IN VITRO CULTIVATION OF HETERAKIS GALLINARUM AND COOPERIA PUNCTATA

by

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TABLE OF CONTENTS

INTRODUCTION

The purpose of this study was an attempt to cultivate Heterakis gallinarum (Shrank, 1788) in vitro and to complete the life cycle of Cooperia punctata (Linstow, 1907) in vitro.

Heterakis gallinarum, the cecal-worm of fowl, belongs to the Phylum Nemathelminthes, Class Nematoda, Order Ascaridida, Family Heterakidae. Cooperia punctata, an intestinal worm of cattle, belongs to the Phylum Nemathelminthes, Class Nematoda, Order Rhabditida, Family Trichostrongylidae.

Heterakis gallinarum is economically important because it is the carrier of Histomonas meleagridis, the causative agent of enterohepatitis (blackhead) of turkeys. Cooperia punctata is economically important because in heavy infections it causes losses in young cattle.

Completion of the life cycle of these parasites in vitro would provide better understanding of their in vivo requirements; such knowledge may reveal a weak link in the life cycles where effective treatments could be employed.

REVIEW OF THE LITERATURE

Heterakidae, Ascaridiidae, and Ascaridae

No attempts to cultivate Heterakis gallinarum in vitro are reported in the literature. Malviya (1966) maintained Heterakis gallinarum eggs in tap-water for 175 days and conducted infectivity tests of the eggs from culture. Glocklin and Fairbairn (1952) and Fairbairn (1954) kept adult Heterakis gallinarum alive for 3 days in Krebs-Ringer solution. The worms consumed oxygen at an appreciable rate, but high oxygen tensions (266-380 mm) were toxic.

The closest taxonomic groups to Heterakis gallinarum on which in vitro cultivation studies are reported are in the Families Ascaridiidae and Ascaridae.

Ackert, et al., (1938) reported an increase in length of Ascaridia galli in incubating hen's eggs and in a starch-dextrose solution on cornmeal agar covered with sterile saline solution. Adult Ascaridia galli were maintained in saline solution by Guevara, et al., (1963).

Hoeppli, et al., (1938) kept Ascaris larvae alive in a medium containing horse or guinea-pig serum; no development was reported.

Pitts and Ball (1955) maintained Ascaris lumbricoides var. suum larvae in vitro for 100 days at 25 C in a complex medium containing hog serum, raw hog liver extract, glucose, and yeast extract. No ex-sheathing was observed.

Pitts (1962) supplemented Eagle's balanced salt solution with rabbit serum to obtain a 100% increase in the size of Ascaris lumbricoides var. suum larvae. First-stage exsheathing was observed.

Cleeland and Laurence (1962) hatched larvae from infective eggs obtained from the dissected uteri of female Ascaris lumbricoides var. suum. They reported growth, changes in internal structure, and ex-sheathing of the larvae in medium-199 in a deep culture tube system maintained at 37 C.

By the supplementation of Eagle's medium or medium-199 with calf or bovine serum, Cleeland (1963) was able to show an increase in the length of Ascaris.

Advancement of Ascaris lumbricoides var. suum larvae from second stage to third stage in a two media system was reported by Levine and Silverman (1969). They used serum-supplemented Caenorhabditis briggsae medium and serum-supplemented Eagle's medium.

Trichostrongylidae

No reports are given in the literature on the completion of the life cycle of a nematode of the Family Trichostrongylidae in vitro. However, since the attempts of Glaser and Stoll (1938a,b) to cultivate Haemonchus contortus, many reports are given on the partial completion of the life cycles of various species of nematodes of the Family Trichostrongylidae.

Stoll (1940) reported the advancement of Haemonchus contortus from third-stage larvae to fourth stage by incubation in salt solution. Silverman (1959) reported on the cultivation of parasitic stages of Haemonchus contortus and Ostertagia spp.

Leland (1961) reported the in vitro cultivation of the parasitic stages of Cooperia punctata, Cooperia oncophora, Ostertagia ostertagi,

and Ostertagia circumcincta in Ae medium.

In attempting to produce axenic cultures of Hyostrongylus rubidus, Diamond and Douvres (1962) found their cultures to be contaminated with Oesophagostomum quadrispinulatum; development to fourth stage was observed for both species.

Douvres and Alicata (1962) cultured Cooperia punctata from artificially exsheathed third-stage larvae to sexually immature fifth-stage adults in vitamin supplemented media (media SM-1, Douvres,1962).

Leland (1962) was the first to report the in vitro cultivation of Cooperia punctata from egg to egg.

Leland (1963) cultivated parasitic stages of Cooperia punctata, Ostertagia ostertagi, and Ostertagia circumcincta from third-stage larvae to fifth-stage (egg-laying) adults in Ae medium.

Development of Haemonchus contortus from third-stage larvae to fourth stage in salt solution containing carbon dioxide (40%) was shown by Sommerville (1964, 1966).

Hansen, et al., (1966) cultured Haemonchus contortus from third-stage exsheathed larvae to the beginning of the fourth molt in a chemically defined medium supplemented with a proteinous growth factor.

Leland (1967) reported the cultivation of Cooperia pectinata from artificially exsheathed third-stage larvae to egg-laying (infertile) or sperm-containing adults in Ae medium.

Leland (1967) reported the in vitro cultivation of Cooperia punctata from egg to egg (infertile). Non-parasitic stages were cultured in A-s medium (Ae less serum component); parasitic stages were cultivated in Ae medium. Infertile eggs were produced by virgin females; infertile eggs were also observed in male-containing cultures.

The in vitro cultivation of Cooperia oncophora from third stage to fifth stage in Ae medium was reported by Leland (1968).

Leland (1969) cultivated Hyostrongylus rubidus from artificially exsheathed third-stage larvae to egg-laying or sperm-containing adults in Ap medium (Ae medium with calf serum replaced by pig serum).

Leland (1967) indicates the in vitro fertilization as the final step to the completion of the life cycle of Cooperia punctata in vitro.

MATERIALS AND METHODS

Source and Preparation of Organisms for Inoculations

Naturally infected chickens were the source of Heterakis gallinarum. Adult worms flushed from the cecal contents were stored in saline solution at 4 C until removal of eggs. Eggs were removed from adult worms using the procedures of Hansen, et al., (1954). The worms were combined with a digestion fluid of pepsin and hydrochloric acid (0.1%; 0.5%) and ground using a mortar and pestle. The mixture was passed through an 80-mesh screen, allowing the intact eggs and fluid to fall into glass petri dishes. When the eggs had attached (natural adhesiveness) to the bottom of the dishes (2 minutes) they were washed 3 times with saline. The saline was replaced with 0.1% formaldehyde. Incubation was for 14 days at 27 C, with a change of formaldehyde twice weekly. Embryonated eggs were de-shelled in a solution of Clorox (1.5%) and sodium hydroxide (1.5%) overnight and washed twice in BSSA. Sterile technique was used thereafter. Larvae (second-stage) were hatched from the vitelline membrane by grinding in a Tenbrooke tissue grinder. They were then placed in BSSA for 1 hour. The volume of the larval suspension was adjusted to give a concentration of approximately 200 larvae in 0.2 ml inoculum.

Vermiculite cultures were made with feces taken by rectal collection from a donor calf containing a monospecific infection with Cooperia punctata. Third-stage infective larvae were isolated from 14-day-old vermiculite cultures by the Baermann technique. Larvae were washed 3 times in tap-water and stored at room temperature in tap-water in

screw-cap jars. Prior to inoculation of media, larvae were washed 3 times in tap-water and 2 times in sterile distilled demineralized water. The larvae were then treated with Clorox (2.0%) for 10 minutes, centrifuged 5 minutes at 100 g, washed 2 times in sterile distilled demineralized water, 2 times in BSSA, and allowed to stand 30 minutes in BSSA. The volume of the larval suspension was adjusted to give the desired number of larvae (normally 1000-1500) in 0.2 ml inoculum.

Media

Media used were Ae (Leland, 1963) or modifications of Ae (see below). Standard Ae medium contained the following:

50 ml	chick-embryo extract (CEE_{50})
15 ml	serum from helminth-free calves
15 ml	cystine-fortified sodium caseinate
5 ml	vitamin mixture
5 ml	liver extract
1 ml	antibiotic mixture
9 ml	Balanced Salt Solution-Antibiotic (BSSA)
100 ml	

Modifications of Ae medium were made by 1) replacement of calf serum with chicken serum for Ac medium; 2) replacement of calf serum with pig serum for Ap medium; 3) replacement of calf serum with pig serum and BSSA blank with 3.1 ml of a ten-fold concentration of medium 199 and 5.9 ml sterile distilled demineralized water for Ap199 medium; 4) replacement of calf serum with BSSA blank for A-s medium; and 5) for Ae-pre medium, CEE_{50} was mixed with sodium caseinate, quick frozen

at -70 C, and stored at -20 C for 14 days (the mixture was then thawed, the remainder of Ae components added, and refrozen at -70 C).

Sterile technique was used in all procedures. All glassware was tissue cultured cleaned (Leland, 1963) and chilled before use. Chick-embryo extract was prepared the day media were formulated. Components were combined in the order given.

Chick embryos (11-day) were aseptically removed from eggs and washed in BSSA at 4 C. An equal weight-volume ratio of embryos and BSSA was blended 3 minutes in a Waring blender and allowed to extract 1 hour at 4 C. The preparation was centrifuged 20 minutes at 4 C and 1800 g and allowed to stand an additional 20 minutes at 4 C. The extract (CEE_{50}) was drawn off and pooled.

Serum was obtained from blood taken aseptically from appropriate donor animals. After blood was allowed to clot 1-6 hours at room temperature, it was refrigerated overnight at 4 C. The serum was then drawn off, centrifuged for 1 hour at 4 C and 1800 g, and stored at -20 C.

Sodium caseinate was formulated by combining 4 gm vitamin free casein (Nutritional Biochemical Incorporated), 50 mg cystine, and 150 ml distilled water. After addition of 1 N sodium hydroxide until the solution was complete, 1 N hydrochloric acid was used for back titration to pH 7.2. The volume was adjusted to 200 ml with distilled demineralized water and the solution autoclaved at 15 lbs for 15 minutes. Storage was at -20 C.

Eagle vitamin concentrate (Microbiological Associates Incorporated) containing 0.1 mg/ml biotin, choline, folic acid, nicotinamide, pantothenic acid, pyridoxal, thiamine, and 0.01 mg/ml riboflavin was stored

at -20 C.

Liver extract (2.0%) was formulated by the addition of 1 mg liver concentrate (no. 202-20, Sigma Chemical Company) to 50 ml BSSA. After the solution was passed through a Millipore filter(0.45μ), it was stored at -20 C.

The antibiotic mixture of 2 gm crystilline dihydrostreptomycin sulfate (Squibb), 2 million units buffered potassium penicillin G (Squibb), and 500,000 units Nystatin (mycostatin, Squibb) in 50 ml sterile distilled demineralized water was stored at -20 C.

Balanced salt solution-antibiotics (BSSA) was formulated by adding 100 ml Earle's concentrate, 4 ml phenol red, and 20 ml sterile sodium bicarbonate to 878 ml sterile distilled demineralized water. The pH was adjusted to 7.2 - 7.3 with sterile carbon dioxide gas. After the addition of 5 ml antibiotic mixture, the pH was again adjusted to 7.2 - 7.3 with sterile carbon dioxide gas or sodium bicarbonate. Storage was at 4 C.

Sterile carbon dioxide gas or sterile sodium bicarbonate was used to adjust the pH of the various media to 7.2 - 7.3. An automatic pipette connected to a Salvarsan flask was used to dispense 2 ml medium to each screw-cap culture tube (16 x 125 mm). Tubes containing media were sealed and quick frozen at -70 C in an alcohol-dry ice bath. Storage was at -20 C.

Heterakis gallinarum were cultured in Ac, Ae, Ap, Ap199 media and BSSA as a control. Cooperia punctata were cultured in Ae, A-s, and Ae-pre media.

Culture Manipulations

Each tube of medium (38.5 C) was inoculated with 0.2 ml of a larval suspension (see inoculation procedures). Incubation was in roller drums (1/5 rpm) at 38.5 C. Either weekly or twice weekly worms were washed in BSSA and transferred to tubes of fresh medium.

Near the end of the study, the time interval between subsequent transfers of Heterakis gallinarum was extended to 4 weeks. In an attempt to assist molting of larvae from second to third stage, glass homogenizing beads (tissue culture cleaned, Leland, 1963) were placed in tube 129 for 19 days. Worms in tubes 111 and 129, initially inoculated into Ap199 medium, and tube 132, initially inoculated into Ap medium, were later transferred to Ae medium when the supply of the original medium was exhausted.

Manipulation of cultures receiving ^{3}H-thymidine was as described above except for procedures used in the addition and removal of the isotope (see radioisotope techniques below).

For combining different aged cultures, worms 15 days old (tubes 190, 191, 192, 193, 194, and 195) were placed respectively in tubes containing worms 29 days old (tubes 200, 201, 202, 203, 204, and 205). Subsequent culture manipulations were as described above.

Manipulation of the series of cultures (tubes 170, 182, 183, 184, 185, 186, 187, 188, 189, 196, 206, 207, and 208) containing worms showing embryonation, hatching, and development of larvae were as described above except for the number of larvae inoculated into each tube; the level of inoculum in this population density study ranged from approximately 200 larvae per culture (tube 196) to 2100 larvae per culture (tube 186).

First- and second-stage larvae cultivated in vitro were, upon hatching, transferred to Ae medium (38.5 C), or BSSA, Ae, or A-s medium at 27 C. Eggs and first- and second-stage larvae removed from tube 196 were placed on slides for photographing.

Measurements (taken by ocular micrometer) of Cooperia punctata isolated from feces by the Baermann technique at 24 hours (first-stage), 48 hours (second-stage), and 14 days (third-stage) were used to classify larvae that were produced in vitro.

Radioisotope Techniques

Tritium-labeled thymidine (100uC, sp. act. 17.9 C/mM, New England Nuclear) was added to each tube (29 to 42 day old cultures of Cooperia punctata in Ae medium). Following the exposure times of from 30 minutes to 49 days, ^{3}H-thymidine was removed from cultures by washing 5 times in BSSA. Unincorporated ^{3}H-thymidine was removed from worms by the addition of thymidine to cultures or by treatment of prepared slides with 5.0% trichloracetic acid (4 C).

The methods of slide preparation used were 1) fixation of live worms by treatment with formal-acetic acid (10% formaldehyde, 3% acetic acid) for 24 hours or fixation of live worms by treatment with 2.5% phosphate buffered glutaraldehyde at 4 C for 24 hours, followed by crushing worms on the slides; or 2) air drying and heat fixation of sperm (or egg) smears made from live worms, followed by treatment with 5.0% trichloracetic acid at 4 C for 5 minutes.

Autoradiographic techniques used were modifications of the procedures of Nollen (1969) and Gude (1968). In a darkroom, Kodak Nuclear Track

Emulsion NTB2 (Eastman Kodak Company) was melted in a water bath at 45 C, combined with an equal volume of distilled demineralized water, and allowed to stand for 15 minutes at 45 C. Two slides placed back to back were dipped into the liquid emulsion, allowed to drain, and air dried. Ten such slides and a small bag of silica gel were placed in small black slide boxes. The edges of the boxes were taped with black plastic tape. Each box was wrapped in aluminum foil and stored for the exposure of the nuclear emulsion at 4 C for 10-25 days.

Following exposure, development of the nuclear emulsion was for 6 minutes in Kodak Microdol-X. Fixation was in Kodak Acid Fixer for 1 minute. The slides were washed 1 minute in tap-water and air dried. Autoradiographs were microscopically examined for silver grains which indicate the location of incorporated ^{3}H-thymidine.

Background counts were made on clean slides coated with nuclear emulsion (with storage for 10-25 days at 4 C) and on areas adjacent to biological material on prepared slides.

Test of Infectivity for Heterakis gallinarum larvae maintained in culture

A 25-day-old chicken was inoculated by the surgical implantation of 60 Heterakis gallinarum larvae (maintained in culture for 190 days) into the left cecum.

An incision was made through the body wall of an etherized bird to expose the left cecum. The larvae were injected into the cecum, the incision sutured, and the bird revived. Beginning 25 days after implantation, feces were examined daily by the salt floatation technique for the presence of eggs. Examinations were continued for 20 days.

RESULTS

In Vitro Cultivation of Heterakis gallinarum

Data concerning the in vitro cultivation of Heterakis gallinarum are presented in Table I.

All larvae inoculated into BSSA control (tube 115) were dead by day 7; no increase in size beyond the length of second-stage larvae (235μ) reported by Clapham (1933) was observed. Larvae were maintained in culture for a maximum of 248 days (tube 111).

Some precipitation was present in all tubes during the study. Many larvae died soon after inoculations. Dead larvae became entrapped in precipitation which accumulated on the walls of the culture tubes. In the transferring procedure these larvae were eliminated from the culture. This accounts for the small number of larvae present in each tube at the end of the study.

Partial development of the pre-anal sucker of larvae (see Clapham, 1933, for a description of the pre-anal sucker development) was noted at day 16 in tube 126, day 40 in tube 103, day 44 in tube 111, and day 88 in tubes 129 and 132. No development of the pre-anal sucker was observed on larvae from tubes 107 or 115.

By the end of the study, larvae in all cultures (except tube 115) had increased in length from the 235μ reported by Clapham (1933) as the average length of second-stage larvae. The average length of larvae (in each tube) ranged from 367.5μ (tube 103) to 410.8μ (tube 111). Larvae from tube 107 (terminated at day 36 because of loss of carbon dioxide gas from the tube) averaged 270μ. Larvae cultivated in Ac

TABLE I. In Vitro Cultivation of Heterakis gallinarum

Tube No.	Medium	Duration of Culture (Days)	Survival of Worms (No.)	Avg. Length (μ)
103	Ac	204[1]	10	367.5
107	Ae	36[1]	4	270.0
111	Ap199-Ae[2]	248	16	410.8
126	Ac	190	11	369.0
129	Ap199-Ae[2]	209	21 (+60)[3]	382.5
132	Ap-Ae[2]	217	13	387.0
115	BSSA[4]	7	*	235.0[5]

[1] Terminated due to loss of carbon dioxide.

[2] Transfer to Ae due to lack of Ap199 and Ap media.

[3] 60 larvae removed at day 190 for infectivity test.

[4] BSSA (Balanced salt solution-antibiotics) = control.

[5] Reported by Clapham (1933) as the average length of second-stage larvae.

* Not counted.

medium were shorter (367.5μ in tube 103 and 369μ in tube 126) than larvae maintained in Ap or Ap199 and Ae media (410.8μ in tube 111, 382.5μ in tube 129, and 387μ in tube 132).

The addition of glass homogenizing beads to culture 129 did not affect further molts.

Test of Infectivity for Heterakis gallinarum Maintained in Culture

Beginning 25 days after surgical implantation of larvae in the cecum of the test bird, 3 examinations of the feces were made daily to detect the presence of eggs: examinations were continued for 20 days. No Heterakis gallinarum eggs were found in the feces taken from the surgically infected bird.

Pre-mixing CEE_{50} with Sodium Caseinate

Data relevant to pre-mixing CEE_{50} with sodium caseinate are presented in Table II.

No precipitation was observed after thawing the frozen CEE_{50}-sodium caseinate mixture. Addition of the remainder of the Ae components resulted in a medium (Ae-pre) in which Cooperia punctata advanced to fifth-stage adults and produced eggs. Eggs were detected earlier in Ae cultures (25 days) than in Ae-pre cultures (29 days). The average numbers of eggs produced in vitro per tube were 196 in Ae cultures and 8.5 in Ae-pre cultures.

The average percentages of third- and fourth-stage larvae were greater in Ae-pre cultures (19.3% and 53.7%) than in Ae cultures (15.6% and 52.7%). The average percentage of adults was 31.7 in Ae cultures

TABLE II. Pre-mixing CEE_{50} with Sodium Caseinate (Cooperia punctata)

Tube No.	86	87	88	89	90	91	92	93	Avg Ae-Pre[1]	Avg Ae[2]
Medium	Ae-pre	Ae-pre	Ae-pre	Ae-pre	Ae	Ae	Ae	Ae	Ae-pre	Ae
Total worms recovered	2658	2500	2624	3000	2557	2317	2078	2078		
No. adults	728	815	703	630	623	763	732	709	719.0	708.0
" males	345	401	328	238	278	338	331	323	328.0	317.5
" females	383	414	375	392	350	425	401	386	391.0	390.5
" fourth stage	1323	1314	1399	1777	1516	1153	1050	1063	1453.2	1195.5
" third stage	607	371	522	593	413	401	296	306	523.3	354.0
Eggs produced in vitro	10	4	17	3	182	209	267	126	8.5	196.0
Age eggs detected[4]	32	32	29	35	28	25	25	32		
Per cent adults	27.4	32.6	26.8	21.0	24.6	32.9	35.2	34.1	26.9	31.7
" " males	13.0	16.0	12.5	7.9	10.9	14.6	15.9	15.5	12.4	14.2
" " females	14.4	16.6	14.3	13.1	13.7	18.3	19.3	18.6	14.6	17.5
" " fourth stage	49.7	52.6	53.3	59.2	59.3	49.8	50.5	51.2	53.7	52.7
" " third stage	22.8	14.8	19.9	19.8	16.2	17.3	14.2	14.7	19.3	15.6
Avg length males (mm)[3]	4.61	4.62	4.22	3.49	3.49	4.58	4.69	5.20	4.24	4.49
" " females (mm)[3]	5.04	5.56	5.25	3.80	4.82	5.94	5.63	5.94	4.91	5.58

[1] Average of all Ae-pre cultures.

[2] Average of all Ae cultures.

[3] Average length (mm) of ten largest males or females from each culture.

[4] Days.

and 26.9 in Ae-pre cultures. The length of adult male worms from Ae cultures averaged 4.49 mm while those from Ae-pre cultures averaged 4.24 mm. The length of female worms from Ae cultures averaged 5.58 mm while those from Ae-pre cultures averaged 4.91 mm.

Attempts at Labeling Worms with ^{3}H-thymidine

The best method for preparing sperm for autoradiography slides was method 3 (crushing live worms on slides followed by fixation).

Autoradiographs of sperm revealed no incorporation of ^{3}H-thymidine. No increase in the number of silver grains above background counts was observed in areas of emulsion over sperm or any worm material.

Combining Different Aged Cultures

Hundreds of eggs were produced in each of the 6 cultures (containing approximately 2000-3000 larvae per tube). However, no embryonation was observed.

Population Density Studies

Data concerning population density studies are presented in Tables III and IV.

In the series of 12 cultures (tubes 170, 182, 183, 184, 185, 186, 187, 188, 189, 206, 207, and 208), cultures 183, 184, 187, 196, 206, and 208 contained eggs laid in vitro which embryonated, hatched, and developed from first to second stage (see Plates I, II, and III). Culture 182 contained embryos which did not hatch.

TABLE III. Population Density Study (Cooperia punctata cultures showing embryonation, hatching, and development in Ae medium)

Tube No.	170	182	183	184	185	186	187	188	189	196	206	208
Total worms recovered	1350	1366	1442	1543	1742	2041	872	393	945	190	881	355
No. adults	273	391	345	320	275	313	285	65	345	83	348	131
" males	161	225	154	156	144	165	136	32	159	37	174	67
" females	112	166	191	164	131	148	149	33	186	46	174	64
" fourth stage	609	547	710	731	936	1183	278	134	321	55	319	137
" third stage	468	418	387	492	531	545	309	194	279	52	214	87
Age embryos detected [1]	-	57	57	70	-	-	57	-	57	48	42	33
No. eggs produced	2046	1788	1031	1937	687	760	2915	528	1222	862	2084	537
No. first stage [2]	0	0	21	25	0	0	0	0	6	103	121	3
" second stage [2]	0	0	13	18	0	0	0	0	1	50	15	2
Per cent adults	20.2	28.6	32.9	20.7	15.7	15.3	32.6	16.5	36.5	43.7	39.5	36.9
" " males	11.9	16.4	10.6	10.1	8.2	8.1	15.6	8.1	16.8	19.5	19.8	18.9
" " females	8.2	12.1	13.2	10.6	7.5	7.3	17.1	8.3	19.7	24.2	19.8	18.0
" " fourth stage	45.1	40.7	49.2	47.4	53.7	58.0	31.8	34.0	29.5	27.4	36.2	38.6
" " third stage	34.7	31.3	26.8	31.8	30.4	26.7	35.4	49.4	34.0	28.9	24.3	24.5
Avg length males [3]	5.06	5.16	5.10	4.81	4.81	5.30	4.90	4.68	5.18	4.73	5.00	4.50
" " females [3]	5.54	5.33	5.57	5.90	5.03	5.70	5.46	4.89	5.72	5.66	5.82	4.97

1 Age (days) when embryos were first detected in culture.

2 Number of first- or second-stage larvae remaining in original culture tubes. Refer to Table IV for larvae (first- and second-stage) removed from original cultures (listed above) and transferred into fresh media listed (at temperatures listed).

3 Average length (mm) of ten largest male or female worms from each culture.

Tube 207 (not listed) was a BSSA control. All larvae were dead at day 24. No development was observed.

TABLE IV. Larvae (Cooperia punctata) produced in vitro and transferred into fresh media[1]

Tube No.	Total No. First and Second stage transferred[1]	After Transfers				
		to Medium	at Temperature (C)	Worms Recovered		
				No. First Stage	No. Second Stage	No. Third Stage
196	96	BSSA	27	58	38	0
196	79	Ae	27	64	15	0
196	67	Ae	38.5	58	9	0
206	67	Ae	38.5	35	32	0
206	67	A-s	27	36	21	4
208	5	Ae	27	3	2	0

[1] First- or second-stage larvae were removed from original culture tubes (Ae at 38.5 C) (see Table III) and transferred to fresh media at temperature listed. Nearly all larvae transferred were first stage.

First- and second-stage larvae removed from cultures 187, 196, 206, and 208 and transferred into fresh Ae medium at 38.5 C, Ae at 27 C or BSSA at 27 C did not develop past second stage. Of the 61 in vitro hatched first- and second-stage larvae removed from culture 206 and transferred into A-s medium at 27 C, 4 developed to third stage.

The greatest numbers of first- and second-stage larvae hatched per culture were in tubes containing 43.7% adults (tube 196) and 39.5% adults (tube 206). No embryonation was present in cultures where the percentages of adult females and males was less than 10.6 or 10.1, respectively.

No relationship was observed between the number of larvae inoculated per culture tube (see inoculation procedures) and the presence of embryonation or hatching. However, embryonation was present only in tubes containing less than 1742 worms. In this experiment, no relationship was observed between the length of adult worms and the presence of embryonation or hatching.

The ages of cultures at the time embryonated eggs were first detected were 33 days for culture 208, 42 days for culture 206, 48 days for culture 196, 57 days for cultures 182, 183, and 187, and 70 days for culture 184.

DISCUSSION

In vitro cultivation of Heterakis gallinarum

Second-stage larvae inoculated into BSSA at 38.5 C died within 7 days, probably due to lack of nutrients.

Uribe (1922) reported the average length of embryos in infective Heterakis gallinarum eggs to be 180μ; Clapham (1933) reports the average length of second-stage larvae to be 235μ. Larvae inoculated in all media had increased in length (except for those in BSSA), indicating the media to be of value in cultivation.

The maximum increase in length of larvae was observed in culture 111 (Ap199-Ae media), where larvae averaged 410.8μ. This represents nearly a doubling in average length.

In vitro development of the pre-anal sucker was first noted in culture 126 (Ac medium) at day 16, and from day 44 to day 88 in other cultures (see data). Clapham (1933) reports in vivo development of the pre-anal sucker as occurring in fourth stage (96 hours post-infection), when the average length of larvae is 3-4 mm. Although growth and development is shown in vitro by this study, the rate and sequence of events is apparently altered.

In contrast to results with other species (Leland, 1963) increases in length were greater in media containing host heterologous serum (Ap199, Ap, and Ae media) than in medium containing host homologous serum (Ac medium).

Addition of a solid substrate (glass beads) did not affect further molts; some other stimulus is apparently required for further development.

Reports of the increase in length of other Ascaroidea (Ascaridia galli and Ascaris lumbricoides var. suum) in vitro are given by Ackert, et al., (1938) and Pitts (1962). However, there are no reports of the exsheathing of Ascaridia galli cultured in vitro. Cleeland and Laurence (1962) showed exsheathing of Ascaris lumbricoides var. suum larvae. Levine and Silverman (1969) reported the advancement of Ascaris lumbricoides var. suum from second stage to third stage in vitro.

Test of Infectivity of Heterakis gallinarum Maintained in Culture

No Heterakis gallinarum eggs were found in feces taken from the surgically infected bird. This indicates an apparent loss of infectivity of larvae maintained in culture for 190 days. Malviya (1966) reported that 147-day-old Heterakis gallinarum embryos were unable to develop in hosts. The extended maintenance in culture of non-parasitic stages of H. gallinarum results in a loss of infectivity. This indicates that the culture media were not adequate for development to this point in the life cycle even though there was an increase in size.

Pre-mixing CEE_{50} with Sodium Caseinate

No precipitation was observed after thawing the CEE_{50}-sodium caseinate mixture, indicating that CEE_{50} may be frozen for storage without danger of precipitation formation. However, the smaller percentage of adults, the fewer number of eggs, and the overall smaller size of adult worms cultivated in Ae-pre medium indicate Ae is a superior medium for the cultivation of Cooperia punctata.

In Ae-pre medium, the CEE_{50}-sodium caseinate combination was frozen

for storage, thawed for the addition of the remaining Ae components, refrozen for storage, and thawed for inoculations; the number of freezings and thawings may have caused a partial inactivation of some required constituent of the CEE_{50}.

Attempts at Labeling Worms with ^{3}H-thymidine.

The purpose of this study was to determine if fertilization was taking place in vitro since female worms are able to produce eggs in the absence of males (Leland, 1967). Males containing radioactively labeled sperm were to be cultivated with virgin females. Any resulting eggs were to be tested for radioactivity, indicating fertilization in vitro. Concurrent with this study were cultures showing embryonation, hatching, and development (see above). Because fertilization had taken place in vitro and incorporation of ^{3}H-thymidine was not observed, no further efforts were made to label Cooperia punctata sperm.

Combining Different Aged Cultures

This experiment was designed to see if male and female worms were not mating because they were maturing at different times in vitro. However, the embryonation shown in the population density study (see above) indicates this not the case.

No embryonation was observed in any eggs from the combined cultures. In the population density study (see above) no embryonation was observed in cultures where the total number of worms was above 1742 per tube. Since the total number of worms per tube in the combined cultures was 2000-3000 per tube (double the standard 1000-1500 worms per tube), the high popu-

lation density may have been inhibitory to fertilization or embryonation.

Population Density Study

Embryonation was observed in 8 of 12 tubes; hatching was observed in 7 of 8 tubes showing embryonation. The number of larvae inoculated per tube (below 1742/tube) apparently did not affect embryonation. High percentages of adults were apparently required for embryonation.

First- and second-stage larvae produced in vitro and transferred into fresh Ae medium (38.5 C or 27 C) did not develop to third-stage. Of 61 first- and second-stage larvae transferred into A-s medium at 27 C, 4 developed to third-stage. The serum component of Ae medium was apparently inhibitory to the non-parasitic stages of Cooperia punctata as reported by Leland (1967).

This study records the first in vitro cultivation of Cooperia punctata from artificially exsheathed third-stage larvae to fifth-stage adults which laid eggs that embryonated, hatched, and developed to third stage. The life cycle of no other nematode of the Family Trichostrongylidae has been completed in vitro.

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APPENDIX

EXPLANATION OF PLATE I

Fig. 1. *Cooperia punctata*. A cluster of eggs laid in vitro by worms cultivated in vitro, X 54.5.

Fig. 2. *Cooperia punctata*. Close-up of cluster of eggs (see above). Two embryonated eggs are seen, X 165.

THIS BOOK CONTAINS NUMEROUS PICTURES THAT ARE ATTACHED TO DOCUMENTS CROOKED.

THIS IS AS RECEIVED FROM CUSTOMER.

PLATE I

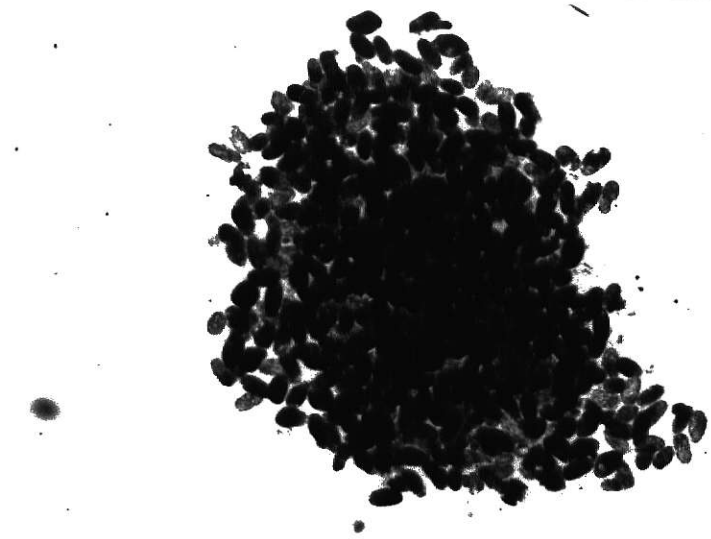

Fig. 1.

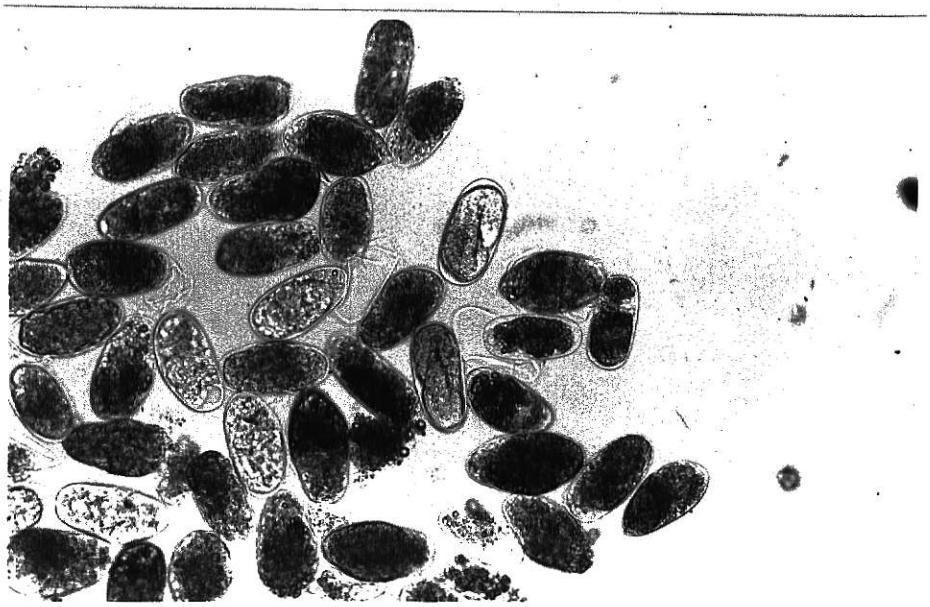

Fig. 2.

EXPLANATION OF PLATE II

Fig. 3. Cooperia punctata. In vitro produced embryo in the process of hatching. Several empty shells of larvae that hatched, X 330.

Fig. 4. Cooperia punctata. In vitro cultivated embryo (artificially hatched), X 330.

PLATE II

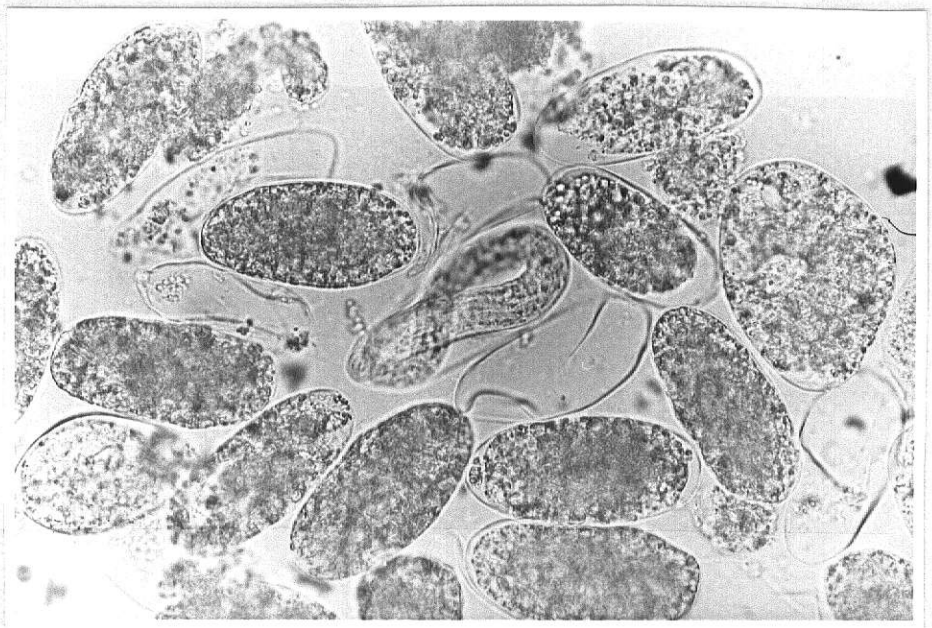

Fig. 3

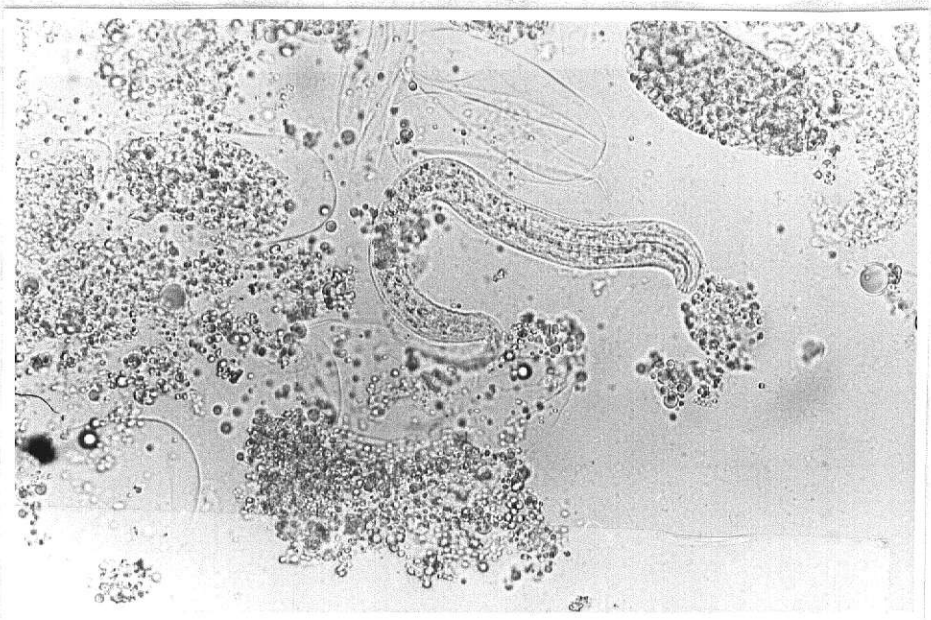

Fig. 4

EXPLANATION OF PLATE III

Fig. 5. Cooperia punctata. Three first-stage and one second-stage larvae hatched in vitro from eggs produced in vitro, X 330.

Fig. 6. Cooperia punctata. First-stage larvae produced in vitro. Fifth-stage adult (cultivated in vitro) included to show size relation, X 330.

PLATE III

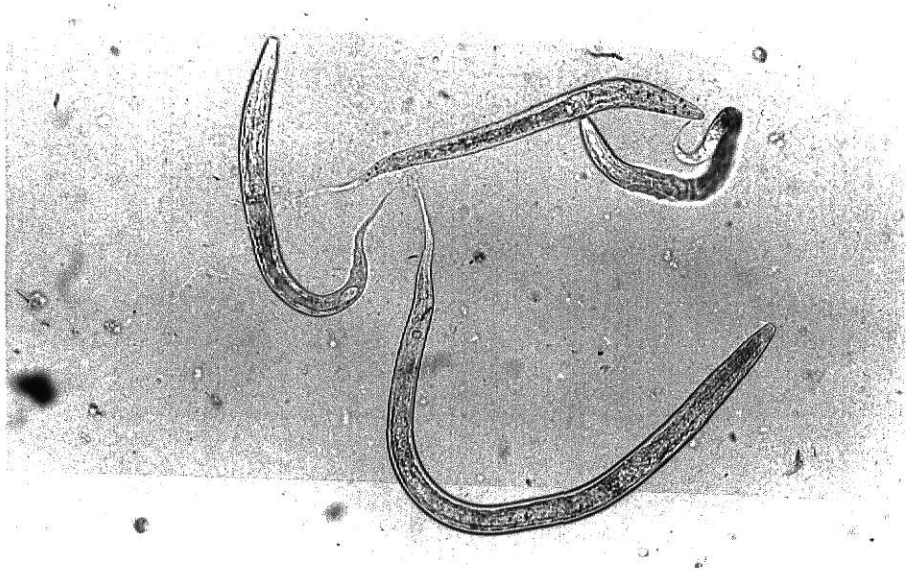

Fig. 5

Fig. 6

IN VITRO CULTIVATION OF *HETERAKIS GALLINARUM* AND *COOPERIA PUNCTATA*

by

GARY LEE ZIMMERMAN

B. S., Kansas State University, 1967

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

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1970

Heterakis gallinarum larvae were cultivated in vitro in Ae medium (Leland, J. Parasit. 49: 600-611, 1963) or modifications of Ae for a maximum of 248 days. Modifications of Ae included replacing calf serum with chicken or pig serum, or replacement of calf serum with pig serum and a replacement of a portion of the BSSA blank with medium-199. Larvae nearly doubled in length in addition to showing partial development of the pre-anal sucker. There was some evidence that larvae maintained in culture for 190 days lost their infectivity.

Attempts at pre-mixing CEE_{50} with sodium caseinate resulted in a medium (Ae-pre) in which Cooperia punctata developed from third-stage larvae to egg-laying adults; Ae, however, was shown to be a superior medium in terms of growth and egg production.

Attempts to label sperm of C. punctata with tritium-labeled thymidine were unsuccessful.

Third-stage C. punctata larvae were cultivated in vitro to adult worms which laid eggs that embryonated, hatched, and developed to third stage. Parasitic stages were cultivated in Ae medium; free living stages were cultivated in A-s medium (Leland, J. Parasit. 53: 1057-1060, 1967). Although few larvae developed to third stage in this study, it does prove that the life cycle of C. punctata can be completed in vitro. This is the first record of a nematode of the Family Trichostrongylidae completing its life cycle in vitro.