THE UNIVERSITY OF MANITOBA

A STUDY ON THE SEXUAL ATTRACTION AND EFFECT OF CYCLOHEXIMIDE AND HYDROXYUREA ON MATING BEHAVIOUR AND ITS DEVELOPMENT IN THE FREE-LIVING NEMATODE PANAGRELLUS SILUSIAE (DE MAN 1913) GOODEY, 1945 (NEMATODA : CEPHALOBIDAE)

BY

RONALD CHI-TUN CHENG (B.Sc. Hons.)

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TO MY

PARENTS

ABSTRACT

Female Panagrellus silusiae produces a substance that attracts males. Males do not produce attractant, nor is there homosexual attraction. The attractant is waterborne and forms a gradient in agar. The attractant is first produced by females during the fourth juvenile stage and males first respond to the attractant during the fourth juvenile stage. Female Panagrellus redivivus (strain B) also produce an attractant, but the attractants of each species only attract males of that species.

Female Panagrellus silusiae that have undergone postembryonic development in hydroxyurea do not attract males, and males so treated do not respond to normal females. Nematodes placed in hydroxyurea as adults have normal gonads and retain the mating attraction system. Hydroxyurea has no effect upon copulatory behaviour of adult animals. Treatment of adult females with cycloheximide stops production of attractant, although production resumes within 24 hours after removal of the females from cycloheximide. Cycloheximide treatments of males has no effect upon the response to attractant by males or on copulation. It is proposed that sexual

attraction depends on complete development of the reproductive system, but that copulation does not.

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ABBREVIATION.

expts	:	experiments
L_2	:	second stage juveniles of *Panagrellus silusiae*
L_3	:	third stage juveniles of *P. silusiae*
L_4	:	fourth stage juveniles of *P. silusiae*
S^2	:	variance of number of males of 10 experiments

INTRODUCTION

Little is known of the mechanism by which specific information encoded in the genetic material is expressed as genetically controlled behaviour. One experimental approach to these mechanisms is the examination of behavioral mutants to determine the morphological or biochemical basis of the altered behaviour. Another approach is examination of development of behavioral patterns that occur only during specific phases of the life cycle of an organism. In this latter class of behavioral characteristics are those associated with mating in dioecious organisms. Behaviour such as sexual attraction and copulation are usually restricted to the adult phase of the life cycle. Nematodes are good experimental animals for the study of such behaviour because of their relatively short, simple life cycle, their approximate eutely, and the ease with which large numbers of synchronously growing animals can be obtained. (Samoiloff and Pasternak 1968).

Sexual attraction has been demonstrated in several free-living and parasitic nematode species. Males are attracted to females of the same species in

Ancylostoma caninum (Roche 1966), Pelodera teres (Jones 1966), Heterodera rostochiensis and H. schachtii (Green 1966), and Cylindrocorpus longistoma and C. curzii (Chin and Taylor 1969). Mutual attraction between males and females has been reported for Panagrolaimus rigidus (Greet 1964) and Trichinella spiralis (Bonner and Etges 1967). Sexual attraction in nematodes appears to be mediated via a waterborne species specific chemical. Green (1966) found that the Heterodera sex attractants are water soluble and pass through dialysis membranes, and that the attractants produced by females of Heterodera schachtii did not volatilize enough to form perceptible vapour gradients (Green 1971).

Postembryonic development of the free-living nematode Panagrellus silusiae (de Man 1913) Goodey 1945 has been studied at the morphological (Samoiloff and Pasternak 1968, 1969; Sin and Pasternak 1970; Samoiloff 1970; Pasternak and Samoiloff 1972) and biochemical (Chow and Pasternak 1969; Pasternak and Samoiloff 1970; Samoiloff and Smith 1971) levels. As molting fourth stage juvenile females undergo dissolution of the old cuticle during ecdysis and no such dissolution occurs during other molts (Samoiloff and Pasternak 1969),

Samoiloff (1970) proposed that the dissolved cuticular material was involved with sexual attraction. Such an attractant would be produced only by females, be waterborne, be capable of forming a gradient from the females, and should be produced at the time of ecdysis only.

The first part of this investigation was designed to determine if mating attractant is produced by P. silusiae females and if so, at what stage in their postembryonic development it is produced. Positive results of the first part of this investigation enable further studies on the mating attraction system (production of attractant by females and the response to attractant by males) from a developmental point of view. The second portion of this investigation reports the effects of the growth inhibitors hydroxyurea and cycloheximide (actidione) on sexual attraction and copulation in P. silusiae.

MATERIALS AND METHODS

A. Nematode Culture

Panagrellus silusiae and *Panagrellus redivivus* were maintained xenically on 4% Czepex Dox agar in plastic Petri plates at room temperature. Virgin adult worms were obtained by growing juvenile worms individually in depression slides containing Nigon's agar (Nigon 1949). Other worms used in this study were selected directly from the stock cultures.

B. Test for sexual attraction in adult worms.

Tests for sexual attraction were done on V-shaped 2% agar strips modified from those described by Chin and Taylor (1969). The arms of the V were 1.5cm long by 0.5cm wide, leading to a circular zone 1cm in diameter at the end of each arm. These circular terminal zones were covered with 1cm circle of Whatman number 2 filter paper which supported a polyethylene ring 0.5cm high cut from "BEEM" capsules forming a chamber from which animals could not escape, though material could diffuse from the chamber and down the agar arms. Two drops of 2% agar were placed in both chambers and animals tested for production of attractant were placed only in one chamber (test chamber). The inoculation

zone, at the apex of the V, contained the animals tested for response to the attractant. (Fig. 1)

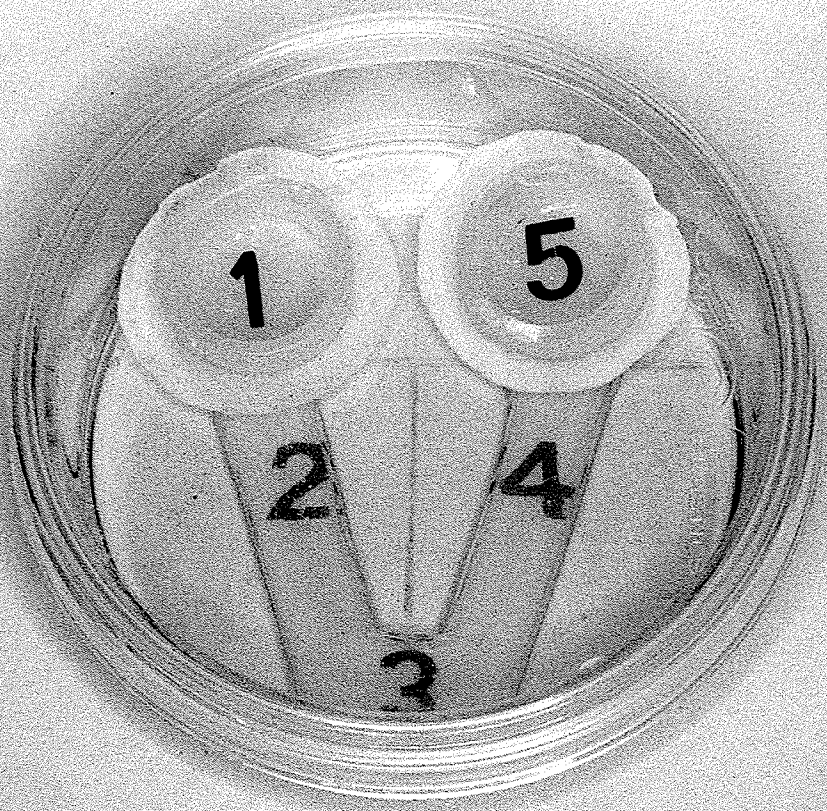
A group of 20 males, virgin females, or gravid females was placed in the test chamber and water was placed in the control chamber at the end of the other arm. After 24 hours to permit diffusion of attractant from the test chamber, 10 males or females were placed in the inoculation zone at the apex of the V. Seven intraspecific pairing experiments were carried out with P. silusiae. The location of the inoculated worms was recorded at hourly intervals for 4 hours. Each experiment was repeated 10 times. Similar tests were carried out with P. redivivus males in the inoculation zone and P. silusiae females in the test chamber. Each of these interspecific pairing experiments was repeated 10 times.

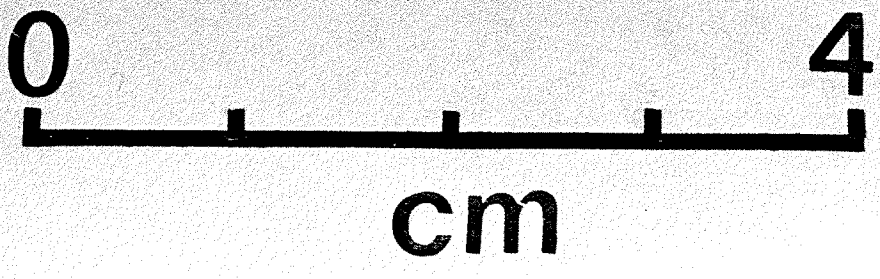
C. Test for the presence of a mating attractant gradient.

To determine if the attractant formed a gradient in the agar strip, 20 females were placed in the test chamber and after 24 hours four agar blocks (1 X 5 X 5 mm) were cut from the agar under the test chamber, the arm leading to the test chamber (the test arm), and the arm leading to the control chamber (the control arm). These blocks were placed at the periphery of a 35mm Petri dish containing a layer of

Figure 1. Photograph of the V-shape 2% agar strips apparatus used for the study of sexual attraction.

1 = test chamber
2 = test arm
3 = inoculation zone
4 = control arm
5 = control chamber


1
5
2
4
3


0
4
cm

2% agar 0.5 mm thick. Each block was placed 90 degrees from adjacent blocks (Fig. 2). Twenty-five males were placed in the inoculation zone in the center of the Petri dish and 8 hours later the number of males in each quadrant was recorded. Each experiment was repeated 10 times.

D. Test to demonstrate that the attractant is waterborne.

A glass quadrant Petri dish 100 mm in diameter was used in which each quadrant was separated from the others by a glass wall (Fig. 3a). Two opposite quadrants were partially filled with Nigon's salt solution. One of these quadrants was inoculated with 50 adult P. silusiae females. 24 hours later, 50 adult P. silusiae males were placed in the opposite quadrant. After a further 24 hour the distribution of males was recorded. Fluid exchange between these quadrants was then permitted via a wick of Whatman number 2 Filter paper (Fig. 3b) 1mm wide. The distribution of males was recorded after 4 hours. Each experiment was repeated 10 times and the results were compared with those of the control experiments.

Figure 2. Diagram of the apparatus used in testing for the gradient of mating attractant. Agar blocks 1 - 4 were obtained at increasing distances from P. silusiae females, although females were not in direct contact with any of the blocks. Males were placed in the inoculation area and their distribution was recorded after 24 hours.

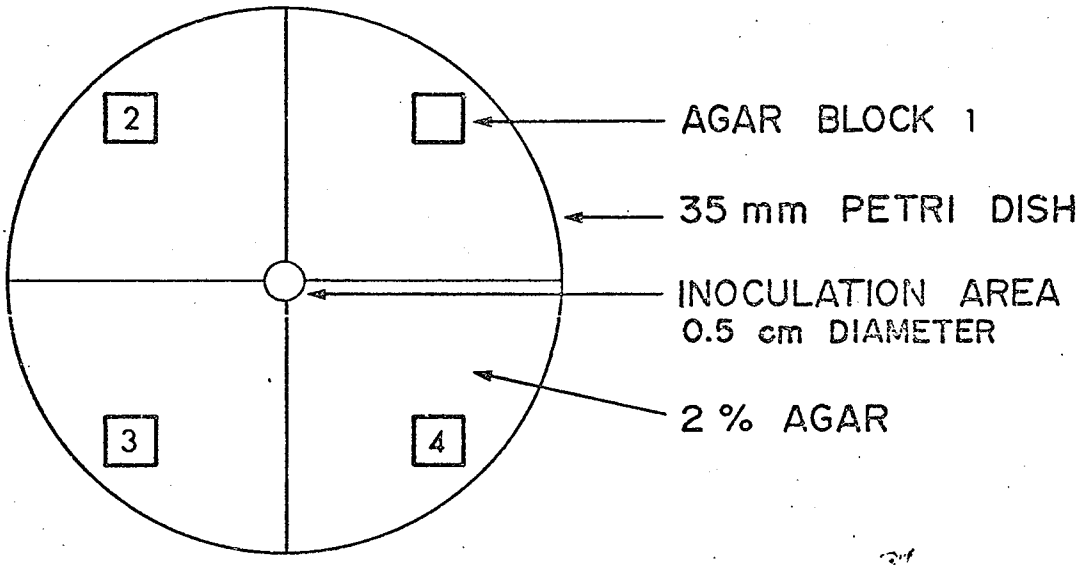
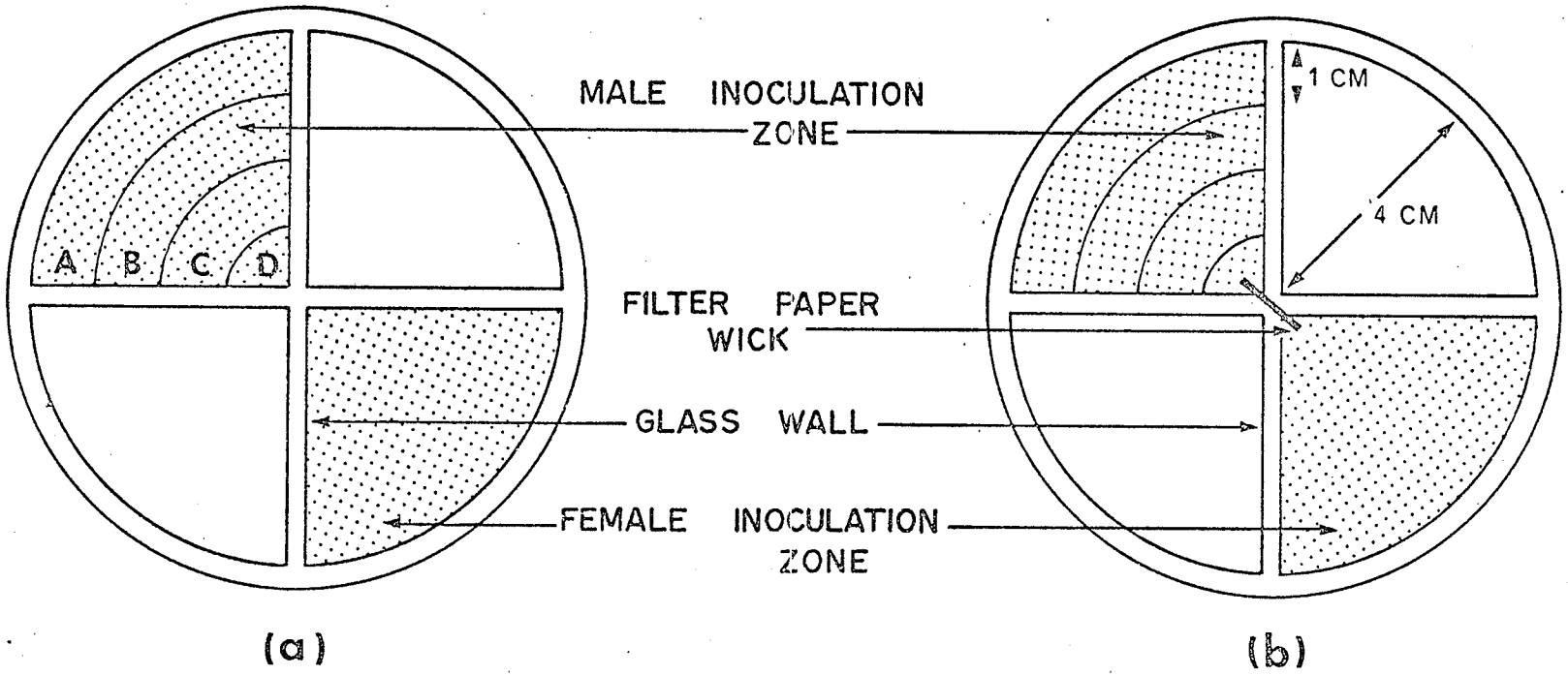

2
AGAR BLOCK 1
35 mm PETRI DISH
INOCULATION AREA
0.5 cm DIAMETER
2 % AGAR
3
4

Figure 3. Diagram of the apparatus used in the test to demonstrate that attractant is waterborne. Shaded quadrants contain salt solution. Males were placed in quadrant divided into radians 1cm thick (A,B,C,D). Females were placed in the other quadrant. After 24 hours the distribution of males was observed. Males and females quadrants were then connected (b) by a wick of filter paper and distribution of male observed 4 hours later.


MALE INOCULATION ZONE
A
B
C
D
FILTER PAPER WICK
GLASS WALL
FEMALE INOCULATION ZONE
1 CM
4 CM
(a)
(b)

E. Test for sexual attraction in juvenile P. silusiae.

To test for the production of attractant by juvenile P. silusiae, 50 L_2, L_3, or L_4 juveniles were placed in the test chamber and 10 adult males were placed in the inoculation zone. As the sex of living juveniles cannot be recognized before the fourth juvenile stage, L_2 and L_3 juvenile populations in the test chamber contained both males and females; all L_4 juveniles in any one experiment were of one sex. The distribution of adult males was recorded 8 hours after inoculation and each experiment repeated 10 times.

To test for the response of juvenile worms to mating attractant, 20 adult females were placed in the test chamber and 10 L_2 or L_3 juvenile worms were placed in the inoculation zone 24 hours later. The location of these juvenile worms was recorded and they were picked up by a micropipet, placed separately in Nigon's agar, and grown to adult stage to determine their sex. The response of L_4 juveniles to the attractant was tested by placing 10 male or female L_4 juveniles in the inoculation zone and 20 adult females in the test chamber. The distribution of the L_4 juvenile worms was recorded 8 hours after inoculation. Each experiment was repeated 15 times.

F. Hydroxyurea and Cycloheximide.

Experimental worms were grown in three-spot depression slides containing Nigon's agar (1949). L_2 juveniles were removed from stock cultures using an isolation procedure described by Samoiloff and Pasternak (1968) (Appendix 1). These juveniles were grown either individually to provide virgin animals or in populations of about 200 per depression to provide animals for sexual attraction experiments.

Hydroxyurea and cycloheximide were purchased from Calbiochem, Los Angeles, California. Hydroxyurea was used at a final concentration of 400μg/ml and cycloheximide was used at 200μg/ml. Juvenile worms grown in hydroxyurea reached adult size within 72 hours or more. Adult worms were exposed to hydroxyurea for 24, 48 or 72 hours. all hydroxyurea-treated animals were washed in distilled water immediately before testing. Cycloheximide-treated adults were maintained in inhibitor for 24, 48 or 72 hours. Half the cycloheximide-treated animals were washed and tested immediately, while the remainder were washed and placed in distilled water over Nigon's agar for 24 hours before testing.

Hydroxyurea, a specific inhibitor of DNA synthesis (Young and Hodas 1964), was used on both adult and developing juvenile worms since in P. silusiae it prevents the development of the reproductive tract without markedly slowing general growth (Pasternak and Samoiloff 1970). Furthermore, since hydroxyurea does not block formation of the copulatory apparatus of males or the gonopore of females, copulatory behaviour as well as sexual attraction was studied in worms lacking functional gonads.

Cycloheximide, an inhibitor of protein synthesis (Siegel and Sisler 1963), was used only on adult worms since larval growth and gonad development do not occur in this compound (Pasternak and Samoiloff 1970). Experiments were also carried out to determine if cycloheximide effects on sexual attraction are reversible as in other organisms (de Kloet 1966).

After completion of the test for mating attraction the animals used for the test were fixed and stained with propionic orcein (Mulvey 1960) for examination of their gonad morphology (Appendix II).

Copulatory behaviour was observed in depression slides containing Nigon's salt solution into which were placed 20 virgin males and 20 virgin females. Observations were made of clumping of worms before copulation, copulation, and fecundity of the matings for the following test combinations:

(i) 72 hour hydroxyurea treated males with control females;

(ii) hydroxyurea grown males with control females;

(iii) hydroxyurea grown males and hydroxyurea grown females;

(iv) hydroxyurea grown females with control males;

(v) 72 hour cycloheximide treated males with control females;

(vi) control males with 72 hour cycloheximide treated females; and

(vii) control males with control females.

RESULTS

A Sexual Attraction in Adult Worms.

Male P. silusiae were attracted only to females (Table I). The number of males that migrated towards the test chamber containing either virgin or gravid females was significantly greater ($p<0.001$) than the number of males migrating towards the control chamber. There was no immediate response of P. silusiae males to females in the test chamber but the response was detectable after 2 hours after inoculation (Fig 4b & 4c). By the end of the fourth hour about 40% of the males were found directly in the agar block underneath the test chamber, which demonstrates that males remain near the source of attractant. The various test combinations are listed in Table I. The number of females migrating towards the test chamber containing females was not significantly different from control experiments (Fig. 5), indicating that females do not respond to the attractant. No evidence was found to indicate that males produce any attractant (Table I) (Fig. 6). There is no evidence of homosexual attraction (Fig. 6b). Panagrellus redivivus females attracted P. redivivus males, but neither P. silusiae nor P. redivivus males responded to females of the other species. (Table I) (Fig. 7)

Table I

Test of response of adult Panagrellus silusiae or Panagrellus redivivus in inoculation zone to secretions from adult P. silusiae or P. redivivus in test chamber.

Test chamber	Inoc. zone	Total no. of adult animals of 10 expts. at the end of 4 hours found in:			Variance of no. of animals in test arm	P (X)
		Test arm	Inoc. zone	Control arm		
A. Intraspecific pairings						
1. P. silusiae						
Distilled water	male	44	6	50	2.93	$0.7 > X > 0.5$
Male	male	48	10	42	1.29	$0.7 > X > 0.5$
Gravid female	male	82	8	10	1.06	$X \ll 0.001$
Virgin female	male	88	4	8	0.62	$X \ll 0.001$
Distilled water	female	39	18	43	1.21	$0.7 > X > 0.5$
Female	female	47	7	46	3.23	$0.95 < X < 0.9$
Male	female	36	16	48	1.37	$0.2 > X > 0.1$
2. P. redivivus						
P. redivivus female	P. redivivus male	65	18	17	2.72	$X \ll 0.001$
Distilled water	P. redivivus male	45	19	36	2.50	$0.5 > X > 0.3$
B. Interspecific pairings:						
P. redivivus female	P. silusiae male	47	9	44	0.90	$0.8 > X > 0.75$
P. silusiae female	P. redivivus male	44	16	40	2.04	$0.7 > X > 0.5$

Figure 4a. Control experiment. 4 hourly distribution of adult males in response to agar and distilled water in both chambers.

Figure 4b. 4 hourly distribution of adult males in response to gravid female in the test chamber.

Figure 4c. 4 hourly distribution of adult males in response to virgin females in the test chamber.

Diagram of the V-shaped 2% agar strip apparatus and the locations of the various regions in which the distribution of worms were recorded.

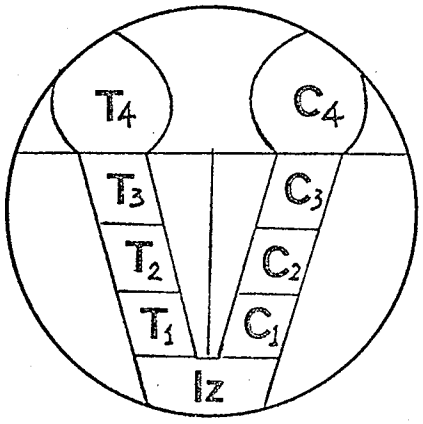


fig 4a

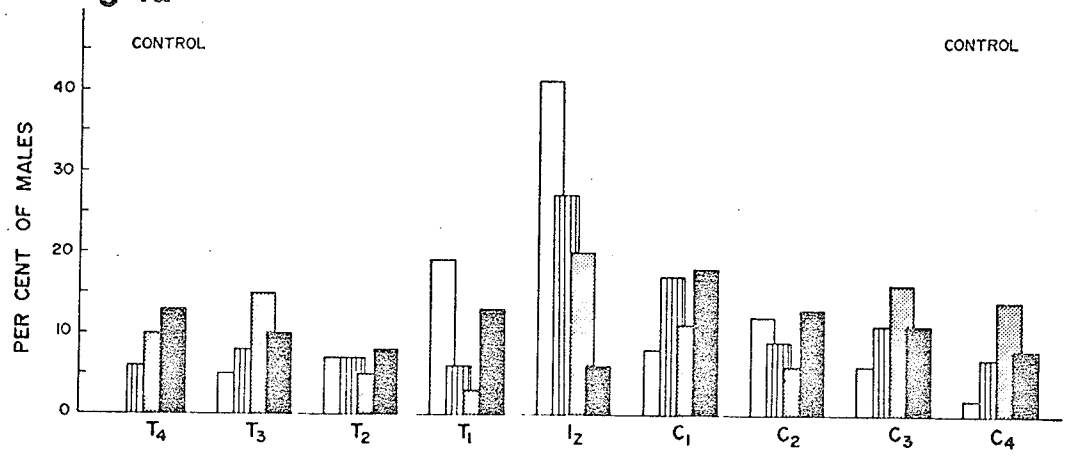


fig 4b

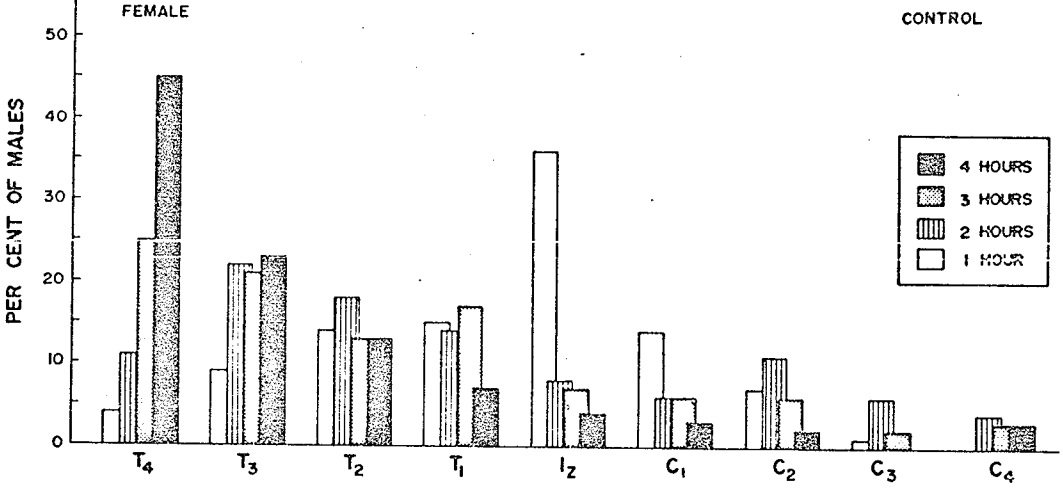


fig 4c

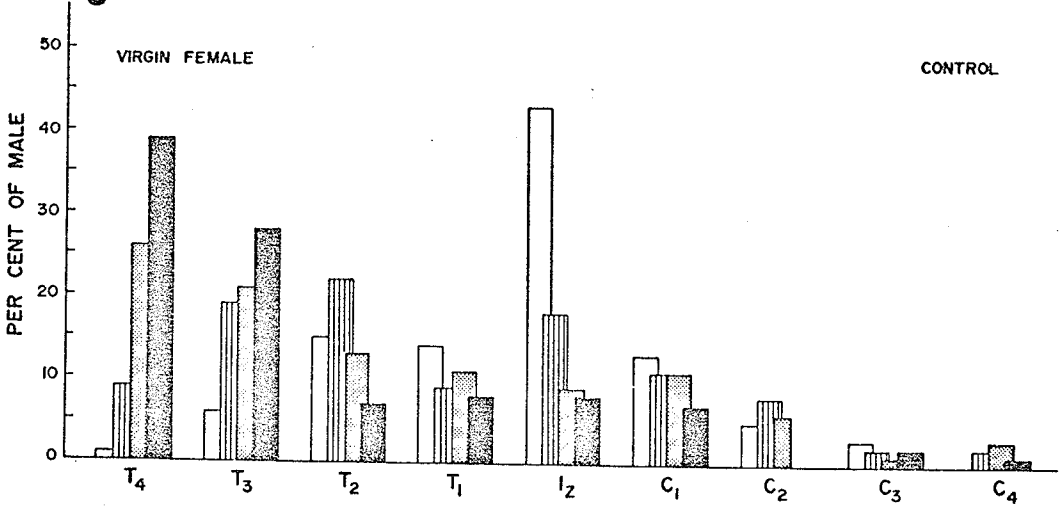


Figure 5a. Control experiment. 4 hourly distributions of adult females in response to agar and distilled water in both chambers.

Figure 5b. 4 hourly distributions of adult females in response to adult females in the test chamber. (homosexual attraction experiment)

fig 5a

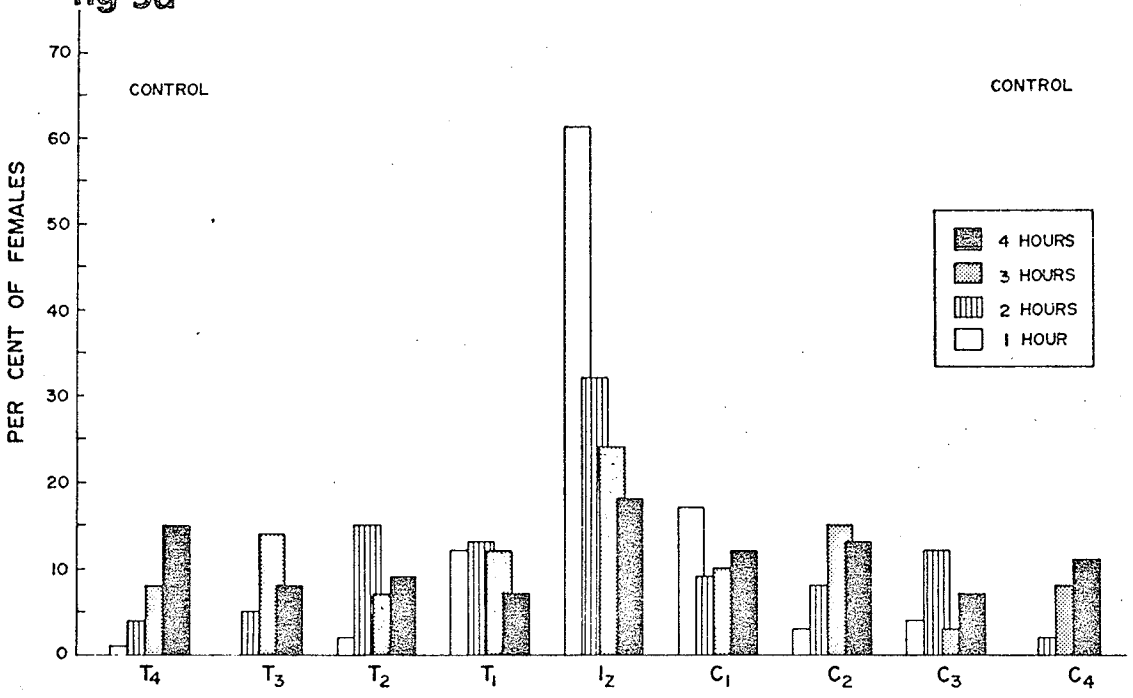

CONTROL
CONTROL
PER CENT OF FEMALES
70
60
50
40
30
20
10
0
4 HOURS
3 HOURS
2 HOURS
1 HOUR
T4
T3
T2
T1
IZ
C1
C2
C3
C4

fig 5b

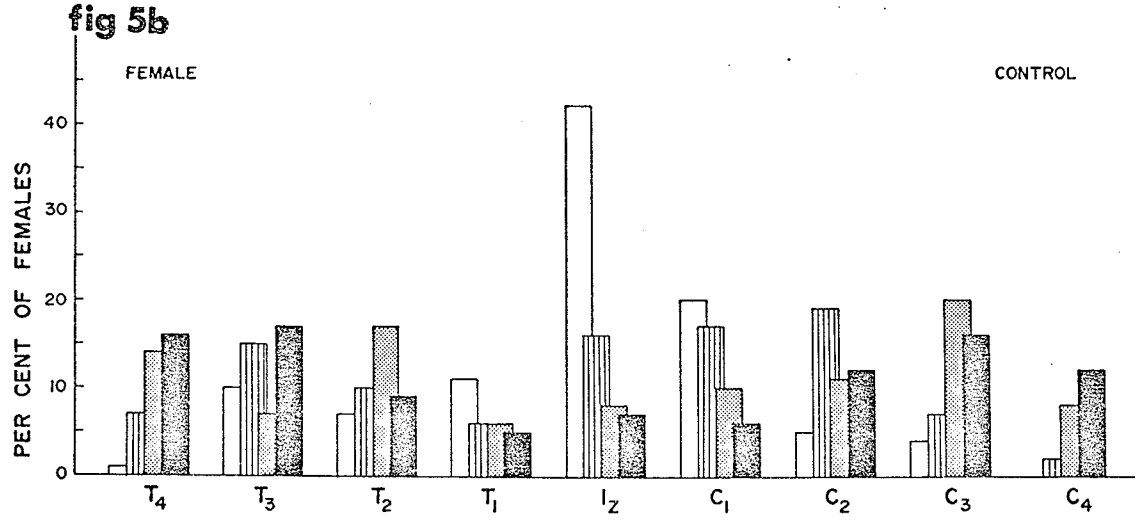

FEMALE
CONTROL
PER CENT OF FEMALES
40
30
20
10
0
T4
T3
T2
T1
IZ
C1
C2
C3
C4

Figure 6a. 4 hourly distributions of adult females in response to adult males in the test chamber.

Figure 6b. 4 hourly distributions of adult males in response to adult males in the test chamber. (homosexual attraction experiment)

fig 6a

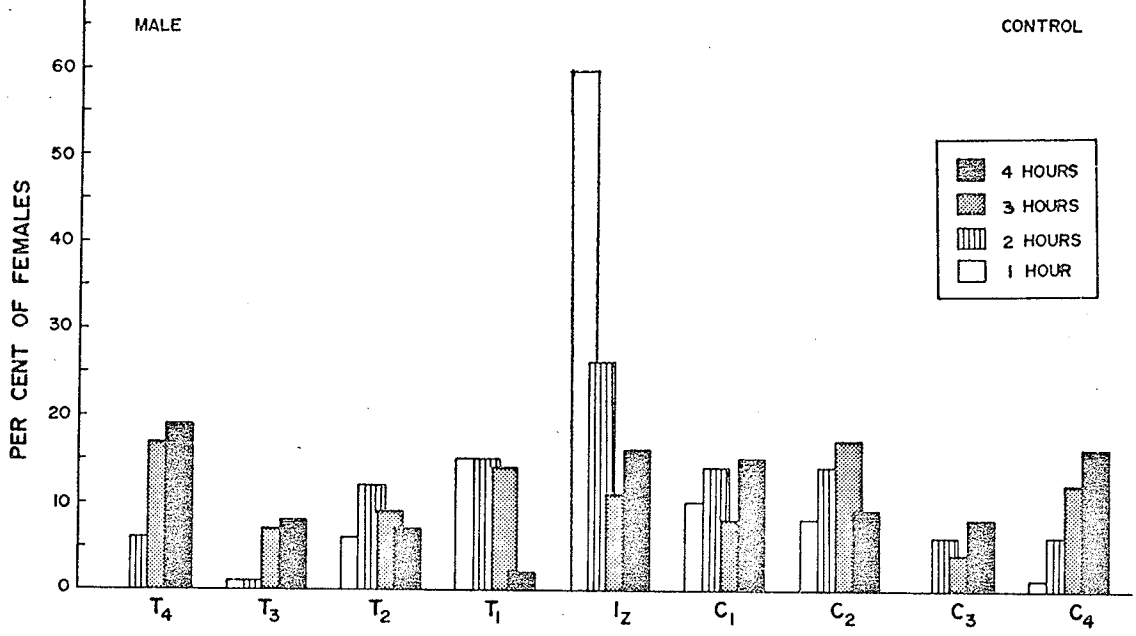

MALE
CONTROL
PER CENT OF FEMALES
0
10
20
30
40
50
60
4 HOURS
3 HOURS
2 HOURS
1 HOUR
T4
T3
T2
T1
IZ
C1
C2
C3
C4

fig 6b

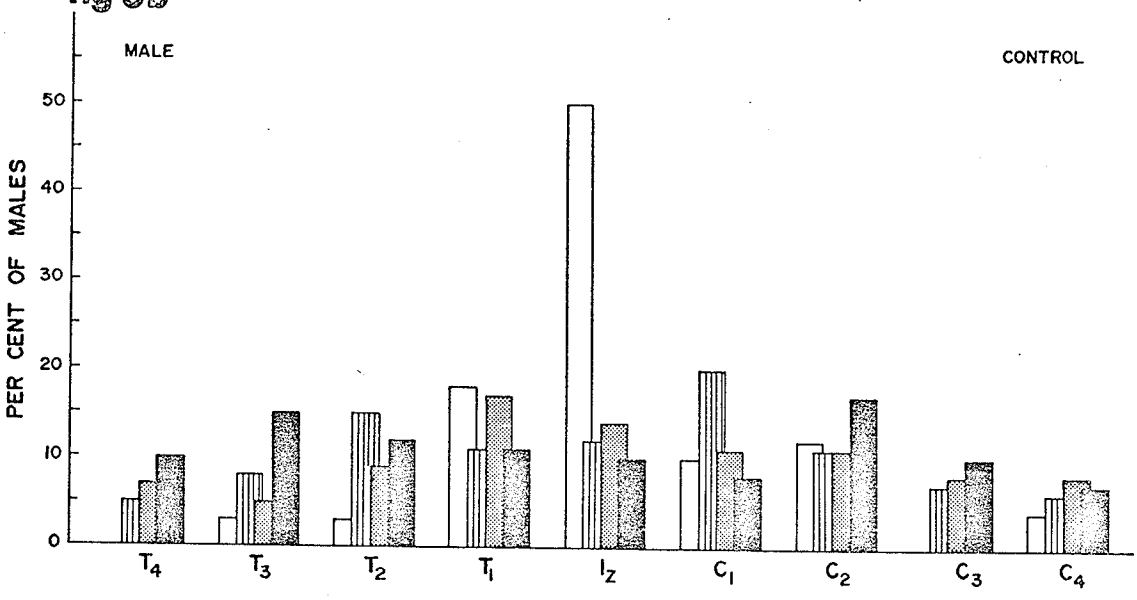

MALE
CONTROL
PER CENT OF MALES
0
10
20
30
40
50
T4
T3
T2
T1
IZ
C1
C2
C3
C4

Figure 7a. Interspecific attraction experiment. 4 hourly distributions of P. silusiae adult males in response to P. redivivus adult females in the test chamber.

Figure 7b. Interspecific attraction experiment. 4 hourly distributions of P. redivivus adult males in response to P. silusiae adult females in the test chamber.

fig 7a

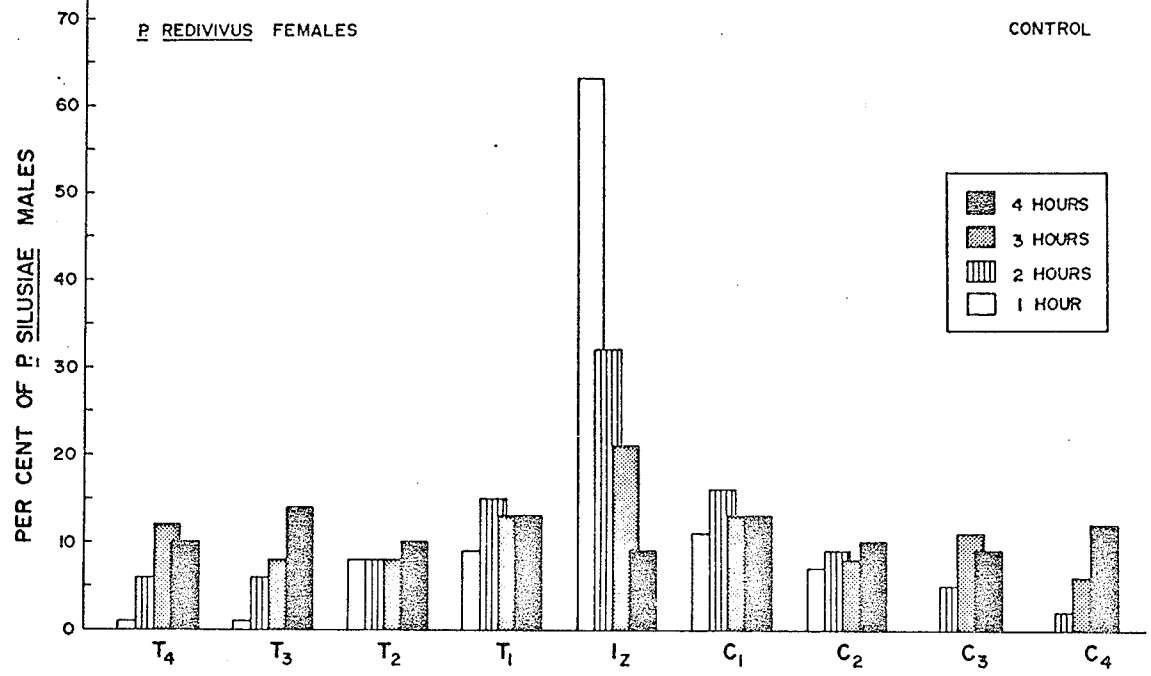
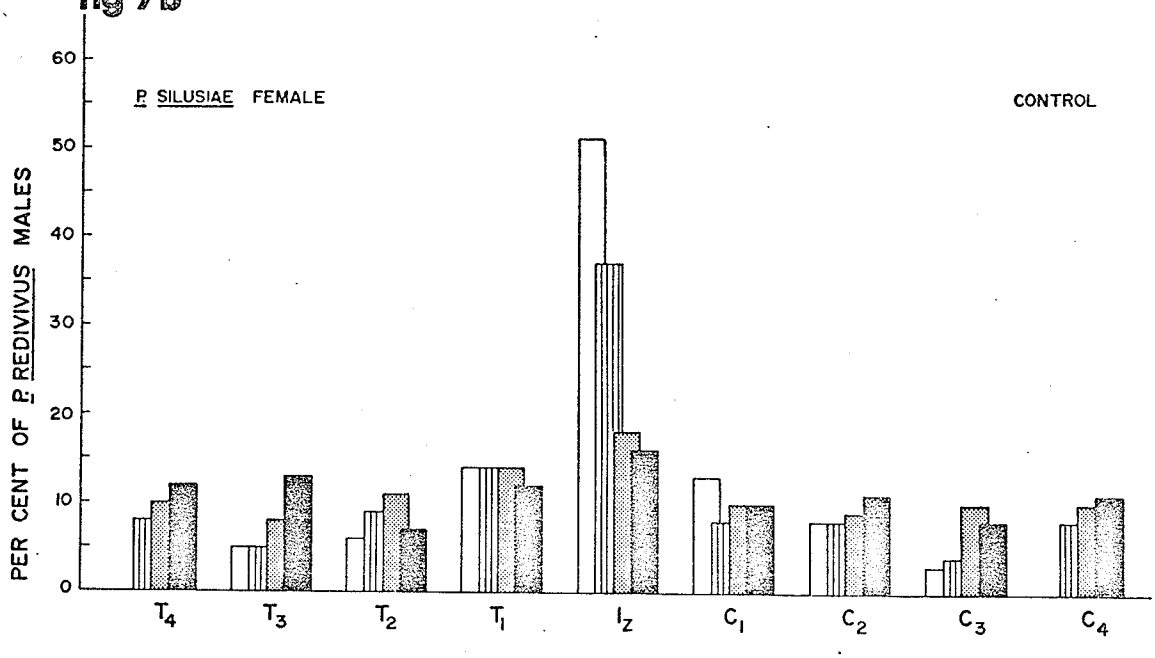

P. REDIVIVUS FEMALES
CONTROL
PER CENT OF P. SILUSIAE MALES
70
60
50
40
30
20
10
0
4 HOURS
3 HOURS
2 HOURS
1 HOUR
T4
T3
T2
T1
IZ
C1
C2
C3
C4

fig 7b


P. SILUSIAE FEMALE
CONTROL
PER CENT OF P. REDIVIVUS MALES
60
50
40
30
20
10
0
T4
T3
T2
T1
IZ
C1
C2
C3
C4

B. Mating Attractant - Gradient Formation

The attractant produced by P. silusiae females formed a gradient in agar. Agar blocks taken from the region underneath the test chamber containing females attracted 33.6% of the total males inoculated (Table II). Agar blocks taken from regions further away from the test chamber attracted correspondingly fewer males. The overall distribution of males was significantly ($p<0.001$) different from that of control experiments.

The attractant produced by P. silusiae females is waterborne. In the quadrant Petri dish experiment, no aggregation of males in the innermost radian was observed 24 hour after inoculation of males (Table III). If the attractant is airborne, one might expect a gaseous gradient to form such that increasing numbers of males would have been attracted and found towards the radian which is nearest to the quadrant containing females. This was never observed during the 24 hours after the males were inoculated. After the two quadrants were connected by the wick a significant number of males migrated towards the innermost radian where liquid exchange between the two quadrants occurred (Fig. 8). Males were observed swimming vigorously around the tip of the wick and occasionally one or two males were found

Table II

Number of P. silusiae males migrating to agar blocks which had been exposed to secretions from P. silusiae females in the test chamber demonstrating the presence of a gradient of the water borne mating attractant in the agar strip.

Agar blocks No.	source	Total no. and % of males found in the quadrants 8 hours after inoculation. (10 expts.) No.	%	Variance of no. of males of 10 expts.
1	From region directly underneath the filter paper disk of the chamber.	84	33.6	3.37
2	1cm from test chamber	72	28.8	6.84
3	2cm from test chamber	46	18.4	4.04
4	1cm from the control chamber in the control arm	26	10.4	0.93
Total no. of males of 10 expts. remaining in the inoculation area		22	8.8	3.29
	Total	250	100.0	

$X^2 = 36.72$

$P(X) \ll 0.001$

Table III

Distribution of total number of adult P. silusiae males from 10 quadrant Petri dish experiments to demonstrate that the attractant produced by P. silusiae females is waterborne.

Distance of radian from the female-containing quadrant in cm.	Area of radian in cm^2	Total no. of males per radian at the end of the 4th hour reading				Density of average no. of males of 10 expts. in each radian at the end of the 4th hour reading	
		Control	S^2*	Connected by wick	S^2*	Control	Connected by wick
0	0.78	26	1.15	65	2.50	3.33	8.33
1	2.35	96	3.82	120	8.44	4.08	5.10
2	3.92	150	15.77	145	14.5	3.82	3.69
3	5.50	228	13.51	170	6.00	4.14	3.09

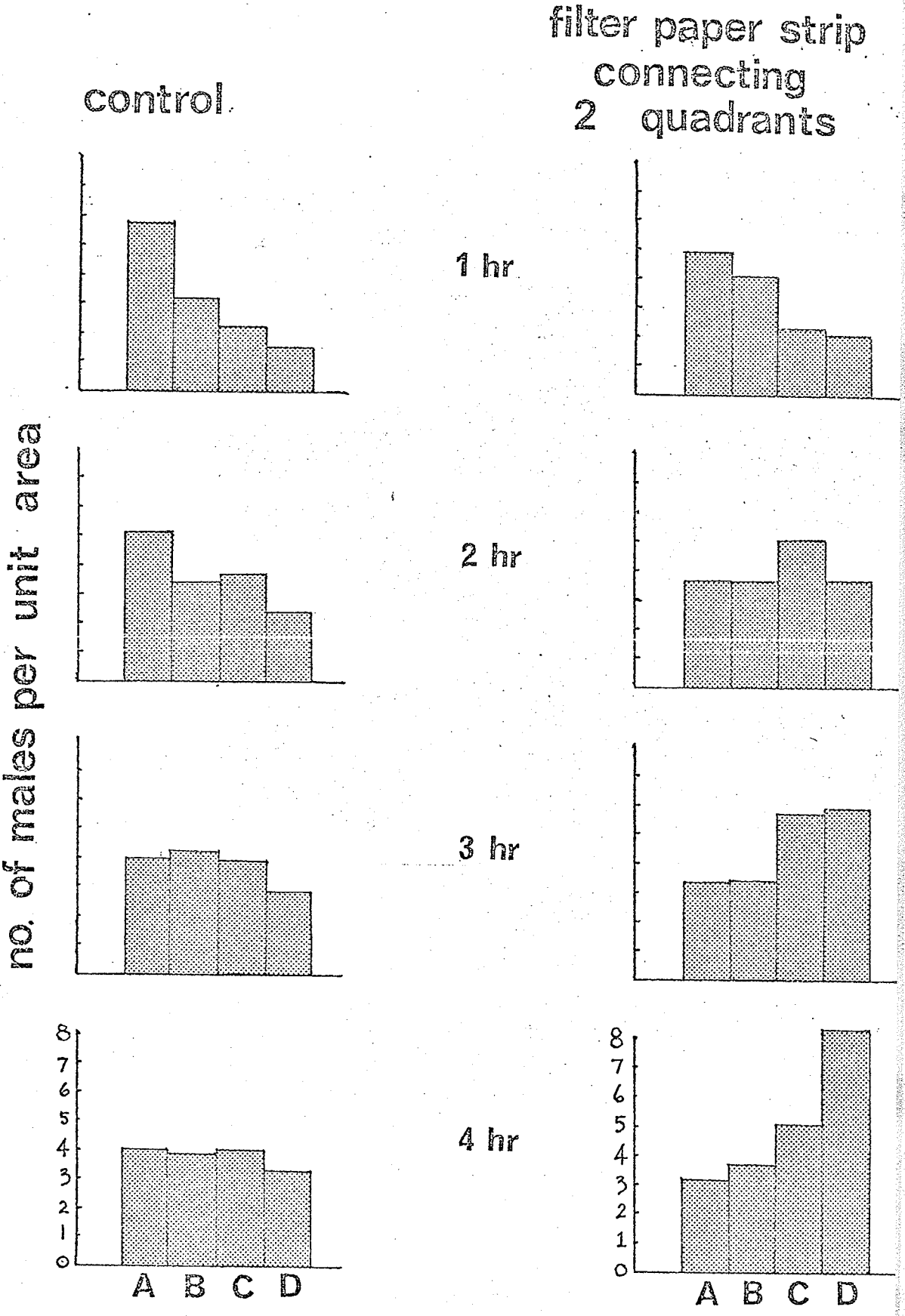
$X^2 = 79.42$

$P(X) = <0.001$

* S^2 denotes the variance of no. of males of 10 expts.

Figure 8. Histograms showing the density of male P. silusiae (average of 10 experiments) in each radian at the end of 1 hour for a period of 4 hours.

	Distance of radian from the female-containing quadrant in cm.
Radian D	0
Radian C	1
Radian B	2
Radian A	3


control
filter paper strip connecting 2 quadrants
no. of males per unit area
1 hr
2 hr
3 hr
4 hr
0
1
2
3
4
5
6
7
8
A B C D

wriggling along the film of water on the wick towards the female quadrant.

C. Sexual Attraction in Juvenile Worms.

The response to and production of mating attractant in juvenile P. silusiae is shown in Table IV. The number of adult males migrating towards the test chamber containing L_4 females was significantly greater ($p<0.001$) than that migrating towards the control chamber. The number of L_4 males migrating towards the test chamber containing adult females was also significantly greater ($p<0.001$) than the number migrating towards the control chamber. No significant attraction was detected in other juvenile stages.

D. Effects of hydroxyurea and Cycloheximide on Sexual Attraction and Copulatory Behaviour.

The previous results demonstrated female P. silusiae first produce the attractant during the fourth (final) juvenile stage and it is also during the final juvenile stage that males become competent to respond to the attractant. The main developmental event of the L_4 stage is the maturation of the reproductive system. Females grown from the L_2 stage in hydroxyurea did not attract untreated males, as shown in

Table IV

Response of *P. silusiae* adult males in inoculation zone to *P. silusiae* juveniles in test chamber and *P. silusiae* male and female juveniles in inoculation zone to *P. silusiae* adult females in test chamber.

Test chamber	Inoc. zone	Total no. of adults or juveniles of 10 or 15 expts. after 8 hours found in:			Variance of no. of animals in test arm	P (X)
		Test arm	Inoc. zone	Control arm		
I.						
L_2	adult male	34	34	32	2.48	$0.9 > X > 0.8$
L_3	adult male	24	45	31	0.93	$0.5 > X > 0.3$
L_4 male	adult male	27	43	30	1.34	$0.7 > X > 0.5$
L_4 female	adult male	52	36	12	2.40	$X \ll 0.001$
II.						
Adult female	L_2 male	16	33	19	0.93	$0.7 > X > 0.5$
Adult female	L_2 female	27	35	20	1.52	$0.5 > X > 0.3$
Adult female	L_3 male	20	36	24	1.24	$0.7 > X > 0.5$
Adult female	L_3 female	25	24	21	2.09	$0.7 > X > 0.5$
Adult female	L_4 male	88	39	23	1.12	$X \ll 0.001$
Adult female	L_4 female	46	55	49	2.06	$0.8 > X > 0.75$

L_2 - second stage juveniles
L_3 - third stage juveniles
L_4 - fourth stage juveniles

Table V. Adult females placed in hydroxyurea for 24, 48 or 72 hours produced attractant. Adult females treated with cycloheximide for 24, 48 or 72 hours did not attract males (Table VI) when tested immediately after cycloheximide treatment but did attract males when tested 24 hours after cycloheximide treatment.

Males grown from the L_2 stage in hydroxyurea were not attracted to normal females (Table V). Hydroxyurea or cycloheximide treatment of adult males for 24, 48 or 72 hours had no effect on their response to mating attractant; males treated as adult were attracted to untreated females (Table V and VI)

Examination of the gonads of females grown in hydroxyurea from L_2 stage (Fig. 9c & d) revealed that only 8.3% had normal gonads while 74.2% had incomplete gonads and 17.5% had undeveloped gonad primordia. Only 5.5% of the hydroxyurea-grown males had normal gonads (Fig. 9a) and these males were found on the test arm or dead on the inoculation zone. Of the hydroxyurea-grown males 52% had incomplete gonads (Fig. 9b) and 42.5% showed no gonad development past the L_2 stage. The hydroxyurea-grown worms with incomplete gonads suggested that hydroxyurea blocked the morphological organization

Table V

Migration of P. silusiae adult males (grown in Hydroxyurea or treated with Hydroxyurea as adults), in response to P. silusiae adult females (grown in Hydroxyurea or Hydroxyurea treated).

Sexual attraction system test combinations		Total no. of males found in:			Variance of no. of males in test arm	P (X)
Adult males in inoc. zone	Adult females in test chamber	Test arm	Inoc. zone	Control arm		
HU. grown	Control	48	59	43	1.60	$0.7 > X > 0.5$ *
HU. grown	HU. grown	39	62	49	1.54	$0.3 > X > 0.25$ *
Control	HU. grown	62	34	54	1.98	$0.5 > X > 0.3$ *
24 hours in HU.	Control	47	32	21	2.23	$0.005 > X > 0.001$
48 hours in HU.	Control	50	35	15	1.55	$X \ll 0.001$
72 hours in HU.	Control	37	44	19	1.78	$0.02 > X > 0.01$
72 hours in Distilled water	Control	47	29	24	0.76	$0.01 > X > 0.005$
Control	24 hours in HU.	58	24	18	2.40	$X \ll 0.001$
Control	48 hours in HU.	52	21	27	1.73	$0.005 > X > 0.001$
Control	72 hours in HU.	49	25	26	1.65	$0.01 > X > 0.005$
	72 hours in Distilled water	64	17	19	1.37	$X \ll 0.001$

HU. = Hydroxyurea

* Number of males migrating to the test chamber was not significantly different ($P > 0.05$) from that migrating to the control chamber.

Table VI

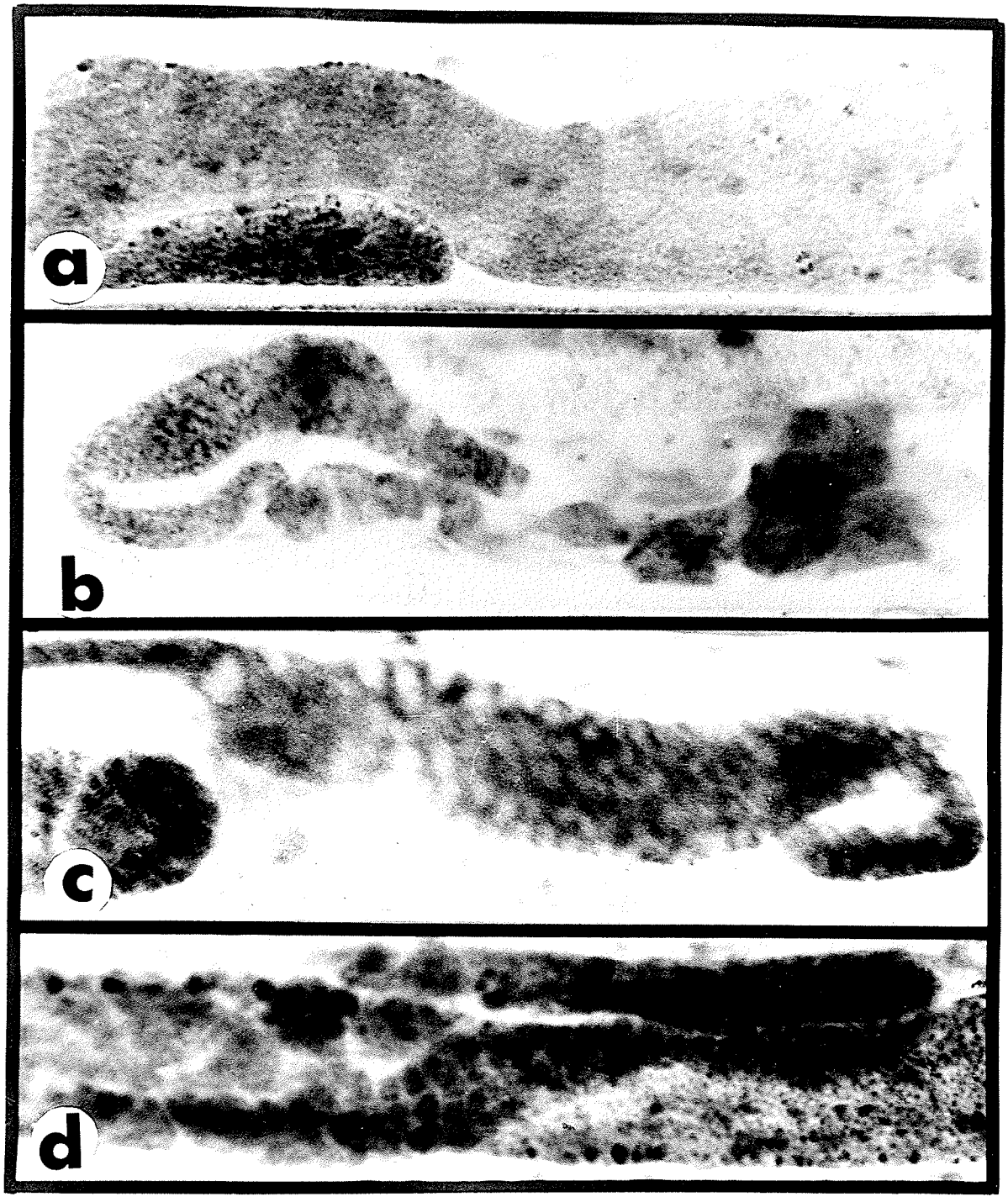
Migration of P. silusiae males (control or actidione treated) to females (control or actidione treated).

Time in actidione (hr.)	Time of wash after actidione (hr.)	Total Number of Males found in: Test arm	Inoc. zone	Control arm	P (X)
a. Treated Females - Untreated males					
	Control	129	58	53	$X \ll 0.001$
24	0	92	62	86	$0.8 > X > 0.75$*
24	24	105	72	63	$0.005 > X > 0.001$
48	0	69	82	89	$0.2 > X > 0.1$*
48	24	99	74	67	$0.02 > X > 0.01$
72	0	49	46	55	$0.5 > X > 0.3$*
72	24	56	58	36	$0.05 > X > 0.025$
b. Untreated females - treated males					
	Control	125	54	61	$X \ll 0.001$
24	0	92	88	60	$0.01 > X > 0.005$
24	24	108	65	67	$0.05 > X > 0.025$
48	0	97	92	51	$0.01 > X > 0.005$
48	24	116	73	61	$X \ll 0.001$
72	0	81	102	57	$0.05 > X > 0.025$
72	24	83	98	59	$0.05 > X > 0.025$

* Number of males migrating to the test chamber was not significantly different ($P > 0.05$) from that migrating to the control chamber.

Figure 9. Normal and abnormal gonad development in P. silusiae. (1200X)

a. Normal adult male gonad.
b. Abnormal gonad of adult male grown in hydroxyurea since L_2 stage.
c. Normal adult female gonad.
d. Abnormal gonad of adult females grown in hydroxyurea since L_2 stage.


a
b
c
d

DISCUSSION.

A. Sexual Attraction, Mating Attractant and Postembryonic Development.

Sexual attraction in P. silusiae resembles that of Ancylostoma caninum and Cylindrocorpus spp. in that only females attract males. Previous studies of sexual attraction dealt with virgin females (Greet 1964; Jones 1966; Chin and Taylor 1969) or non-virgin females (Roche 1966; Bonner and Etges 1967), but not both. This work shows that both virgin and gravid P. silusiae females produce the attractant. Males do not attract females nor do they attract other males. Bonner and Etges (1967) suggested that a repelling influence might exist among males of Trichinella spiralis. No evidence of such repulsion in P. silusiae was found.

The mating attractants of Cylindrocorpus longistoma and C. curzii are species specific (Chin and Taylor 1969). The mating attractant of P. silusiae is also species specific. P. silusiae and P. redivivus do mate (Balakanich & Samoiloff, unpublished result) and produce viable young and their status as a separate species has been questioned (Behme and Pasternak 1969; Hechler 1971; Anderson, personal communication) Work on this problem is being continued (Balakanich, pers. comm.).

The attractant of P. silusiae is capable of forming a gradient in agar in 24 hours, which indicates a moderate molecular weight. The specificity of the attractant also suggests molecular complexity. As the attractant can be retained in agar, its chemical nature can be investigated. (Appendix III)

Green (1967) demonstrated that females of Heterodera spp. attracted males by secreting substances which were in solution rather than gases through the air, although Greet, Green, and Poulton (1968) found volatile and non-volatile components of mating attractant. Results of tests with P. silusiae indicate that the attractant produced by females is waterborne.

Sexual attraction is an interesting facet of postembryonic development in P. silusiae. Production of attractant during the L_4 stage in females and continuation beyond the completion of the final molt refutes the concept that mating attractant is only produced during ecdysis of the final female molt (Samoiloff 1970). But production of attractant by only L_4 and adult females demonstrates the synthesis of a chemical product with both stage and sex specificity. Males of P. silusiae respond to attractant

during the L_4 stage, unlike males of Pelodera teres which only respond to attractant after the completion of the final molt (Jones 1966).

As sexual attraction occurs first during the L_4 stage, I propose that two sex-specific processes must be involved, namely, production of a sex-specific chemical at a specific developmental stage in the female, and development of a sex-specific behavioral response at a specific developmental stage in the male. Stage-specific protein patterns have been demonstrated in P. silusiae (Chow and Pasternak 1969), but biochemical sex specificity has not. As the maturation of the gonads of both sexes occurs during the L_4 stage, sexual attraction and the maturation of the reproductive system in P. silusiae may be developmentally coupled. Furthermore, as the processes of growth, molting and gonad development can be uncoupled from one another with inhibitors of macromolecular synthesis (Pasternak and Samoiloff 1970; Boroditsky 1972), the developmental events required for both the production of the attractant and the response to it can be determined.

B. Mating Attraction System, Gonad Development and Effects of Inhibitors.

Results of the effects of inhibitors on mating behaviour and its development of P. silusiae indicate a developmental link between sexual attraction and gonad development. The mating attraction system does not appear in worms grown in hydroxyurea. The reproductive system, which is the only tissue to undergo extensive cell proliferation during P. silusiae postembryonic development (Sin and Pasternak 1970), also does not completely develop in hydroxyurea-grown worms. The primary action of hydroxyurea is the inhibition of DNA synthesis although during P. silusiae postembryonic development synthesis of RNA and protein is also significantly lowered (Pasternak and Samoiloff 1970). These latter inhibitors are insufficient to stop body growth or the development of the copulatory structures and may represent RNA and protein synthesis that normally occurs in the complete gonad. Neither the production of mating attractant by females nor the response to attractant by males is blocked by hydroxyurea after gonad development has occurred.

Production of mating attractant by adult females is stopped by cycloheximide treatment. It is unlikely that the attractant itself is protein since preliminary chemical

analysis reveals that the attractant is a substituted hydrocarbon, and furthermore the attractant is not destroyed by pepsin (Cheng and Samoiloff, unpublished). It is likely that synthesis of attractant is enzyme mediated, but, for the direct synthesis of attractant to be inhibited by cycloheximide, these enzymes would have to be quite labile and would require continuous synthesis. Another alternative is that protein synthesis is required for the control of the production of attractant; production of attractant may be under hormonal regulation and such control may require protein synthesis.

The response to mating attractant by males is inhibited in animals grown from the L_2 stage in hydroxyurea but not by treatment with hydroxyurea or cycloheximide after gonad maturation. The most probable mechanism for the development of this response is either the activation or de novo formation of a specific receptor site or its neuronal connections. Hydroxyurea treatment during development could directly stop de novo formation or block activation of the receptor either directly or by inhibition of a hormonal trigger from some other organ.

The results obtained with these inhibitors are

consistent with the concept that the gonads play a major role in initiating the development of sexual attraction either by directly producing compounds involved in mating attraction or in regulating sexual attraction via a hormonal mechanism.

C. Copulatory Behaviour in P. silusiae.

Greet (1964) proposed that copulation is initiated by a tactile stimulus separate from sexual attraction. The observation that copulation is not affected in these inhibitors even when sexual attraction is stopped strongly supports this hypothesis that sexual attraction and copulation are separate events. Males and females of P. silusiae copulate only after the final molt (Samoiloff, unpublished) indicating that the developmental mechanisms for sexual attraction and copulation are different. P. silusiae adult males initiate the tail curling behaviour of copulation upon contact with adult females, although females gently treated with pepsin do not evoke the response.

All the evidences support the proposal that sexual attraction and copulation are separate events and that sexual attraction depends on complete development of the reproductive system, but that copulation does not.

CONCLUSION.

1. Female *Panagrellus silusiae* produce a mating attractant that attracts males of the same species.
2. The production of the mating attractant by the female first occurs during the L_4 stage and continues in adult stage of both virgin and gravid females.
3. The mating attractant is waterborne, species specific, not digested by pepsin and forms a gradient in agar.
4. Male *Panagrellus silusiae* do not produce attractant. They first respond to the attractant produced by L_4 stage or adult females of the same species during the L_4 stage.
5. The mating attraction system of *Panagrellus silusiae* depends on the complete development of the gonad, i.e. sexual attraction and maturation of the reproductive system of *P. silusiae* are probably developmentally coupled.
6. Sexual attraction and copulation are separate events.

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APPENDIX I

Procedures of Isolation of larval Panagrellus silusiae.

Cultures of P. silusiae were grown and kept in five to seven 100 X 15mm plastic Petri dishes containing distilled water and 2% agar.

The contents of each plate was passed through 4 layers of Kimwipes tissue wipes in order to filter out the agar. The agar-free nematode suspension was then passed through a 125ml separatory funnel filled with a mixture of glass microbeads (0.50mm and 0.30mm in diameter, 1:1).

The first 10-20 ml of effluent contained exclusively early L_2 larvae (284 $\pm$ 31 s.e.). The L_2s were concentrated by centrifugation for 5 minutes at 1400 X g and resuspended in Nigon's agar (Dougherty 1960) and distilled water in depression slides.

APPENDIX II.

Gonad Staining Technique.

The staining procedures, modified from Mulvey's (1960) technique, are listed as follows:-

1. Male and female adult *Panagrellus silusiae* were handpicked with a micropipet and washed twice in distilled water.
2. Heat killed on a slide and fixed for 10 min. in Carnoy's fluid (absolute alcohol, chloroform and glacial acetic acid in the ratio of 6:3:1).
3. The fixed nematodes were air dried and stained with three or four drops of propionic orcein.
4. A cover slip was placed over the drops and the slide was warmed for a few minutes at 40°C.
5. Excess stain was absorbed and the mount sealed with nail polish.

The stained whole mount specimens were examined 8 to 12 hours after staining. Selected control and treated gonad development were photographed using a Carl Zeiss photomicroscope.

APPENDIX III.

Preliminary Studies on the Biochemical Nature of the Mating Attractant.

A. Thin Layer Chromatography

100 young adult males or females were kept in a test chamber for 48 hours. The agar block underneath the test chamber was removed and placed into a "BEEM" capsule with perforated cover on it. The capsule was placed inside an automatic freeze-dryer (model no. 10-010, The Virtis Co., INC.) at -50°C and at a vacuum of 50 μ for 8-12 hours.

The powder-like residue inside the BEEM capsule was collected and dissolved in a minimum amount of distilled water which was used as sample solution for thin layer chromatography.

Thin layer chromatography was used for the detection of amino acids. Solvent used consisted of butanol, acetic acid and water in a ratio of 60:20:20.

The experiment was repeated 5 times. The control, male and female samples showed no difference and no definite

APPENDIX III A

Figure 1. Thin layer chromatogram of mating attractant developed in BAW.

BAW = Butanol:acetic acid:water, 60:20:20.

Control
22/6
22/6
22/6

amino acid spot was observed after ninhydrin spray. (Fig 1)

B. Infrared Radiation.

300 young adult males or females were kept in a test chamber for 48 hours and freeze-dried as described previously. The powder-like residue was extracted three times in methanol, acetone and ether. These solvents were evaporated off and the residue redissolved in 0.5ml of methylene chloride.

Two infrared radiation spectra, female against control and male against control, (Fig. 1 and 2) were obtained with a single-beam infrared sodium chloride spectrometer (Perkin-Elmer 137).

Both spectra suggested the following:

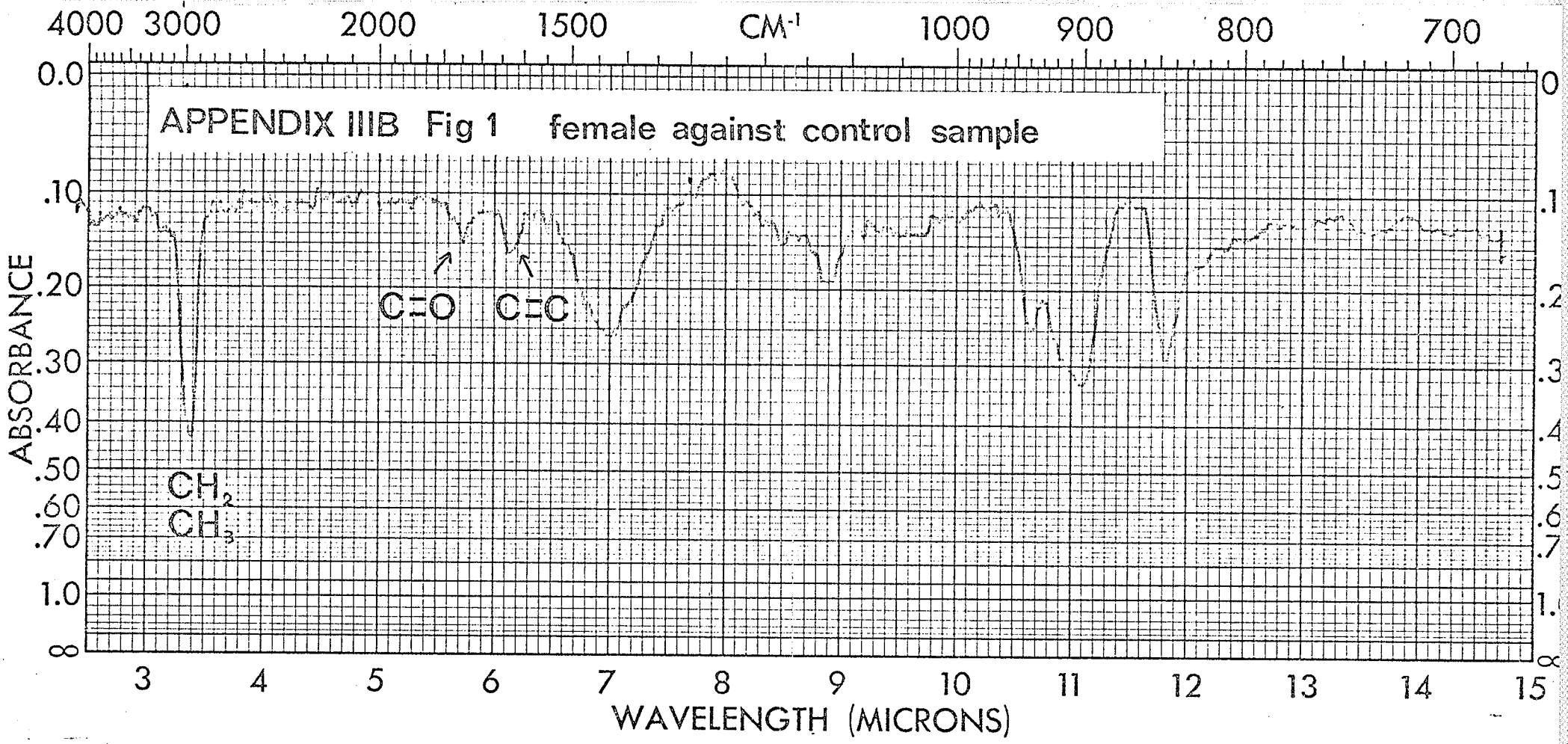
1. a CH_3 and/or CH_2 peak at $3000cm^{-1}$
2. a possible -C=O peak at $1750cm^{-1}$
3. a possible -C=C- peak at $1650cm^{-1}$

However, there was no difference in peak between males and female sample.

APPENDIX III B

Figure 1. Infrared radiation spectrum of female against control sample

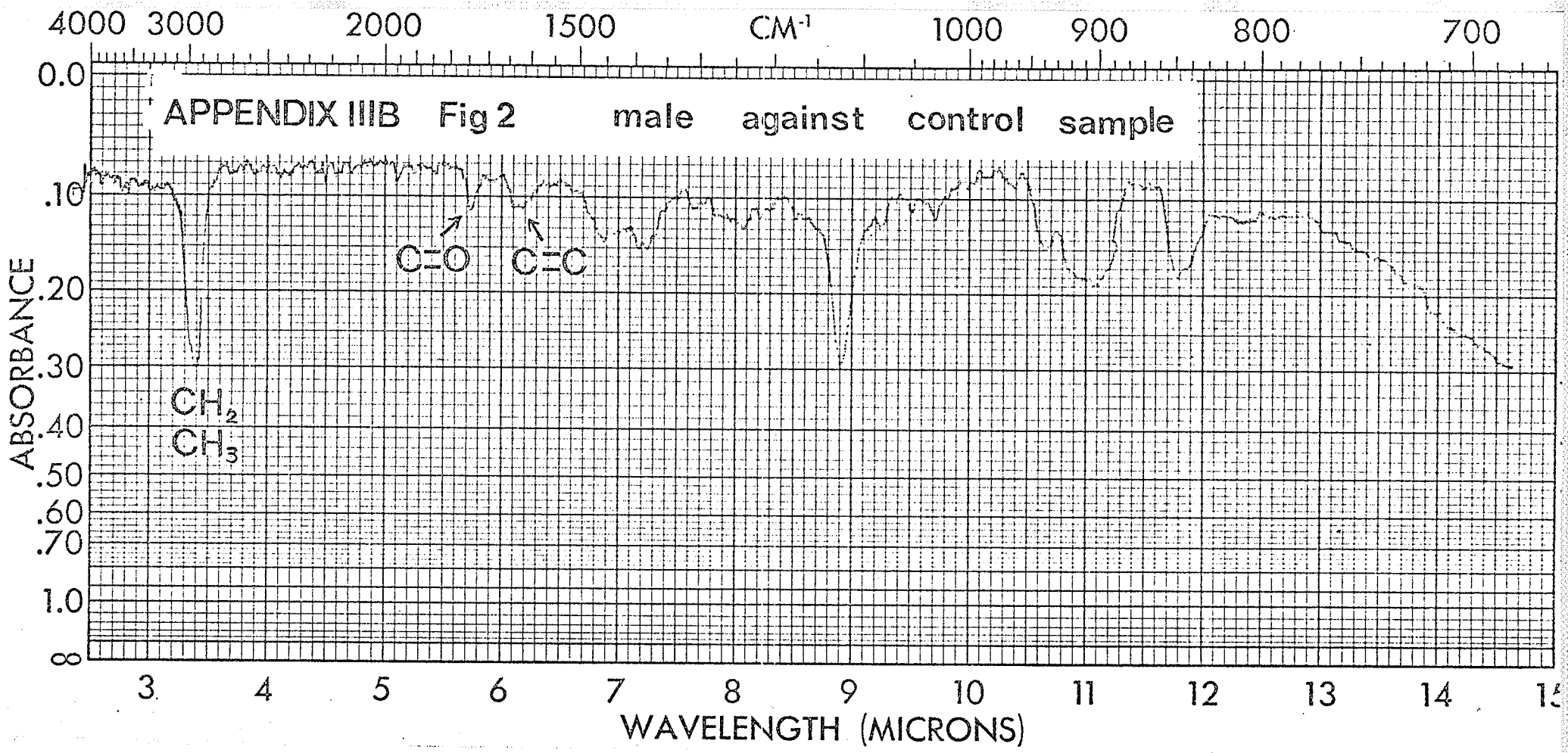
solvent:methylene chloride


APPENDIX IIIB Fig 1 female against control sample
4000 3000 2000 1500 CM-1 1000 900 800 700
0.0 .10 .20 .30 .40 .50 .60 .70 1.0 ∞
ABSORBANCE
C=O
C=C
CH2
CH3
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

APPENDIX III B

Figure 2. Infrared radiation spectrum of male against control sample.

solvent:methylene chloride.


4000 3000 2000 1500 CM-1 1000 900 800 700
APPENDIX IIIB Fig 2 male against control sample
C=O
C=C
CH2
CH3
ABSORBANCE
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
∞
3 4 5 6 7 8 9 10 11 12 13 14
WAVELENGTH (MICRONS)

c. Ultraviolet absorption.

100 young adult males or females were kept in a test chamber for 48 hours. The agar block underneath the test chamber was immersed in 2ml distilled water so that the mating attractant can diffuse from the agar block into the surrounding water. These were the sample solutions employed for the ultraviolet absorption analysis. A Bechman spectrophotometer (ACTA III) was used and three spectra were obtained from a male against control sample, female against control sample and a female against male sample.

A peak at λ_{max} = 200 was observed in both the male against control and the female against control sample. This peak persisted in the female against male sample. The groups which fall into the region of this peak of the absorption spectrum include -C=C- (λ=190), >C=0 (λ=195) >C=S (λ=205) and -COOR (λ=205).

APPENDIX III C

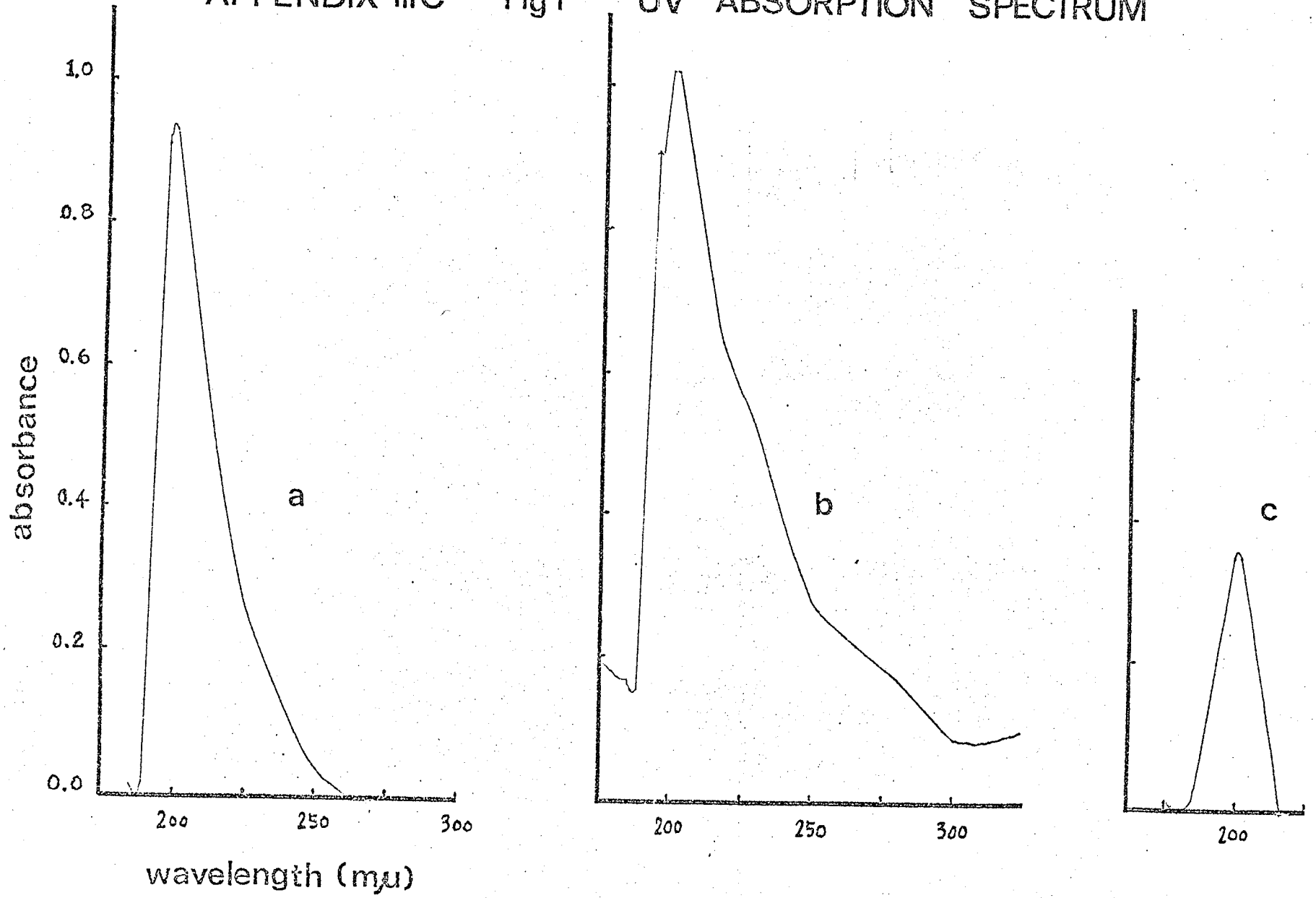
Figure 1. Ultraviolet absorption spectra

a. female against control sample

b. male against control sample

c. female against male sample

APPENDIX IIIC Fig 1 UV ABSORPTION SPECTRUM



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