response in 70 day old female chickens than in males as judged by the degree of worm destrobilization and by the cellular infiltration, and this may possibly be due to the influence of low levels of oestrogens. It was suggested that oestrogens and androgens probably have their effect in mature birds by acting on some intestine environmental factor, such as the availability of lipid.

It was pointed out in Chapter 2 that in mature birds worm destrobilization was associated with a more diffuse worm distribution, which implied that the factor(s) responsible for age resistance may affect the intestinal environment. Support for this theory was obtained by the transplanting of destrobilated scoleces into susceptible chickens, in which restrobilation of both primary and secondary scoleces occurred. This experiment provides strong evidence to suggest that destrobilization is caused by an adverse alteration of the cestode's environment.

The hormone therapy experiment suggested that a nutritional factor, such as lipid, may be involved and it is interesting to note that lipid-poor serum sometimes caused fragmentation of worms *in vitro*. However, no changes in lipid content, or in protein and polysaccharide content, in worms about to destrobilate were detected, so a direct nutritional effect is unlikely. Nevertheless, it was found that the largest worms were the last to destrobilate, so it would appear that worm viability is directly related to destrobilization. The physiology of the small intestine is extremely complex and the requirements of cestodes, as judged by *in vitro* studies, are also complex, so it is likely that an alteration of a large number of different environmental factors could affect worm viability.

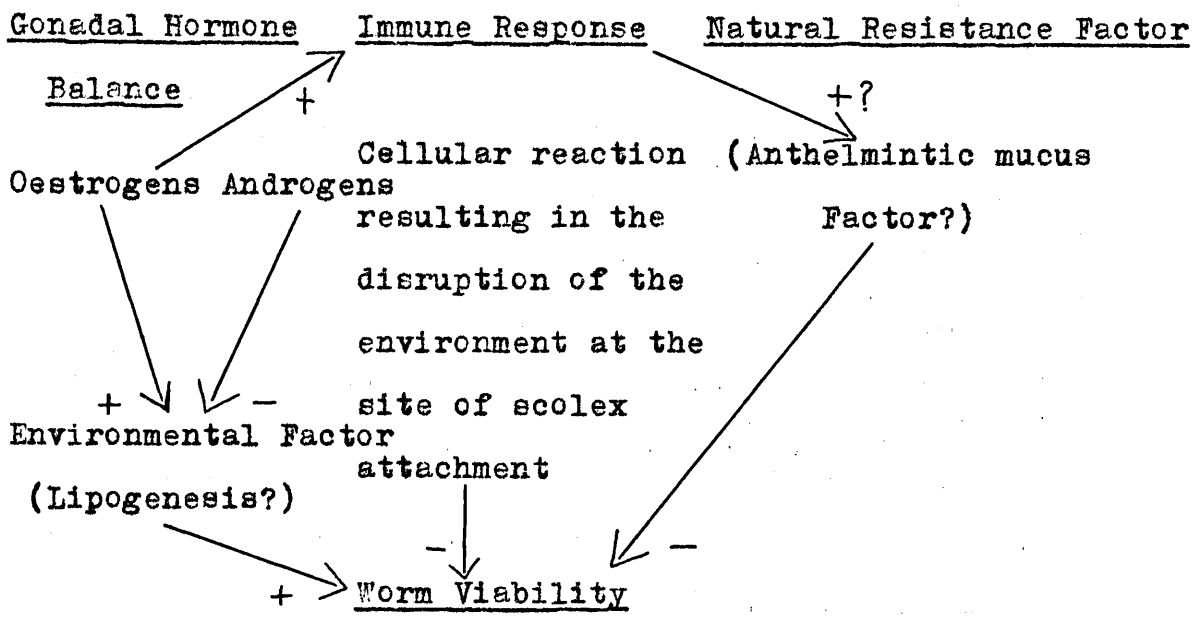
It has been established that worm destrobilization is caused by an alteration in the intestinal environment. The immune response evoked by *R. cesticillus* causes destrobilization and probably has its effect at the site of scolex attachment, since the cellular reaction is restricted to this area. The antigenicity of the scolex was demonstrated by immunofluorescence (Chapter 5). Smyth (1972) suggested that membrane-digestion might occur at the cestode scolex - host mucosa interface and presented considerable circumstantial evidence to support this hypothesis. If membrane-digestion takes place in *R. cesticillus* one would expect an immune reaction at the site of scolex attachment to cause considerable disruption of nutrient supplies and developmental stimuli.

The immune response may well contribute to age resistance to R. cesticillus, but there is almost certainly another factor involved, since immunosuppression with dexamethasone only depressed destrobilization values of 14 day old secondary infections in 70day old birds to about 50% (Chapter 4). This is about the same destrobilization value as that obtained with 14 day old primary infections in birds of this age, which suggests that the immune response plays little part in the destrobilization of short term infections. It is interesting to note that Hymenolepis microstoma destrobilates after about 9 days of infection in the rat (an abnormal host), but there appears to be no serological response and immunosuppressants did not prevent destrobilization (Goodall, 1972).

It would appear, therefore, that a physiological age resistance factor probably plays a part in the destrobilization of R. cesticillus. It is quite likely that this is the anthelmintic factor shown to be present in mucus extracts (Chapter 7) and is probably largely responsible for the rapid destrobilization of short term infections in adult male birds. This factor seems to be negated in some way by the oestrogens of mature female birds. The immune response probably plays a part in the elimination of worms in the growing chicken, in which the infection is present long enough to provide sufficient antigenic stimulus. The immune response is likely to play a greater part in the natural situation, in which repeated infection occurs.

The possible relationships of the various factors involved in the effect of host age on R. cesticillus are

represented schematically as follows:-



Age resistance can be regarded as a form of host specificity and Sandground (1929) suggested that in well adapted host-parasite systems age resistance is almost or entirely absent. From the studies presented here it would appear that R. cesticillus in the chicken is not a particularly well adapted host-parasite system and it is interesting to note that the progressive destrobilization of Hymenolepis diminuta and H. microstoma, demonstrated by Hopkins, Subramanian and Stallard (1972 a b) and Goodall (1972) respectively, occurred in abnormal hosts.

It is possible that the 'Apollo' strain of chicken used in these studies is unusually resistant to R.cesticillus. Strain differences in resistance to this cestode were reported by Todd, Hansen, Kelley and Wyant (1950), who found that hybrid De Kalb 105 cockerels withstood R. cesticillus infections better than pure bred New Hampshires,

but contained more worms.

A detailed comparison of the susceptibility of different strains of chickens to R. cesticillus was not carried out in the present study, but it was noted that destrobilization occurred in the 'White Link' strain, which is closely related to 'Apollo', and also in the unrelated brown-egger 'Ranger' strain. This suggests that destrobilization of R. cesticillus in the chicken is probably a normal feature of this host-parasite system.

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