ON VARIOUS ASPECTS OF REPRODUCTION AND EARLY DEVELOPMENT IN ARENICOLA MARINA

David Ian Dickson Howie

A Thesis Submitted for the Degree of PhD
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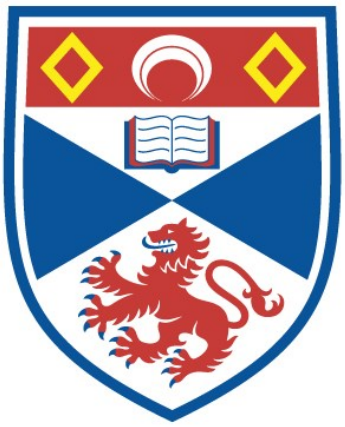
University of St Andrews



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ΑΙΕΝ ΑΡΙΣΤΕΥΕΙΝ

SUPERVISOR'S CERTIFICATE

I certify that David Ian Dickson Howie has fulfilled the conditions laid down in the regulations for the Degree of Ph.D., under Ordinance No. 16 of the University Court of the University of St. Andrews, and that he is accordingly qualified to submit this Thesis for the degree of Doctor of Philosophy.

Signed:

RESEARCH CAREER.

The research work recorded in this Thesis was carried out between the years 1950 and 1954. During this time I was a research student in the Gatty Marine Laboratory of the Department of Natural History in the United College of the University of St. Andrews and subsequently, a member of the staff of the University Zoological Department, Trinity College, Dublin University. I was enrolled as a research student on 1st October, 1950, as a graduate of the University of St. Andrews.

DECLARATION

I hereby declare that the work recorded in this Thesis has been carried out by me except where otherwise stated, and that it is of my own composition. I further declare that it has not been submitted in any previous application for a higher degree.

Signed:

[redacted]

On various aspects of reproduction and early development in Arenicola marina.

BY

David I. D. Howie.

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INTRODUCTION

The work described below started as an experimental study of artificial fertilisation and early development in Arenicola marina. It was hoped that the eggs and larvae of Arenicola would respond to techniques of fertilisation and rearing which had been developed at the Gatty Marine Laboratory for certain other marine invertebrates. However, in spite of many attempts at artificial fertilisation and larval rearing over three breeding seasons, only limited success was achieved. Because of this, the scope of the work was subsequently extended to include other aspects of the reproductive biology of Arenicola.

During the investigation of spawning and the spawning stimulus, a new approach to the problems of ripening and fertilisation was opened up by experiments in which spawning was induced artificially. This proved a profitable line of research and it forms a major part of the thesis.

I. REPRODUCTION

(a) Literature

Considerable literature exists on the reproductive biology of Arenicola marina. This may be summarised under various headings.

1. The breeding season:

Prior to 1916 it was generally accepted that breeding occurs in the Spring. Kyle (1896) stated that spawning takes place at St. Andrews between January and March and again between July and September. Gamble and Ashworth (1898) believed that on the Lancashire coast the laminarian variety spawn in the Spring, but that the littoral variety spawn in late Summer (see footnote p.12). In the same area, Ashworth (1904) reported that both varieties breed in the Spring. Worms containing ripe eggs, said to be 160μ in diameter, were found in February and March. The ova were all discharged by the first or second week of April. A description of oviposition was given (see p. 4). Ashworth goes on to report that in the Firth of Forth area genital products were present in both forms in July 1903. He states "I am inclined to believe that in some locations at any rate Arenicola marina also breeds in August and September." This view coincides with that of Cunningham and Ramage (1888), who worked on the Firth of Forth. They alone among this group of authors failed to report a Spring spawning. Genital products were "minute and immature" in February.

Since 1916 workers have been unanimous in describing a breeding season of about a fortnight's duration occurring between late September and early November. A slight divergence of view is shown by Smidt (1951) who postulated an "inconsiderable" spawning in the Summer at Esbjerg. Evidence for this was a slight decrease in the percentage of worms containing genital products during July and August, together with the finding of larval stages (1 - 3 chaetigers) in June 1943 and June 1947. However, Smidt states that the main spawning occurred at the end of September (in 1947), and that the worms were empty throughout Winter and Spring. This agrees with Pirlot's (1933) observations that genital products were absent in Winter and Spring (on the Belgian coast) and spawning occurred during the first fortnight of October in 1928, 1932 and 1933. According to Newell (1948), there was no evidence of a Spring spawning at Whitstable. In the Autumn the percentage of worms containing genital products dropped from 98% to 9% between 18th October and 31st October. Spawning commenced on 16th October and reached its maximum on 25th October. By 31st October all the worms were spent.

2. Spawning:

The spawning of male Arenicola has been observed on the shore. Storrow (1925) describes white patches of sperm which appeared between ripple marks on the sand at low tide. Newell (1948) gave a more detailed account. Small puddles of white fluid appeared on the wet sand of the flats (at Whitstable) half an hour

after dead low water, and "sperms were seen being pumped out of the exits from the tail shafts of the worm burrows."

According to the literature, oviposition has not been seen on the shore. McIntosh (1915) stated that gelatinous green egg capsules found on the shore might be those of Arenicola marina. Williamson (1916) also suggested that greenish masses, which in this case were found in an aquarium, might belong to this species. However, they were occasionally found some way up the sides of the aquarium and other polychaets were present. Prior to both these reports, Cunningham and Ramage (1888) had examined similar egg masses and suggested that they belonged to Scoloplos armiger. Gravier (1922) agreed with this suggestion, stating that the egg masses of Scoloplos armiger had for long been attributed to Arenicola marina.

Gamble and Ashworth (1898) observed spawning in the laboratory. Ova and spermatozoa escaped via the nephridiopores. "During the discharge of ova from the female the eggs were caught by the slimy mucus covering the body, and .... collect in strings around the body." Formation of gelatinous egg capsules was not observed. Blegvad (1923) stated that a reddish yellow layer of eggs was despoited loose on the sand within a radius of 20cm. of the burrow. The eggs were "whirled up on movement of the water". Pirlot (1933) agrees with Gamble and Ashworth (1898) in that eggs were found aggregated in mucus and with Blegvad (1923) in that they were readily liberated by agitation of the water. Newell (1948)

observed oviposition in an aquarium on one occasion. Eggs passed out through the head shafts of the burrows, near which they remained until disturbed.

Egg puddles, similar to sperm puddles, have once been found on the shore (Miss Nan Duncan, at Port Erin, I.O.M. - personal communication). Despite a close watch on the shore Newell (1948) failed to find eggs. He assumed that this was due to their similarity in colour to the sand. However, eggs were obtained when sand samples were stirred up in water and passed through a plankton net. This suggested that eggs are spawned onto the surface of the sand in the manner described in laboratory observations. An account of spawning in Arenicola claparedii given by Okuda (1938) may be significant in this respect. Oviposition was observed in the laboratory Eggs accumulated in the burrow united together "by a thin chorion". In this way a cylindrical egg tube was formed round the parent. Fertilisation and larval development, up to the 1st. chaetiger, took place in the burrow. Okada (1941) states that oviposition and fertilisation of the eggs of Arenicola cristata also takes place in the burrow. Gelatinous egg capsules are formed, which appear on the surface of the sand several hours after the original discharge of the genital products.

3. The influence of environmental conditions on spawning:

According to Pirlot (1933), the onset of spawning in Arenicola marina is not related to weather conditions. There

were violent storms during the breeding season in 1928 and 1932, while in 1933 the weather was calm. Pirlot noted that spawning occurred during spring tides in each of these years, but irrespective of whether the tides were new moon or full moon springs. "L'influence astrale semble s'être exercée d'une façon indirecte, par l'intermediare de la marée." However, Newell (1948) states that while spawning (at Whitstable) begins during springs, the spawning crisis occurs during the following neap tides.

Okada (1941) found that in Arenicola cristata "the periods of spawning, with about 4-day intervals, coincide well with component tides of about 4-day periodicity". The two maxima of activity during each lunar month fall during neap tides. Okada goes on to suggest that spawning is controlled by a physiological rhythm arising as a cumulative effect of the tidal periodicity and that it is affected by nutrition. Temperatures above 20°C. were said to be favourable. Okuda (1938) suggests that spawning in Arenicola claparedii is also controlled by a complex of factors, which include tides, sea temperature and calm water.

4. Environmental conditions and spawning in other marine organisms:

Much of this literature concerns organisms exhibiting sexual periodicity. According to Scott (1911), Amphitrite ornata spawns at new or full moon springs. Spawning in the laboratory coincides with spawning on the shore but only on the day of collection or the following day. This suggested that the periodic sexual reflex has acquired a physiological

basis in the organism which is not, however, strongly developed. Amirthalingham (1928) came to a similar conclusion after studying the rhythmic development of the gonad in Pecten opercularis.

The annual breeding swarm of the Palolo worm shows strict lunar periodicity. Mayer (1908) investigated the reaction of the Atlantic palolo, Eunice fucata, to the removal of various environmental factors, during the breeding season. Swarming was not inhibited by the removal of tidal influences nor by the exclusion of light. However, when the tidal effect and moonlight were removed at the same time, spawning was inhibited. This latter observation was challenged by Treadwell (1909). See also Korringa (1950)

Grave (1922) found that both sexes of Chaetopleura apiculata spawned in the laboratory when moved from running to quiet water. While giving warning of the danger of drawing an analogy between laboratory and natural conditions, Grave suggested that spawning normally takes place at slack water during low tide. Increased pressure from low to high tide provides the initial stimulus. The suggestion has been made by Orton, Southward and Dodd (1956) that spawning in Patella vulgata may be stimulated by shock in the form of wave action during rough weather.

Orton (1926) discussed the environmental factors which might provide the immediate stimulus to spawning in Ostrea edulis. The oysters studied by Orton generally spawned at full moon springs, but in August 1925 they spawned at both new and full moon springs. He suggested that the factors influencing spawning are

those exerting their greatest effect during spring tides, i.e. rise in pressure at high tide and rise in temperature at low tide. In an earlier paper, Orton (1920) had reviewed the relationship between sea temperature and breeding in marine animals. Examination of breeding records from Plymouth and Naples and personal observations led him to state "most animals under normal conditions begin to breed either at a definite temperature, which is a physiological constant for the species, or at a definite temperature change." Evidence was brought forward to suggest that under stenothermal conditions, e.g. in the tropics, marine organisms breed continuously. There was no evidence that food and salinity factors stimulate spawning. Many subsequent investigators have supported these views. Nelson (1928) and Yamamoto (1951) found that Ostrea virginica and Peoten yessoensis, respectively, spawn only after the attainment of a fixed sea temperature. In both cases subsequent spawning is dependent on further temperature increases, of the order of 2.0°C. and 0.5°C. respectively. Carr (unpublished data, 1949) found that a slight temperature increase induces spawning in ripe specimens of Patina pellucida and Dales (1950) states that spawning in Nereis diversicolor is correlated with a rise in temperature. Moore (1934), found that the spawning of Echinus esculentus is correlated with a definite temperature change, in this case when the temperature starts to rise from the Winter minimum.

Table 1

Environmental factors influencing the onset of spawning in various organisms.

AUTHORS	DATE	SPECIES	Periodic spawning	Non-periodic spawning	FACTORS INITIATING SPAWNING: Physiol. rhythm	Tides	Moonlight	Temperature	Rough weather	Calm weather	Nutrition
Okada K.	1941	Arenicola cristata	×		×	×					
Scott	1911	Amphitrite ornata	×		×	×	×				
Amirthalingham	1928	Pecten operularis	×		×						
Mayer	1908	Eunice fucata	×			×	×	×			
Crave	1922	Chaetopleura apiculata	×			×	×			×	
Orton	1926	Ostrea edulis	×			×	×	×			
Pirlot	1933	Arenicola marina		×		×	×	×			
Okuda	1938	Arenicola claparedii		×		×		×		×	
Orton	1920	Various		×				×			
Nelson	1928	Ostrea virginica		×				×			
Yamamoto	1951	Pecten yessoensis		×				×			
Moore	1934	Echinus esculentus		×				×			
Carr	(unpub)	Patina pellucida		×				×			
Dales	1950	Nereis diversicolor		×				×			
Orton, Southward & Dodd	1956	Patella vulgata		×				×	×		

The views of various authors on the environmental factors which might influence the onset of spawning are summarised in Table 1.

4. The mode of life of the larvae:

Post larval stages of Arenicola marina have been found and described by Kyle (1896), Ashworth (1904), Benham (1893) and Blegvad (1923). In each case the larvae were found in tow nettings. This led Thamdrup (1935) to postulate the existence of a lengthy pelagic larval stage in the life history. Thorson (1946), despite numerous plankton hauls during the breeding season, never found pelagic larvae. He suggested that larvae found by previous authors in the plankton occurred there accidentally, due to stirring up from the bottom. Newell (1948, 1949) also carried out repeated plankton searches without success. However, 2 - 4 chaetiger and post larval stages were located high up on the flats at Whitstable. It was suggested that the eggs were carried up to this area by the incoming tide and were trapped there as the tide retreated. Smidt (1951) reported that out of numerous plankton samples, 1 - 3 chaetiger stages were obtained on a few occasions. Similar stages were taken more frequently from "whirled up bottom material", in one case due to a heavy swell. Both Newell and Smidt examined newly hatched larvae in the laboratory. These were capable of vigorous swimming when disturbed but they rapidly regained the bottom. Normally the larvae crawled about on the substratum. As a result of these

observations both authors agree that there is no pelagic stage in the life history and Newell suggests that the larvae spend the Winter in the top few centimetres of sand. Referring to migrations in the lug worm population at Whitstable, Newell (1948) states "the occurrence of post larval stages in the Spring plankton would then perhaps be best explained as neither accidental (Thorson, 1946) nor as the end product of a very extended pelagic phase (Blegvad, 1923; Thamdrup, 1935) but as short migrations to places suitable for the continuation of the adult mode of life."

A non-pelagic life is probably common to the Arenicolidae. Guberlet (1934) states that after fertilisation the eggs of A. pusilla adhere to the sand, where development takes place. Okuda (1938) found that the eggs of A. claparedii develop in the egg tube. Finally, in A. cristata, Okada (1941) noted that development takes place in the egg capsule up to 3 - 5 chaetigerous segment stages. He states, however, that they then leave the egg capsule "to grow to be free swimming larvae".

(b) Observations at St. Andrews and Dublin

While collecting specimens for experimental work, observations were made on the breeding season, from 1949 to 1952 at St. Andrews and in 1953 at Dublin.

1. Areas of collection:

At St. Andrews, worms were collected from three areas. The positions of these are indicated on the map (text fig. 1).

Area A: a region between L.W.N.T. and L.W.S.T. close to a low reef of rocks known as the "Burn Stools". The laminarian variety were obtained in this area. *

Area B: this is immediately in front of the laboratory, lying approximately between H.W.N.T. and L.W.N.T. The littoral variety were obtained in this area. *

After the breeding season in 1950, the littoral population almost disappeared from Area B. Furthermore, observations in Area A were too readily interrupted by rough weather and onshore winds causing poor spring tides. A third collecting area was therefore chosen.

Area C: this is a large area of gently shelving sand, situated immediately off the rocks forming the southern boundary of the West sands. The region is densely populated with Arenicola from just below H.W.N.T. to L.W.S.T.

* The morphological criteria given by Ashworth (1904) for the separation of the species Arenicola marina into laminarian and littoral varieties is at present being re-investigated by Wells (personal communication). The terms are used here mainly to indicate whether the worms were obtained from a high (littoral) or low (laminarian) tide zone.

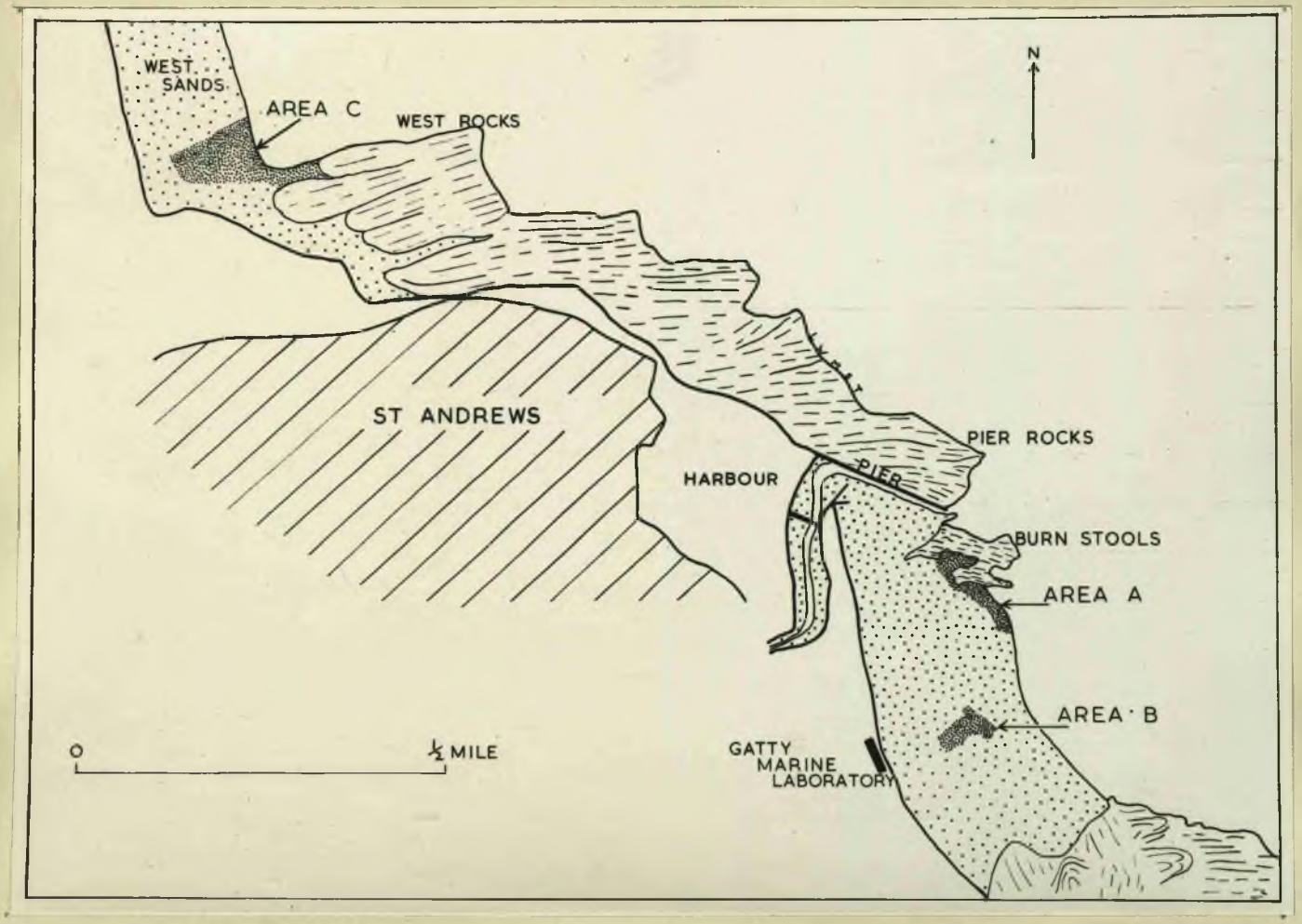


Fig. 1, Map of a portion of St. Andrews bay - showing the collecting areas.

Collections in Dublin Bay were made at Booterstown, three miles south of the city and at Seapoint, two miles further south. The former is an area of extensive flats while at the latter the tide has a short horizontal rise before reaching the sea wall.

2. The breeding season:

Examination of worms in the Spring:

Worms were collected during the Spring and early Summer of 1949, 1950 and 1951 (Table 2) and samples of coelomic fluid were examined microscopically. The first collections were made on 22nd and 24th June 1949 from Area B. 22 Worms were examined, none contained genital products. Immature genital products were present in laminarian worms from Area A, on 28th June, though not in all. By July, all laminarian worms and the majority of littoral worms contained genital products, though in the latter they were less well developed. In the following year collections were made in February, March and early May. There were no genital products in any of the worms at these times. Immature eggs and sperm first appeared in mid June (Table 2).

Although fewer collections were made in 1951, 1952 and 1953, the collecting areas were visited frequently. There was no evidence of a Spring spawning. Worms examined on 24th April and 15th May 1951 contained immature eggs, but it was not till 23rd May that all the worms contained genital products. In late

April 1953, 100 worms were examined from Area C. None contained genital products.

Autumn spawning:

Collections were made during the Summer and Autumn of the years 1949 - 1953 inclusive. Spawning was not always observed in all the areas of collection, but the duration of the breeding season could be deduced from a drop in the percentage of worms containing genital products. During the Summer all the worms in Area A were mature, i.e. contained genital products In Area B, the maximum percentage of mature worms was 55 - 58%, and in Area C, 70%.

and at no other time??

The spawning crisis:

Sperm puddles were observed all over the shore, i.e. the crisis described by Pirlot (1933), on the following dates:-

1950 - 24th to 26th October inclusive (Area A).
1951 - 2nd November (Area A).
1st and 2nd November (Area C).
1952 - 18th and 19th October (Area A).
16th and 17th October (Area C).

All these dates fell during full moon spring tides. Experience showed that the males spawned at low tide, only when the sand had a surface covering of water. In Area B, the sand surface was always smooth and dried out at low tide. In consequence, spawning was never observed in that area.

Table 2

Examination of the coelomic fluid of Arenicola in Spring and early Summer.

Date	Area of collection	Total worms examined	Genital products	
			Present	Absent
24.6.49	B	11	0	11
28.6.49	A	8	6	2
12.7.49	A	6	6	0
27.7.49	A	84	84	0
5.8.49	B	28	23	5
4.2.50 - 1.5.50	B	44	0	44
8.2.50 - 8.3.50	A	32	0	32
9.6.50 -15.6.50			some genital products	
4.7.50	A	19	19	0
4.7.50	B	20	18	2
6.4.51	A	13	0	13
6.4.51	B	5	0	5
24.4.51	A	4	2	2 *
15.5.51	C	11	6	5 *
23.5.51	A	13	13	0

* Genital products very infrequent in the body cavity.

NOTE: - Genital products in all cases immature.

Duration of the breeding season:

Area A: Observations were frequently curtailed by rough weather and poor tides. When collections were possible, the percentage of mature worms indicated the stage which had been reached in the breeding season. In 1949 no significant observation or collection was possible between 24th October, when all specimens were mature, and 20th November, when all were spent (Table 3). In 1950, only the crisis was observed, while in 1951, although spawning was slight when first noticed (2 sperm puddles, 1st November), it was evident that some considerable spawning had occurred previously as 23% of the worms were already spent. Spawning was general on the following day (Table 5). No further collections were possible. Large collections were made in 1952. On the first day of the spring tide period commencing 18th October, spawning was general. Already, however, 58% of the worms were spent. On the following day only 13.5% of the worms contained genital products (Table 6).

Area B: As noted above, spawning was never observed in this area. From counts of mature worms it was deduced that spawning took place from 25th October onward, in 1949 (Table 3) and during the second fortnight in October 1950 (Table 4). These periods corresponded approximately to the breeding season in Area A.

Area C: Collections could be made at all times in Area C. A number of sperm puddles were observed at about L.W.N.T. on 29th and 31st October, 1951. General spawning took place on 1st and 2nd November and a few sperm puddles were still to be seen on 7th

November. Spawning was not finally completed until the 13th and 14th, when only 4 sperm puddles were observed and 97.5% of the worms were spent (Table 5). Large samples were taken in 1952. 468 Worms were collected between 9th and 14th October, 70% of these were mature. As this was equivalent to the maximum percentage of mature worms throughout the Summer (see p. 14), no spawning had occurred so far. On 16th and 17th October, spawning was general. Activity had decreased by the 20th and 21st, only a few sperm puddles were observed though not all the worms were spent (Table 6).

Observations at Dublin:

Collections were made in Dublin Bay commencing in late September 1953. The Seapoint area closely resembled Area A at St. Andrews. in that immature specimens were rare prior to the breeding season. At Booterstown 12 - 16% of the worms were always immature (Table 7). Spawning was not observed except for a single sperm puddle at Booterstown on 28th October. Counts of worms showed that spawning took place at Seapoint around 21st October and was practically complete on 4th November. At Booterstown the percentage with genital products showed no decrease on 12th November when collecting had to be discontinued.

Weather conditions during the breeding season:

In 1949 spawning occurred during a prolonged period of storms. In 1950 and 1951, the weather was calm at the start of the breeding season and later deteriorated, while in 1952 the

weather was calm throughout.

Spawning in the laboratory:

Specimens kept in the laboratory (see p.48) spawned intermittently throughout the breeding season as observed on the shore (Tables 5 and 6). In 1951, one male and one female spawned somewhat later, their spawning coinciding with the spring tide period following that on which the crisis occurred. In 1952, 2 males spawned two weeks before the spawning crisis, while a male and a female failed to spawn until a month after the crisis, i.e. during the following full moon springs.

Two weeks before the 1952 breeding season, ten males and eleven females were dissected and the nephridia examined. Spermatozoa were found in the nephridia of two of the males and in one female, the nephridial vesicles contained a few eggs.

Discussion:

No evidence has been found to support Kyle's (1896) report of a Spring spawning at St. Andrews. This is the only case where it has been possible to draw a comparison - in the same area - with an author writing at that time. As noted in the literature, authors reporting a Spring spawning may have been influenced by the belief that egg masses of Scoloplos armiger, which are common in the Spring, were those of Arenicola. On the other hand, Ashworth's (1904) report cannot be so summarily dismissed. His description of oviposition, which he observed in the Spring, agrees closely with accounts by recent authors and that

given in the present work (see p. 24). Furthermore, Ashworth's discovery of eggs and spermatozoa in the nephridia in the Spring agrees with observations made in the present work just before the breeding season in the Autumn. No observations subsequent to Ashworth's have been made on the Lancashire coast. - Miss Duncan - Jahr. / Mar. 97.

It has been established that at St. Andrews the onset of spawning is independent of weather conditions (p. 17) but that it does coincide with periods of spring tides. This agrees with observations made by Pirlot (1933), except that at St. Andrews spawning is always related to full moon springs. This, together with the fact that spawning in adjacent populations at St. Andrews coincided within a few days, might suggest that here light and tidal influences exert a direct control on spawning. However, there is some evidence that the relationship between tidal periods and spawning is retained in the laboratory. Also, in adjacent populations at Dublin one population had practically completed spawning while there was no sign of the other commencing. These observations suggest that any environmental control is indirect and that the onset of spawning may be controlled by a physiological rhythm in the organism (see p. 194).

Table 3

The percentage of worms containing genital products in October and November, 1949.

Date	Area A		Area B	
	Total worms	%age ripe	Total worms	%age ripe
24.10.49	36	97%	-	-
25.10.49	-	-	27	55.5%
4.11.49	-	-	82	22.0%
5.11.49	2	50%	-	-
9.11.49	-	-	58	15.5%
20.11.49	31	0%	-	-

Table 4

The percentage of worms containing genital products during October and November, 1950, and the dates on which spawning was observed.

Date	Area A			Area B		
	Spawning observed	Total Worms	%age ripe	Spawning observed	Total Worms	%age ripe
12.10.50	-	40	100%	-	-	-
18.10.50	-	-	-	-	36	47%
24.10.50	X	-	-	-	35	17%
25.10.50	X	-	-	-	-	-
26.10.50	X	17	47%	-	-	-
1.11.50	-	-	-	-	8	0%
4.11.50	-	-	-	-	64	3%
9.11.50	-	8	0%	-	-	-

Dates underlined fell during spring tides.

Table 5

The percentage of worms containing genital products during October and November, 1951, and the dates on which spawning was observed.

Date	Lab.	Area A			Area C		
		Spawning observed	Total Worms	%age ripe	Spawning observed	Total Worms	%age ripe
16.10.51	-	-	14	100%	-	-	-
18.10.51	-	-	-	-	-	21	100%
29.10.51	-	-	3	100%	X	-	-
31.10.51	-	-	-	-	X	89	47%
1.11.51	-	X	48	67%	-	-	-
2.11.51	X	X	-	-	X	43	51%
4.11.51	X	-	-	-	-	-	-
5.11.51	X	-	-	-	-	-	-
7.11.51	X	-	-	-	X	34	26.5%
8.11.51	X	-	-	-	-	-	-
9.11.51	X	-	-	-	-	-	-
12.11.51	X	-	-	-	-	-	-
13.11.51	-	-	-	-	X	80	2.5%
14.11.51	-	-	-	-	X	31	3.0%
17.11.51	X	-	-	-	-	-	-

Lab. = Spawning observed in the laboratory.

Dates underlined fell during full moon springs.

Table 6

The percentage of worms containing genital products during October and November, 1952, and the dates on which spawning was observed.

Date	Lab.	Area A			Area C		
		Spawning observed	Total Worms	%age ripe	Spawning observed	Total Worms	%age ripe
2.10.52	X	-	-	-	-	-	-
14.10.52	-	-	-	-	-	468	70%
16.10.52	-	-	-	-	X	201	52%
17.10.52	-	-	-	-	X	311	48%
18.10.52	X	X	164	42%	-	-	-
19.10.52	X	X	75	37%	-	-	-
20.10.52	-	X	22	13%	X	119	28%
21.10.52	X	-	-	-	X	116	18%
22.10.52	X	-	-	-	-	-	-
31.10.52	X	-	-	-	-	-	-
17.11.52	X	-	-	-	-	-	-

Table 7

The percentage of worms containing genital products during October and November, 1953, and the dates on which spawning was observed.

Date	Seapoint		Booterstown	
	Total Worms	%age ripe	Total Worms	%age ripe
6.10.53	19	89%	-	-
8.10.53	17	100%	-	-
14.10.53	-	-	33	88%
21.10.53	14	50%	21	72%
28.10.53	-	-	19	84%
4.11.53	22	23%	19	84%
12.11.53	-	-	29	86%

Dates underlined fell during full moon spring tides.

Summary:

1. There is no evidence of a Spring breeding season at St. Andrews.

2. Immature genital products were first found in the body cavity in June 1949 and 1950 and in late April 1951.

3. The breeding season, at St. Andrews and Dublin, takes place in the Autumn, sometime between 16th October and 14th November.

4. The spawning crisis may or may not be preceded by a subsidiary spawning.

5. It is suggested that the onset of spawning may be controlled by a physiological rhythm in the organism.

3. Spawning and the fate of the eggs:

The spawning of male Arenicola was observed on the shore and in the laboratory. As noted previously, spawning on the shore only takes place when the sand has a surface covering of water. Seminal fluid is ejected forcibly, in strings from the head holes of the burrows, forming puddles in the surface water. Spawning in the laboratory differed in no essential detail. During general spawning, activity is greatest immediately after the ebb and just before the flow of the tide. not clear!

Despite a close watch on the shore, oviposition was not observed nor were egg puddles found. During the 1951 season, several females spawned in large sinks in the laboratory. The bottom of each sink was covered with sand to a depth of about

3 inches. This in turn was covered with water from the laboratory circulation system. The first specimen spawned on the morning of 8th November. The head of the worm occasionally protruded from the head hole and the eggs were stripped off the body in a thin sheet of mucus as the head was withdrawn. In this way an egg puddle accumulated at the head hole (Plate 1). These eggs were successfully fertilised. It was noted that unlike spawning in the males, the eggs were not forcibly ejected from the nephridiopores but merely accumulated round the worm. Subsequent observations on oviposition, under similar conditions, confirmed this account. Before this worm spawned, samples had been taken, by pipette, from its burrow. These samples contained a number of eggs which were also successfully fertilised.

A female spawned which had been kept in a large crystallising basin with about 500ml. sea water, the bottom of which was covered with a thin layer of sand. The worm formed an open 'burrow' round the edge of the basin and in this the eggs were deposited. They were imbedded in fine mucus and partly surrounded the worm.

Plankton hauls were made using an apparatus called the 'sledge' (Fig. 2), devised at the Gatty Marine Laboratory. It was designed to collect organisms and eggs from the surface layers of sand. It consisted of an open frame set on steel runners for sliding along the bottom. On the front of the frame a chain was fixed so that it rubbed along and stirred up the surface sand. A plankton net was tied to the back of the frame.

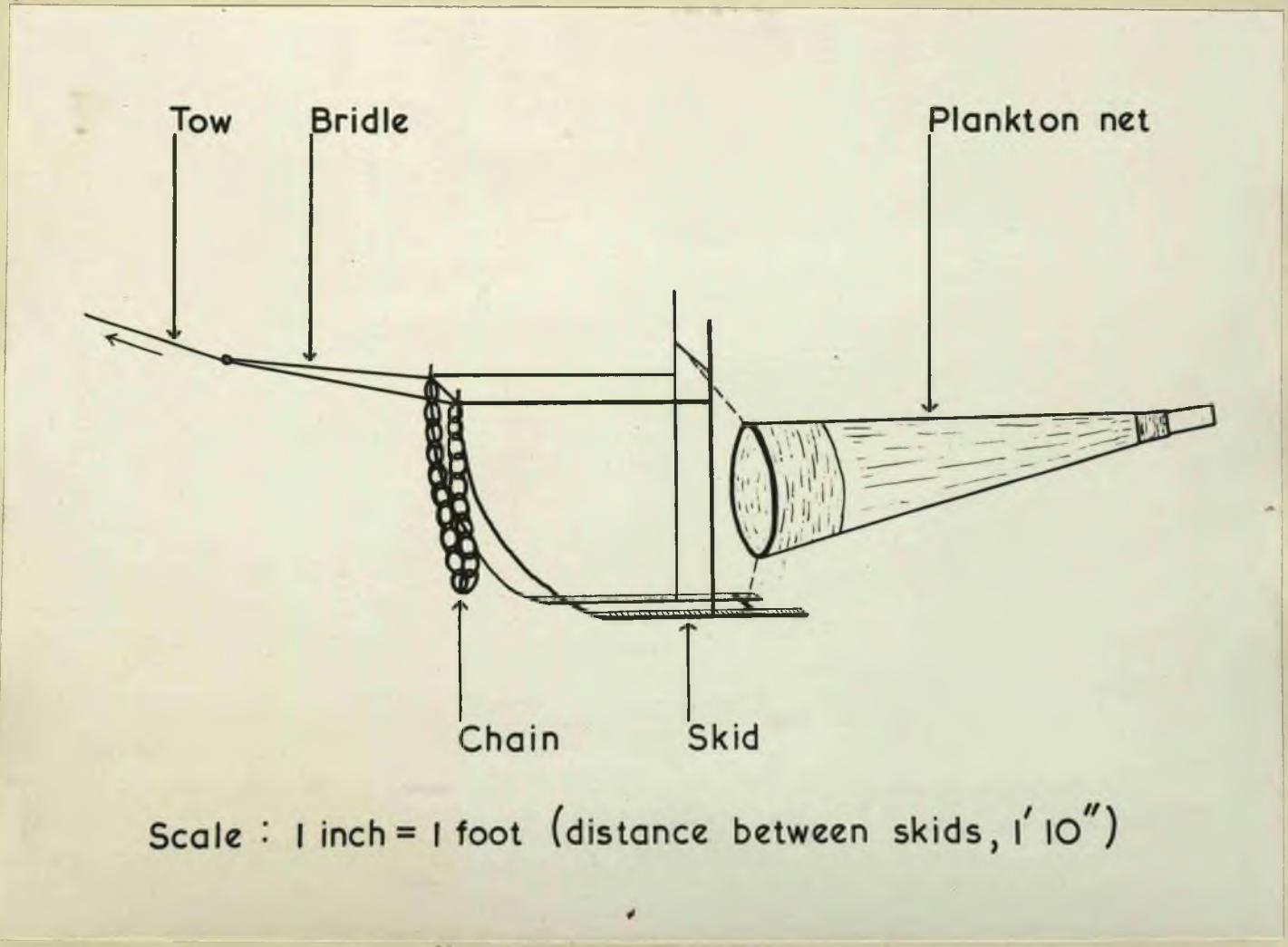


Fig. 2, 'The sledge' (partly to scale). An apparatus for towing along the bottom and designed to collect eggs and larvae from the superficial layers of sand.

When the apparatus had been towed over a sandy bottom, the plankton tin was found to contain a quantity of fine silt in which were various larval forms, including young annelids. In 1950, hauls were made at high tide over Area A, six just before the breeding season (October 19th to 23rd) and five during the breeding season (October 24th and 25th). Thereafter rough weather prevented the 'Argonaut' going close inshore until November 10th, when hauls were made over Area C. The samples obtained were examined under a binocular dissecting microscope. Neither eggs nor larvae of Arenicola were found.

During the same season, other methods were adopted to find eggs and larvae. On October 26th samples of surface sand, to a depth of 1 inch, were collected at scattered points in Area A. Later, on 1st November (Area B) and 9th November (Area A) sand samples were taken from the shafts of worm burrows. All these samples were elutriated by passing a jet of sea water through the sand in an elutriation flask. The water which came over was filtered through fine bolting silk. Neither eggs nor larvae were recovered by this method.

In 1952, the sand surrounding the worm burrows was sampled. A glass tube was used to lift a core containing the head or tail shaft. In addition, Newell's (1949) method for finding eggs and larvae was used. A pit was dug in the sand, and from it, radiating channels. Water from the surrounding sand drained into the pit and was subsequently sieved through a

plankton net. Both methods were used (the latter four times) on 1st and 2nd November in Area C and both failed to produce either eggs or larvae.

Discussion:

This section is largely a repetition of previous work. Observations made while the males were spawning on the shore differ only in minor detail from previous reports. Newell (1948) noted that spawning takes place half an hour after dead low water, whereas the greatest activity at St. Andrews is immediately after the tide leaves the worms and just before the flow. Newell also states that sperm is pumped from the tail shafts, whereas here it appeared at the head holes of the burrows.

Previous reports, from laboratory observations, suggest that eggs are spawned in the same way as sperm, so that they collect at the mouths of the burrows. A similar phenomenon has never been observed at low tide on the shore. Newell (1948) suggested that eggs were not found on the shore due to their similarity in colour to the sand. This is not an acceptable suggestion if it refers to egg puddles similar to those observed in the laboratory (Plate 1). Two possibilities remain with regard to oviposition:-

(a) females may spawn in the manner observed in the laboratory but only while covered by the tide, or,

(b) the eggs may be retained in the burrows after oviposition in the way described by Okuda (1938) for Arenicola claparedii.

If the first of these possibilities is true it appears certain, from the known sampling capabilities of the 'sledge', that eggs and larvae would have been obtained over the spawning area. However, hauls made during the breeding season were unsuccessful.

It seems doubtful whether accounts of oviposition in the laboratory can be related to spawning under natural conditions. Reports by previous authors do not mention the depth of sand in their aquaria in which oviposition was observed. In the aquaria used at St. Andrews when the depth of sand was greater than 3 inches the organic material tended to decompose rapidly. In order to maintain healthy conditions it was therefore impossible to have a depth of sand sufficient for the worms to make a normal burrow. Thus, during oviposition, the head of the worm frequently protruded from the mouth of the burrow and the eggs were stripped off as the head was withdrawn. This may not occur in the deeper natural burrow. If this is true, it is unlikely that the eggs would reach the surface as they are not forcibly expelled from the nephridiopores. It may be then that they are retained in the burrows after oviposition.

Apart from this negative evidence, it is worth noting that the earliest larval stage located by Newell (1948) in the young worm area at Whitstable (at H.W.N.T.) was the 2nd chaetiger. No younger larva has ever been found on the shore. Newell reported finding dividing eggs but these were found, not in the young worm area, but in the area where the adults were spawning.

This suggests that the eggs were not carried up the shore immediately after oviposition. Furthermore, Newell states that there is a correlation between the greatest spawning intensity and neap tides, so that the eggs may be carried by the tide to a suitable habitat at H.W.N.T. This would not hold good at St. Andrews or in Belgium, where the crisis occurs during spring tides. If the eggs were immediately released from the burrows they would be carried to the dry region between H.W.N.T. and H.W.S.T. The weight of evidence suggests that the eggs are retained in the burrows till after hatching. The larvae may then gain the surface and be carried up to the young worm areas during neap tides.

Some doubt must remain. The method described above (p.25) in which cores of sand containing the head or tail shafts of the burrows were examined, failed to yield eggs or larvae. However, the method was inefficient as only the top 2 - 3 inches of the vertical shafts were sampled.

No information was gained on the mode of life of the larvae. Observations made while rearing larvae in the laboratory (Section 11) confirm Newell's statement that there is no true pelagic larval stage in the life history.

PLATES - SECTION I

Plate 1

Female *Arenicola* in a laboratory sink containing a 3 inch layer of sand. An egg puddle can be seen round the entrance to one of the burrows and a few eggs are also present round the tail of the worm emerging from its burrow (left).

11. FERTILISATION AND LARVAL DEVELOPMENT.

(a) Literature

1. Artificial fertilisation in Arenicola and other annelids.

With the exception of Child (1898), previous authors have found that coelomic eggs* of Arenicola marina cannot be fertilised. Child gave an account of maturation and fertilisation, stating that eggs were taken from the coelom and fertilised immediately in sea water. Subsequently, Pirlot (1933) pointed out that Child's investigation was carried out before the important revision of the diagnostic features of Arenicola marina made by Gamble and Ashworth (1898) and Ashworth (1904). In consequence, there is some doubt whether Child was dealing with this species.

Pirlot (1933) described his attempts to bring about artificial fertilisations with the coelomic gametes of Arenicola marina. The spermatozoa showed no activity in sea water and the eggs failed to ripen. They were treated in various ways to induce ripeness and activity (see pp.33 & 38) and, as a result, spermatozoa did attach themselves to the chorion of the egg. However, fertilisation did not take place. In explanation, Pirlot suggested that maturation of the eggs is initiated in the nephridia. Thus eggs can only be fertilised once they have passed through the nephridia in the course of oviposition. Miscrosoopic examination of eggs taken from the nephridia during spawning, supported this theory. 'Les oocytes s'y montrent en voie de maturation; les vésicules germinatives

* Eggs taken from the coelom artificially.

sont nettement plus chromatiques que d'habitude et leurs parois sont plissées et presques effacées.'

In contrast with Pirlot's observations, Newell (1948) found that coelomic sperm* soon acquired motility when transferred to sea water, especially if the water was slightly hypotonic. The results of fertilisations attempted by Newell may be summarised as follows:-

(a) Coelomic oocytes with coelomic sperm - produced fertilisation and two cleavages.

(b) Coelomic oocytes and spawned sperm - the oocytes again did not develop beyond the 4-cell stage.

Newell pointed out that these results discount Pirlot's theory that eggs are not capable of fertilisation until they have passed through the nephridia. Nevertheless, they suggest that there is a bar to the subsequent development of the embryos. As results are similar irrespective of whether fertilisation is made with spawned or coelomic spermatozoa, it would appear that the bar to development is in the (coelomic) eggs.

The Arenicolidae as a whole vary in the ease with which artificial fertilisation can be brought about. Ashworth (1904) experienced no difficulty with A. claparedii. Ripe ova were artificially fertilised by adding to the sea water in which they were contained a small quantity of spermatozoa from the coelom

* Sperm taken from the coelom artificially.

of a mature male. On the other hand, Child (1900) could not bring about fertilisation with the coelomic gametes of A. oristata - even at the height of the breeding season.

Occasional references are found in the literature to difficulties in making artificial fertilisations with other annelids. Wilson (1936a) reported repeated failure with eggs and spermatozoa from the body cavity of Audouinia tentaculata. In this case spawned eggs could be fertilised with coelomic sperm. In general, however, artificial fertilisations can be made without difficulty in annelids. Fuchs (1911) obtained ripe sperm and ova from Nephtys hombergi, by slitting open the body cavity with a pair of scissors. Males and females slit in this way were placed separately in sterile finger bowls containing outside sea water to allow the gametes to exude. The worms were then removed and fertilisations made. Fuchs refers to the belief, current at that time, that eggs from the body cavity of an organism show irregular segmentation. He states that this is not true in the case of Nephtys hombergi. Wilson (1936b), also with N. hombergi, found that similar methods gave cultures of strongly swimming larvae. Earlier, Wilson (1932a), reported that artificial fertilisations can be made with ripe specimens of Owenia fusiformis. These were laid on glass trays and the posterior segments slit to release the eggs and sperm. The eggs were washed by decanting in filtered outside sea water and retained for an hour or two before fertilisation. After fertilisation they were again washed to

remove excess sperm. Wilson (1933) successfully brought about artificial fertilisations in Notomastus latericus by the same method. Investigations of sperm entry in ova of Nereis limbata by Lillie (1912) and Chambers (1933) suggest that artificial fertilisation can be made without difficulty in this species. More recently, Dales (1950) made artificial fertilisations in Nereis diversicolor. Ova and spermatozoa were obtained by slitting mature individuals at the base of the parapodia, where there was least blood. Fertilisations were made with a small quantity of sperm which was later decanted off.

2. Precautions in artificial fertilisation experiments.

Factors inhibiting fertilisation were reviewed by Lillie and Just (1924). Embryologists were said to agree that contamination of the eggs of some marine invertebrates, with coelomic fluid or tissue exudates, reduces the percentage fertilisation. Lillie (1914) had previously demonstrated that the eggs of Arbacia contaminated in this way could be washed and subsequently fertilised. A second factor inhibiting fertilisation is the presence of metallic ions in the medium. Hoadley (1923) showed that eleven heavy metals inhibit fertilisation, while Lillie (1921) demonstrated that $CuCl_2$ in a dilution of 2 p.p.m. (parts per million) completely inhibits fertilisation in Arbacia. On the other hand, Rothschild and Tuft (1950) and Rothschild (1950) in an investigation of the dilution effect on the rate of O_2 consumption in dense suspensions of sea urchin spermatozoa,

water. No details of dilution were given. Newell (1948) found that coelomic sperm morulae become active in sea water without treatment, though activity is enhanced if the water is made slightly hypotonic.

Much of the literature on the physiology of fertilisation is concerned with the activation of sperm suspensions. Lillie's (1914, 1919) 'Fertilizin' theory involves the production of a specific secretion (fertilizin) by the cortex of the unfertilised egg, which increases the chances of a meeting between egg and sperm. Fertilizin was said to have the following effects upon spermatozoa of the same species:- (a) activation; (b) aggregation (i.e. chemotaxis up an increasing gradient of egg secretion) and (c) agglutination or clumping together of the spermatozoa. Agglutination in a concentrated solution of egg secretion was said to have no significance, as such, in fertilisation. The reactions involved normally bring about the attachment of the sperm to the egg (Lillie and Just, 1924). With homologous sperm, agglutination is reversible and non-toxic.

Gray (1922), in suggesting a physical approach to the problem of fertilisation admitted the existence of egg secretions but suggested that they are (a) non-specific and (b) that their only effect lies in their ability to increase the activity of sperm suspensions. Gray (1920) had shown that sperm activity can be regulated by alteration of the hydroxyl ion concentration of the medium and he suggested (1922) that

fertilizin stimulates activity merely by virtue of being a weak base. Sperm aggregations were said to be due to the movement of spermatozoa towards a region of optimum CO_2 concentration produced by their own activity. In cases where aggregations are temporary, dispersal results from the abatement of activity, and in consequence CO_2 production, in an increasingly acid medium. Finally, Gray goes on to state that "agglutination depends primarily on the presence of free ions." Marked agglutination can be produced with the trivalent cation Cerium and with Calcium. Lillie and Just (1924) were aware that agglutination could be produced with alkalies and other agents but this was said to be mere physical adhesion, which is toxic and non reversible.

Gray (1928c) examined the effect of egg secretions on the sperm of Echinus miliaris and Echinus esculentus. The spermatozoa of the former immediately become active in sea water. The only effect of the presence of egg secretions is to prolong the high level of activity as represented by the O_2 consumption. The sperm of the latter, at first relatively inactive in sea water, is immediately activated when egg secretions are present and again the high level of activity is prolonged. These results suggested that egg secretions inhibit the senescence of motile spermatozoa. Carter (1930, 1931a) repeated this work and found that thyroxine produces the same effects but to a lesser degree than egg secretions.

Carter (1931a) found that unripe sperm of the sea urchin is at first inactive in sea water, but that it gradually ripens if allowed to remain dry for some hours before dilution. A similar observation was made by Dodd (1951), who found that sperm from the limpet can be activated by allowing the teased gonad to lie for some time in a virtually dry condition. According to Carter (1931a) unripe sea urchin sperm immediately becomes active if egg secretions are present in the water. A similar effect can be brought about by thyroxine and by increasing the alkalinity of the medium. With the latter, however, the sperm ages more rapidly. The pH value at which the sperm can be activated varies with the ripeness of the sperm.

In a later paper, Carter (1932b), demonstrated that the phenomenon of agglutination can be produced by thyroxine. It resembles agglutination in alkaline sea water (previously reported by Gray, 1920; and Lillie and Just, 1924) in that it is permanent. Unripe sperm agglutinates with the addition of 1% N/10 NaOH and strongly with 2% N/10 NaOH (pH 9.4). Carter's general thesis expounded in this and subsequent papers (1931b; 1932a; 1932b,c and d; and 1935) is that an active component of egg secretions which, among other properties, could activate spermatozoa, is related chemically to thyroxine.

Hartmann and Schartau (1939) extracted Echinochrome A, a red pigment, from the eggs of Arbacia pustulosa. This is capable of causing activation and chemotaxis in the sperm of that species in dilutions of 1:2,500,000,000 and 1:2,000,000,000

respectively. The chemotactic effect is not repeatable with the sperm of Arbacia punctulata or Strongylocentrotus purpuratus, a fact that was confirmed by Cornman (1941). Previously a review of the literature had led Morgan (1927) to state that there is no evidence for chemotaxis in spermatozoa. Cornman (1941) was able to extract a protein complex from the eggs of A. punctulata, which both activates and agglutinates the sperm of that species. The activity factor appears to be in the protein moeity. There were indications that activity and agglutinating factors in egg secretions can be separated by dialysis. Tyler (1940, 1941) gave a method for the extraction of fertilizin* from the gelatinous coat of the sea urchin egg with which it was said to be chemically identical. This is contrary to Lillie's (1914, 1919) theory that fertilizin is derived from the cortex of the egg. Tyler (1955) states that fertilizin is a complex of amino acids and sugars, that may be termed a glycoprotein. Referring to the work by Cornman (1941), Tyler concludes that the activating agent is normally bound to the agglutinating agent and confirms that they may be separated by dialysis, the former being a substance of relatively low molecular weight whose exact chemical nature is unknown. Tyler (1948, 1955) also drew attention to the analogy between agglutination and immunological phenomena. It was suggested

* Tyler restricts the use of the word 'fertilizin' to the agglutinating agent.

that the mutual multivalence theory of antigen - antibody reactions holds for the agglutination of sperm by fertilizin. If this is so, the molecule of fertilizin must possess two or more combining groups available for uniting separate cells.

Although the majority of this work has been performed with echinoderms, Tyler (1955) notes that agglutination of sperm in homologous egg water has been reported in a variety of other groups (including annelids) in which, however, it tends to be permanent or long lasting.

4. Artificial fertilisation.

Pirlot (1933) describes a variety of media in which coelomic eggs of Arenicola marina were treated in an attempt to induce artificial maturation. Sea water alkalised with increasing quantities of sodium hydroxide or to which had been added calcium chloride or potassium chloride, produced elevation of the perivitelline membrane, but the germinal vesicle remained intact. Fertilisations attempted in these media were unsuccessful. Eggs treated with an emulsion of nephridia (see p. 29) showed no sign of maturation and again fertilisations were unsuccessful.

The literature suggests that a number of methods whereby, (a) ripeness may be induced in eggs which, untreated, cannot be fertilised, and (b) sperm entry can be facilitated. Lillie (1914) suggested that fertilizin is essential to the ripening of the egg, but that it is only produced in the last stages of

that process. Later, Carter (1932a) demonstrated that the percentage development in slightly unripe eggs of two echinoids can be improved by treatment with secretions of ripe eggs or thyroxine. There is no evidence that this reaction, like many others, can be reproduced by alteration of the H ion concentration of the medium. Woolfsohn (1907) reports Loeb as finding that eggs of Asterias forbesii do not mature immediately in sea water. The substances in sea water required to produce or accelerate maturation are O_2 and free OH ions. Loeb also reported that if eggs of Lottia gigantea are removed from the ovary they fail to mature in sea water. However, fertilisation is possible if these eggs are treated with 1ml N/10 NaOH in 50ml sea water for 4 - 5 hours. Woolfsohn (1907) found that the eggs of 4 species of Aomaea very rarely mature in sea water if taken from the ovary. When treated with N/10 NaOH in concentrations ranging from 0.4 - 1.5ml/50ml sea water, for periods of 1 - 3 hours, they subsequently matured in from 2 - 18 hours after return to normal sea water. The highest percentage successful fertilisation was obtained when the eggs were treated with 1.5ml N/10 NaOH/50ml sea water for 1 hour, good larvae resulted. Maturation can also be induced by a number of fat solvents.

Lillie (1919) reported that within certain limits an increase in alkalinity of the medium favours fertilisation. Lillie and Just (1924) include a report by H.W. Smith that the eggs of Arbacia and Asterias are readily fertilisable between a

pH of 8.0 and 9.8 to 10.0. It was thought that the plasma membrane of the egg becomes more permeable in alkaline media. Hyperalkaline media were used by Loeb (1903, 1904) to bring about heterogeneous hybridisation. Lillie and Just (1924) noted that the reaction is only favoured in the actual presence of alkali. This is not the case in the sea urchin/crinoid crosses made by Godlewski (1906), in which the eggs can be washed and fertilised after exposure to alkali. Hörstadius (1923) found that alkalinity is favourable to the maturation of the eggs of Pomatoceros triqueter. The percentage fertilisation varies between 0.6% at pH 7.0 and 86.6% at pH 9.6.

5. Sperm entry - egg membrane lysins.

Loeb (1916), in support of his 'cytolytic' theory of fertilisation, based on evidence that all parthenogenetic agents have a cytolytic effect, suggested that spermatozoa carry a lysin effective in the fertilisation of the egg. Lillie and Just (1924) rejected this concept. Although Loeb apparently overestimated the significance of the postulated lytic agent, abundant evidence is now available to show that spermatozoa do, in fact, carry such a substance. The dispersal of the cementing substance (Hyaluronic acid) of the follicle cells of the mammalian egg, by Hyaluronidase, has been investigated by a number of workers (see Meyer and Rapport, 1952). There have also been reports of lytic agents carried by invertebrate spermatozoa. Tyler (1939) demonstrated the presence of a substance capable of

dissolving the egg membrane in extracts of the sperm of Megathura crenulata and in Haliotis cracherodii. This substance was found to have distinct protein properties and evidence was brought forward to show that its activity is dependent on the presence of sulphydryl groups in the molecule. A lytic agent was also detected in the sperm of Arbacia punctulosa by Hartmann et al (1940), while Tyler (1948) reports Monroy and Ruffo as finding that the gelatinous coat of the sea urchin egg can be dissolved by both homologous and heterologous sperm extracts. These were prepared as for the preparation of Hyaluronidase from bull testis. A lytic agent extracted from the sperm of Mytilus edulis, by Berg (1950), was found to be unrelated to Hyaluronidase. Hyaluronidase has no effect on the cementing substance of the egg of the mussel.

The literature indicates that the vitelline membranes of invertebrate eggs can be readily attacked by enzymes. Tyler (1939) found that, while the membrane of the keyhole limpet is resistant to concentrated acid, it can be dissolved by enzymes other than sperm lysins. Moore (1930a, b) has shown that fertilisation takes place in Strongylocentrotus purpuratus and Dendraster excentricus without membrane formation after treatment with trypsin. Hultin (1948a, b) has reported that such treatment renders eggs more easily cross fertilised.

6. Larval development.

Inability to fertilise artificially the eggs of Arenicola marina, and the infrequent occurrence of naturally shed eggs have made the study of larval development in this species difficult. The first description of the early larval stages of Arenicola marina was given by Blegvad (1923). Fertilised eggs were found in an aquarium. The external features and the habits of the larvae were described. Four days after fertilisation a free swimming trochophore hatches which shows the typical ciliated bands of this type of larva. The 1st, 2nd and 3rd chaetigerous segments appeared on the 2nd, 4th and 7th days after hatching respectively. Many died at this stage and no further development was observed. No account was given of the conditions under which the culture was maintained. Later, 5 chaetiger larvae were described from a separate brood.

A more detailed account of larval development was given by Newell (1948, 1949). Fertilised eggs were again found in an aquarium. Early cleavage and hatching stages were described. A fertilisation membrane appears 1½ to 2 hrs. after fertilisation and the first cleavage 1 hr. later. A study of three cleavages sufficed to show that cleavage is spiral and similar to that described by Child (1900) for A. cristata, and Ashworth (1904) and Okada (1941) for A. claparedii. Advanced cleavage and the internal structure of the trochophore could not be made out since later developmental

stages are opaque. The external features and the habits of the larvae closely resemble the description given by Blegvad (1923). Hatching takes place four days after fertilisation. Whole mounts failed to reveal more than the apical tuft, prototroch, telotroch, ventral ciliated band and a pair of reddish brown eye spots. At this stage the brood died due to unsatisfactory conditions in the culture.

Among larvae collected in the Fucus zone at Whitstable (Newell, 1948, 1949, see p.10) were 2nd chaetiger stages. In these the ciliated bands, apical tuft and eye spots are still present. The 1st chaetigerous segment bears a slightly curved simple seta and a spatulate seta. The larvae are capable of swimming when disturbed but quickly settle, becoming attached by their posterior ends. Generally they crawl about the substratum and show muscular movements, particularly of the prostomium. Early larvae were also found by Pirlot (1933) and Smidt (1951), but were not described.

Newell (1949) found that 3rd and 4th chaetiger larvae were similar to the 2nd chaetiger stage, with the ciliated bands still present. Again the larvae were capable of swimming but were enveloped in mucus. Early post larval stages were also described. The proboscis appears to engulf food in the same way as in the adult. It was noted that the "larva was enclosed in a thick mucus tube, a good deal longer than the total length of the larva", and that it did not inhabit a burrow. Newell found that the description of post larval stages given by

Thorson (1946) was distinctly different from his own observations. However, Benham's (1893) description contained fewer discrepancies. These were as follows:-

(a) The larvae differed in general appearance.

(b) Benham's larvae had fewer segments in the tail region.

(c) There were only 2 capillary setae in the anterior notopodial bundles and the notopodial bristles were all of one kind. Notopodial lobes were absent.

(d) There were fewer crochets in the neuropodial region.

The descriptions of post larval stages by Ashworth (1904) and Newell (1949) agree closely.

7. The rearing of annelid larvae in the laboratory.

According to the literature, no larvae of Arenicola marina have survived in the laboratory longer than the 5th chaetigerous segment stages reared by Blegvad (1923). These larvae were not fed and the culture conditions were not described.

Allen and Nelson (1910) were the first authors to suggest a systematic approach to rearing marine larvae in the laboratory. Culture media and methods for the maintenance of pure cultures of unicellular marine algae were described. These algae were used as food for a variety of larval forms. Larvae were reared in "tank water" which was filtered through charcoal, and Berkefeld filter candles. It is worth noting

that when tank water and outside sea water were compared as media for algal cultures, the latter gave better results. Water was stored in sterile glassware and the instruments used for handling larvae were also sterilised. A small number of larvae was kept in a large volume of medium, e.g. 70/80 per 2000ml. Bruce, Knight and Parke (1940) found that oyster larvae could be reared successfully in outside sea water. This was changed at frequent intervals. They emphasised the importance of maintaining a constant temperature. Wilson (1932a, b; 1933; 1936a) reared a variety of annelid larvae in plunger jars containing outside sea water, while Dodd (in the Press) found that stirring the larvae improved the results of rearing experiments with Patella vulgata. The latter author also emphasised the importance of a constant temperature.

In general, the purpose of investigations in which larval annelids have been reared has been to describe the development stages. Little attempt has been made to discover the most suitable food materials and culture methods for these larvae. Allen and Nelson (1910) found that Pomatoceros triqueter survived through metamorphosis, became attached and grew to normal size when fed on the diatom Phaeodactylum tricornutum (Nitzschia closterium). In one experiment the larvae of Sabellaria alveolata survived for three months on this diatom. Wilson (1932a) reared larvae of Owenia fusiformis through metamorphosis, on Phaeodactylum, in three experiments out of many.

Phaeodactylum was again used by Wilson (1936a) to rear larvae of Audouinia tentaculata. The most advanced stage reared was nine months old and possessed 40 chaetigerous segments. After metamorphosis these larvae were kept in flat bottomed glass dishes in fine sandy gravel which had been washed and boiled. Day (1934) described the larvae of Scolecolepis fuliginosa. These were reared through metamorphosis on Phaeodactylum. Wilson (1933) was less successful with larvae of Notomastus latericus. These were reared to F + 23 and 7 chaetigers on Phaeodactylum. Wilson was of the opinion that at this stage most of the yolk was used up and that the larvae could not be reared much further on plain Phaeodactylum.

Fuchs (1911) and Wilson (1936b) found that the larvae of Nephtys hombergii could not be reared in the laboratory. Fuchs states that development ceased after 14 days and the larvae only survived for 3 - 4 weeks. Wilson reports that the larvae "ate Nitzschia and Thalassiosira and probably other organisms as well", but failed to develop. Wilson (1936b) again had no success with Pectinaria koreni. In this case it was suggested "that diatoms were too large for them". Earlier, Wilson (1932b) had reared larvae of Nereis pelagica in plunger jars containing outside sea water which was filtered only through bolting silk. The larvae fed on diatoms which grew naturally in the jar. The larvae settled and formed tubes. Some individuals survived for one year, of which the largest was 1.5cms. in length (fixed), and had approximately 60

chaetigerous segments.

Dodd and Howie (unpublished data) compared the food values of various marine micro-organisms and dead organic materials for the bottom living larvae of Cirratulus cirratus. Growth rates were compared in several experiments for each food material. Fisher's 't' test was applied to test the significance between means of the growth rate results. The growth rate on dried liver powder (245μ/week) was at least twice that on any of the other foods employed. Larvae fed on this material were kept alive for over 2 years. The next most successful foods were dried egg powder, and the diatoms, Chaetoceros decipiens and Phaeodactylum tricornutum. Growth rates on these materials could not be separated statistically. Other materials, less successful, but which gave some growth, were - Chlamydomonas sp. I, Skeletonema costatum and Coccolithus sp. - in that order.

(b) Fertilisation experiments

1. Fertilisations with spawned eggs. (1951)

During the breeding season six females spawned in the laboratory and eight fertilisations were made. In some cases, oviposition took place in sinks with about 3 inches of sand on the bottom in which the worms had been kept for up to 2 weeks. In others, eggs were obtained from worms which spawned in trays immediately after they were brought into the laboratory. In one case a female spawned after it had been kept for some time at 15°C in the constant temperature room (see p. 24).

Methods: Fertilisations were made with spawned or coelomic sperm. The latter was activated by diluting approximately 1ml in 50ml sea water, to which was added 0.5 - 1.0ml N/10 NaOH (see p.71). Cultures were kept at 15°C. Other conditions and the treatment of the gametes varied from experiment to experiment.

Experiment 1: A few eggs were obtained from a burrow in the sand tank. These were placed in a small crystallising basin containing 50ml sea water from the laboratory circulation system (circulation water). Fertilisation was made at once, using a few drops of a suspension of coelomic sperm.

Experiment 2: A large number of eggs was obtained from two worms. The majority of eggs from the first spawning (A - table 8) were divided between two jars, 9 and 10, each containing 2 litres circulation water.

In jar 9 no attempt was made to clean the eggs, which were surrounded with sand and mucus. In jar 10, the eggs were washed by decanting and were subsequently kept in suspension by means of a glass rod stirrer. Three or four drops of coelomic sperm were added to each jar. Finally, a number of eggs from this spawning (A) was placed in a/250ml beaker, jar 11; these were treated in the same way as the eggs in jar 9 but in this case the fertilisation was made with naturally spawned sperm.

Eggs from the second spawning (B, jar 12 - table 8) were treated exactly as those in jar 9.

Experiment 3: A female spawned on 9th November, soon after collection. The eggs were transferred to a beaker containing 1 litre of circulation water. Fertilisation was made with coelomic sperm activated by alkali. The suspension in this case was very dilute.

Experiment 4: A female spawned in the sand tank on 12th November, but no males were available to make a fertilisation owing to bad collecting conditions. The eggs were pipetted off, and after careful washing were kept in the constant temperature room overnight. On the following day they were again washed and transferred from circulation water to outside sea water*. Naturally spawned sperm was added and the eggs

* Water collected approximately 3 miles offshore in St. Andrews Bay and transported to the laboratory in glass carboys.

were stirred.

Experiment 5: The only remaining female spawned in the constant temperature room on 17th November. The worm was removed, and the circulation water in which it had been lying was decanted. 1 Litre filtered outside sea water was added carefully so that the eggs were not disturbed from the mucus in which they had been spawned. Fertilisation was made with naturally spawned sperm.

Results:

The results of these experiments are related to experimental conditions in Table 8. During the four days after fertilisation the following cultures became heavily contaminated with ciliates and bacteria:- jars 9, 11 and 12 (experiment 2); jar 1 (experiment 3) and jar 3 (experiment 5). The eggs in these cultures had not been washed before fertilisation. Subsequent changes of water, and stirring from F + 3, in the case of jar 1 (experiment 3), were insufficient to control the contamination. In the other cultures the eggs had all been thoroughly washed before fertilisation (except in experiment 1 where only a few eggs were present). The eggs in these cultures had also been stirred.

All the cultures gave a high percentage of segmentation stages (Table 8). However, in experiment 1 and jars 9, 10 and 12 (experiment 2), no normal larvae hatched. In jars 9 and 10 a few irregular larvae hatched, which exhibited a constriction in the mid region giving them the appearance of a large 2 cell stage. They failed to develop further. In all the remaining

jars there was a large hatch of regular larvae on F + 4/5. 200 Larvae were selected for rearing experiments from jar 11 (experiment 2); more than 150 larvae were obtained from jar 1 (experiment 3) and more than 700 larvae from jar 2 (experiment 4). There was also a large hatch in jar 3 (experiment 5), but larvae which were not immediately removed from the original jar were killed by a rapid deterioration of the conditions in the culture.

2. Fertilisations with spawned eggs (1952)

Methods: The preparation of the gametes and the conditions of culture were more uniform in these experiments. Eggs were obtained from females which spawned in trays under the laboratory circulation system. The eggs were removed and washed by decanting. 5 Broods of eggs were fertilised, 2 were changed from circulation to filtered outside sea water before fertilisation, 2 on F + 1 and 1 on F + 2. In one case (jar 5/22) Berkefeld filtered outside sea water was used. Four broods were fertilised with spawned sperm found on the shore while in the final experiment (5/22) the sperm came from a male which spawned as a result of injection of extract (see Section III).

During the period prior to hatching the water in the cultures was changed daily and the embryos were transferred to clean glassware at least once during this period.

Table 8

Fertilisations with spawned eggs (1951), conditions related to results.

	Exp. No.		1	2A			2B	3	4	5
	Jar No.			9	10	11	12	1	2	3
	Sperm	Spawned				x			x	x
		Coelomic	x	x	x		x	x		
Conditions - F to F + 5	Water	Circulation	x	x	x	x	x	x		
		F.O.S.W. *							x	x
	Eggs washed before fertilisation				x				x	
	Times water changed			1	1	2	1	2	2	2
	Stirred (days)				4			1	4	
	Condition of culture	Clean	x		x				x	
		Contam.		x		x	x	x		x
Results	%age dividing		high	86	64	91	100	95	100	100
	Hatch of normal larvae					x		x	x	x
	Survival	Chaetae				6		6	12	5
		Months				4		3	4	$1\frac{1}{2}$

* filtered outside sea water.

Results:

Results are related to experimental conditions in Table 9. Despite the precautions outlined above, four cultures became contaminated by ciliates or bacteria. Only jar 5/22 (Table 9) remained clean. In all but one case the percentage segmentation was high (Table 9) but the hatch was poor. The maximum in any fertilisation was 25 larvae, at least half of which were abnormal. No attempt was made to rear these larvae.

Discussion of Results:

A feature of the 1951 experiments was the fact that all the cultures gave a high percentage of segmentation yet only half of them produced regular larvae. The only experimental condition related to this result appears to be the type of sperm used. All three fertilisations with spawned sperm gave regular larvae while only one of the five fertilisations with coelomic sperm gave regular larvae. It may be, that though coelomic spermatozoa became active after treatment with alkali, they were still unripe and in consequence, in the majority of cases, the nuclei failed to function.

The various physical conditions imposed on the 1951 experiments appeared to bear little relation to their success or failure. Failure to wash the eggs before fertilisation resulted in heavy contamination of the culture by bacteria and ciliates. However, this did not affect the immediate results. All the cultures gave a high percentage segmentation and of the four

giving a hatch of regular larvae, three were heavily contaminated. The eggs used in the experiments had been spawned into circulation water. They had, therefore, been exposed to any inhibitor of fertilisation which might be present in the circulation system. There was therefore no apparent advantage in a change to outside sea water. This proved to be the case: of the four cultures which gave regular larvae, two had been kept in circulation water and two had been changed to outside sea water before fertilisation.

While it was true that contamination of a culture did not affect the immediate results, nevertheless the increase in ciliates and bacteria may be so rapid that it cannot be controlled by changing the water. In consequence, as in jar 3 (experiment 5), the larvae may be killed after hatching. In view of these observations, it is probable that a combination of the following factors, allied to fertilisation with naturally spawned sperm, contributed to the survival of the most successful brood, (jar 2 - Table 8):-

(a) the use of filtered outside sea water,

(b) washing the eggs before fertilisation, and

(c) some stirring.

Although the percentage segmentation was again high in the 1952 experiments and regular larvae hatched in four of the five experiments, nevertheless the results in terms of numbers of larvae which hatched, were relatively poor. In view of the fact that all the fertilisations were made with naturally spawned sperm,

Table 9

Fertilisations with spawned eggs (1952), conditions related to results.

	Jar No.		1/19	2/20	3/20	4/21	5/22
Conditions - F to F + 5	Spawned sperm		x	x	x	x	x
	F.O.S.W. (days)		2	3	4	3	4 *
	Eggs washed before fertilisation		x	x	x	x	x
	Times water changed		3	4	4	4	4
	Culture	Clean					x
		Contam.	x	x	x	x	
Results	%age dividing		97	81	77	48	68
	Hatch of normal larvae			x	x	x	x
	Survival (months)			½	½		½

* Berkefeld filtered outside sea water

there are only two apparent explanations for the results. First, there may have been some deterioration in the condition of the spawning females. The small trays in which the worms were kept were not as suitable for this purpose as the sand tanks used in the previous year. Worms which spawned in similar trays in 1951 had only been in the laboratory overnight. All the worms from which eggs were obtained were collected on 17th October. Spawning took place intermittently from 19th October. With the exception of the last experiment, the percentage segmentation decreased the longer the worm had been kept in the laboratory before it spawned (Table 9). Secondly, the conditions obtaining in the 1952 experiments were in some respects more rigorous than those in the previous year. The water in the cultures was changed daily and there was at least one transfer to clean glassware before the eggs hatched. Reference will be made below (p.103) to the detrimental effect of removing the larvae from the bottom. Dividing eggs, like the larvae, tend to adhere to the bottom of the container. Although only a subjective observation and contrary to accepted principles, it may be that frequent changes involving separation of the eggs from the bottom may have a detrimental effect on their development.

Summary:

1. These experiments have shown that:-

(a) Spawned eggs can be successfully fertilised with spawned sperm under laboratory conditions.

(b) Spawned eggs can be successfully fertilised with coelomic sperm. Larvae from such a fertilisation were reared to 6 chaetigers and kept alive for 3 months. This confirms the views that in artificial fertilisations with coelomic eggs and coelomic sperm, any inhibition of fertilisation or development is a property of the eggs. (as noted by Runnström 1949, p. 568

2. Spawned eggs do not appear to age rapidly: they can be successfully fertilised after a lapse of 24 hours and normal larvae are produced.

3. Experimental conditions are suggested under which spawned eggs may be fertilised and the early larval stages reared.

3. Experiments in artificial fertilisation I

I. A single experiment was carried out in 1951 with eggs from the coelom of a female which had spawned in the laboratory. The body cavity contained a good deal of blood together with a few residual eggs. Coelomic fluid containing 29 eggs was extracted from the worm with a glass pipette. The eggs were washed in filtered outside sea water and placed in a petri dish.

Fertilisation was made with sperm spawned in the laboratory. The culture was kept at 15°C.

Results: On the following day, of the 29 eggs, 8 had reached an advanced stage in division (in excess of 32 cells), 4 others were probably dividing. The water in the petri dish was changed. No change could be detected in the eggs on the two succeeding days. On F + 4 one egg was elongating, but this and the remainder failed to hatch. In the parallel experiment with eggs spawned by this female (jar 3, table 8), there was a large hatch of regular larvae.

II. A similar experiment was carried out on 22nd October 1952. In this case eggs were obtained from two spawned females. Eggs were extracted by pipette, washed and placed in 100ml Berkefeld filtered sea water. Fertilisation was made with sperm from an injected male (see Section III).

Results: On F + 1, 29 hours after fertilisation, 4.5% of the eggs were dividing. The stage in development was in excess of 32 cells. Thereafter development appeared to continue though only a single larva hatched. This was removed to a separate jar but died on F + 9. In the parallel experiment using eggs spawned by the same worm (jar 5/22, table 9), 68% segmented. Of these three hatched.

Discussion:

The above experiments show that coelomic eggs can be fertilised, in this case, with spawned sperm. Newell (1948)

was therefore correct in contradicting Pirlot's (1933) suggestion that the passage of the eggs through the nephridia is essential for fertilisation. It must be noted, however, that in the present experiments the percentage of segmenting eggs in the coelomic egg fertilisations was small relative to the comparable experiments with spawned eggs. This may be due to the fact that the coelomic egg fertilisations were made with residual eggs - and the fact that these eggs had not been shed might indicate that they were abnormal. On the other hand there may be some truth in Pirlot's statement; the lower percentage of dividing eggs may be because the eggs had not passed through the nephridia and in consequence were not fully ripe.

The data from these experiments should be taken together with the results of fertilisations of coelomic eggs after the injection of extracts (see pp. 181-189).

Summary:

1. Fertilisations were made with the coelomic eggs which remained in the body cavities of three spent females.

2. A number of these eggs developed beyond the 32-cell stage; one larva hatched and survived to F + 9. The most advanced developmental stage previously obtained in fertilisations of coelomic eggs was the 4-cell stage (Newell, 1948).

4. Experiments in artificial fertilisation II

Although there is some doubt whether Child (1898) was dealing with the species Arenicola marina (see p.29), the possibility cannot be ignored that he may have identified the species correctly and that artificial fertilisations are possible. Experiments have been described above, in which limited success was obtained with coelomic eggs from spawned females. It was thought, therefore, that a further attempt should be made to bring about artificial fertilisations by direct methods.

Methods:

Experimental conditions were designed to avoid factors known to inhibit fertilisation. Berkefeld filtered outside sea water was used. Fertilisations were made immediately after the worms were brought into the laboratory, or, in a few cases, on the sands. Genital products were removed from the coelom by glass pipette or syringe, care being taken to avoid blood vessels. Samples contaminated with blood were discarded. The eggs were washed several times by decanting before being placed in beakers of 2 - 5 litres capacity.

In almost every case the coelomic sperm showed no activity in sea water. The methods for ripening sperm used by Carter (1931a) and Dodd (1951, in the press) were adopted. Spermatozoa were allowed to lie for up to 2-3 hours in a few ml of sea water, or 'dry' in coelomic fluid. Samples were kept at 15°C. They were then diluted in sea water and their activity

examined microscopically. Although a number of samples was treated before each experiment, on many occasions active sperm suspensions were not obtained. In these cases violent agitation of the seminal fluid with a pipette usually produced a thin suspension. A few drops of the most active suspension were added to the eggs which were kept in motion with a glass rod stirrer. Cultures were kept at 15°C.

Except where otherwise stated the conditions described above apply to all the experiments described in this section.

Experiments:

In 1949, worms were generally taken from area A (see p. 12). When tides prevented collection in area A, they were taken from area B. Experiments carried out immediately before, during and after the main breeding season are listed below. The number of experiments during the breeding season (24th October to 20th November) was limited by rough weather.

9.10.49	3 fertilisations	with	eggs	from	5 females.
21.10.49	1 fertilisation	"	"	"	6 "
24.10.49	2 fertilisations	"	"	"	16 "
28.10.49	1 fertilisation	"	"	"	1 female.
4.11.49	1 fertilisation	"	"	"	1 "
5.11.49	2 fertilisations	"	"	"	2 females.
10.11.49	4 fertilisations	"	"	"	1 female.

Experiments in 1950, listed below, were made between 9th October and 18th October. This was before spawning occurred on the shore (24th October). However, experiments using other

methods had produced fertilisation in a few eggs on 5th October (see p. 83). Other work (see pp. 80-81) had indicated that stirring inhibits fertilisation or development. In consequence these cultures were not stirred.

Experiments in 1950:

9.10.50	1 fertilisation	with	eggs	from	3	females.
11.10.50	3 fertilisations	"	"	"	3	" *
12.10.50	1 fertilisation	"	"	"	4	"
12.10.50	1 fertilisation	"	"	"	2	"
18.10.50	1 fertilisation	"	"	"	?	"

* Conducted at room temperature.

Results:

The 1949 experiments were unsuccessful, although on numerous occasions sperm haloes formed round the eggs. In 1950 a few eggs were fertilised in all but one experiment. Development ceased at the 4-cell stage except in one case where 8-cell stages were obtained. The percentage development was always less than 1%.

Discussion:

The genital products used in the experiments on 5th and 9th November 1949 were taken from the only surviving gravid individuals out of 82 and 58 worms respectively. It must be assumed that this material had reached the maximum degree of ripeness possible in the body cavity. Nevertheless, as noted above, the fertilisations were unsuccessful. The results obtained in 1950 are comparable to those obtained by Newell (1948).

It appears then that development cannot be induced or prolonged in a high percentage of coelomic eggs by direct methods. Nevertheless some doubt remains as to the validity of the results. Sperm suspensions were always thin and individual spermatozoa were relatively inactive. Although the literature indicates that the bar to fertilisation is a property of the eggs, it seemed advisable that further fertilisations should be attempted if more active sperm suspensions could be obtained. Some method of forecasting the breeding season was also desirable so that a concentrated effort could be made when the worms were due to spawn.

It is perhaps remarkable that no eggs were fertilised in the 1949 experiments while a small percentage segmented in 1950. As noted above (p. 61) the 1950 experiments were carried out prior to the breeding season and presumably, therefore, the genital products were less ripe than those used in 1949 (p. 62). Experimental conditions were similar in the two groups of experiments except that in 1949 the eggs were stirred while in 1950 there was no stirring. This lends support to the suggestions (pp. 56 & 81) that the disturbance caused by stirring and separating the eggs from the bottom, inhibits fertilisation or development.

5. Prediction of the breeding season.

The gradual development of the genital products in the coelom suggested two methods whereby a forecast of the breeding season might be obtained.

Egg measurements: The average size of the eggs could be measured at regular intervals during the Summer and plotted against time. The graph could be extrapolated to the known average diameter of ripe eggs (190μ - Newell, 1948) to give the approximate spawning date. When this possibility was examined it was found that the eggs grew rapidly after their first appearance in the coelom; subsequently the growth rate levelled off (fig. 3). This agrees with an observation by Newell (personal communication). The graph was therefore of no value for forecasting spawning.

Sperm counts: As the males mature, the sperm cells in the body cavity metamorphose from spermatocytes, which are grouped in small rosettes, to spermatozoa which are grouped in sperm plates or morulae. This is a gradual process; the rosettes are not entirely replaced by morulae until the worms are about to spawn. It was argued, that if a graph of percentage morulae against time proved to be a straight line, this could be extrapolated to 100% to give a forecast of the breeding season.

Method of estimating percentage morulae: Sperm samples, taken from 6 - 10 males, are each diluted in a little sea water and examined microscopically. All the morulae and rosettes lying within 9 - 10 arbitrarily chosen fields under the 1/6th power of the microscope are counted. From the totals the percentage morulae in each sample is calculated and these are averaged. This is done at regular intervals.

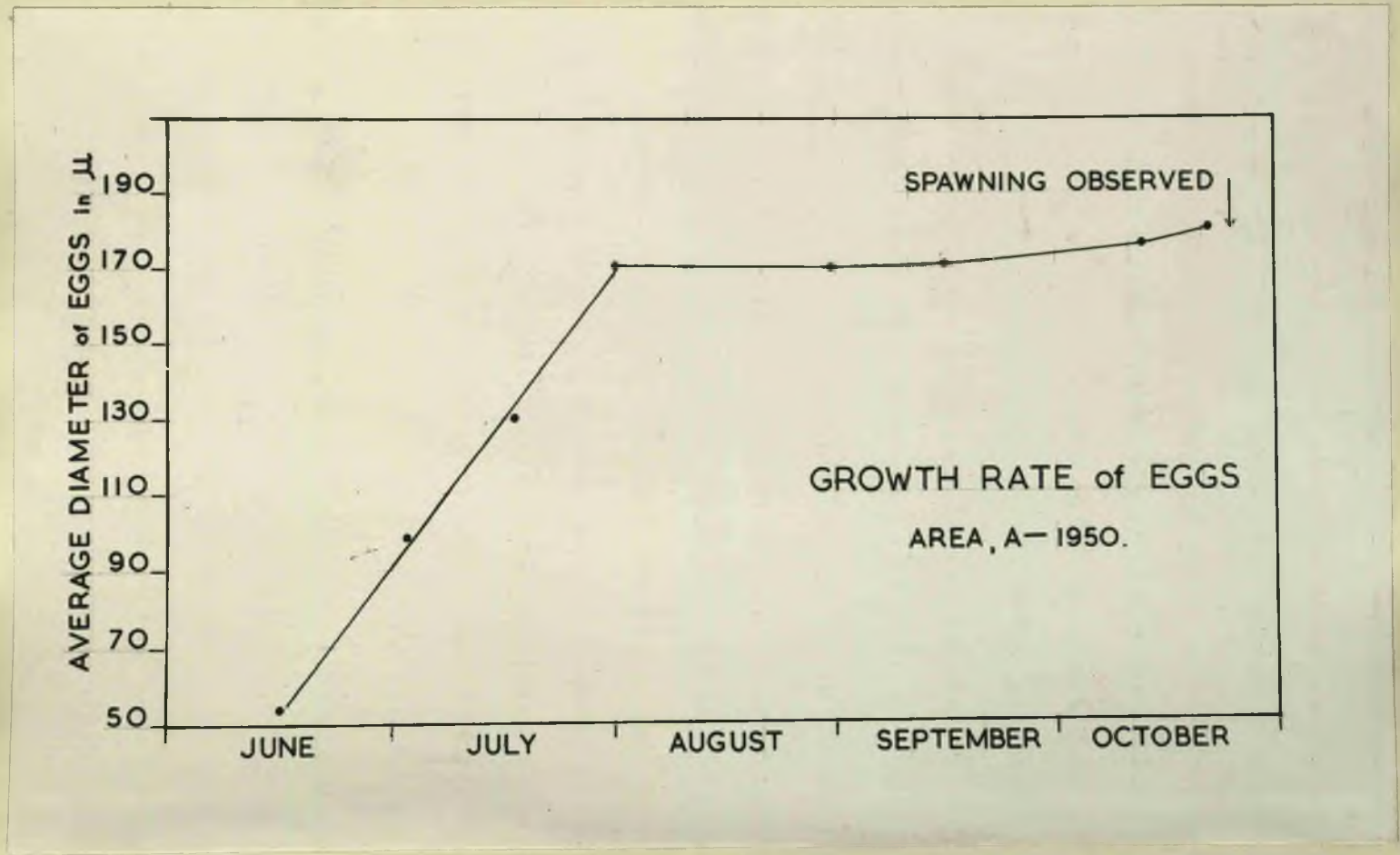


Fig. 3, The average growth rate of eggs in the coelom during the Summer.

Results: The points obtained by plotting average percentage morulae against time lie approximately in a straight line. A period of 2 - 3 weeks can be forecast during which the main breeding season occurs (figs. 4 & 5). The method is of assistance in view of the fact that the breeding season may vary between early October and mid November.

6. Activation of coelomic sperm.

Two series of experiments were carried out. In the first a variety of physical conditions * were investigated under which sperm might be ripened, and in the second, the effect of alkalisation was tested.

Method for assaying activity: Samples were examined microscopically. Estimation of activity was subjective, and is expressed on an arbitrary scale from nil activity (0), to maximum activity (5), the latter being equivalent to the activity of naturally spawned sperm. The method was similar to that used to estimate the activity of sperm spawned due to injection of tissue extracts (pp. 121). The rapidity with which the morulae disintegrated was also taken into account.

Experiments to test the effect of various physical conditions on ripening:

18 Experiments were carried out from 29.8.50 to 25.10.50, in which sperm was kept under the following conditions:-

* Conditions of temperature and dilution.

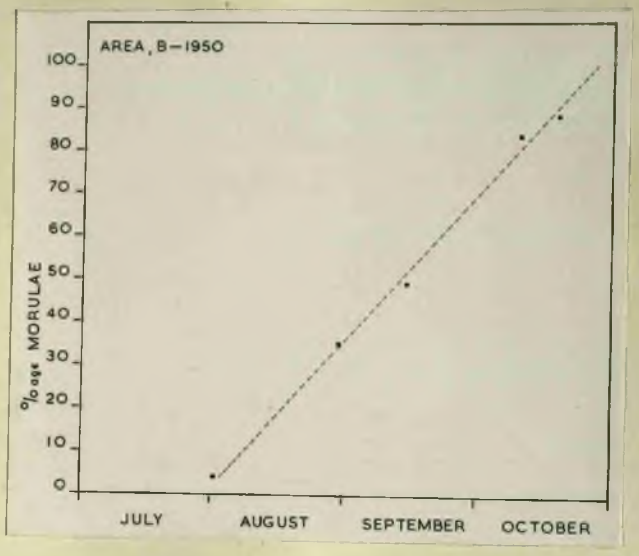


Fig. 4, Forecast of the breeding season Area A, 1950. Extrapolation (to 100% morulae) of the points obtained by plotting the average percentage sperm morulae, in the coelom, against time.

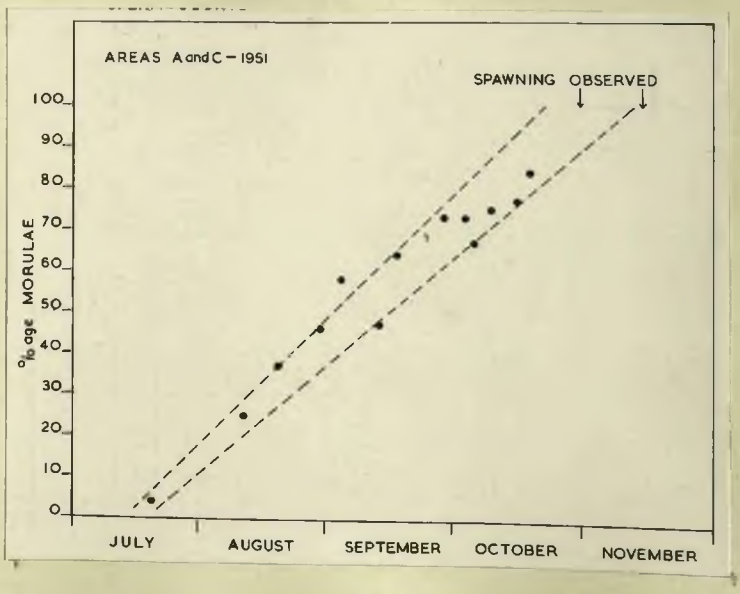


Fig. 5, Forecast of the breeding season Areas A and C, 1951. Method as for figure 4.

(a) In coelomic fluid, at a constant temperature (15°C.).
(b) " " " , at room temperature (15 - 18°C.).
(c) Diluted with 5 - 10 ml B.F.O.S.W. at a constant temperature (15°C.).
(d) " " " " " " at room temperature (15 - 18°C.)
(e) " " 50ml " at a constant temperature (15°C.).

Results:

Samples were examined immediately after removal from the worms and these were either inactive (0) or very slightly active (1). Thus only those samples showing activity in excess of 1 gave an indication of a ripening effect due to the treatment employed.

Almost all the methods gave occasional activity in excess of 1 (table 10) but none gave consistent results or activity significantly greater than the others.

Experiments with alkali I.

The literature indicates a variety of chemical methods by which sperm, inactive in sea water when untreated, might be activated. These were:-

(a) by secretions of ripe eggs,
(b) by various egg extracts,
(c) by thyroxine, and
(d) by alteration of the H ion concentration.

Ripe eggs of Arenicola could not be obtained and there is no evidence for the general applicability of thyroxine. There

Table 10

The activity of samples of coelomic sperm kept under various conditions of temperature and dilution.

Treatment	Total samples	Samples classed against activity						Average act.
		0	1	2	3	4	5	
Undiluted at 15°C.	15	3	9	3	-	-	-	1.0
Undiluted at room T°.	12	3	5	2	1	1	-	1.3
5-10ml sea water at 15°	23	8	6	6	3	-	-	1.2
5-10ml sea water at room T°.	3	1	2	-	-	-	-	0.7
50ml at 15°.	5	4	-	1	-	-	-	0.4

N.B. Mixed samples from more than one worm are treated as 1 sample.

is, on the other hand, much evidence that alkalisation of the medium might be generally effective - with the important drawbacks, that the active life of the spermatozoa might be shortened and that toxic agglutination might occur.

Preliminary experiments:

Concurrently with the experiments on 'physical conditions', 10 samples of coelomic sperm were treated with alkali, for comparative purposes.

Method: Sperm samples (of approx. 1.0ml) were placed in 10ml or 20ml Berkefeld filtered outside sea water, to which N/10 NaOH was added in quantities varying from 0.05 - 2.0ml. The samples were kept at 15°C. and activity was assayed after a lapse of 2½ - 4½ hours.

Results: In all but one case the activity was greater than that given by the best of the 'physical' methods (p. 65) tested on the same day. Six samples out of ten gave activity in excess of 1 (table 11) and the increase in activity with greater concentration of alkali or dilution of the sperm suggested that the results could be improved.

Experiments with alkali II.

The possibility that the activity of dense suspensions might be increased by dilution, found support in work by Gray (1928a), Southwick (1939) and Rothschild and Tuft (1950). Southwick (1939) found that a drop of sperm in a drop of sea

Table 11

The activity of sperm samples treated with various concentrations of alkali.

Concentration of alkali	Samples tested	A_1	A_2
0.1ml N/10 in 10	6	1.3	1.3
.05ml " " "	1	1.0	0
0.2ml " " "	2	2.0	1.0
0.2ml " " 20	1	3.0	2.0

A_1 = average activity of alkalised sperm.

A_2 = activity with the best 'physical' method (p. 65) tested on the same day.

water, on a slide, soon became inactive. It could be revived as many as eight times by successive additions of sea water. Gray (1928a) states that the specific activity of spermatozoa is a linear function of the cube root of the volume of sea water in which it is free to move. Rothschild and Tuft (1950), on the other hand, thought that this was only true of very dense suspensions in which the O_2 tension was inadequate. However the O_2 consumption of the spermatozoa increased when suspensions in which there was no lack of O_2 were diluted. In this case the addition of sea water increased the available quantity of an element (copper or zinc) which was thought to be essential to the metabolism of the spermatozoa. Tyler (1955) has pointed out that while dilution will temporarily increase the activity of spermatozoa - their total active life will be decreased.

The method described above (p. 68) was modified as follows: 1ml of coelomic sperm was diluted in 50ml of sea water. Circulation water was used in this case. The concentration of alkali was kept at the same level as in the majority of the preliminary experiments by adding 0.5ml N/10 NaOH.

After 10 mins. the sperm tails were intensely active but the morulae did not disintegrate. It was thought that if the supernatant fluid was decanted off and fresh sea water added, the physical shock might aid the breakdown of the morulae. This proved to be the case. The spermatozoa broke free and an active suspension was formed.

Experiments: The method was repeated on numerous occasions and the following variations were tested, (a) the effect of increasing the concentration of alkali from 0.5ml to 1.0ml in 50ml sea water, and (b) the effect of different types of sea water as media, e.g. Berkefeld filtered outside sea water, circulation water and water collected on the shore.

Results: When circulation water was used activity was always of a high order. Results were improved by increasing the concentration of alkali (table 12). A remarkable, indeed astounding feature of the results was the consistently poor reaction when outside sea water and shore water were used (table 12).

Further information was obtained on the nature of the reaction. First, although decanting aided the production of free suspensions, suspensions were eventually produced in alkaline media without decanting. Secondly, breakdown of the morulae was never passive. Individual spermatozoa broke free by their active movements. It appeared therefore that alkalisation stimulated activity directly and not indirectly by first breaking down the binding substance of the morulae. Finally, there was no correlation between the apparent ripeness of the sperm, as calculated by sperm count,(pp. 64-65) and its activity when alkalised (table 13).

When suspensions were required for fertilisations the following method was used:- Approximately 1 ml of coelomic sperm was placed in 50ml of circulation water, to which was added 0.5 or 1.0ml N/10 NaOH. After 10 mins. the supernatant fluid was

Table 12

The activity of coelomic sperm treated with N/10 NaOH in various types of sea water.

Type of water	Conc. alkali/ 50ml sea water	No. of samples	Average reaction
C	1.0ml	23	4.1
C	0.5ml	37	3.2
B	0.5ml	12	1.2
B	1.0ml	6	1.1
S	0.5ml	9	1.1
Control	0	3	0.8

C = Circulation water.
B = B.F.O.S.W.
S = Filtered shore water.

Table 13

Comparison of the activity of sperm samples treated with alkali and their apparent ripeness calculated by sperm count (%age morulae).

Sample no.	1	2	3	4	5	6	7	8
%age morulae	31	32	36	39	43	47	53	55
Activity	3	5	5	1	3	4	3	5

decanted and Berkefeld filtered outside sea water added. This aided formation of the suspension and reduced the amounts of alkali and circulation water added to the eggs. Once activated, the addition of Berkefeld filtered outside sea water did not reduce the activity of the suspension. The possible effect of using alkalised sperm suspensions for fertilisation experiments, is discussed elsewhere (p. 81).

Experiments with alkali III.

Controlled experiments were carried out, (a) to discover the range of alkalinity over which activity could be induced in coelomic sperm, (b) to compare the activity of suspensions in Berkefeld filtered outside sea water and circulation water with equal additions of alkali, and (c) to compare the pH shift in Berkefeld filtered outside sea water and circulation water when various volumes of alkali are added.

Methods: In each experiment sperm was taken from a number of males, mixed, and 1ml of the mixture was added to each of a number of small dishes containing 50ml sea water. N/10 NaOH was added to each dish in volumes varying between 0.01ml and 4.5ml. After 10 mins. the alkaline medium was decanted off and fresh sea water added. The activity of the suspensions was then assayed.

When pH shift was measured the pH of each dish was recorded prior to the addition of alkali and again before decanting. A Cambridge pH meter was used. After decanting, the activity of the suspensions was again assayed.

Untreated controls were kept in either Berkefeld filtered outside sea water or circulation water. They were decanted and their activity was assayed at the same time as the alkalised samples.

Experiments: Experiments were conducted in September 1951 and September 1952.

Experiments 1 - 3: Sperm activity was assayed in concentrations of alkali varying between 0.1ml and 4.5ml N/10 NaOH/ 50ml circulation water.

Experiments 4 - 8: Sperm activity was assayed in concentrations of alkali varying between 0.01ml and 3.0ml N/10 NaOH/ 50ml sea water. (Both B.F.O.S.W. and circulation water were used.)

Experiments 7 & 8: Activity and pH shift were measured in concentrations of alkali varying between 0.25ml and 3.0ml N/10 NaOH, in both types of sea water.

Results: In circulation water, the average activity of the suspensions increased with increasing concentration of alkali (table 14). Optium results (taken as activity in excess of 4) were obtained in concentrations from about 1.25ml to 2.75ml N/10 NaOH/ 50 ml sea water. In higher concentrations activity decreased. At 4.5ml, activity was equivalent to suspensions treated with the minimum quantity of alkali. As the method of assaying activity was subjective, accuracy of estimation could only be expected to the nearest ½ unit (fig. 6).

Table 14

Activity of sperm suspensions related to the concentration of N/10 NaOH added and pH.
(C = Circulation water. B = B.F.O.S.W.)

Exp. No.	Water	Volume of alkali (in ml) / 50ml sea water.																			Assay of Activity.
		.00	.01	.05	.10	.20	.25	.50	.75	1.0	1.25	1.5	1.75	2.0	2.25	2.5	2.75	3.0	3.5	4.0	4.5	
1	C	1.0			2.0		2.5	4.0	4.0	4.5		4.0										
2	C	2.0								3.5				4.0				2.5	3.5	3.0	2.5	
3	C	2.0								3.5				4.0				3.5	3.5	2.5	2.5	
4	C	1.5		2.5	2.5	3.0		3.5														
	B	1.5		2.5	2.5	2.5																
5	C	2.5	2.5	2.5	2.0			4.0		4.0				4.0								
	B	2.0	1.0	1.0	2.5			2.5		3.0				2.5								
6	C	1.0						2.5		3.0		4.0		4.0		4.0		3.5				
	B	1.0								3.0		3.5		4.0		3.5		3.5				
7	C	2.5					3.0		3.5		4.0		5.0		5.0		4.0					
	B	2.0					2.5		2.5		4.0		5.0		4.5		3.5					
8	C							2.5		3.5		4.5		5.0		5.0		4.0				
	B							3.0		3.0		4.0		4.0		4.0		3.5				
Av.	C	2.0	2.5	2.5	2.0	3.0	3.0	3.0	4.0	3.5	4.0	4.0	5.0	4.0	5.0	4.5	4.0	3.5	3.5	3.0	2.5	
Av.	B	1.5	1.0	2.0	2.5	2.5	2.5	3.0	2.5	3.0	4.0	4.0	5.0	3.5	4.5	4.0	3.5	3.5				
	C	.04					.19	.25	.81	1.2	1.3	1.7	1.6	2.1	1.7	2.0	2.1	3.0				pH Shift
	B	.05					.74	.41	1.0	1.3	1.6	1.7	2.3	2.0	2.4	2.3	2.3	3.2				

Similar results were obtained when Berkefeld filtered outside sea water was used (table 14, fig. 7). The optimum range for addition of alkali was from 1.25ml to 2.5ml. In both types of water clumping of the sperm took place in concentrations of alkali beyond the optimum. The controls gave relatively high values for activity. This was probably due to the shock of decanting.

The increase in pH shift with increasing concentration of alkali was also almost identical in the two types of water. Berkefeld filtered outside sea water shows slightly higher values (table 14, figs. 6 & 7). In both cases the graphs level off at 1.5ml N/10 NaOH. For additions of N/10 NaOH between 1.5ml and 2.75ml the pH shift only varies between 1.7 and 2.1 for circulation water and between 1.7 and 2.4 for Berkefeld filtered outside sea water. The absolute value of pH varies between 9.4 and 10.0 in this range. At a level of 3.0ml added alkali, there is a sudden increase in the pH shift to 3.0 or 3.2 (figs. 6 & 7).

The pH of the controls was 8.08 for circulation water and 7.94 for Berkefeld filtered outside sea water.

Discussion:

There appears to be a direct relationship between pH shift and activity; the graphs are almost parallel (figs. 6 & 7). The flattening of the pH curve corresponds to the range of optimum conditions for activity, i.e. at a shift of 2, $\pm$ 0.4.

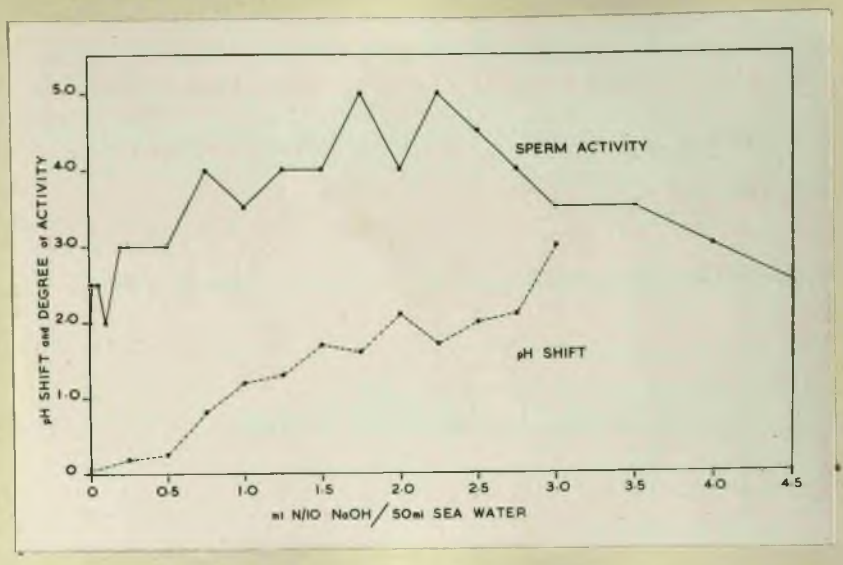


Fig. 6, The activity of sperm suspensions in circulation water, related to the concentration of N/10 NaOH added and the resulting pH shift.

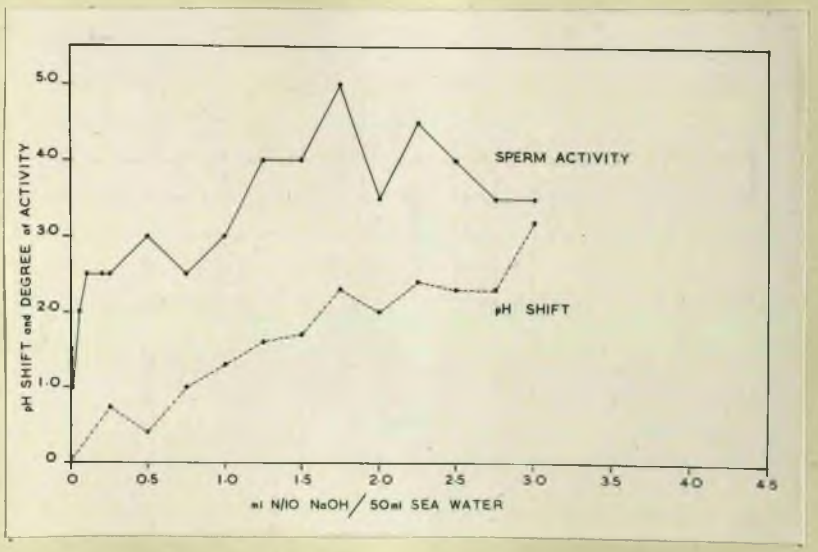


Fig. 7, The activity of sperm suspensions in Berkefeld filtered outside sea water, related to the concentration of N/10 NaOH added and the resulting pH shift.

The decline in activity is marked by a sudden rise in pH, which suggests that the natural buffer in the sea water had been overcome with consequent release of OH ions in the medium. This coincided with the clumping of the spermatozoa; presumably the toxic and irreversible agglutination referred to by Lillie and Just (1924). They suggested that agglutination of this kind was due to mere physical adhesion of the spermatozoa and that it does not correspond to agglutination by fertilizin. This statement is true if the mutual multivalence theory for the action of fertilizin is accepted (see p. 38). There is no possibility of univalent OH ions forming a chemical link between the molecules of one spermatozoan and the molecules of another.

The concentration of alkali required to induce maximum activity and the pH shift at which agglutination took place were considerably higher than those producing the same effects in unripe sperm of echinoids (Carter, 1932b).

In contrast with previous experiments (p. 71) there was no significant difference between the activity of the spermatozoa in the two types of sea water. There was some indication that circulation water was slightly better buffered than outside sea water but this was insufficient to account for the divergence of results in the experiments referred to above. Work by Rothschild and Tuft (1950) may provide an explanation. They noted that the dilution effect does not always operate when dense suspensions are diluted to approximately 4×10^8/ml in natural sea water. The metallic ion content of sea water is marginal for the metabolic

requirements of sperm suspensions of this density (see p. 70). In this case alkalisation might have no effect. This situation would not arise with water from the laboratory circulation system, which having passed through metal pipes and a pumping engine, always contains a high concentration of metallic ions.

Summary:

1. Coelomic sperm fails to ripen and gain activity merely by being kept for a period of time after removal from the body cavity. The conditions of temperature and dilution under which the sperm was kept had little effect on the result.

2. Activity can be induced by alteration of the H ion concentration of the medium.

3. Optimum results are obtained by placing the sperm in 50ml sea water to which is added 1.25ml - 2.75ml N/10 NaOH. This corresponds to a pH rise of 2, $\pm$ 0.4. A direct relationship exists between the degree of activity and pH shift.

4. At concentrations of alkali above the optimum, agglutination of the sperm coincides with a sharp rise in the OH ion concentration.

5. In alkali the breakdown of the morulae to free spermatozoa is never passive, consequently alkali causes direct activation of the spermatozoa.

6. The degree of activity is unrelated to the apparent ripeness of the sperm as calculated by the percentage morulae.

7. Experiments in artificial fertilisation III.

(a) With sperm activated by alkali:

The method for the production of active sperm suspensions with N/10 NaOH, has been described (p. 71). Treatment of the eggs, and experimental conditions were the same as those described for previous experiments (p. 60). The experiments listed below were those conducted immediately before or during the breeding season.

Experiments (1950):

12.10.50:	1 fertilisation	with	eggs	from	4	females	(unstirred).
15.10.50:	1	"	"	"	3	"	"
18.10.50:	1	"	"	"	3	"	(stirred).
	2 fertilisations	"	"	"	4	"	(unstirred).
21.10.50:	1 fertilisation	"	"	"	5	"	"
	1 fertilisation	"	"	"	3	"	"
25.10.50:	1 fertilisation	"	"	"	3	"	"

Spawning commenced 24.10.50.

Experiments (1951):

Spawning commenced 29.10.51.

31.10.51: 3 fertilisations with eggs from several females.
1.11.51: 1 fertilisation with eggs from several females.

The eggs were not stirred in these experiments.

Results:

In 1950 the eggs developed to 8-cell stages in all the experiments where they were not stirred. The best percentage development was 15%, of which less than 2% were thought to be normal.

After several hours there was no sign of development in cultures where the eggs were stirred. Stirring was discontinued and a few dividing eggs were later observed. The experiments in 1951 were totally unsuccessful.

(b) With naturally spawned sperm:

During the 1950, 1951 breeding seasons Newell's experiments with spawned sperm and coelomic eggs were repeated. Sperm was collected on the shore, or from sperm aggregations spawned in the laboratory, and added to the eggs with as little delay as possible. Eight fertilisations were attempted in two of which the eggs were stirred.

Results:

Cell division was observed in cultures which were not stirred. In one, 16-cell stages were observed but in the remainder development ceased at 4-cells. As in the previous experiments there was no development in cultures in which the eggs were stirred. In one of these, cell division took place when stirring was stopped.

Discussion:

The results of coelomic egg fertilisations, described above, in which alkalised and naturally spawned sperm suspensions were used, did not differ materially from earlier fertilisations using relatively inactive suspensions (pp.60-63). There appears to be no doubt that the failure of fertilisations in which coelomic genital products are used is due to some lack of ripeness or other property of the eggs.

A large number of fertilisations with coelomic eggs have been attempted on or about the spawning date, without success. Nevertheless it has been shown in this work that the eggs of Arenicola marina do ripen in the body cavity because residual coelomic eggs from a spawned female can be fertilised and will develop at least to hatching stages (p.58). It would appear then that the onset of ripeness is extremely sudden and that it does not take place until oviposition begins.

With regard to experimental conditions, it has been demonstrated that stirring has an adverse effect on the chances of fertilisation. The effect of the use of alkalised sperm suspensions can be assessed by the results of fertilisations of spawned eggs with alkalised and naturally spawned sperm suspensions already described (pp.48 & 57). These indicate that the use of alkalised sperm in no way reduces the percentage of eggs fertilised but the normality of development may be affected.

8. Artificial maturation of coelomic eggs.

The foregoing experiments left no doubt that it is necessary to ripen coelomic eggs if artificial fertilisations are to be successful - even during the breeding season. Methods were investigated for either inducing maturation or weakening the egg membrane. In the latter case it was argued that if sperm entry could be facilitated, maturation might be induced. The effect of each method was assessed by carrying out

fertilisation experiments. The conditions were those described for previous experiments (p. 60).

Methods:

Artificial maturation:

(a) Treatment with extract of nephridia: When these experiments were carried out the possibility that the eggs may be ripened in the nephridia had not been entirely disproved. Eggs were removed from a number of females and kept in their coelomic fluid. The worms were dissected and their nephridia removed. These were then teased and thoroughly mixed with the fluid containing the eggs. After varying periods of time the eggs were washed by decanting and sperm added.

(b) Treatment with N/10 NaOH: This was the method described by Woolfson (1907). Coelomic eggs were immersed in 50ml Berkefeld filtered outside sea water containing 0.5ml - 4.0ml N/10 NaOH for periods varying between ½ hour and 3 hours. They were then washed by decanting in fresh sea water and fertilisations attempted. Four fertilisations were attempted in alkaline medium throughout.

Histolysis or digestion of the egg membrane:

(a) With blood and body fluid: Several females were opened and the mixture of eggs, blood and body fluid which exuded was collected. This was allowed to lie for between ½ hour and 6 hours, and on one occasion, overnight. The eggs were then recovered and washed repeatedly to remove contamination by coelomic fluid. Fertilisations were attempted.

(b) With crude extracts of whole worms: Eggs were extracted from a number of females and kept in coelomic fluid while the worms were broken down in a macerator. The emulsion which resulted was strained through fine bolting silk. The eggs were immersed in the filtrate, which due to its glutinous nature had first to be diluted with 2 or 3 volumes of sea water in order that the eggs could be recovered. The length of treatment varied between 15 mins. and 2 hours. The eggs were washed and sperm added.

(c) Digestion with trypsin: The natural material in pig's pancreas was used. 1mgm of dried pancreatic tissue was ground down in a mortar with 30ml sea water and 1ml of this emulsion was added to 50ml sea water containing coelomic eggs. The eggs were kept in this medium for 15 mins. to 1 hour and were washed repeatedly prior to fertilisation.

Experiments: Experiments carried out in 1950 mainly involved treatment of eggs with nephridial extract. Most of the experiments were carried out between 1st October and 2nd November 1951 (spawning commenced 29th October). A number of samples were treated by each method during this period (table 15). In each experiment sperm was added to untreated eggs as a control.

Results:

Microscopic examination revealed no change in the appearance of the eggs after treatment with nephridial extract. Nevertheless this treatment was the first to give cell division

when a fertilisation was made on 5th October 1950. A few eggs developed to 16-cell stages. Subsequent experiments gave development to only 2 and 4-cell stages. Equivalent results were obtained with untreated eggs (p.80).

Similar results were recorded after treatment of the eggs with N/10 NaOH. The eggs seemed unaffected even in concentrations of 4ml N/10 NaOH / 50ml sea water. Cell division was observed in a few samples treated with up to 2.0ml alkali. Many of the divisions were irregular and the maximum stage reached was 8 cells. Fertilisations in which sperm was added prior to decanting the alkali, or made entirely in alkaline medium, were unsuccessful.

A certain percentage of eggs histolysed in blood and body fluid rounded off and the nuclear vesicle became less distinct, i.e. they took on the appearance normally associated with spawned eggs. However, the egg membrane inflated to as much as twice the diameter of the cytoplasm. The frequency and intensity of these effects increased with more prolonged treatment. The maximum number of affected eggs (40%) was obtained after treatment for 4 hours. Results of fertilisations were the same as with other methods, i.e. the maximum developmental stage attained was 8 cells. The ratio of number of samples treated, to number in which cell division occurred (1 : 0.7) was rather higher than by any other methods.

When eggs were placed in an emulsion of whole worms the effect was negligible. In one sample a few eggs became spherical

Table 15

Methods of ripening coelomic eggs - period of treatment and number of egg samples tested.

Treatment	Period of treatment							Total Samples	Total worms used
	15-30 mins.	1hr	2hr	3hr	4hr	6hr	0		
Emulsion of nephridia	6	1	1					8	34
N/10 NaOH in sea water.									
0.5ml in 50ml		1	1				4	6	10
1.0ml in 50ml		3	4	1				8	26
2.0ml in 50ml	2	1	2					5	26
3.0ml in 50ml	1	1						2	10
4.0ml in 50ml	1	1						2	10
Histolysis with:-									
Blood and body fluid	5	14	20	5	9	1	2	56	131
Whole worm	3	2	3					8	32
Pig's pancreas	1	1						2	10

Samples were tested between (a) 5.10.50 and 12.10.50, and (b) 1.10.51 and 2.11.51.

0 = fertilised in alkalised sea water or allowed to histolise overnight.

and inflated membranes were formed. Fertilisation results were negative. The same was true after treatment with extract of pig's pancreas.

Discussion:

None of the above methods resulted in a higher percentage of fertilisations than was obtained with untreated eggs. Histolysing with blood and body fluid caused the eggs to assume, at least to some degree, the appearance of ripe material. However, even if this appearance is genuine, the method requires modification to control its effect on the egg membrane; although membrane elevation in itself need be no deterrent to fertilisation. Various authors have shown that insemination can take place after the initiation of membrane formation by cytolytic parthenogenetic agents, so long as the process is not too far advanced (Lillie and Just, 1924).

Although there are indications that a solution to the problem of artificial fertilisation might be found along these lines, this work was discontinued. A description is given below (Section III) of a different approach to this problem.

(c) Larval development

Methods: The larvae examined, were reared from fertilisations made in the laboratory (pp. 48-51). Photographs and camera lucida drawings were made using live larvae narcotised in 8% $MgCl_2$. This inhibits muscular activity though the cilia continue to beat. For histological examination the larvae were fixed for 12 hours in cold Bouin's fluid made up in sea water. They were then washed and stored in 70% alcohol. Prior to imbedding, larvae were lightly stained in eosin. This rendered them visible in the wax. They were carried through the processes prior to imbedding in tubes, the lower ends of which were covered with fine bolting silk, and were subsequently transferred to the final wax using a warm pipette. A few well orientated sections were obtained by imbedding large numbers of the early stages in each block. Later and larger stages were orientated by aligning them in the wax with a hot needle. Sections were cut at 5μ or 10μ and stained in Ehrlich's haematoxylin or Heidenhain's iron alum haematoxylin.

Hatching stages: Larvae hatch approximately four days after fertilisation (98 - 108 hrs.). They swim with a rotary motion but tend to stay close to the bottom. When at rest they are almost circular in dorsal view and have a diameter of 200μ (fig. 8a). Slight muscular elongations occur. In most respects the larvae is a typical trochophore. The pretrochal region bears an apical tuft of fine cilia and a pair of reddish brown eye spots.

The prototroch is broad, being wider laterally (85μ) than dorso-ventrally (35μ) (figs. 8a,b), and is composed of numerous short, rapidly beating cilia. The telotroch is also composed of short cilia but is narrower than the prototroch. In a lateral view of the living larva (fig. 8b) the outline of the gut rudiment can be distinguished. In this view a small tuft of anal cilia can be seen but no ventral ciliated band. However, sections reveal that a ventral ciliated band is in fact present but that it stops short of the telotroch posteriorly. A plug of yolky endodermal cells fills the centre of the larva, this is surrounded by two cell layers, the outer forming the body wall. (figs. 8c,d). Anteriorly the pharynx rudiment can be distinguished. A streak or groove indicates the position of the presumptive mouth (figs. 8c,d).

1st and 2nd days after hatching (H + 1 and 2):- The larvae alternate between short periods of swimming and resting on the bottom. Elongation has taken place to between 220μ and 260μ. The pretrochal region shows the greatest muscular activity and bears, in addition to the apical tuft, irregular tufts of short cilia. If the larva is allowed to dry out under a coverslip the mosaic of large cells forming the yolk plug, and the layer lining it, become increasingly distinct.

The first chaeta can be seen in fixed larvae (H + 2) though it has not yet penetrated the body wall. Spatulate notopodial chaetae are always the first to appear in each segment.

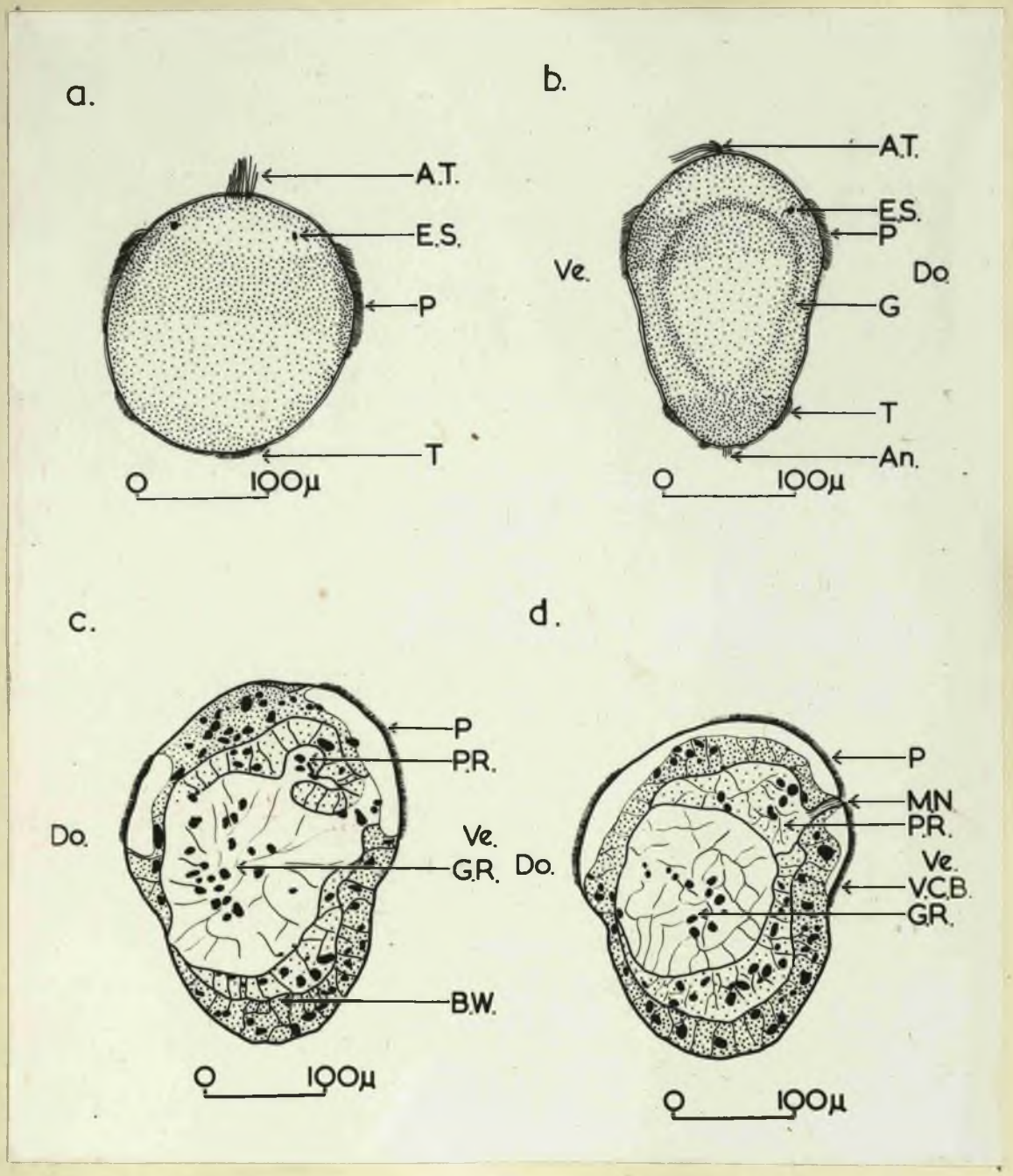


Fig. 8, Camera lucida drawings of hatching stages. (a) dorsal view, (b) lateral view, (c) oblique horizontal L.S., and (d) oblique vertical L.S.

An. anal tuft, A.T. apical tuft, B.W. body wall, Do. dorsal, E.S. eye spot, G. gut wall, G.R. gut rudiment, M.N. mouth notch, P. prototroch, P.R. proboscis rudiment, T. telotroch, Ve. ventral, V.C.B. ventral ciliated band.

H + 3 and 4:- The larvae, though still capable of swimming, generally adhere to the bottom. On H + 3 a segment is demarcated between the prototroch and telotroch. The first chaeta penetrates the body wall on H + 4 (fig. 9a). These larvae average 280μ in length - elongating to between 300μ and 320μ. The prototroch is now widest ventrally and the ventral ciliated band joins the telotroch posteriorly. Sections show that spaces have developed in the middle of the yolk plug.and the mouth groove is more distinct.

H + 5 and 6:- The larvae spend an increasing time adhering to the bottom. When three jars were decanted on H + 3, 43% of the larvae came over, when the same jars were decanted on H + 6, only 30% of the larvae came over. The second segment has appeared in a few larvae, in some cases bearing a chaeta (fig. 9b). By this time all types of chaetae are represented on the first segment, i.e. spatulate and simple notopodial chaetae and the neuropodial crochet. The gut is well defined making independent peristaltic movements within the larva. In vertical longitudinal sections the oesophagus appears as a passage dorsal to the rudiment of the pharynx. The latter is still closed. An anal invagination and a short portion of the hind gut are distinct. The larvae vary between 275μ and 365μ in length.

H + 9 to 13:- Growth rate is now variable and appears to depend on the degree of disturbance suffered by the larvae. Those which were washed and frequently changed to clean glassware developed the 3rd chaetigerous segment between H + 11 and H + 13 (fig. 9c). Such larvae are still capable of swimming but the

cilia of the apical tuft and telotroch are reduced.

Undisturbed larvae, examined at the same time, had settled permanently and built mucous tubes within which they crawled actively back and forth. No 4 chaetiger larvae came over when the containers were decanted. The maximum extended length of these larvae is 495μ (395μ when removed from the tube). The main regions of the gut can be distinguished, i.e. pharynx, oesophagus and intestine. Cilia have almost entirely disappeared. Of the prototroch only a dorsal tuft remains, while the apical tuft and telotroch are absent.

Washed larvae, although developing a 4th chaetiger (472μ) on H + 14, are not so far advanced (fig. 9d). The prototroch and telotroch, though disappearing dorsally, are still present. The ventral ciliated band extends half way to the telotroch. The larvae are still capable of swimming or at any rate of gliding along the bottom. There is little external sign of differentiation in the gut region. In sections the circular and longitudinal muscles stain heavily in Heidenhain's haematoxylin. The mouth opens into a large buccal cavity with the oesophagus entering dorsally. A deep slit points posteriorly from the buccal cavity. This permits eversion of the pharynx though it is not yet functional (see p. 91). On H + 14 the oesophagus is partially open, though the mid gut is still plugged by endoderm. The latter stains heavily in Heidenhain's haematoxylin, suggesting that a quantity of yolk is still present. Posteriorly the hind

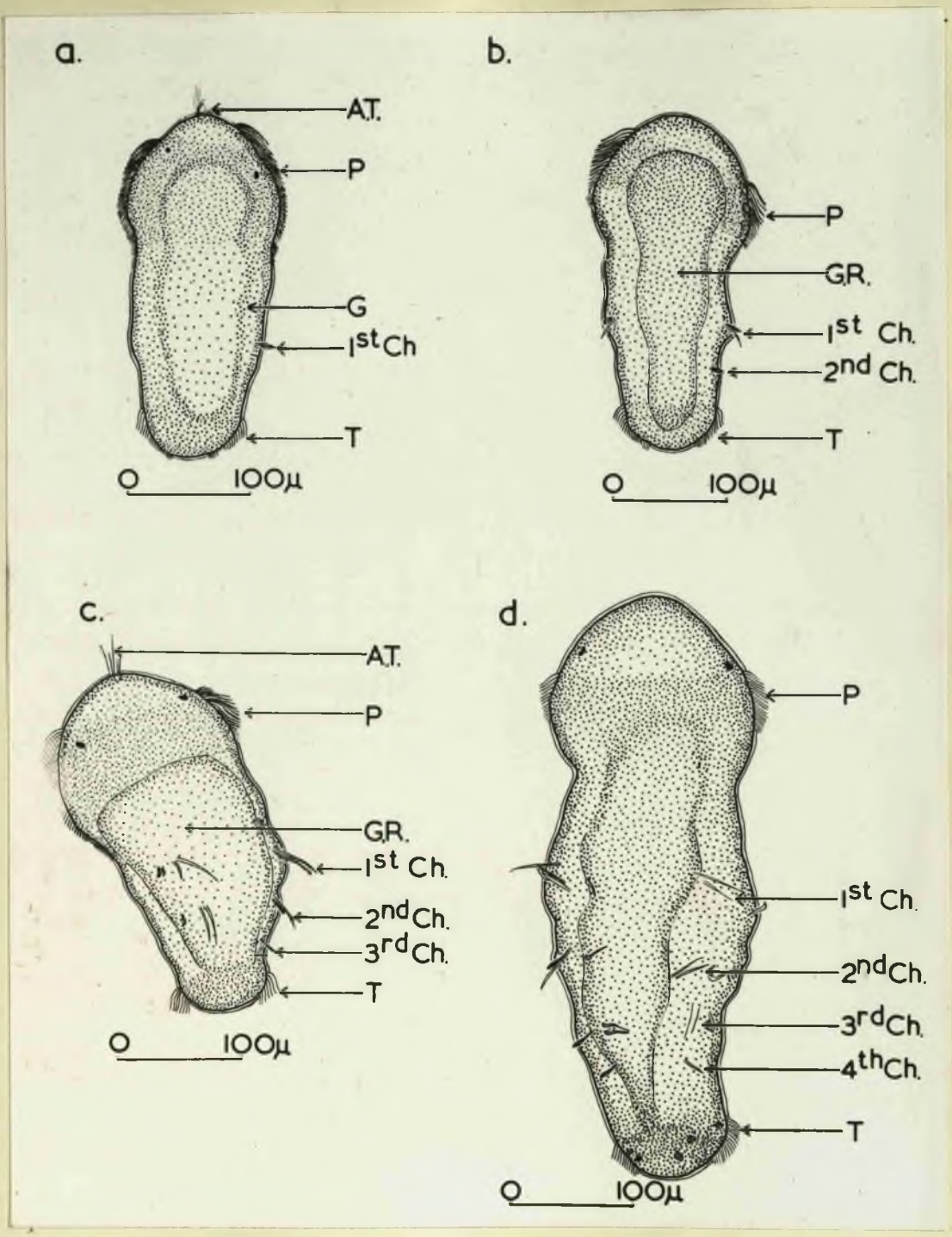


Fig. 9, Camera lucida drawings of larval stages. (a) Dorsal view 1st chaetiger stage (H+4), (b) ventral view 2nd chaetiger stage (H+6), (c) dorsal view 3rd chaetiger stage (H+11) - slightly flattened to show chaetae, and (d) dorsal view 4th chaetiger stage (H+14) - also slightly flattened.
Ch. chaetigerous segment, otherwise as in Fig. 8.

gut is open and has separated from the body wall leaving a space or body cavity.

5th and 6th chaetigerous segment stages:- The 5th chaetigerous segment is first seen on H + 16 and is present in the majority of larvae by H + 20. The age at which feeding commences varies considerably (table 16). In some larvae the pharynx is functional at the 5th chaetiger stage but in others not until the 6th chaetiger develops (plate 2). While watching live larvae it was noted that periodically the lower or posterior lip of the mouth was revolved outward, scraped along the bottom and then withdrawn. This suggests that in these early stages the method of feeding is by 'browsing'. Histological examination of the morphology of the buccal mechanism lends support to this suggestion (fig. 10, plate 3). Contraction of the longitudinal muscles of the body wall and of the basketwork of fibres surrounding the pharynx posteriorly, would cause the lower lip to be turned outward. The necessary elongation of the inner lining is permitted by the 'pharynx folds' (fig. 10). This method of feeding would also be consistent with the habit of the larvae which at this stage are tube dwelling and not burrowing. Howie (unpublished work) made almost identical observations on the structure and function of the feeding mechanism in larvae of Cirratulus cirratus.

Further examination of larvae at the 5th/6th chaetiger stage reveals a bilobed region anterior to the oesophagus which stains lightly in Ehrlich's haematoxylin. It is suggested that this is the cerebral ganglion.

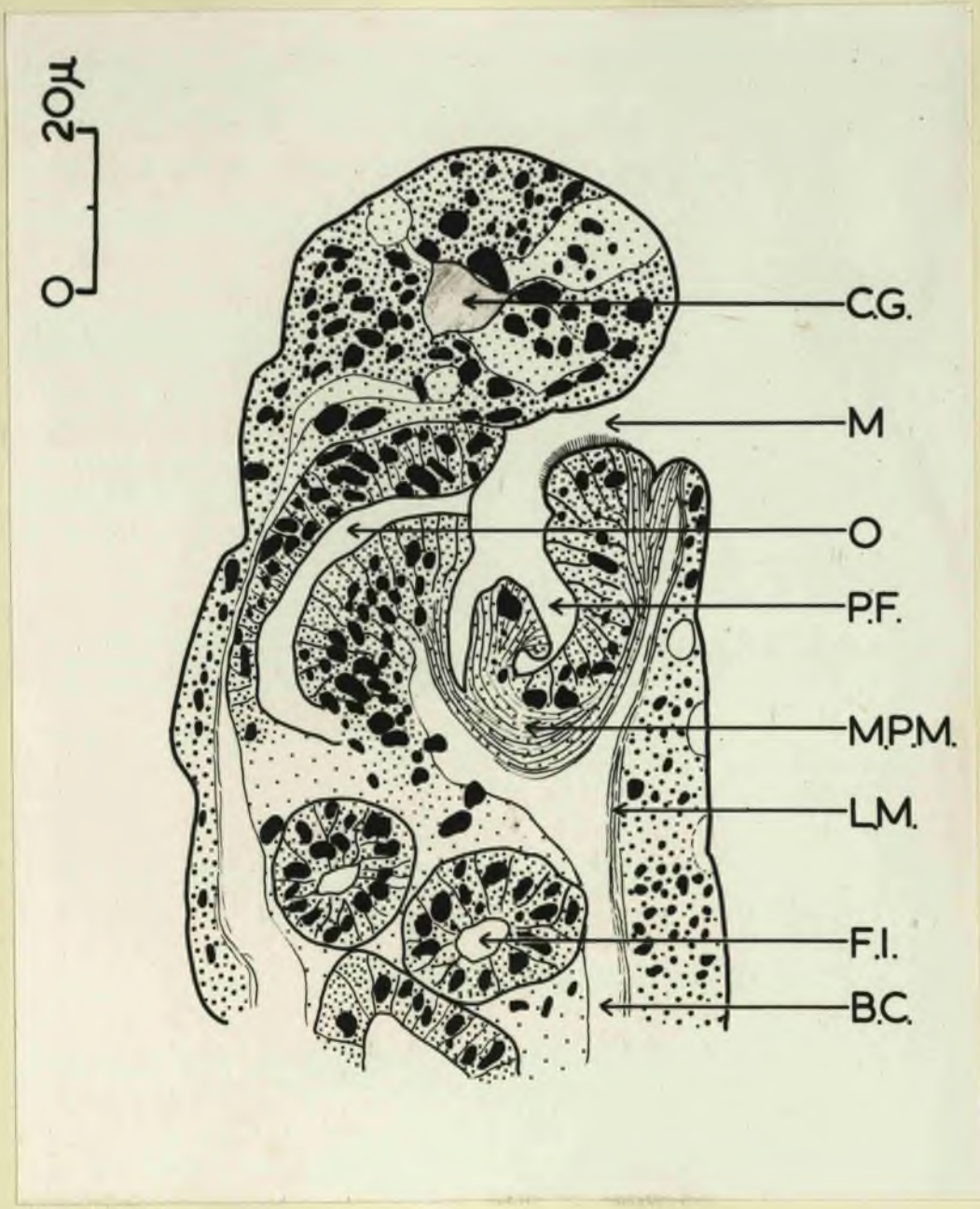


Fig. 10, The mouth and head region of a 5th/6th chaetiger stage (L.S. - see also Plate 3).

B.C. body cavity, C.G. probable location of the cerebral ganglion, F.I. folds of gut, L.M. longitudinal muscle, M. mouth, M.P.M. muscles of the proboscis mechanism, O. oesophagus, P.F. pharynx fold.

Further development:

Once feeding commences the rate of development is increasingly variable. Larvae do not progress beyond the 5th/6th chaetiger stage and about 400μ without feeding. Apart from the complete disappearance of external ciliation and the regular addition of segments there is little change in the external appearance of the larvae up to the 12th chaetiger stage (cf. plates 2 and 4). There is no sign of the characteristic tail of later post larval and adult stages. The bottom living, tube building habit was retained for as long as the larvae were kept alive in the laboratory (12th chaetiger stage, 1,300μ in length, see plates 4 and 5).

Discussion:

This account of the early development of Arenicola marina expands previous descriptions given by Blegvad (1923) and Newell (1948, 1949). It differs from Blegvad's description in that the rate of development (at 15°C.) was precisely half as rapid as that given by Blegvad (p. 42). Blegvad makes no mention of the temperature at which his cultures were maintained. Newell's description agrees closely with the present account. There is a slight difference in the description of the ciliation at the hatching stage. Newell (1948, p.570, Fig. 7) shows the prototroch as approximately 25μ in width. In the present work the prototroch was found to vary in width between 35μ dorso-ventrally and 85μ laterally. In Newell's figures of the 2nd chaetiger

larva the prototroch is 70μ in width. A second difference concerns the ventral ciliated band which Newell shows as complete in hatching stages; in the present work it was found to stretch only part way from prototroch to telotroch at this stage.

(d) Feeding Experiments

Feeding experiments were carried out using larvae of Arenicola marina with the intention of developing a technique for rearing these larvae and providing an account of larval development. The method and food materials used were similar to those used by Dodd and Howie (unpublished data, see p. 47). It was also intended to test the relative growth values for Arenicola marina of the food materials used.

There were two series of experiments - 3 controlled experiments and 4 uncontrolled experiments. Larvae were derived from fertilisations described above (pp. 48-51 , see also table 17). Some larvae were taken direct from the original jar in which the fertilisation had been made - at ages varying between H + 26 and H + 109 (table 17). These larvae had derived some nutriment from the debris of unfertilised eggs (fig. 11). In other cases the larvae were removed from the original jar immediately after hatching. These were washed and kept without food until selected for feeding experiments.

The commencement of feeding:

Tests were made to determine the age at which the larvae

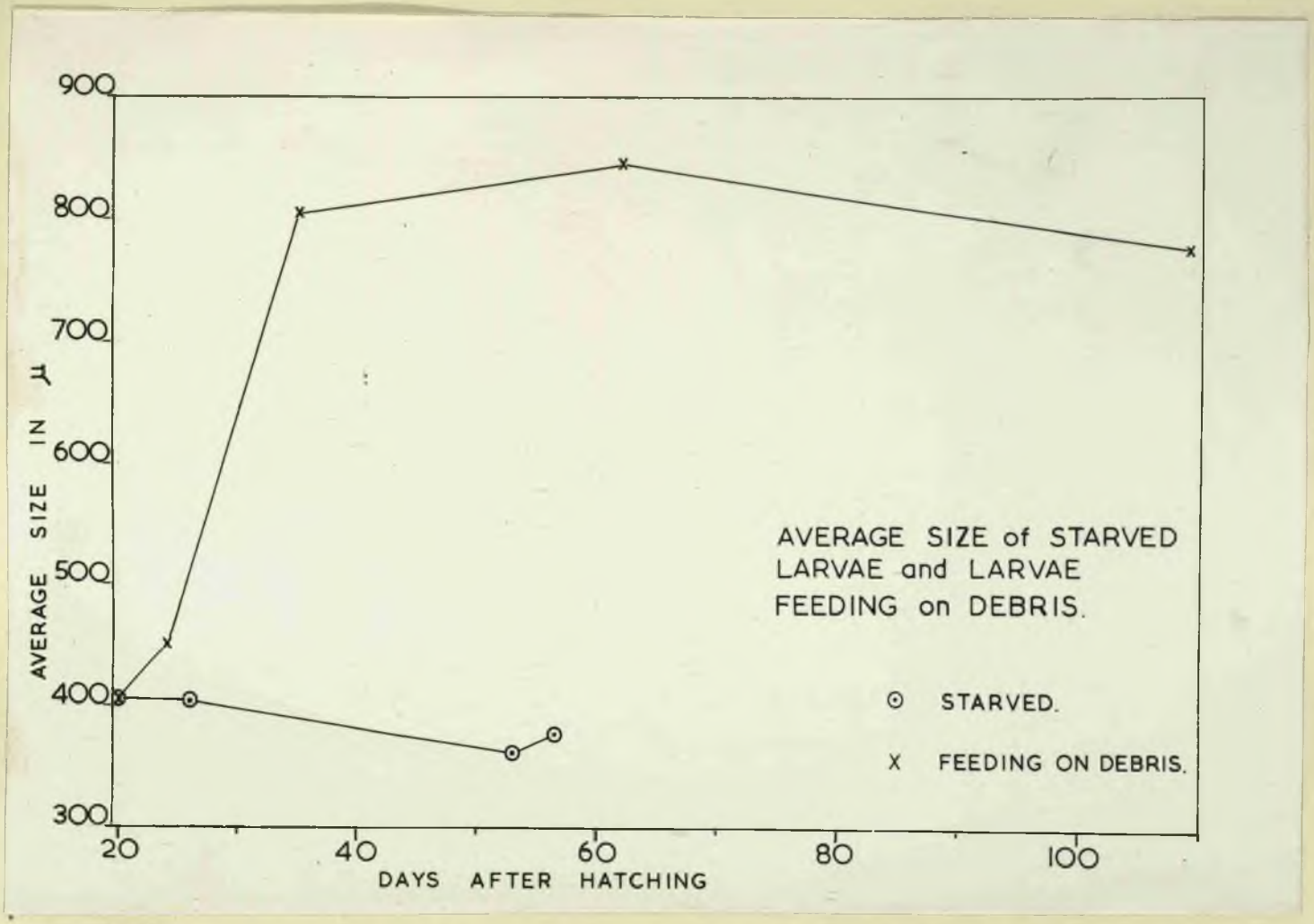


Fig. 11, The average size of starved larvae and larvae feeding on the debris of unfertilised eggs in fertilisation jars.

commenced feeding. Colloidal graphite or carmine particles were given to selected larvae in petri dishes. Experiments were started with larvae from jar 11 (table 16) 10 days after the larvae hatched, but graphite was not observed in the gut (plate 2) till 35 days after hatching (5th/6th chaetiger stage). In a later experiment, one larva from jar 2 took graphite as early as H + 18. Feeding commenced relatively early in all the larvae from this jar (table 16).

Preparation of the larvae for experiments on feeding:

Prior to each experiment the selected larvae were cleaned as follows: larvae which had been removed from the original jar immediately after hatching, were washed approximately 8 times by decanting. This was done over a period of up to 18 days during which time two changes of sterile glassware were made. In this way ciliates and other contaminating organisms were gradually eliminated Larvae remaining in the original jars had formed tubes of mucus and debris (Plate 5). When these larvae were used they were taken from the jar together with their tubes and placed in a clean beaker. They then left their tubes or could be prodded out of them. The free larvae were then treated as described above. No jar was retained for feeding experiments in which organisms, other than the larvae, could be detected.

Experimental conditions:

The larvae were kept in sterilised glassware and Berkefeld filtered outside sea water. Petri dishes (capacity 50ml) or Boveri dishes (capacity 40ml) were used as rearing jars. The

Table 16

Experiments with graphite or carmine particles to determine the age at which larvae commenced feeding.

Fertilisation (Table 8)	Days after hatching	No. of larvae		Stage
		Feeding	Total	
11	35	5	9	5/6 chaetiger
1	30/31	2	4	5/6 chaetiger
	35	4	4	
2	18	1	10	5 chaetiger
	23	3	10	5/6 chaetiger
	24	4	10	
	26	6	10	

Table 17

Derivation, age and initial size of larvae used in feeding experiments.

Feeding experiment	Commenced	Fert. Jar.No.	W or O *	Age of larvae	Number measured	Av. size
1st Controlled	7.12.51	11 & 1	W	H + 26	30	406μ
2nd Controlled	21.12.51	2	O	H + 35	40	804μ
3rd Controlled	8. 1.52	2	W	H + 53	50	360μ
1st Uncontrolled	9. 1.52	11&1&2	W	H+54/59	30	379μ
2nd Uncontrolled	17. 1.52	2	O	H + 62	21	847μ
3rd Uncontrolled	1. 2.52	2	O	H + 77	-	-
4th Uncontrolled	4. 3.52	2	O	H +109	5	775μ

* W = Larvae washed and starved.

O = Larvae taken from the original fertilisation jar.

latter had the advantage of sloping sides on which larvae could be detected under a low power binocular microscope.

The frequency with which water, food and jar were changed, and the quantities of food supplied, varied from experiment to experiment. The larvae were transferred to fresh jars by means of sterilised glass pipettes. All the experiments were conducted at 15°C.

The food materials used were - (a) pure cultures of marine algae, supplied by the Marine Laboratory, Plymouth, and (b) dried organic materials, ground and sieved through 200 mesh/inch bolting silk. The algae were cultured in Erdschreiber medium and sub-cultured once per month.

Growth rates were measured by taking the average length of the larvae in each jar, at intervals. Measurements of fully extended larvae were made under the microscope using a screwgauge micrometer.

The controlled experiments:

I. Similar groups of 10 larvae were selected (table 18). Food materials were administered as follows: Jar 11. 1. L - liver powder (2.5mg. /change of medium); Jar 11. 1. N - *Phaeodactylum tricornutum* (40 drops of a rich culture at each change); Jar 11. 1. C - Starved control.

It was intended that the larvae should be changed to fresh food, water and glassware every third day. This proved impossible due to damage inflicted on the larvae by detaching them

too frequently from the bottom (see pp. 103-104). Instead, the water was changed by decanting every second day for the first 3½ weeks. Only one change of glassware was made in that time. Under these circumstances the food added was excessive. Much ungrazed material remained on the bottom when decanting. The amount of Phaeodactylum administered at each change of water was reduced to 20 drops.

II. 4 Groups of larvae were selected. The food materials administered were: Jar 2.1.L - liver powder (0.5mgm/4 days); Phaeodactylum tricornutum (20 drops/4 days); Jar 2.1.Ch - Chlorella sp. (20 drops/4 days) and Jar 2.1.C - starved control.

The water was changed every 4th day by decanting.

III. 5 Groups of 10 larvae were selected. These were fed as follows: Jar 2.2.L - liver powder (0.5mgm/change); Jar 2.2.N - Phaeodactylum tricornutum (20 drops/change); Jar 2.2.Np - nettle powder (0.5mgm/change) and Jar 2.2.C - starved control.

In the first 3 weeks, three changes of food, water and jar were made. Thereafter the water was changed, and food added, as required.

Table 18

1st Controlled Experiment - Average length (in μ) and survival of larvae.

Date	11. 1. L		11. 1. N		11. 1. C	
	Av.Length	No.	Av.Length	No.	Av.Length	No.
7.12.51	411	10	405	10	402	10
15.12.51	-	0	432	9	420	9
7. 1.52			416	9	358	8
17. 1.52			374	5	336	5
25. 1.52			361	4	327	4
6. 2.52			354	4	358	1
18. 2.52			320	3	290	1

Table 19

2nd Controlled experiment - Average length (in μ) and survival of larvae.

Date	2. 1. L		2. 1. N		2. 1. Ch		2. 1. C	
	Av.Length	No.	Av.Length	No.	Av.Length	No.	Av.Length	No.
22.12.51	810	10	842	10	720	10	845	10
7. 1.52	580	6	622	4	571	8	602	7
17. 1.52	516	2	518	4	536	7	422	6
25. 1.52	610	1	526	4	514	6	492	6
5. 2.52	-	0	505	3	488	3	434	4

Table 20

3rd Controlled Experiment - Average length (in μ) and survival of larvae.

	2. 2.Lp		2.2.Na		2.2.S		2.2.Np		2.2.C	
	Length	No.	Length	No.	Length	No.	Length	No.	Length	No.
9. 1.52	353	10	367	10	349	10	360	10	370	10
21. 1.52	335	9	348	7	344	9	339	10	340	8
2. 2.52	357	5	336	6	308	3	361	6	347	8

Results: No growth was recorded in any of the experiments (tables 18,19 & 20) although nodules of food were observed in the larvae between four days and a week after the start of each experiment. Larvae survived for up to 2½ months (Experiment I, table 18), but in no case was the decline in size, or the mortality rate significantly less rapid than in the controls (tables 18 - 20).

Towards the end of Experiment III the average size of larvae fed on liver powder and nettle powder showed a slight increase (table 20). This was due to the death of smaller larvae. In general the larvae appeared to maintain themselves better than in the previous experiments. This is probably due to the fact that the average length of these larvae was of the order of 350μ and these are the smallest which can survive. In consequence they die off without any previous drop in the average size. In the first experiment there was an immediate mortality among larvae fed on liver powder. Ciliates had not been entirely eliminated in the cleansing process. These thrived on liver powder, killing the larvae in five days despite three changes of water. There were two main causes for the earlier mortalities in the experiments as a whole. First, some larvae secreted an abnormally short mucous tube which covered only 1 or 2 segments of the body (plate 4). These tubes appeared to constrict the larvae so that they eventually split in two. The second cause was fouling of the tubes with food and waste material (see p. 103). Survival rates tended to be better in larvae fed on Chlorella (table 19) which did not settle

on the bottom, and in the starved controls (tables 18,19 & 20) where there was no fouling by food material.

Uncontrolled Experiments:

I and II: Apart from the absence of starved controls experimental conditions were largely the same as in the controlled experiments (pp. 96-97). Groups of 15 - 20 larvae were selected for Experiment I and groups of 5 larvae for Experiment II. The foods used in Experiment I were liver powder, Skeletonema costatum and Chlorella sp., and these, plus Flagellate 4, in Experiment II. 0.5mg of the dried materials and 20 drops of the algal cultures were administered at each change. Changes of food, water and glassware were made only when it was necessary to free the larvae from fouling of the tubes or the bottom.

Results: Although the larvae cleared the food from the area immediately surrounding their tubes, there was no significant growth on any of the food materials (tables 21 & 22).

III: In this experiment the larvae were provided with a substratum of sterile sand about 2 mm. in depth. Two Boveri dishes were set up with five larvae in each. These were taken from the original jar 2 and were not removed from their tubes, but were placed immediately on the sand in the rearing jar. The grain size of the sand was such that it would pass through 64 mesh/inch bolting silk. A little liver powder was pipetted onto the sand in the vicinity of the worm tubes.

Table 21

1st Uncontrolled experiment - average size of larvae in μ.

Date	Liver powder	Skeletonema	Chlorella
10. 1.52	401	375	363
22. 1.52	357	342	348
4. 2.52	343	308	343

Table 22

2nd Uncontrolled experiment - average size of larvae in μ.

Date	Liver powder	Skeletonema	Chlorella	Flagellate 4
17. 1.52	987	887	752	762
22. 1.52	1089	814	652	688
4. 2.52	790	675	619	697

Results: The larvae soon left their tubes and crawled through the sand, gathering grains of all sizes around them. In this way galleries were formed in which they crawled actively back and forth. This contrasted with the behaviour of larvae on a plain glass surface where they remained immobile. Liver powder was taken in as the galleries were extended. It was compacted in the gut to a greater extent than in previous experiments. The jars remained clean and uncontaminated.

An attempt was made to change the larvae (with their tubes) to fresh jars, once a fortnight. Locating the larvae among the sand grains proved extremely difficult, and when isolated the tube tended to obscure the larva so that accurate measurement was not possible. However, the larvae appeared to maintain themselves better under these conditions than under those previously employed. The approximate average size of the larvae at the start of this experiment was 850μ. Two months later the average size of three of the worms from one jar was approximately 750μ, i.e. a loss of 100u. In the 2nd controlled and 2nd uncontrolled experiments, larvae which were initially the same size and fed on liver powder, lost 300μ in 4 weeks and 200μ in 2½ weeks respectively (tables 19 & 22). A single individual in this experiment survived for over three months.

IV: It was thought that the difficulty in locating and measuring the larvae might be overcome by using sand of a finer grain size. Accordingly, sand which passed through 200 mesh/in. bolting silk was used. Three Boveri dishes were set up, with

five larvae in each. The food materials were, liver powder, Phaeodactylum and Chlorella. These were pipetted onto the sand in the vicinity of the larvae. The food and water were changed once a week and a complete change to clean glassware was made after a fortnight.

Results: The change to a smaller grain size was not successful. The larvae failed to utilise the fine grains for building tubes and they proved more difficult to see. After the first week the 200 mesh sand was replaced by that passing 64 mesh. The larvae failed to recover and survived for less than three weeks.

Summary and Discussion:

No growth was recorded in any of the feeding experiments. However, the results with Arenicola should be examined in the light of similar experiments with Cirratulus cirratus (Dodd and Howie, unpublished data - see p. 47). In view of these it would appear that the lack of growth was not due to an inadequacy of the food materials.

After settling on a clean glass surface the larvae secrete a short mucous tube by which they adhere to the bottom and from which they cannot move. They are therefore limited to the food within reach of the point at which they settle. If excess food is added the larvae use the food granules to extend their tubes, creating an unhealthy situation in which food and waste products are in close proximity. In order to maintain clean conditions the larvae had to be removed from their tubes at intervals and transferred to fresh glassware. Larvae changed once or twice in

this way were unable to secrete new tubes, failed to adhere to the bottom and soon died.

In the uncontrolled experiments the intention was merely to rear the larvae. There was no attempt to assess the relative values of the food materials. In the experiment in which a substratum of sand (passing through 64 mesh/inch bolting silk) was supplied, the larvae obtained a greater supply of food (liver powder) and maintained themselves better than on a smooth surface. The culture kept free from contamination. The larvae seemed less able to utilise finer sand for tube building. The importance of the substratum in the settling and metamorphosis of the pelagic larvae of various polychaets has been shown by Day and Wilson (1934) and Wilson (1937, 1948). It has been reported that larvae can, to a varying degree, postpone metamorphosis and continue the pelagic habit until a suitable substratum is encountered. There is no reason to suppose that the substratum is of any less importance to larvae of non-pelagic habit which have no distinct metamorphosis. The fact that larvae of Arenicola marina are immobilised on a smooth surface and survive better in relatively coarse sand than in fine sand, suggests that this is true of this species.

The maximum development previously obtained in the laboratory was the 5th chaetiger stage (Elegvad, 1923). In the present work, larvae survived for 4 months and grew to 1300μ and 12 chaetigers on the debris in the fertilisation jars (see table 8 and p. 92). This indicates that it should be possible to rear these larvae in the laboratory.

PLATES - SECTION II

Plate 3

The anterior region of a 5th/6th chaetiger larva, (vertical L.S., X1440) see fig. 10, pp. 91/92.

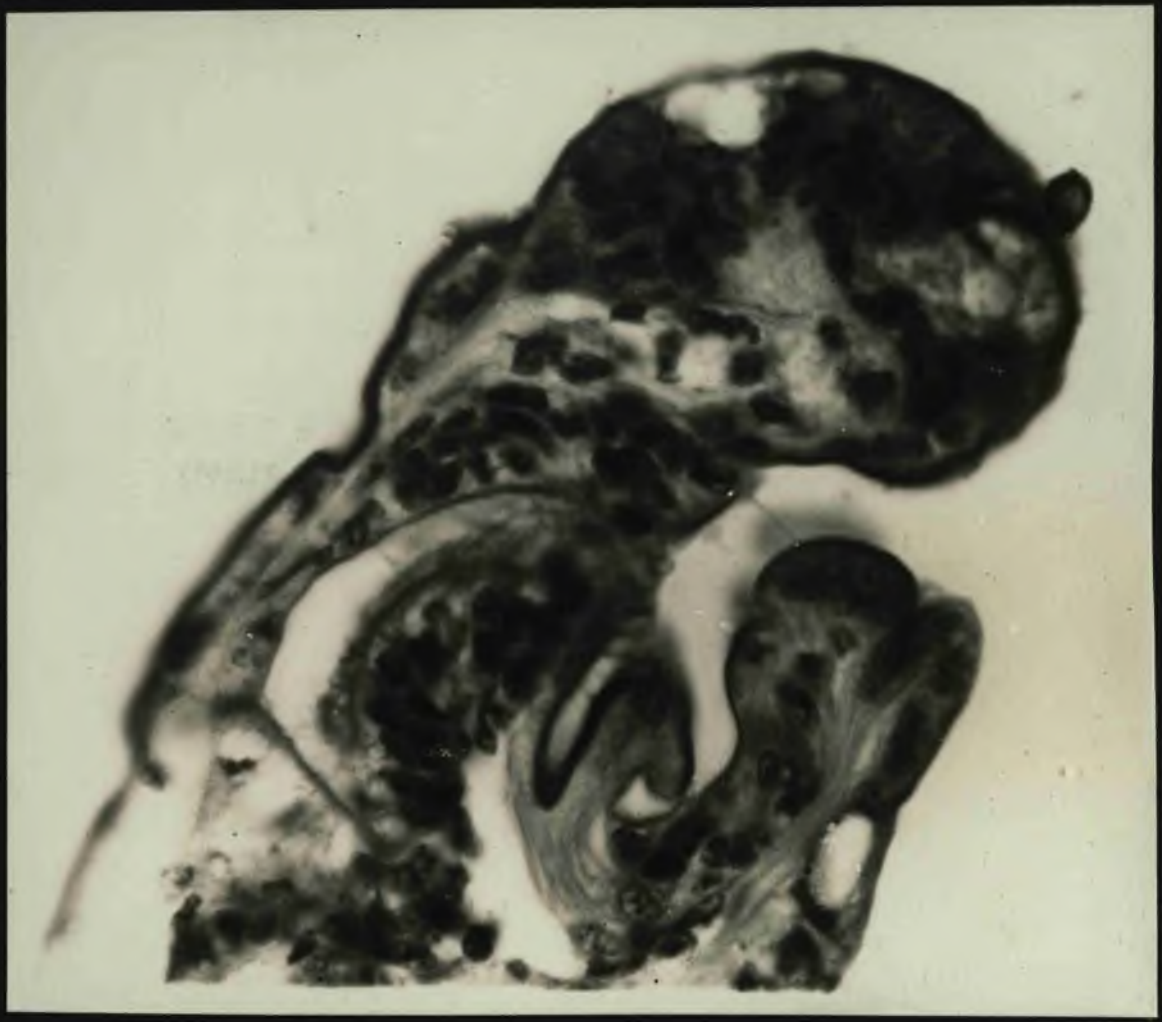


Plate 4

12 Chaetiger larva (X 75) showing the constriction caused by the formation of a short mucous tube.

Plate 5

12 Chaetiger larva (X75) in a tube formed from mucus and the debris of unfertilised eggs.

III. EXPERIMENTAL STIMULATION OF SPAWNING.

(a) Literature.

The literature contains accounts of spawning induced in ripe marine organisms by various organic substances. These substances are generally derived from the genital products of one sex and stimulate spawning in the other sex. Work by Okuda (1938) suggests that females of the species Arenicola claparedii only spawn when stimulated in this way. Spermatophores were seen to flow into the burrows of the females and disrupt on their chaetae, liberating the spermatozoa. At this stage oviposition began.

Lillie and Just (1913) showed that ripe males of the species Nereis limbata spawn when immersed in egg water, i.e. sea water in which eggs have been allowed to lie for a period of time. The properties of the spawning stimulant (table 23) suggest that it may be the same as the sperm agglutinating agent - fertilizin. Townsend (1939), investigating the same species, found that in addition to spawning in egg water, ripe males also spawn in sea water - "charged by a swimming female". The active agent was said to stimulate chemoreceptors in the epitokous region. This stimulus resulted in muscular contraction of the body wall and expulsion of the genital products. A similar spawning reaction was induced by adding a drop of Glutathione (1 : 10 p.p.m.) to the medium. Examination of the chemical properties of the natural agent revealed that they are similar, qualitatively, to glutathione (table 23). Although the natural material is more than 100 times as effective as glutathione, it was suggested that they have a

similar molecular configuration.

Dehorne (1925) reported that males of Nereis diversicolor are positively attracted by secretions of the female and that when the two sexes come in contact sperm is emitted. Dales (1950), however, found that apparently ripe individuals of this form fail to spawn either in each others' presence, or in the presence of genital products.

Stimulation of one sex by the other is also common in the echinoderms. Fox (1924a & b) found that spawning males of Strongylocentrotus lividus induce spawning in females and other males. Females stimulate males only. Fox suggested that secretions of eggs and sperm invoke spawning via the nervous system. Other reports of excitation by genital products (in echinoderms) have been given by Gemmill (1914) for Solaster endica and Asterias rubens, and by Ohshima (1925) for Thyone briareus.

Galtsoff (1930) showed that in physiologically ripe females of Ostrea virginica, the presence of sperm in the medium initiates a series of reactions culminating in ovulation with rhythmic contraction of the adductor muscle. Subsequently, Galtsoff (1938a, 1938b and 1940) reinvestigated the physiology of spawning in this species with particular reference to temperature stimulation. Experiments with females (1938b) showed that whereas thermic stimulation frequently fails, positive results are always obtained when sperm suspensions are added to the medium. The spawning reaction in the female is specific. Live sperm of other

molluscs and substances later found to stimulate males were ineffective. The chemical properties of the active substance in sperm suspensions were investigated (table 23). It is insoluble in water and soluble in various fat solvents, including acetone. Spawning was induced by extracts of ripe testis suspended in boiled sea water and injected into the pallial cavity. These were prepared by the methods developed by Gallagher and Koch (1929) for the preparation of mammalian male hormone. Attempts to purify the lipin by dissolving in acetone and separation of the inert precipitate were unsuccessful. None of the fractions obtained had a specific action on females. The active substance is thermolabile, losing its power after brief exposure to temperatures between 54°C. and 57°C. It was thought that the spawning stimulant did not act directly through the chemoreceptors of the tentacles and mantle since there is a relatively long latent period before the reaction commences (see below). Blood from a spawning female or blood and tissue fluids from a male, produced no response when injected into the tissues of a ripe female.

The spawning reaction in male Ostrea virginica is non-specific (Galtsoff, 1940). Ejaculation can be induced by eggs and egg water, by the eggs of other lamellibranchs and Asterias and by thyroidin, thyroxine, theelin, extract of corpus luteum etc. together with various proteins and amino acids, including glutathione, and finally, by a number of carbohydrates. With all these the latent period is 3 - 65 secs. Sperm in the medium

was found to stimulate spawning in males as well as females; in this case, however, the latent period varied between 6 and 27 mins. It was suggested that sperm only stimulates spawning after assimilation by the gut or gills. The water soluble substances listed above act directly through the neuroreceptors.

Palmer (1937) found that environmental factors and genital products have no influence on the initiation of spawning in Arbacia punctulata. Spawning can be induced by injury. Experiments suggested that this results in the production of a stimulating agent in the injured tissues. Investigation of the transport and mode of action of this agent gave the following information:-

(a) The gonad of the injured urchin contracts, pushing out the genital products.

(b) In injured urchins with the lantern removed, the stimulating agent acts only when the urchin is inverted.

Palmer states " there is no doubt that the contractile fibres of the gonad walls are involved in the shedding reaction" and suggests that the activating agent reaches the gonad wall by gravity and with the aid of ciliary currents in the perivisceral cavity. When whole urchins were ground down and the resultant fluid injected into the perivisceral cavity of ripe individuals, shedding occurred after a latent period similar to that after injury stimulation ($5\frac{1}{2}$ - $7\frac{1}{2}$ mins.). A reaction was induced by extracts of all the structural components of the organism, but

there was no reaction with suspensions of normally shed eggs or sperm, or with blood. On the other hand, extracts of testis, ovary and concentrated eggs or sperm were the most potent; potency being measured by the maximum dilution stimulating spawning. This was thought to be due merely to the greater concentration of cells in these materials than in other tissues. The active substance in extracts is heat stable and unaffected by freezing or by bacterial action. The discovery that the active substance in extracts is inhibited by $MgCl_2$ but that this salt does not inhibit spawning due to injury, led Palmer to state "immediately after injury a humoral substance appeared to be present - more potent than the agent in extracts". Arbacia extracts are non-specific in that they also induce spawning in Echinarachnius and Asterias. Conversely, extracts of these organisms, extracts of the muscles of Rana and Fundulus and injection of calcium and potassium salts all stimulate spawning in Arbacia.

Palmer expresses the view that the problem of injury-induced shedding resolves itself into an analysis of substances capable of stimulating smooth muscle Tissue extracts contain a number of such substances but they are mainly organic and unstable. Histamine might be formed in effective amounts but this is inhibited by histaminase in injured tissues and is attacked by bacteria. Palmer concludes, "the stimulating properties of the extracts seem to be more like those of potassium than any other known constituent of tissues".

Sandeman (1954) obtained similar results. Injection of tissue extracts of Asterias rubens into ripe individuals of that species induced shedding in both males and females. This treatment induced spawning in a higher percentage of individuals than any other method employed; the next best method was to inject potassium chloride. It was noted that whereas this substance stimulated the muscle cells of the gonad wall, there was no such stimulation when the gonad was treated with tissue extract.

Other cognate work: The influence of vertebrate hormones on invertebrates is discussed in Hanstromm's "Hormones in the invertebrates". A report is given of work by Schmidt (1935) and Fennerborn (1936), on the effect of vertebrate hormones on the fresh water polychaet Lycastis rananeubis. Thyroid preparations promote precocious sexual development and increase the rapidity of ripening in the eggs. Preparations of anterior pituitary also provoke unusually early ripening of eggs. Donahue (1939, 1940) tested ovaries of Lytechinus variegatus, Echinometria, Stichopus mobii and Palinurus argus for sterols. All except Palinurus, gave Salkowski reactions. Oestrogenic properties were found when ovariectomised rats were injected.

Some information is available with regard to the lipides present in Arenicola marina. Gillam and Heilbron (1936) examined the absorption spectra of crude and fractionated sterols from various sources. The lug worm extracts gave clear ergosterol -like maxima. The quantity present is of the order of 5 - 12% of the absorbing substance. It was noted that some related

Table 23

The properties of known organic spawning stimulants.

Author	1	2	3	4
Species	Nereis limbata	Nereis limbata	Ostrea virginica	Arbacia punctulata
Source	egg water	egg water	sperm extract	tissue extracts
Water soluble	x	x?		x
Fat soluble			x	
Heat stable				x
Heat labile	x	x	x	
Dialysable		x		
Non-dialysable	x			
Other properties	1. Inactivated by sperm.	1. Ppt'd Acetone 2. Less effect in presence of sperm. 3. Unstable in alkaline sol. & sea water	1. Treatment with Acetone - all fractions ineffective	1. Passes ultra-filter. 2. Antagonised by $MgCl_2$.
Latent period			6 - 27 mins.	$5\frac{1}{2}$ - $7\frac{1}{2}$ mins.
Chemical type	Fertilisin? - (glycoprotein)	Glutathione?- (tripeptide)	Lipide	Potassium?

Authors:- 1. Lillie and Just (1913), 2. Townsend (1939),
3. Galtsoff (1938b, 1940), 4. Palmer (1937).

Table 24

Organic spawning stimulants - sources and mode of action.

Author	Species	Source	Administered in, or by,	Stimulus received by,	Induces spawning by,
1	Nereis limbata	egg water	the medium		
2	Nereis limbata (male)	egg water and water charged by a female.	the medium	chemo-receptors of epitoke	contraction in muscles of body wall.
3	Ostrea virginica (female)	sperm	the medium and injection into pallial cavity	absorption by gills or gut.	Reactions culminating in ovulation & rhythmic contraction add muscle.
3	Ostrea virginica (male)	sperm and eggs	ditto	ditto	normal ejaculation
4	Strongylocentrotus lividus	sperm and eggs	the medium	nervous system	
5	Arbacia punctulata	Tissues	injection into body cavity	gonad wall via perivisceral fluid	Contraction of gonad wall
6	Asterias rubens	Tissues	ditto	?	Shedding without contractin gonad wall

Authors:- 1. Lillie and Just (1913), 2. Townsend (1939), 3. Galtsoff (1938b, 1940),
4. Fox (1924), 5. Palmer (1937), 6. Sandeman (1954).

sterols give absorption bands similar to ergosterol. Other maxima given by lug worm sterols are closely allied to those given by dehydroxyergosterol. Sterols were extracted by hot saponification of the raw biological material with aqueous or alcoholic caustic potash followed by ether extraction. Results given by Wilber and Bayors (1947), in a comparative study of the lipids in some marine annelids, show that the cholesterol : fatty acid, and cholesterol : phospholipide ratios, are relatively high for Arenicola. The total lipide (1.22% of the wet weight) is relatively low. It was suggested that cholesterol is probably a normal protoplasmic constituent of the annelids, as of the vertebrates.

(b) Experiments with tissue extracts I.

The literature summarised above, suggested that the effect of tissue extracts should be investigated in Arenicola marina.

1. Trial experiment - stimulation of spawning in male worms.

Method: 50 Male and female Arenicola were emulsified in a macerator. The emulsion was lightly centrifuged and the mother liquor decanted and filtered through Whatman No. 1 filter paper. The filtrate (crude extract) was used for injection.

On 11.9.52 (i.e. some 5 weeks prior to spawning), 24 male worms were washed and placed in 1½ litre crystallising basins containing circulation water. Each worm was injected with 1.0ml of crude extract, using a 27 s.w.g. hypodermic needle. In half of the worms an equal amount of coelomic fluid was removed prior to injection. Injections were made through the ventral body wall, care being taken to avoid damage to the internal structures. The time of injection was noted.

Immediate reaction to injection: The worms reacted vigorously to the injection. Some made swimming movements while others exhibited extreme contraction of the body wall. In the latter case, the tail tended to contract and split between segments with the ejection of quantities of coelomic sperm. These worms were not however discarded (see. p. 116).

Examination of coelomic sperm I: A drop of coelomic sperm was removed from 4 unbroken, injected worms, 30 mins. after injection, and from a further 5 injected worms an hour later.

When diluted in sea water these samples showed a high average activity. Many of the morulae were broken down to loose sperm.

Spawning: Approximately two hours after injection 9 worms commenced spawning. Sperm was spawned in discrete strings; in some cases from all but the first pair of nephridiopores (plate 6), but generally a variable number of nephridia functioned. Spawning could be accelerated by touching the worm, when the body wall contracted slightly and sperm was emitted with a more forcible puffing action than usual.

During spawning the worms lay moderately extended, with a slight state of tension in the body wall.

Examination of spawned sperm: Occasionally the first sperm spawned contained morulae and rosettes in addition to loose sperm. The free sperm was very active. Intensely active suspensions frequently showed spontaneous and reversible agglutination (plate 7).

Examination of coelomic sperm II: No further coelomic sperm was examined until all spawning had ceased. The proportion of morulae broken down and the activity of the loose sperm did not differ materially from the previous examination. Breakdown of the morulae occurred whether the worm spawned or not.

Discussion:

The extract produced a strong spawning reaction. Although this experiment was not controlled, it can safely be said that no male Arenicola would spawn in the laboratory 5 weeks prior to the

breeding season. This point is amply illustrated by the controls in later experiments (see p.129). Further, a high degree of breakdown and activity is never found in coelomic sperm from untreated worms.

Observations on technique: There was no advantage in reducing the volume of coelomic fluid prior to injection. It was hoped in this way to avoid pressure effects due to the relatively large volume of fluid injected. However, fragmentation of the tail appeared to be caused by some muscle stimulant in the extract and not by increased pressure in the body cavity (table 25). Fragmentation of the tail did not inhibit spawning (see Experiment 3, table 26).

Table 25

The effect of reducing the volume of coelomic fluid prior to injection of extract.

	Coelomic fluid reduced	Coelomic fluid normal
Number of worms showing fragmentation of the tail	3	3
Number of worms spawning	5	4

2. Trial experiment - stimulation of spawning in female worms.

On a priori grounds it was anticipated that more difficulty would be encountered in attempting to stimulate spawning in females.

Method: The volume of extract was reduced from 1.0ml to 0.5ml per injection (see pp. 123-124). 9 Worms were given 0.5ml and 1, 1.0ml. There were no controls. In other respects the method was similar to that with the males.

Results: No spawning took place. Coelomic eggs were examined 7 hours after injection. In 8 of the 10 worms they had retained the appearance of unripe eggs. In one case the eggs were irregular in shape, probably due to cytolysis, and in another the egg membranes showed some separation from the cytoplasm. There was therefore no significant reaction to the injection of extract at this stage in the season. Fragmentation of the tail occurred despite reduction of the volume of injection.

(c) Experiments with tissue extracts II.

1. Introduction:

In this section experiments will be described in which the physical and chemical properties of the spawning stimulant were investigated by treatment or fractionation of the crude extract. The activity of these fractions was assayed by injection of male worms. In subsequent sections experiments will be described in which the most active of these fractions were injected into females and in which some biological properties of the extracts, other than stimulation of spawning, were examined.

2. Methods of Extraction:

Extracts were made by first macerating about 40 sexed worms for not less than 15 mins. Two general methods were employed with the macerated material.

(a) wet method: The macerated material was lightly centrifuged, producing three layers, mother liquor, residue, and at the bottom, sand. Crude extracts were made by filtering the mother liquor (p. 114). Other extracts were derived from both mother liquor and residue by shaking them up with various solvents over a period of days.

(b) dry method: The macerated material was dried in an oven at 40 - 60°C. It was then ground down and extracted with solvents as in the wet method.

Subsequently, in both methods, the solvents were separated from the residue after extraction and filtered through Whatman No. 1 filter paper. The solutes were recovered by distillation (under reduced pressure with alcohol and acetone). Final traces

of solvent were driven off in a fume chamber or oven at 35 - 40°C. When active material was extracted by a solvent the criterion of solubility employed was whether, while suspended in the solvent, it would pass through asbestos ultra-filters (Carlson 'EK' sterilising asbestos filter film).

Extracts, other than crude extracts, were made up for injection in 0.6 M NaCl. The volume of saline added was such that the concentration of the extracted materials was approximately equal to their concentration in the original emulsion. Storage of extracts in the refrigerator did not impair their efficiency.

3. Extraction code:

Description can be facilitated by using a code for labelling the extracts. This was as follows:- a crude extract was labelled - Axla/1, where,

Ax = Arenicola extract, 1952.
1 = the maceration from which the extract was derived.
a = crude extract.
/1 = paper filtered (/2 = asbestos ultra-filtered).

The lower case letter a, indicating a crude extract, may be replaced as follows:-

b = heat treated crude extract.
c = extracted by alcohol, wet method.
d = " " " dry method.
e = " " ether.
f = " " acetone.

Thus an extract labelled, Ax5e/2, is an ultra-filtered ether extract from the 5th maceration. Subsequent additions were

made to this code. These will be explained as they occur.

4. Method of assay:

Extracts were tested for activity by injecting them into male worms. The assays were set up largely in the manner described for the trial experiment (p.114). Worms used in these experiments were always collected on the day of the experiment, collections being made from Area C (see p.12). Prior to the experiment the worms were washed to remove any sand which might mask slight exudations of sperm. Each worm was placed in a 1¼ litre crystallising basin, almost filled with sea water. The experiments were set up on the circulation bench (plate 8). 36 Worms was the maximum number which could be handled in a single assay.

The optimum volume of injection was still in doubt but there was no alteration in the injection technique from that described above (p. 114). After injection the worms were kept under constant observation until spawning took place. The assay criteria are described below.

The experiments were controlled by injecting 2 - 6 worms (depending on the number treated with extract) with 0.5ml of 0.6 M saline.

5. Assay criteria:

Criteria were established by which the reaction to each type of extract could be measured quantitatively and compared. They were derived from observations made during the trial experiment.

There were two objective criteria:-

Criterion S: whether the injected worm spawned or not.

Criterion T: the time elapsing between injection and spawning.

Four other criteria were used. These were measured subjectively, each on an arbitrary scale of 5 units.

Criteria A_1 and A_2: The degree of activity shown by the spawned and coelomic sperm respectively. The scale of activity was graded -

0 = No activity.
1 = Very poor activity.
2 = Poor activity.
3 = Good activity.
4 = Very good activity.
5 = Excellent activity (general and spontaneous agglutination takes place, plate 7).

Sperm activity was examined microscopically. In an experiment in which a number of suspensions were assayed for activity and subsequently reassayed in random order, the possible error in assessing these criteria was found to be less than 6%. The method has the advantage of speed. Measurement of activity by the respiratory rate of standard suspensions (Gray, 1928a,1928b; Carter 1930, 1931a) could not be used here due bothoto lack of time and to the fact that the O_2 consumption of suspensions from different individuals is not necessarily comparable (Carter,1931a).

Criterion D: The quantity of sperm spawned or size of sperm pool. The scale was graded as follows:-

0 = No pool (sperm spawned insufficient to form a discrete pool).
1 = Very poor pool (contrast insufficient to photograph).
2 = Poor pool (plate 9).
3 = Good pool (plate 10).
4 = Very good pool (plate 11).
5 = Excellent pool (worm becomes spent - the dense suspension formed obscures the worm).

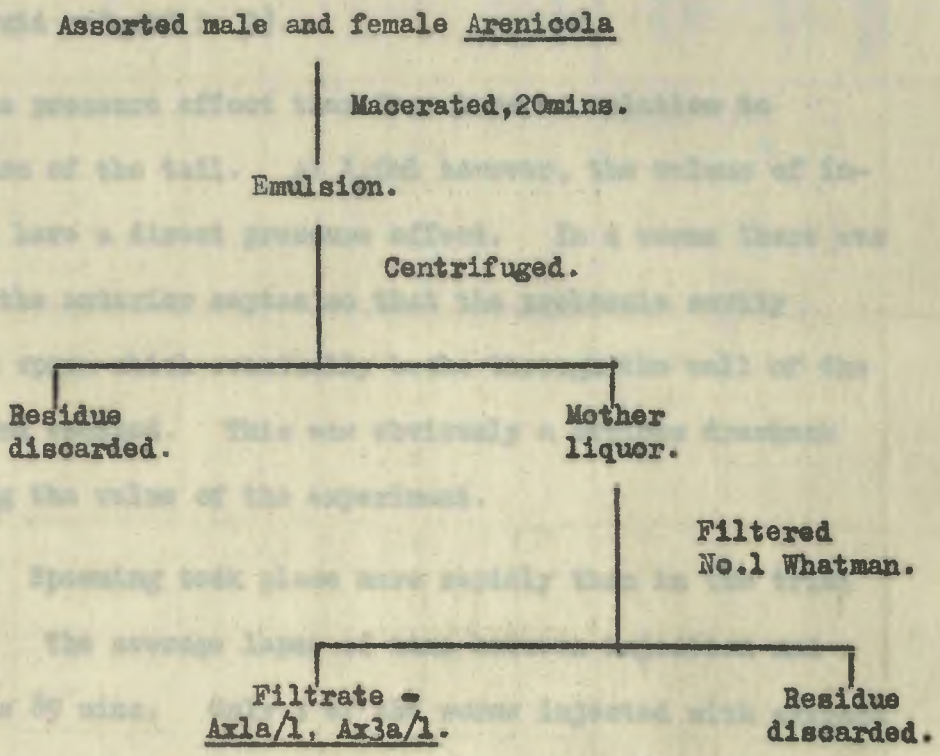
Criterion Br: The degree of breakdown from sperm morulae to free spermatozoa in the coelom.

0 = No breakdown (plate 12).
1 = Practically no breakdown.
2 = Small amount of breakdown (plate 13).
3 = Increased amount of breakdown (plate 14).
4 = Large amount of breakdown (plate 15).
5 = Vast degree of breakdown (breakdown to free spermatozoa almost complete, plate 16).

In most cases a number of assays were carried out with each type of extract. The results are illustrated by histograms in which the average values for criteria A_1, D, Br and A_2 are expressed as a percentage, to correspond with the percentage of worms in which spawning was induced by each extract. The time lapse between injection and spawning (criterion T) will be shown on an inverse scale of minutes.

6. Experiments with crude aqueous extracts.

Method of extraction: Two extracts were prepared, Ax1a/1 and Ax3a/1. The preparation of Ax3a/1 differed from the method shown in the chart given below, in that the emulsion was filtered through fine bolting silk instead of being centrifuged.



Experiment 3: This experiment will be fully described since it was designed to establish details of technique.

Method: The following volumes of Ax1a/1 were injected into each of 3 groups of 6 worms:- 0.25ml, 0.5ml and 1.0ml. There were 6 controls (see p. 120). In 3 worms from each group the coelomic fluid was first reduced by an amount equal to the volume of

injection.

Observations on technique: Fragmentation of the tail was observed as follows:-

(a) 2 of the group which received 0.25ml extract (coelomic fluid reduced).

(b) 3 of the group which received 0.5ml (coelomic fluid unaltered).

(c) 5 of the group which received 1.0ml (coelomic fluid reduced in 3).

The pressure effect therefore bore no relation to fragmentation of the tail. At 1.0ml however, the volume of injection did have a direct pressure effect. In 4 worms there was rupture of the anterior septae so that the proboscis cavity filled with sperm which eventually broke through the wall of the proboscis and escaped. This was obviously a serious drawback in assessing the value of the experiment.

Results: Spawning took place more rapidly than in the trial experiment. The average lapse of time between injection and spawning was 89 mins. Only 3 of the worms injected with extract failed to spawn. The spawned sperm was in many cases extremely active (table 26), although the amounts of sperm spawned were less than in subsequent assays with this type of extract (table 27). None of the controls spawned.

The nature of the spawning reaction was as observed in the trial experiment (p. 115). This applies to all subsequent experiments with male Arenicola and will not be referred to again.

Table 26

Experiment 3: Assay of crude aqueous extract Axla/1.

T.B. or P.B. = Tail or Proboscis breaking.

Criterion T in minutes. Injection volume in ml.

R or N = reduced or normal coelomic fluid.

Worm No.	Vol injected	R or N	Extract	P.B.	T.B.	Criterion S	Criterion T	Criterion A1	Criterion D	Examination of coelomic sperm			
										Time Examined	Criterion Br	Criterion A2	Sperm count & morulae
1a	.25	N	Axla/1			x	77	4	3	8h40m	3.5	0	
b	"	"	"			x	81	5	1	"	1.5	2	
c	"	"	"			x	83	3	2	"	3.5	3	
2a	"	R	"			x	83	3	2	"	3.5	2	25%
b	"	"	"		x	x	85	5	3	"	3.5	2	39%
c	"	"	"		x	x	72	5	2	"	2	3	50%
3a	.5	N	"		x	x	90	5	0	"	2	3	
b	"	"	"	x	x	-	-	-	-	9h30m	1.5	2	
c	"	"	"		x	x	132	5	0	10hrs	1.5	0	
4a	"	R	"			-	-	-	-	"	1	0	48%
b	"	"	"			x	67	5	3	"	3.5	4	75%
c	"	"	"			x	68	5	3	"	1.5	2	79%
5a	1.0	N	"	x	x	x	144	4	0	"	4	3	
b	"	"	"			-	-	-	-	"	0	0	
c	"	"	"	x		x	95	5	3	"	3.5	3	
6a	"	R	"	x	x	x	79	4	0	"	4	2	59%
b	"	"	"	x	x	x	117	4	0	"	3.5	2	49%
c	"	"	"		x	x	60	5	4	"	3.5	2	71%
			Average			83%	89	3.7	1.4		2.6	1.9	
7a	.25	N	Saline			-	-	-	-	10hrs	1	0	
b	.5	N	"			-	-	-	-	"	1	2	
c	1.0	N	"			-	-	-	-	"	1	2	
8a	.25	R	"			-	-	-	-	"	1	0	49%
b	.5	R	"			-	-	-	-	"	0	0	58%
c	1.0	R	"			-	-	-	-	"	2	2	56%
			Average			0%		0	0		1.0	1.0	

Examination of the coelomic sperm was delayed until 8½ - 9 hours after injection as at this time it was doubtful how long spawning would continue. The average activity of the coelomic sperm was less than that later found to be normal for aqueous extracts although in some individuals it was of a high order. It was noted that unlike sperm activated by alkali there was no tendency for the remaining morulae to break down after the sperm had been diluted in sea water. Active breakdown of morulae in the coelomic fluid was never observed.

7. Further experiments with crude or standard extracts.

In the majority of assays a few worms were injected with the crude extract for purposes of comparison. A large number of results were accumulated in this way.

Assay.

Criterion S: 66 Worms were injected, 61 spawned.

Criterion T: The average time elapsing between injection and spawning was 83 mins. There was no tendency for spawning to become more rapid as the breeding season approached (table 27).

Criteria A_1 and A_2: The activity of both spawned and coelomic sperm was of a high order; approaching that of sperm spawned naturally.

Criterion D: In most cases the quantity of sperm spawned was not large (av. D = 2.6). This proved to be the case with every type of extract. The worms seldom spawned out completely. Sufficient sperm always remained in the coelom for an assay of breakdown and activity.

Criterion Br: Breakdown of the coelomic sperm was of a very high order (av. Br = 4.1).

Fragmentation of the tails always took place in some of the worms when crude extracts were injected.

Discussion:

Although the worms must have increased in ripeness between 17th September and 17th October, nevertheless the results of the assays are remarkably uniform (table 27). It is therefore permissable to compare reactions to the injection of different types of extract although these may have been assayed on different dates within this period. In experiments described below the average reaction to the injection of crude aqueous extracts (table 27) is used as a standard with which reactions to other extracts are compared.

Summary:

1. The injection of crude aqueous extracts of whole Arenicola induces spawning in males of that species up to 5 weeks prior to the breeding season.

2. Spermatozoa spawned as a result of injection show comparable activity to those spawned naturally.

Table 27

Assay of crude aqueous extracts.

Experiment No.	Extract No.	Date	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
				% age spawning	T	A_1	D	Br	A_2	
3	Ax1a/1	17. 9	18	83	89	3.7	1.4	2.6	1.9	48%
5	"	18. 9	4	100	101	4.0	3.2	3.5	4.0	74%
6	"	19. 9	8	100	69	3.9	2.7	4.3	4.4	77%
9	"	24. 9	6	100	69	4.3	3.3	4.4	3.8	79%
9	Ax3a/1	24. 9	6	100	78	4.3	3.2	3.8	3.3	73%
11	"	25. 9	6	100	97	3.7	2.0	4.2	3.4	67%
12	"	1.10	6	83	69	3.5	2.7	4.5	4.0	73%
13	"	2.10	4	100	75	4.5	2.7	4.1	3.6	75%
19	"	16.10	6	83	100	3.2	2.2	4.7	4.4	73%
21	"	17.10	2	100	?	3.5	2.0	4.7	4.2	72%
	Average *			92%	83	3.9	2.6	4.1	3.7	72%
	Controls		28	5%	48	0.14	0.04	1.0	0.7	9%

* As the number of worms injected in each experiment varies the final averages shown here and in all subsequent assay tables are the true means of the values given by each worm and not means of the average values for each experiment shown previously in the table.

3. A muscle stimulant is present in aqueous extracts which causes contraction and fragmentation of the tail region.

4. No advantage was gained by reducing the volume of coelomic fluid. This refinement was therefore discontinued after 18.9.52.

5. The volume of extract injected in all experiments after 18.9.52 was 0.5ml since pressure effects were evident when a greater volume was injected.

8. Controls.

In experiments described in this section 52 worms were injected with saline as controls. Only 3 spawned, of these, 2 spawned in experiments carried out during the breeding season. The average activity of the sperm spawned by these worms was poor (A_1 = 2.0) and only in one case was sufficient sperm spawned to form a discrete pool (D = 1.0). The average values of Br and A_2 for all the controls were 1.5 and 0.6 respectively. This is greater than in untreated worms and may be due to the fact that the saline injection was slightly hypertonic relative to the body fluids.

The reactions of the appropriate controls are averaged and tabulated along with the assays of each type of extract.

9. Solubility I - in the aqueous medium.

Method of Extraction: The paper filtered aqueous extracts described above (pp. 123), were subsequently ultra-filtered through asbestos filter film to produce ultra-filtered aqueous

extracts, Ax1a/2 and Ax3a/2.

Experiments with ultra-filtered aqueous extracts:

Experiment 5: 8 Worms received an injection of Ax1a/2.

Experiment 6: 16 " " " " " "

The possibility that the mere presence of particulate material might induce spawning was tested by adding a small quantity of colloidal graphite to the ultra-filtered extract injected into 8 of the worms in experiment 6.

Assay:

14 of the 24 worms injected with ultra-filtered extract showed fragmentation of the tail.

Criteria S and T: Only 4 worms spawned, the average time elapsing between injection and spawning was 192 mins. There was no spawning in the group to which graphite had been added.

Criterion A_1: In 3 of the 4 spawners there were many morulae in the first sperm spawned and the free sperm showed a poor degree of activity (table 28).

Criterion D: Only 2 of the spawners spawned sufficient sperm to form a pool. One of these was a good pool (D = 3) and the other a very poor pool (D = 1).

Criterion A_2: In experiment 5, examination of the coelomic sperm was delayed until 8 hours.after injection. This may explain its complete lack of activity (table 28). However, sperm from only 6 of the 16 worms in experiment 6 showed activity and this was poor (av. A_2 = 0.6).

Criterion Br: Breakdown of the coelomic sperm was minimal throughout.

Summary and Discussion:

A striking difference is apparent between the reaction to Axla/2 and the reaction to the equivalent paper filtered extract, Axla/1 (table 27, fig. 12). The reaction to Axla/2 was only slightly greater than that shown by the controls. Similar results with graphite added indicate that removal of inorganic particulate matter was not the cause of the reduction in the activity of the extract. The removal of the major part of the active constituents by ultra-filtration suggests that they are only sparingly soluble in water.

The fraction of the extract causing fragmentation of the tail is not eliminated by ultra-filtration of the extract.

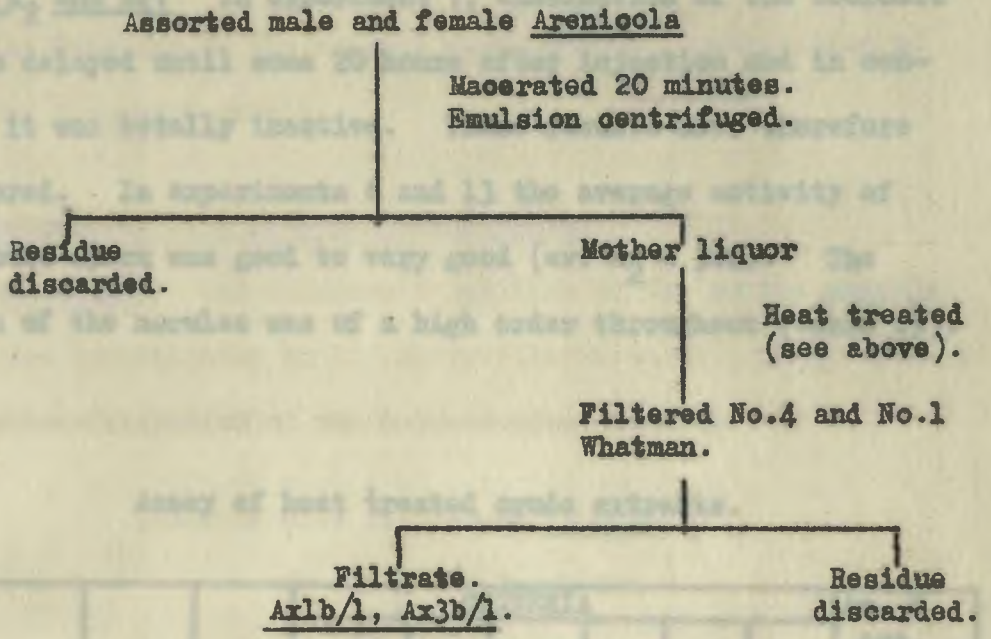
Table 28

Assay of ultra-filtered aqueous extracts

Experiment No.	Extract No.	Date	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2
				% age spawning	T	A_1	D	Br	A_2	
5	Axla/2	18.9	8	37.5	220	0.75	0.37	1.62	0	13.7%
6	"	19.9	8	12.5	110	0.37	0.12	1.06	0.50	10.3%
6	Axla/2 + graph	19.9	8	0	-	-	-	1.62	0.69	11.6%
	Average			16.6	192	0.37	0.17	1.44	0.4	11.8%
	Control			0	-	-	-	0.69	0.12	4.1%

10. Test for heat stability.

Method of extraction: Crude paper filtered extracts were prepared which, in the course of extraction, had been boiled for 10 min. and kept at 84 - 86°C. for 1 hour.



Experiments with heat treated extracts:

Experiment 6: 6 Worms received an injection of Ax1b/1.

Experiment 7: 6 " " " " " "

Experiment 13: 4 " " " " " Ax3b/1.

Assay:

10 Worms showed fragmentation of the tail.

Criteria S and T: Spawning took place later than with the standard extract (fig. 14), 14 of the 16 worms spawned.

Criteria A_1 and D: An examination of the first sperm spawned showed that in 9 cases only loose sperm was present while in 5 a number of morulae were observed. A good degree of activity was recorded (av. A_1 = 3.0). A poor to good pool was produced by most of the worms.

Criteria A_2 and Br: In experiment 7, examination of the coelomic sperm was delayed until some 20 hours after injection and in consequence it was totally inactive. These results have therefore been ignored. In experiments 6 and 13 the average activity of the coelomic sperm was good to very good (av. A_2 = 3.6). The breakdown of the morulae was of a high order throughout (table 29).

Table 29

Assay of heat treated crude extracts.

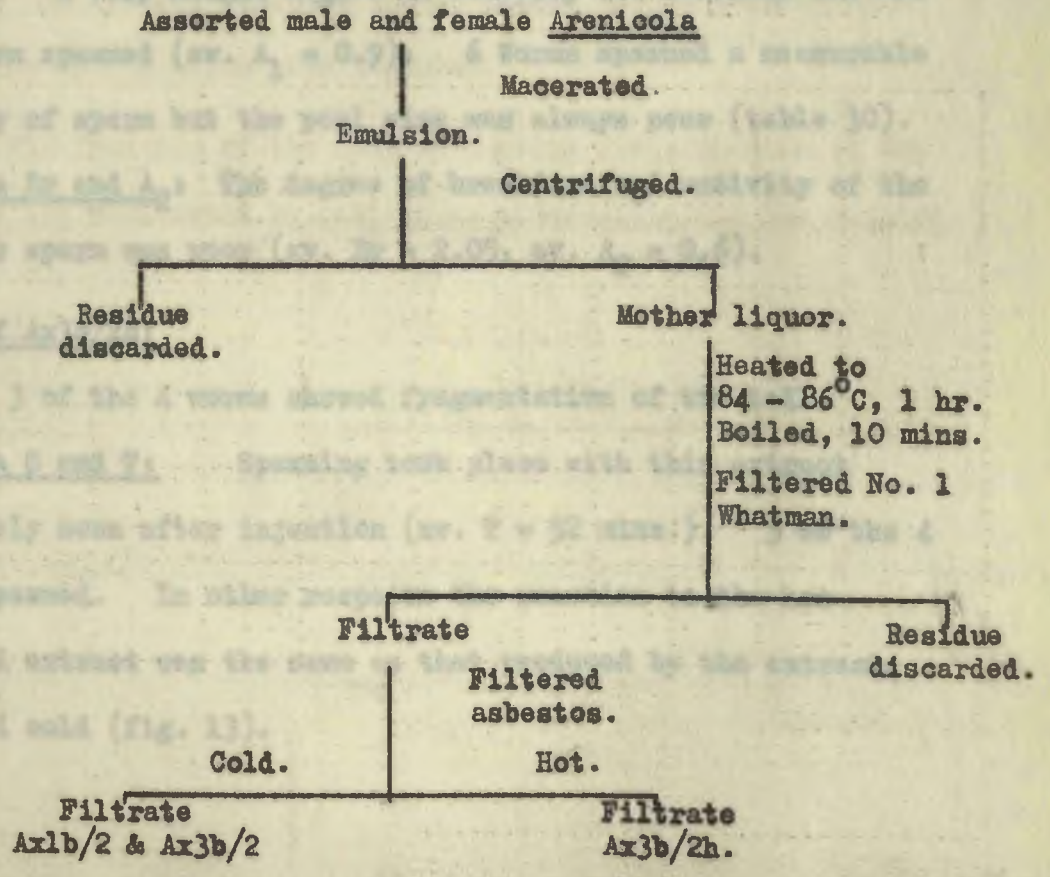
Experiment No.	Extract No.	Date	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
				% age spawning	T	A_1	D	Br	A_2	
6	Ax1b/1	19.9	6	100	102	3.66	2.36	4.16	4.00	71.8%
7	Ax1b/1	23.9	6	66	102	1.66	0.66	2.66	-	33.2%
13	At3b/1	2.10	4	100	82	4.00	2.25	3.25	3.00	62.5%
	Average			87.5	96	3.00	1.69	3.37	3.60	58.3%
	Control		10	0	-	-	-	1.10	0.3	7.0%

Discussion: The reactions to injection of the heat treated extract were similar in all respects to those with the standard extract (fig. 14). Differences lie within the limits of error of the method and the variability of the material. The active constituents are therefore heat stable under the conditions of extraction described.

The fraction of the extract which causes fragmentation of the tail is also heat stable.

11. Solubility II - in the aqueous medium.

Extraction method: The relative solubility of the active material in water was investigated by 1. ultra-filtration of the hot extract and 2. ultra-filtration of the extract after cooling.



Experiments with heat treated ultra-filtered extracts:

Experiment 7: 6 Worms received an injection of Ax1b/2.

Experiment 13: 4 " " " " " Ax3b/2, and

4 " " " " " Ax3b/2h.

Assay of Ax1b/2 and Ax3b/2:

2 of the 10 worms showed fragmentation of the tail.

Criteria S and T: 9 of the worms spawned, the average time between injection and spawning was almost the same as with the standard extract (fig. 13), but there was a considerable spread shown by individual spawning times (41 - 130 mins.).

Criteria A_1 and D: In 4 cases the initial sperm spawned was mainly composed of morulae and in 4 there was no loose sperm present. A very slight degree of activity was recorded for all the sperm spawned (av. A_1 = 0.9). 6 Worms spawned a measurable quantity of sperm but the pool size was always poor (table 30).

Criteria Br and A_2: The degree of breakdown and activity of the coelomic sperm was poor (av. Br = 2.05, av. A_2 = 0.6).

Assay of Ax3b/2h:

3 of the 4 worms showed fragmentation of the tail.

Criteria S and T: Spawning took place with this extract relatively soon after injection (av. T = 52 mins.). 3 of the 4 worms spawned. In other respects the reaction to the hot filtered extract was the same as that produced by the extracts filtered cold (fig. 13).

Summary and discussion:

Both extracts give values comparable to the standard for criteria S and T (fig. 13), while the reaction assessed by criterion D is approximately half standard. This contrasts strongly with the normal ultra-filtered aqueous extract, Axla/2 (fig. 12), where these criteria are minimal.

The reactions assessed by the remaining criteria, A_1, A_2 and Br were minimal and similar to those induced by Axla/2.

Heating the aqueous extract therefore increases the solubility of some fraction of the active material. This may be confirmed, in part, by the greater rapidity of spawning and quantity of sperm spawned when the hot filtered extract was injected. No greater emphasis can be placed on the results with Ax3b/2h as only 4 worms were injected.

The fraction of the extract causing fragmentation of the tail was not eliminated by heating or ultra-filtering the extract.

Table 30

Assay of heat treated, ultra-filtered extracts, filtered cold.

Experiment No.	Extract No.	Date	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
				% age spawning	T	A_1	D	Br	A_2	
7	Ax1b/2	23.9	6	83.5	96	.66	1.16	2.25	.16	21%
13	Ax3b/2	2.10	4	100	75	1.25	0.75	1.75	1.25	25%
	Average			90%	87	0.9	1.0	2.05	0.6	23%
	Control		6	0	-	-	-	1.58	0.33	10%

Table 31

Assay of heat treated, ultra-filtered extracts, filtered hot.

Experiment No.	Extract No.	Date	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
				% age spawning	T	A_1	D	Br	A_2	
13	Ax3b/2h	2.10	4	75%	52	0.75	1.25	1.00	0.75	19%
	Control		2	0	-	-	-	1.25	1.0	11%

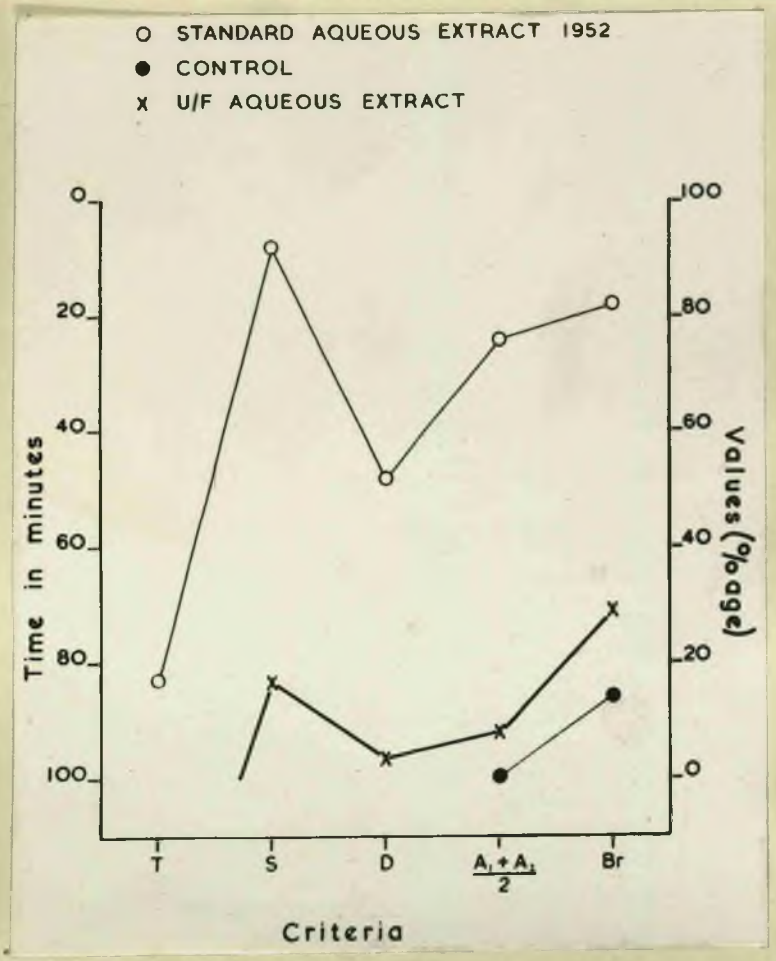


Fig. 12, Solubility I - in the aqueous medium. Assay of ultra filtered aqueous extracts.

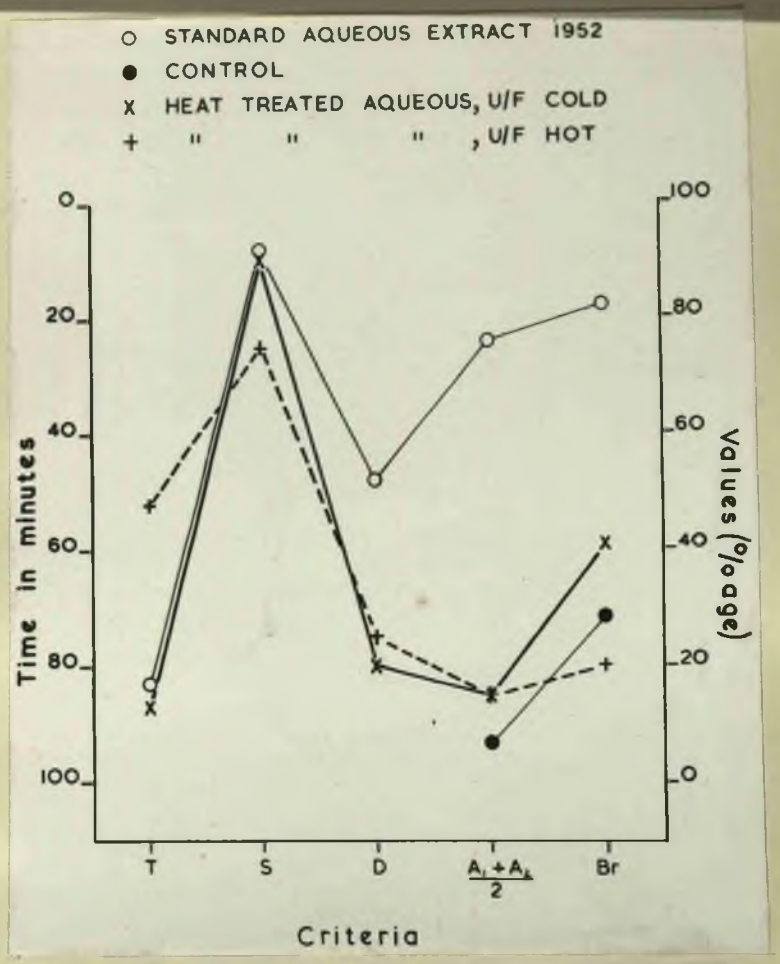


Fig. 13, Solubility II - in the aqueous medium. Assay of heat treated ultra-filtered aqueous extracts.

N.B. In these and all subsequent histograms illustrating assays, the time scale refers only to criterion T. The values of the other criteria are expressed as a percentage of the maximum value of each criterion (see pp. 121 - 122).

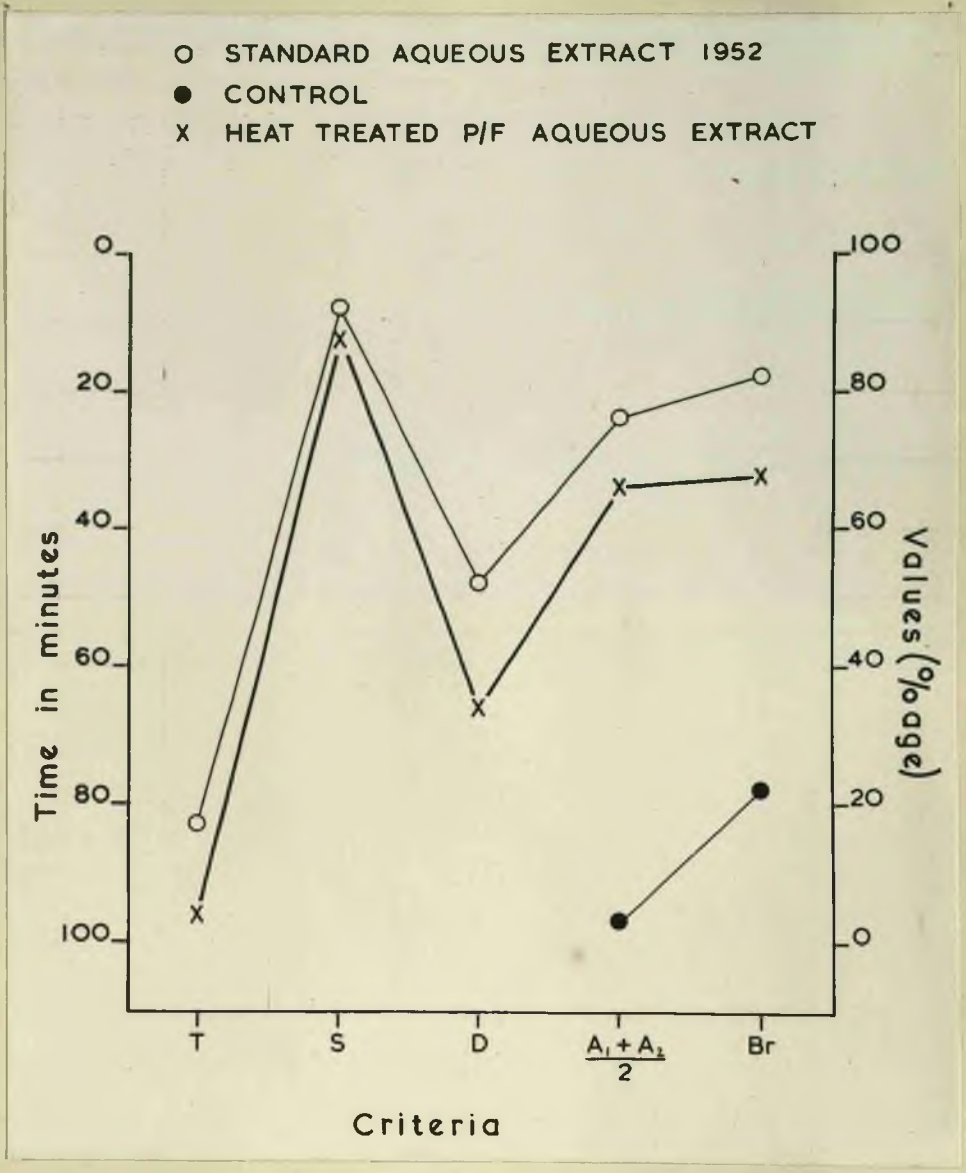
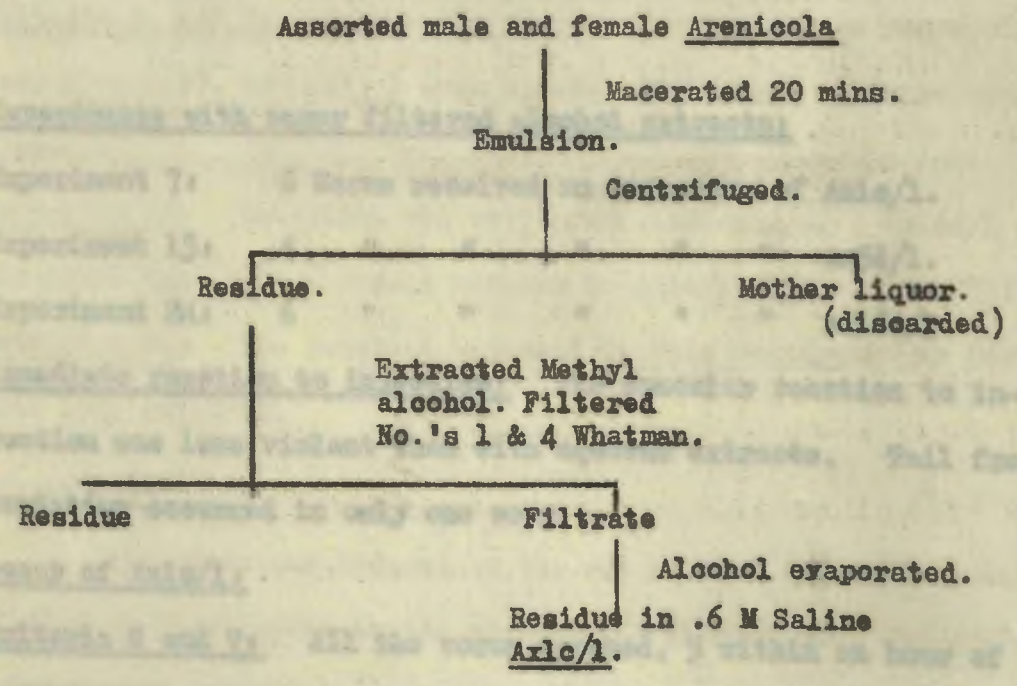


Fig. 14, Test for heat stability. Assay of heat treated paper filtered aqueous extracts.

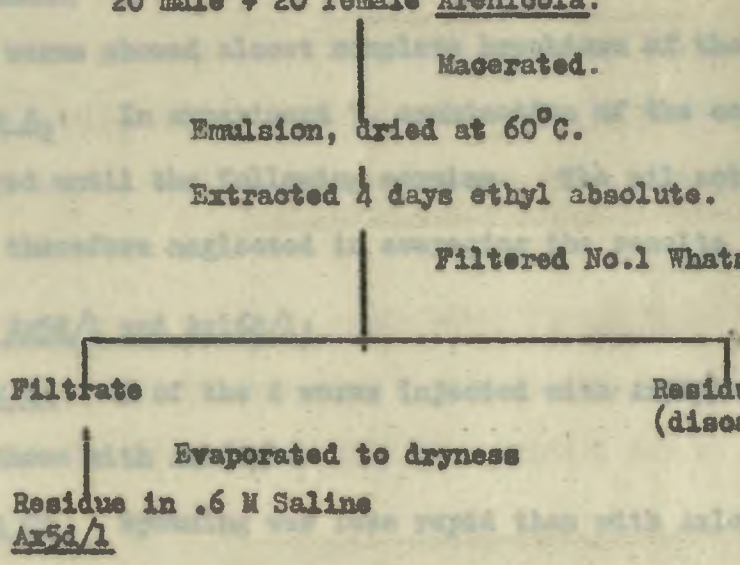
12. Solubility III - in alcohol.

Extraction method: Both wet and dry extraction methods were used (p.118).

Wet Method: The residue, after centrifugation, was covered with about 6 volumes of alcohol (95%) and kept for 4 days, with occasional shaking. The solvent was then separated and filtered through Whatman No. 1 and later Whatman No. 4 filter papers. The process was repeated a second time using the same residue. The filtrates were mixed and the solvent distilled off. The residue after distillation was oily, yellow to brown in colour, and gave off a strong fish oil odour. This material was suspended in 0.6 M saline and injected. Due to the immiscibility of the residue with water, continuous shaking was required to maintain an even suspension while making the injections.



Dry method: After maceration the emulsion was dried (see p.118). A hard residue resulted. This was ground down in a mortar and extracted with absolute alcohol over a period of 4 days. Thereafter the procedure was the same as in the wet method.



Experiments with paper filtered alcohol extracts:

Experiment 7: 6 Worms received an injection of Ax1c/1.

Experiment 15: 4 " " " " " Ax5d/1.

Experiment 24: 6 " " " " " Ax16d/1.

Immediate reaction to injection: The muscular reaction to injection was less violent than with aqueous extracts. Tail fragmentation occurred in only one worm.

Assay of Ax1c/1:

Criteria S and T: All the worms spawned, 5 within an hour of injection.

Criterion A_1: In 5 of the 6 worms the first puffs of sperm showed little activity and were mainly composed of morulae. Subsequently a good degree of activity was recorded (av. A_1 = 3).

Criterion D: The amount of sperm spawned averaged something better than a good pool (av. D = 3.1).

Criterion Br: The degree of breakdown of the coelomic sperm was high; 2 worms showed almost complete breakdown of the morulae.

Criterion A_2: In experiment 7, examination of the coelomic sperm was delayed until the following morning. The nil activity of this sperm is therefore neglected in averaging the results (table 32).

Assay of Ax5d/1 and Ax16d/1:

Criterion S: 3 of the 4 worms injected with Ax5d/1 spawned, but none of those with Ax16d/1.

Criterion T: Spawning was less rapid than with Ax1c/1 (fig. 15).

Criteria A_1 and D: An average degree of activity was recorded (av. A_1 = 2.4), but only 1 worm spawned sufficient sperm to create a pool.

Criterion Br: Breakdown was very poor with Ax5d/1. Ax16d/1 on the other hand gave a result similar to Ax1c/1 (tables 32 & 33).

Criterion A_2: The reaction assessed by this criterion was low with both extracts.

Discussion:

The reactions induced by the wet extract (Ax1c/1) were similar to those given by the standard. Results with the dry extract were relatively poor (fig. 15). There are two possible explanations for this discrepancy. First, due to the miscibility

of water and alcohol, water was present at all stages in the wet extraction. It is possible then, that the active principle was carried mainly in the aqueous fraction. In this case the reaction given by Ax1c/1 would be misleadingly high. Secondly, although the active materials are heat stable in the aqueous medium, it is possible that evaporating to dryness at 60°C. affects their properties. This would account for a relatively poor result with Ax5d/1. The results will be discussed further (p. 144) in the light of those obtained with ultra-filtered alcohol extracts.

Subsequent to these experiments it was found that extracts made at the end of the breeding season were weak in active principle; in view of this, results with Ax16d/1 may be disregarded (see pp. 159).

Table 32

Assay of paper filtered alcohol extract - wet method.

Experiment No.	Extract No.	Date	No. injected	CRITERIA						Av. % age A_1, D, Br, A_2.
				% age spawning	T	A_1	D	Br	A_2	
7	Axlc/1	23.9	6	100	49	3.0	3.1	4.25	0 *	69%
	Control		4	0	-	-	-	1.75	0	9%

* This criterion neglected due to delay.

Table 33

Assay of paper filtered alcohol extracts - dry method.

Experiment No.	Extract No.	Date	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2
				% age spawning	T	A_1	D	Br	A_2	
15	Ax5d/1	9.10	4	75	103	2.37	0.50	1.50	1.50	29%
24	Ax16d/1	19.10	6	0	-	-	-	4.00	0.50	22.5%
	Average		10	33	103	0.95	0.20	3.00	0.90	25%
	Control		6	16.6	?	0.33	0	1.50	1.00	14.15%

Experiments with ultra-filtered alcohol extracts.

Extraction method: After extraction by the wet and dry methods described above, solvent and solute were filtered through asbestos filter film before evaporating to dryness. The extracts Ax1c/2 and Ax5d/2 correspond to the paper filtered Ax1c/1 and Ax5d/1. Experiments were conducted as follows:-

Experiment 7: 6 Worms received an injection of Ax1c/2.
Experiment 15: 4 " " " " " Ax5d/2.
Experiment 17: 4 " " " " " "
Experiment 21: 2 " " " " " "

Assay of Ax1c/2:

Tail fragmentation occurred in two worms.

Criteria S and T: One worm failed to spawn. The time elapsing between injection and spawning was much the same as with the standard extract, but greater than with Ax1c/1 (fig. 16).

Criteria A_1 and D: Poor to good values were recorded for both these criteria. Again the results were poorer than when Ax1c/1 was injected (tables 32 and 34).

Criterion Br: The observations on the preceding criteria apply here.

Criterion A_2: This criterion is again ignored in experiment 7 (see p.140).

Assay of Ax5d/2:

The results (table 35, fig. 16) were almost identical to those obtained from the injection of Ax1c/2.

Discussion:

The poor reaction to the injection of the paper filtered alcohol extract by the dry method (Ax5d/1) cannot be accounted for by the explanations suggested above (pp. 140-141) because the same extract, after ultra-filtration (Ax5d/2) gave good results (table 35). It may be that when the paper filtered extract was tested the solvent had not been completely driven off. The presence of traces of alcohol might inhibit the spawning reaction.

The reaction to the injection of the ultra-filtered alcohol extract by the wet method (Ax1c/2) was significantly lower than when the corresponding paper filtered extract was injected (Ax1c/1; cf. figs. 15 and 16). There may, therefore, be some truth in the suggestion (p. 141) that in alcohol extracts by the wet method some active material is carried in the aqueous fraction. As the active material is insoluble in water (p. 131) the part suspended in the aqueous fraction would be filtered out by the ultra-filter and in consequence the concentration of the active materials would be lower than in the corresponding paper filtered extract.

While the active material is extracted by alcohol, comparison of the reactions induced by ultra-filtered alcohol extracts with those induced by the standard extract shows that while criteria S and T are similar the reactions assessed by the remaining criteria are approximately half that of the standard. This suggests that some part of the active material is not fully soluble in alcohol.

The factor causing extreme muscular contraction and fragmentation of the tail is weak in alcohol extracts.

Table 34

Assay of ultra-filtered alcohol extract - wet method.

Experiment No.	Extract No.	No. injected	Date	CRITERIA						Av. % age. A_1, D, Br, A_2.
				% age spawning	T	A_1	D	Br	A_2	
7	Axlc/2	6	23.9	83	77	2.62	1.66	2.92	-	48%
	Control	4		0	-	-	-	1.75	0	9%

Table 35

Assay of ultra-filtered alcohol extract - dry method.

Experiment No.	Extract No.	No. injected	Date	CRITERIA						Av. % age. A_1, D, Br, A_2.
				% age spawning	T	A_1	D	Br	A_2	
15	Ax5d/2	4	9.10	75	86	3.38	1.12	2.25	1.87	43%
17	Ax5d/2	4	14.10	100	84	1.62	1.25	2.37	0.75	30%
21	Ax5d/2	2	17.10	100	92	3.25	2.0	3.25	2.75	56%
	Average			90	87	2.65	1.35	2.50	1.60	40.5%
	Control	6		0	-	-	-	1.42	1.00	12%

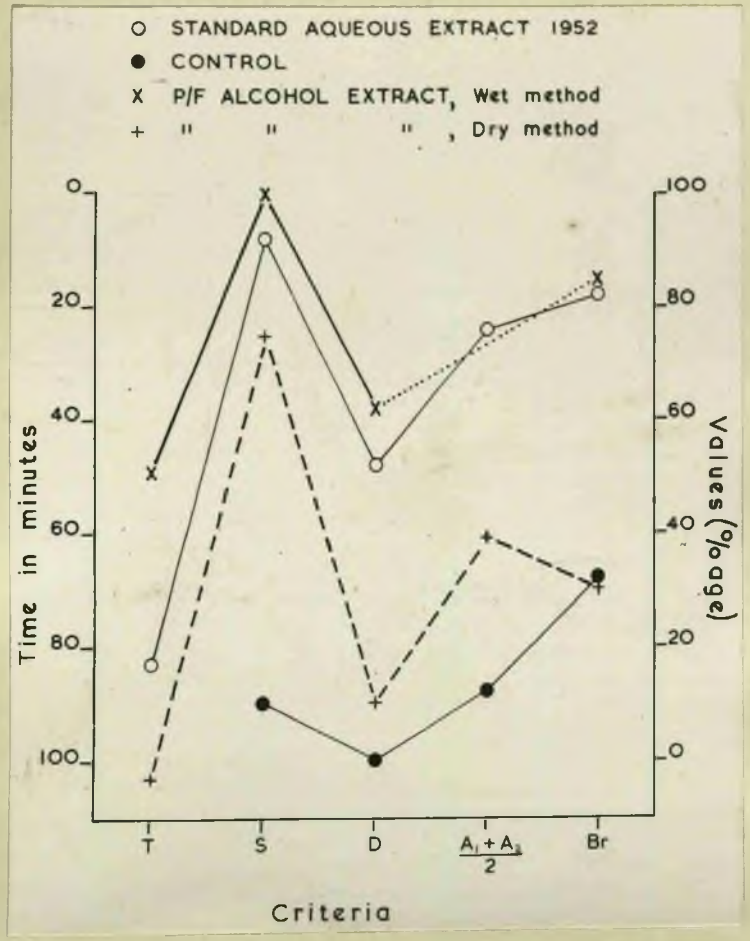


Fig. 15, Solubility III - in alcohol.
Assay of paper filtered alcohol extracts.

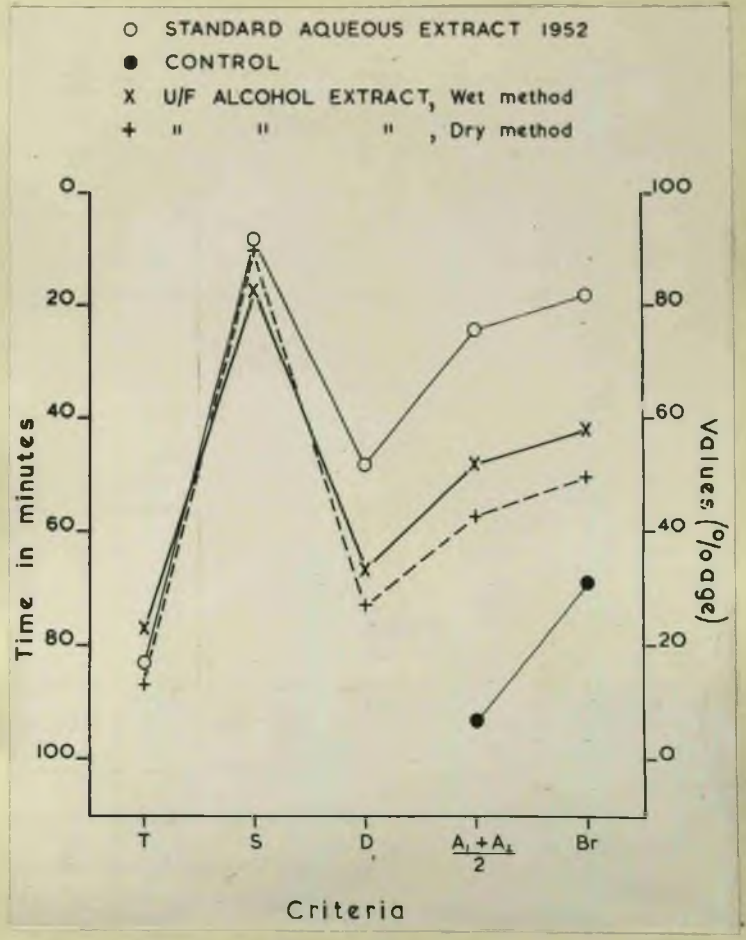
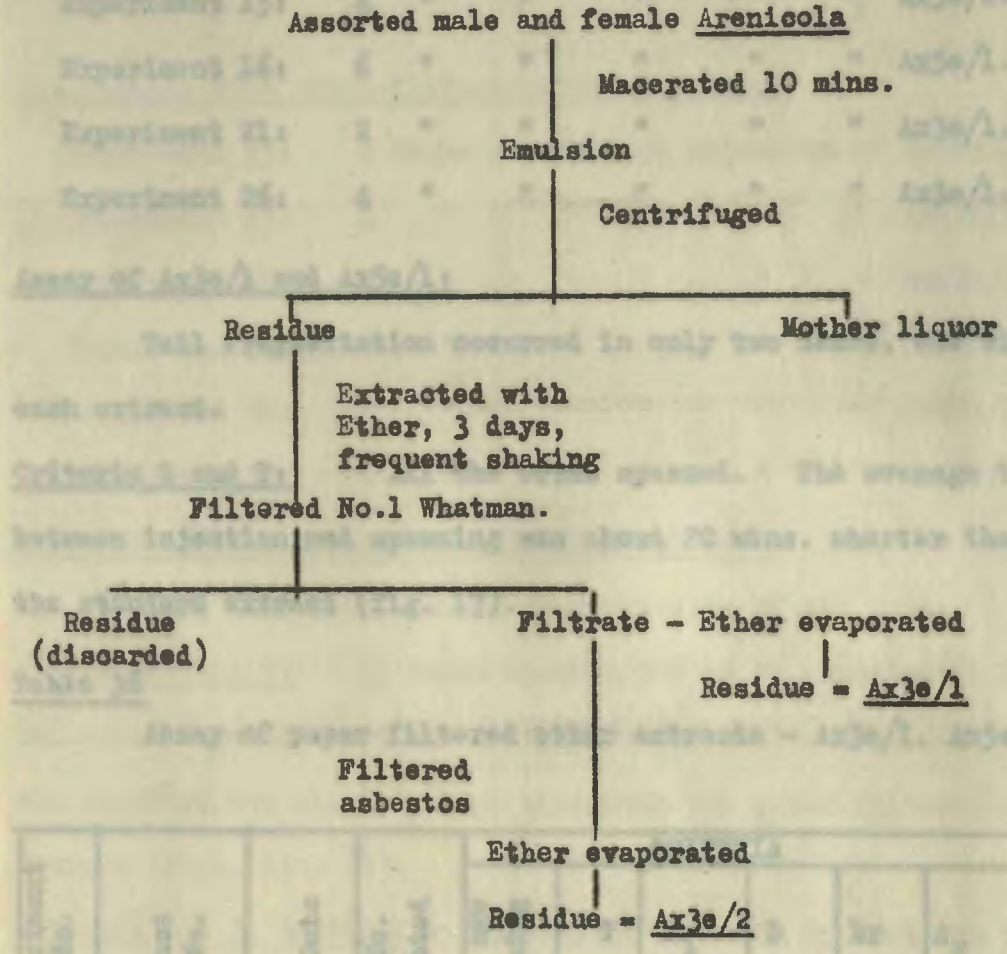


Fig. 16, Solubility III - in alcohol.
Assay of ultra-filtered alcohol extracts

13. Solubility IV - in ether.

Extraction method: Both wet and dry methods were employed, the processes were the same as those with alcohol.

Wet method:



In addition, one extract was produced by a wet re-extraction from crude extract - Ax19e/2.

Dry method: As for the wet method, except that the emulsion after centrifuging was dried before extraction with ether. The following extracts were produced - Ax5e/1, Ax5e/2, Ax13e/2 and Ax15e/2.

Oily residues were produced by both methods and these were again suspended in 0.6 M saline for injection.

Experiments with paper filtered ether extracts:

Experiment 13: 4 Worms received an injection of Ax3e/1.

Experiment 15: 4 " " " " " Ax5e/1.

Experiment 16: 6 " " " " " Ax5e/1.

Experiment 21: 2 " " " " " Ax3e/1.

Experiment 26: 4 " " " " " Ax3e/1.

Assay of Ax3e/1 and Ax5e/1:

Tail fragmentation occurred in only two cases, one with each extract.

Criteria S and T: All the worms spawned. The average time between injection and spawning was about 20 mins. shorter than with the standard extract (fig. 17).

Table 36

Assay of paper filtered ether extracts - Ax3e/1, Ax5e/1.

Experiment No.	Extract No.	Date	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
				% age spawning	T	A_1	D	Br	A_2	
13	Ax3e/1	2.10	4	100	78	4.75	4.00	3.25	2.87	74%
15	Ax5e/1	9.10	4	100	67	4.50	2.87	4.50	3.87	79%
16	Ax5e/1	13.10	6	100	67	3.83	3.17	4.41	3.17	73%
21	Ax3e/1	17.10	2	100	60	5.00	5.00	5.00	4.25	96%
26	Ax3e/1	22.10	4	100	46	4.50	4.50	3.00	2.25	71%
	Average		20	100	65	4.40	3.70	4.00	3.20	76%
	Control		14	0	-	-	-	1.20	0.57	9%

Criteria A_1, D, Br and A_2: The reactions assessed by A_1, Br and A_2 were either equal to or slightly in excess of reactions given by the standard extract. The average pool size was assessed at almost a unit larger (av. D = 3.7) than with standard extracts.

Experiments with ultra-filtered ether extracts:

Experiment 15: 4 Worms received an injection of Ax5e/2.
Experiment 17: 4 " " " " " Ax13e/2.
Experiment 19: 3 " " " " " Ax13e/2.
Experiment 21: 2 " " " " " Ax13e/2.
Experiment 22: 4 " " " " " Ax15e/2.
Experiment 26: 6 " " " " " Ax19e/2.

Assay of Ax5e/2, Ax13e/2, Ax15e/2 and Ax19e/2:

Only four worms showed fragmentation of the tail.

Criteria S and T: 21 Worms spawned out of 23 injected. The reactions assessed by these criteria were practically identical to the standard but slightly less than with the paper filtered ether extract (figs. 17 & 18).

Criteria A_1, D, Br and A_2: Reactions assessed by criteria D and Br were again identical to the standard (fig. 18) but the activity of the spawned and coelomic sperm was relatively poor (table 37).

Summary:

1. Reactions induced by the paper filtered ether extract were better than any previously obtained. The values of criteria S, T and D were in excess of the standard. The active materials might therefore be purified and concentrated by this method of extraction.

2. The ultra-filtered ether extract gave similar reactions to the standard except with regard to sperm activity. Thus apart from a factor which may be responsible for sperm activation, the active materials involved in spawning appear to be fully extractable by ether.

3. The factor causing tail fragmentation was only weakly extracted.

Table 37

Assay of ultra-filtered ether extracts

Experiment No.	Extract	Date	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
				% age spawning	T	A_1	D	Br	A_2	
15	Ax5e/2	9.10	4	100	65	5.00	4.50	4.50	4.12	91%
17	Ax13e/2	14.10	4	100	84	2.12	1.75	1.75	0.50	31%
19	Ax13e/2	16.10	3	100	75	2.17	3.33	4.33	0.33	51%
21	Ax13e/2	17.10	2	100	62	2.75	2.50	5.00	0.00	51%
22	Ax15e/2	18.10	4	75	100	1.25	1.25	4.50	1.75	44%
26	Ax19e/2	22.10	6	83	70	2.17	2.17	4.75	- *	61%
	Average		23	91	76	2.52	2.51	4.11	1.56	53.5%
	Control		20	5	(32)	0.05	0.05	1.25	0.70	10%

* This figure not included due to delay in examining the coelomic sperm.

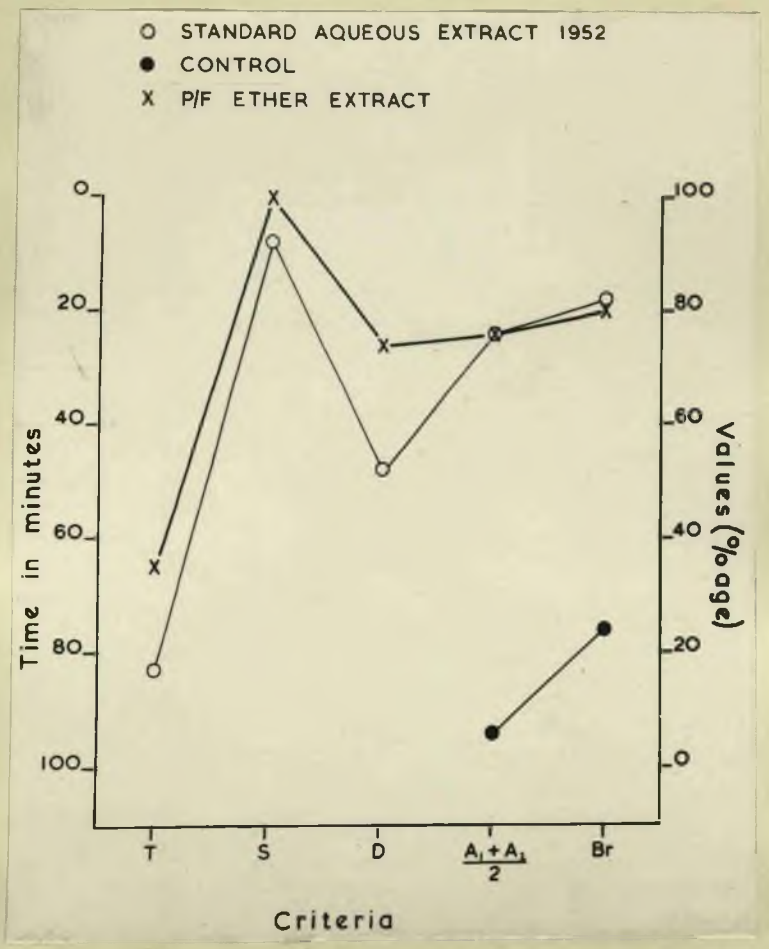


Fig. 17, Solubility IV - in ether.
Assay of paper filtered ether extracts.

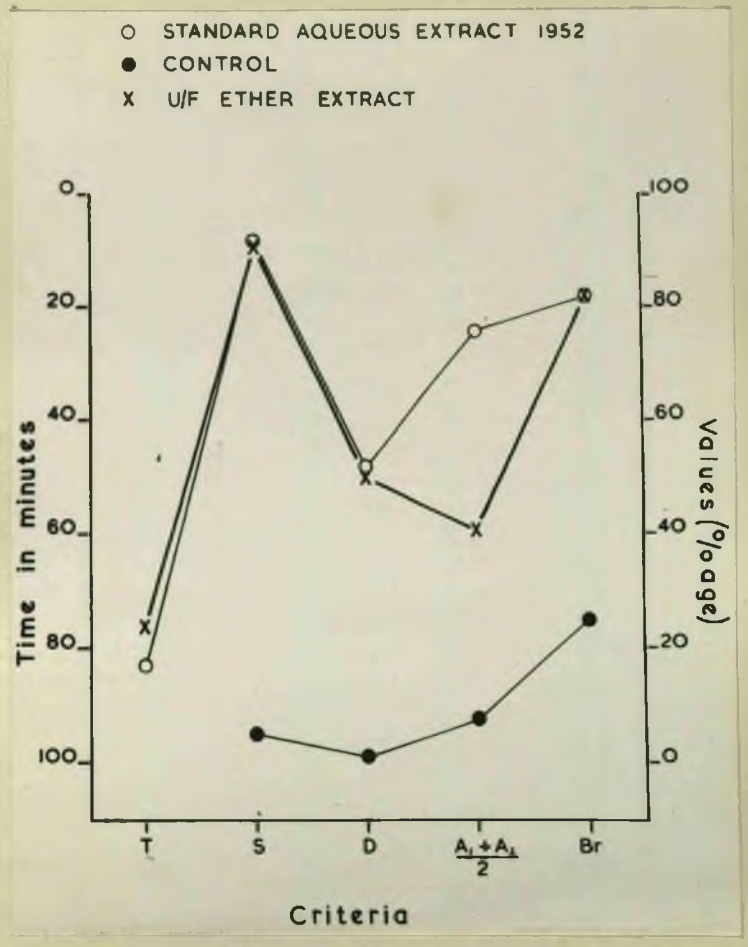
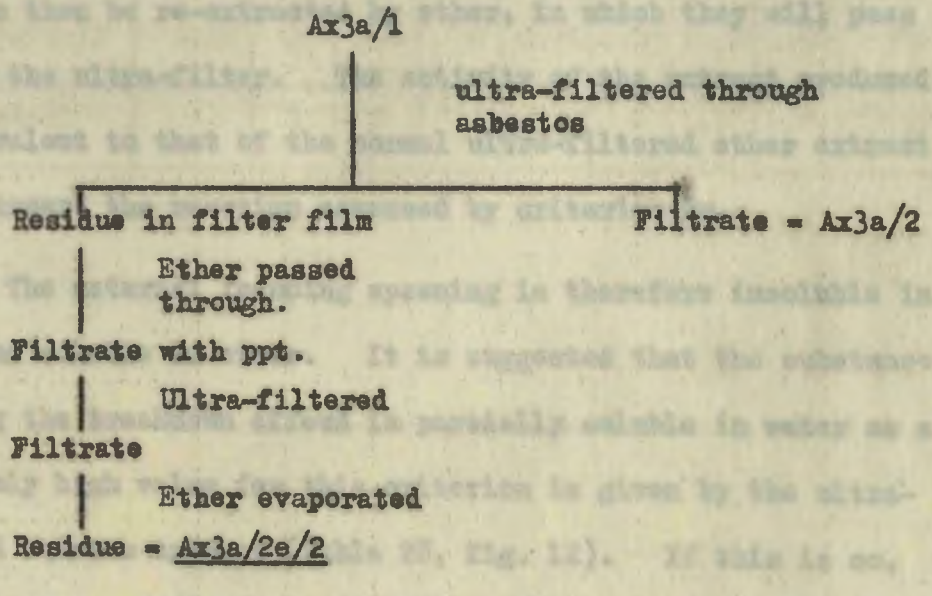


Fig. 18, Solubility IV - in ether.
Assay of ultra-filtered ether extracts.

Experiments with ultra-filtered ether extracts II.

An extract was prepared to cross check two properties of the active material already deduced, (a) insolubility in the aqueous medium, and (b) solubility in ether.

Extraction method: A crude extract was ultra-filtered. The material retained in the asbestos filter film was re-extracted by pouring through it, 20ml hot ether, followed by 10ml cold ether. The filtrate contained a fine precipitate which aggregated on shaking. The solution was again ultra-filtered to remove the precipitate. The filtrate was evaporated and the oily residue suspended as usual in saline.



The material recovered was sufficient for four injections.

Experiment 13: 4 Worms received an injection of Ax3a/2e/2.

Assay of Ax3a/2e/2:

No tail fragmentation occurred.

Criteria S and T: Spawning took place almost immediately after injection (table 38). All the worms spawned.

Criteria A_1, D, Br and A_2: The values of the activity criteria and the quantity of sperm spawned were equivalent to those given by the standard extract. An unusual feature was that although spawning was exceedingly rapid, the activity of the first sperm spawned was of a high order. The breakdown of the coelomic sperm was poor (table 38).

Summary and Discussion:

This assay confirms that when aqueous extracts are ultra-filtered, the active materials are retained by the filter film. They can then be re-extracted by ether, in which they will pass through the ultra-filter. The activity of the extract produced is equivalent to that of the normal ultra-filtered ether extract in all except the reaction assessed by criterion Br.

The material inducing spawning is therefore insoluble in water and soluble in ether. It is suggested that the substance inducing the breakdown effect is partially soluble in water as a relatively high value for this criterion is given by the ultra-filtered aqueous extract (table 28, fig. 12). If this is so, a low value for criterion Br could be anticipated with Ax3a/2e/2.

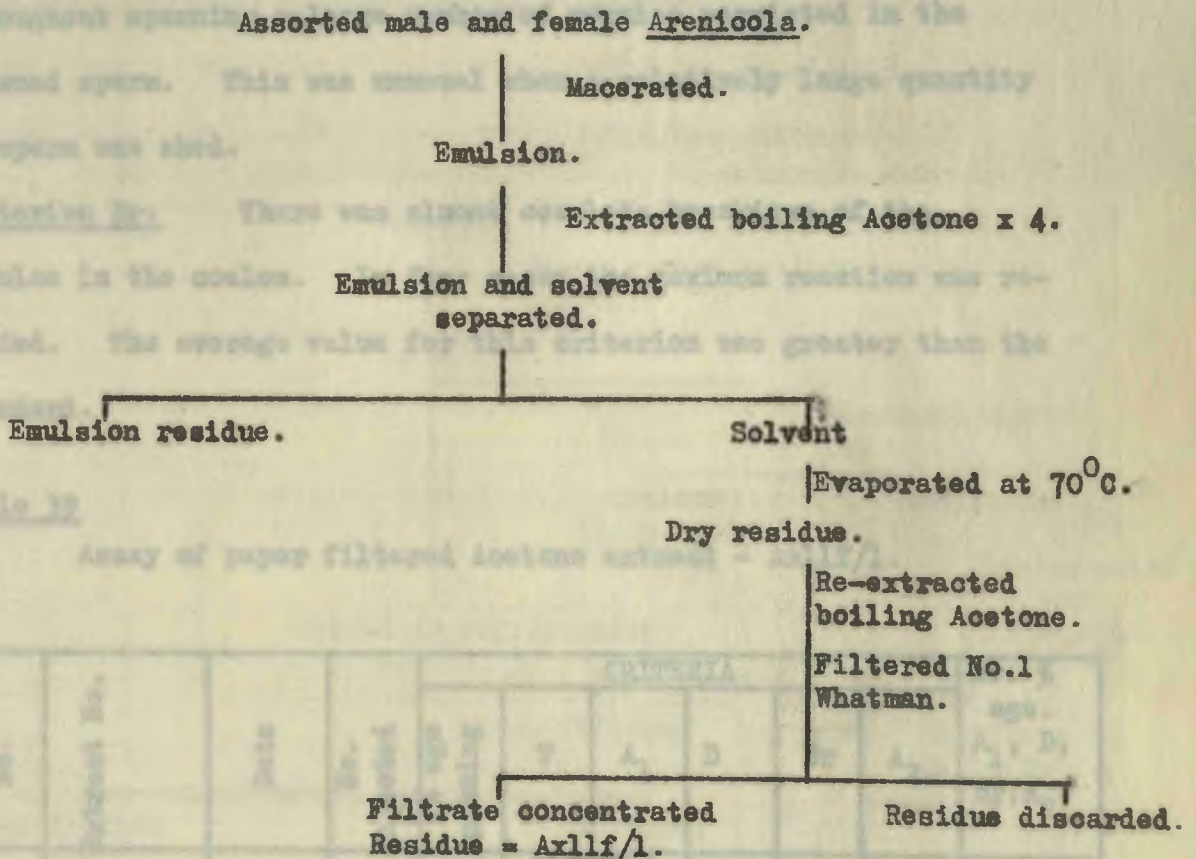
Table 38

Assay of an ultra-filtered ether extract (Ax3a/2e/2) produced by the re-extraction of the material retained in an asbestos film used to filter the aqueous extract Ax3a/2.

Experiment No.	Extract No.	Date	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
				% age spawning	T	A_1	D	Br	A_2	
13	Ax3a/2e/2	2.10	4	100	30	4.75	2.25	1.50	1.50	50%
	Control		2	0	-	-	-	1.25	1.0	11%

14. Solubility V - in acetone.

Extraction method: The emulsion after maceration was extracted with boiling acetone. This was repeated four times. The last fraction was almost colourless. The solvent was separated and evaporated at 70°C. As water may have been present in this extraction the dry residue was re-extracted in boiling acetone and filtered while hot. The filtrate was concentrated at 40°C. and finally evaporated to dryness in a vacuum dessicator.



Experiments with paper filtered acetone extracts:

Experiment 19: 6 Worms received an injection of Axllf/1.

Assay:

Criteria S and T: 5 of the 6 worms spawned. The average time between injection and spawning was only a few minutes longer than standard (fig. 19).

Criterion D: The amount of sperm spawned was similar to that given by the standard (av. D = 2.0).

Criteria A_1 and A_2: The reactions assessed by these criteria were strikingly poor (av. A_1 = 0.5, av. A_2 = 0). When combined they were less than the values for the controls (fig. 19). Throughout spawning a large number of morulae persisted in the spawned sperm. This was unusual when a relatively large quantity of sperm was shed.

Criterion Br: There was almost complete breakdown of the morulae in the coelom. In four cases the maximum reaction was recorded. The average value for this criterion was greater than the standard.

Table 39

Assay of paper filtered Acetone extract - Axllf/1.

Experiment No.	Extract No.	Date	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
				% age spawning	T	A_1	D	Br	A_2	
19	Axllf/1	16.10	6	83	88	0.50	2.00	4.83	0.00	37%
	Control		4	25	(32)	0.25	0.25	1.75	1.25	17.5%

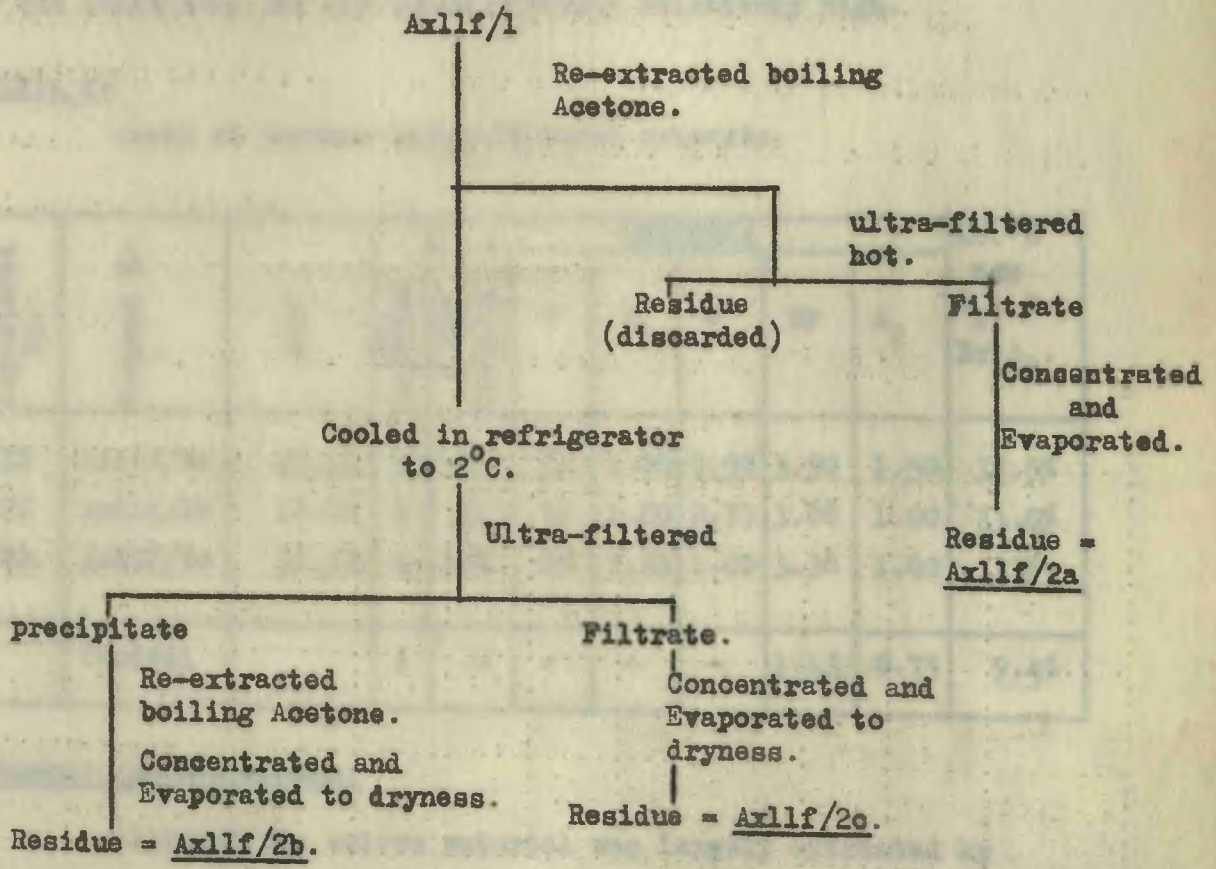
Ultra-filtered acetone extracts.

Extraction method: The dry residue, Axllf/1, was re-extracted in boiling acetone. This solution was divided into two portions. One was ultra-filtered hot, the other was placed in a refrigerator, brought to 2°C. and ultra-filtered at that temperature. In both cases the filtrate was concentrated and the residue recovered. Finally, the material retained in the asbestos film after the cold filtration was re-extracted in boiling acetone and subsequently concentrated and recovered. Thus three fractions were obtained:

Hot ultra-filtered acetone extract - Axllf/2a.

Cold " " " " - Axllf/2b (precipitate).

Cold " " " " - Axllf/2c (filtrate).



Experiments with ultra-filtered acetone extracts.

Experiment 22: 4 Worms received an injection of Axllf/2a.

Experiment 22: 4 " " " " " Axllf/2b.

Experiment 22: 4 " " " " " Axllf/2c.

Assay:

Tail breaking occurred in one worm with Axllf/2b.

The results require no discussion in detail. All the fractions gave a similar result with very small variations in degree (table 40, fig. 20). Results were slightly lower than those given by the paper filtered extract (fig. 19). It will be noted that compared to the paper filtered extract criterion D was relatively low and sperm activity relatively high.

Table 40

Assay of Acetone ultra-filtered extracts.

Experiment No.	Extract No.	Date	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
				% age spawning	T	A_1	D	Br	A_2	
22	Axllf/2a	18.10	4	50	82	1.00	0.50	3.50	1.50	32.5%
22	Axllf/2b	18.10	4	75	72	1.00	0.75	3.88	1.00	33.0%
22	Axllf/2c	18.10	4	100	69	1.25	1.00	3.38	1.00	33%
	Control		4	0	-	-	-	1.13	0.75	9.4%

Summary and discussion:

Although the active material was largely extracted by boiling acetone, the result with the paper filtered extract suggested

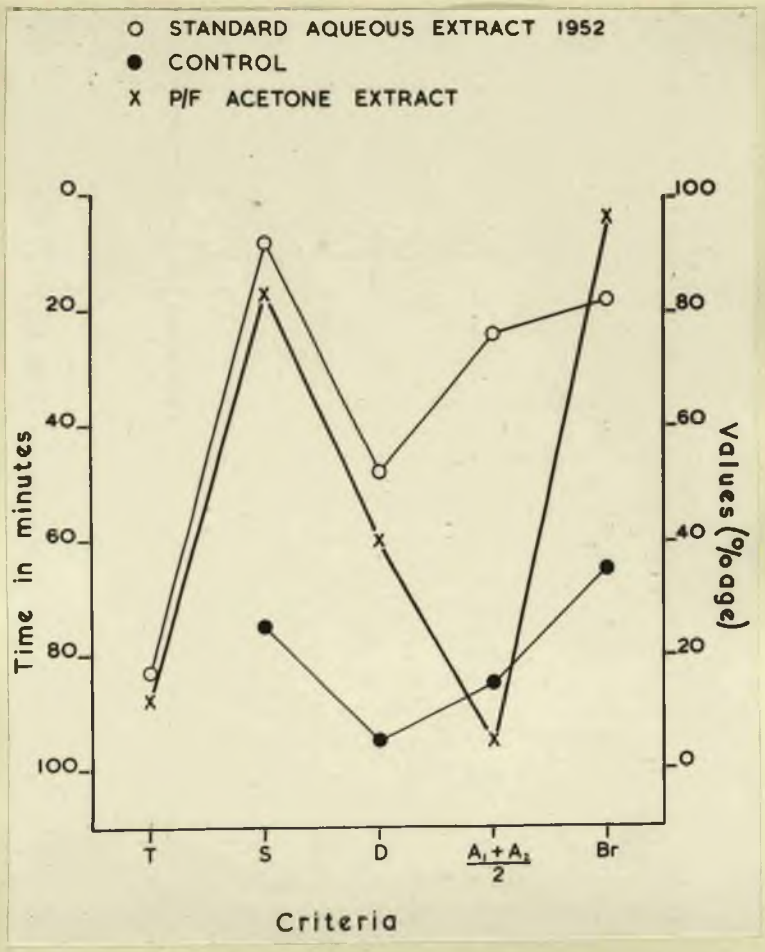


Fig. 19, Solubility V - in acetone. Assay of paper filtered acetone extracts.

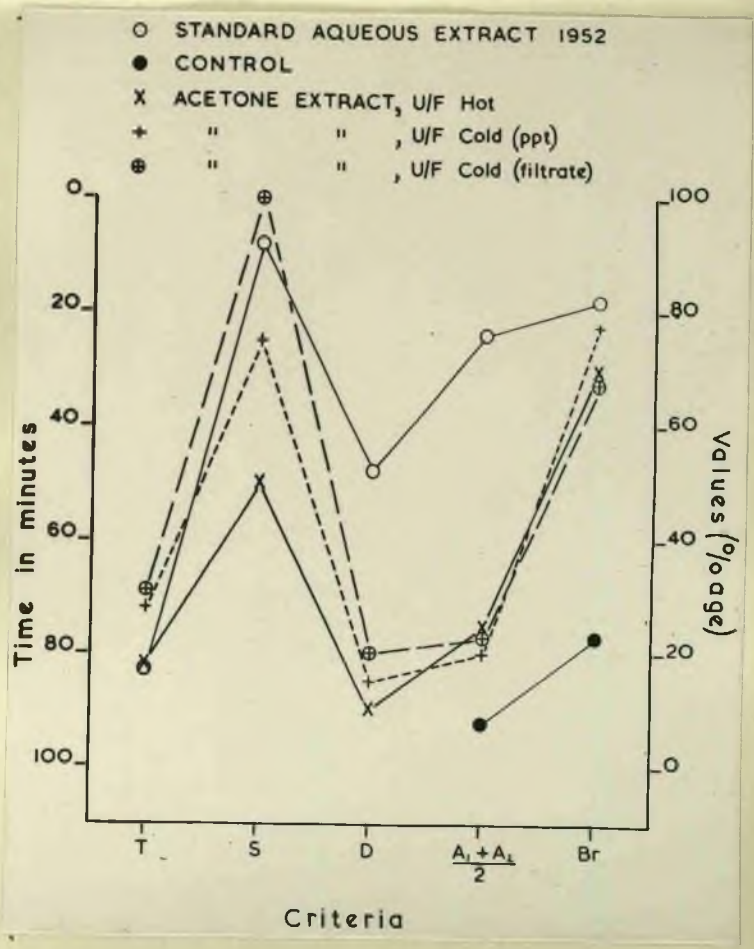


Fig. 20, Solubility V - in acetone. Assay of ultra-filtered acetone extracts, (a) filtered hot, (b) filtered cold - together with (c) an assay of the precipitate which was filtered out in cold filtration.

that a factor inducing sperm activity was absent or inhibited. This was not entirely borne out by the results with the ultra-filtered material. Again criteria A_1 and A_2 were relatively low but the results were not so significant. The whole degree of reaction with the ultra-filtered extract was less than with the paper filtered extract, which suggests that the active material is only partially soluble in acetone. This view is confirmed by the reactions given by the precipitate remaining in the asbestos film after cold filtration which were similar to those given by the filtrate. Further, the hot filtrate gave no better reaction than the cold filtrate.

The results suggest that the active material can be partially extracted by acetone.

15. Review of the physical and chemical properties of the active material.

The following properties have been established:-

(1) The material is almost insoluble in water. The solubility is increased slightly in hot aqueous solution.

(2) It is heat stable in aqueous suspension and is unaffected by evaporation to dryness.

(3) It is soluble in the fat solvents, alcohol and ether, but only partially soluble in acetone.

The active material is therefore a lipide.

Active extracts all tend to induce a "pattern" of reaction similar to that given by standard aqueous extracts. There are

two variations from this general rule.

(A) Heat treated ultra-filtered aqueous extracts and acetone ultra-filtered extracts induce spawning in approximately the standard time but only a trace of sperm is spawned and this is inactive (figs. 13 & 20).

(B) Ether ultra-filtered and acetone paper filtered extracts also stimulate spawning in the standard time. In this case the reaction is maintained and a good (D = 3 approx.) amount of sperm is spawned. Sperm activity is again low or absent (figs. 18 & 19).

These observations may give a clue to the nature of the active material:-

(a) the active material may be composed of more than one substance, these substances varying in their properties, e.g. the substance stimulating sperm activity may be less soluble in hot water, ether and acetone, than the substance initiating the spawning reaction. Or,

(b) the active material may be a single substance, weakly soluble in hot water and acetone. Thus although dilute in extracts of this type, it may be capable of initiating a spawning reaction but neither capable of maintaining it or promoting sperm activity. However, the second type of variation (B - above) is less readily explained on this basis. If the active material is extracted in sufficient quantity to maintain the spawning reaction, there seems no reason why the sperm should be inactive. It may

be, however, that sperm activity is more readily inhibited than other reactions, for instance in the presence of trace amounts of solvent.

16. Further fractionation of the lipide.

To solve the above problem and identify the type or types of lipide involved further fractions of the crude material were investigated. This was interrupted in 1952 by the discovery that extracts made at the start of the breeding season were inactive. Comparative tests were carried out using new extracts and old extracts of known activity. It was found that whereas the old extracts gave a reaction similar to that recorded earlier in the season, the new types continued to give a poor result. It was apparent that this was due to the extracts and not to a lack of response by the worms. Further fractionation was carried out by re-extraction from earlier extracts of known activity, or postponed until subsequent seasons.

17. Separation of simple and derived lipides from complex lipides.

Extraction method: Cold acetone was added to an ether extract. This precipitates the phospholipides. The simple lipides, sterols and steroids remain in solution. A second group of complex lipides, the glycolipides, are not extracted by ether.

Ax5e/2 (in saline)

Extracted with ether.
Solvent separated and evaporated.

Solvent residue.

Added 1 volume of ether and 5 volumes acetone.

Centrifuged.

Precipitate	Mother liquor.
Solvent evaporated.	Solvent evaporated.
Residue = Ax5e/2a (phospholipide fraction)	Residue = Ax5e/2b (simple Lipides, sterols and steroids).

Assay of Ax5e/2a and Ax5e/2b:

Experiment 17: 4 Worms received an injection of Ax5e/2a.

None of the worms spawned. 3 Hours were allowed to elapse between injection and examination of the coelomic sperm. Both the activity and breakdown of this sperm were poor (table 41).

Experiment 17: 4 Worms were injected with Ax5e/2b.

Criteria S, T and D: 3 Worms spawned, the average time was 15 mins. less than standard. The reaction assessed by criterion D was almost as high as the standard reaction (fig. 21).

Criteria A_1 and A_2: The spawned sperm showed a high order of activity immediately spawning commenced. Coelomic sperm activity was poor. As a result the average activity was slightly low relative to the standard reaction. This was typical of the ether ultra-filtered extract from which this extract was derived (fig.18).

Criterion Br: Breakdown of the coelomic sperm was extremely poor.

Table 41

Assay of Ax5e/2a and Ax5e/2b.

Experiment No.	Extract No.	Date	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
				% age spawning	T	A_1	D	Br	A_2	
17	Ax5e/2a	14.10	4	0	-	-	-	0.87	0.25	5.6%
17	Ax5e/2b	14.10	4	75	65	3.37	2.00	1.25	1.50	40.6%
	Control		2	0	-	-	-	1.00	1.00	10.0%

Summary:

Despite the poor breakdown of the coelomic sperm there is no doubt that the spawning stimulant is located in the simple and derived lipide fraction (Ax5e/2b).

18. The Salkowski and the Lisbermann and Burchard reactions.

An ether extract, Ax5e/2, was tested and gave a positive reaction for the presence of sterols with both tests. This does not necessarily indicate that the active material is a sterol but their presence in emulsions of Arenicola was confirmed.

19. Action of Cholesterol.

Cholesterol was injected to test the effect of a simple sterol on male Arenicola. The equivalent of 1% of the body weight, made up in 0.6M saline, was administered to each worm.

Experiment 17: 4 Worms received an injection of cholesterol.

Assay:

Two worms spawned, one soon after injection (9 mins.). The total volume of sperm spawned was in each case negligible and its activity was almost nil. There was little breakdown of the coelomic sperm and this too showed very poor activity (table 42).

Discussion:

Again these results, in themselves, are no certain indication that the active material is a sterol. Taken together with the qualitative sterol tests, they suggest that of the remaining lipide groups, the active material is most probably a sterol.

Table 42

Assay of Cholesterol.

Experiment No.	Extract No.	Date	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
				% age spawning	T	A_1	D	Br	A_2	
17	Cholest.	14.10	4	50	52	0.25	0.25	2.00	1.00	17.5%
	Control		2	0	-	-	-	1.00	1.00	10.0%

20. Experiments during 1953, 1954 at Dublin.

Further fractions were tested at Dublin during 1953 and 1954. Experimental conditions were largely the same as at St. Andrews. Technical difficulties, involving the transport of material and water to the laboratory, resulted in:-

(a) a delay of up to 3 hours between digging and injecting the worms, and

(b) a rise in the water temperature of the order of 2 - 3°C.

The latter depended on the difference between sea and air temperatures on the day of the experiment. Water temperatures during experiments varied between 14 and 16°C.

The magnitude of the reactions with standard aqueous extracts was slightly less than at St. Andrews. This was probably due to the adverse conditions described above. Nevertheless, the assays follow an identical "pattern" to those at St. Andrews (table 43, fig.22)

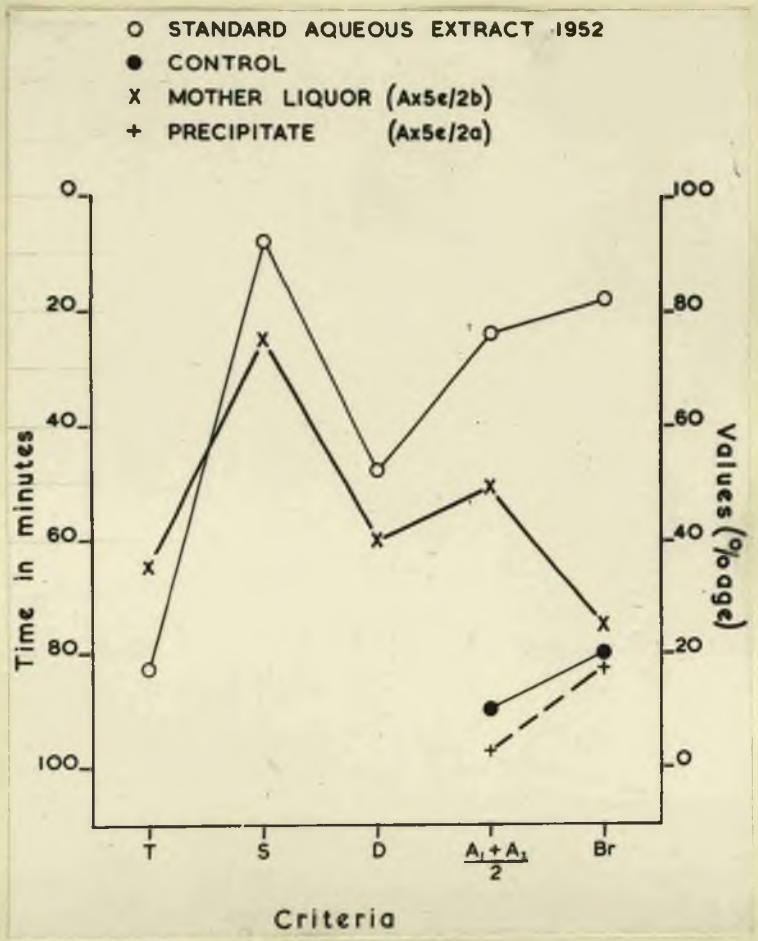


Fig. 21, Assay of the simple lipide and sterol fraction (mother liquor) and the phospholipide fraction (precipitate) after the separation of these fractions by precipitation by cold acetone from and ether solution.

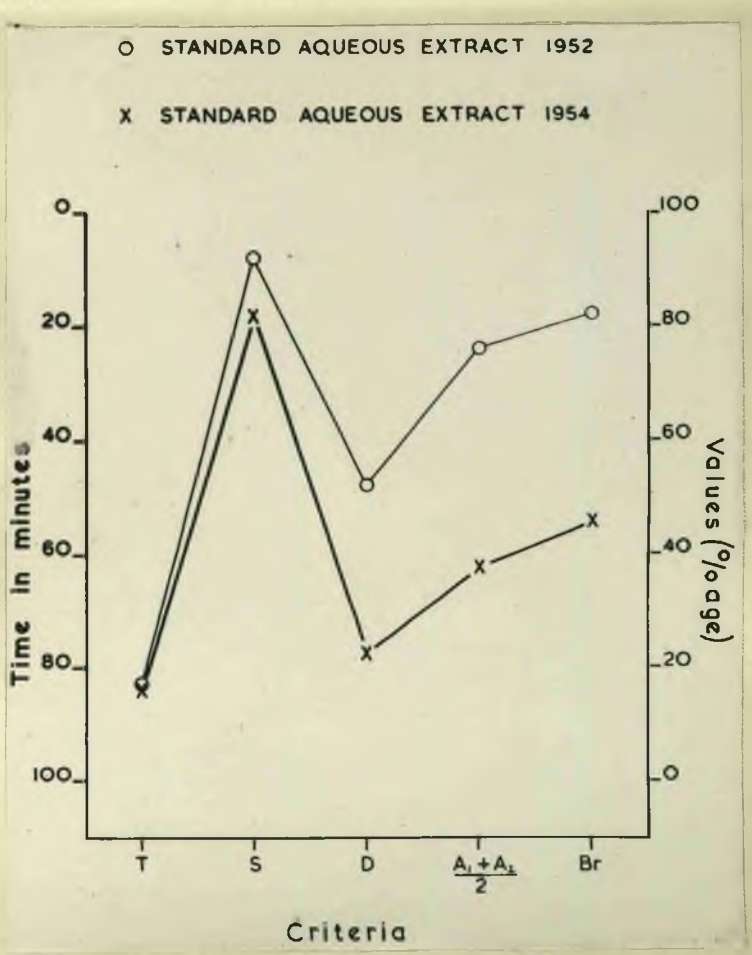
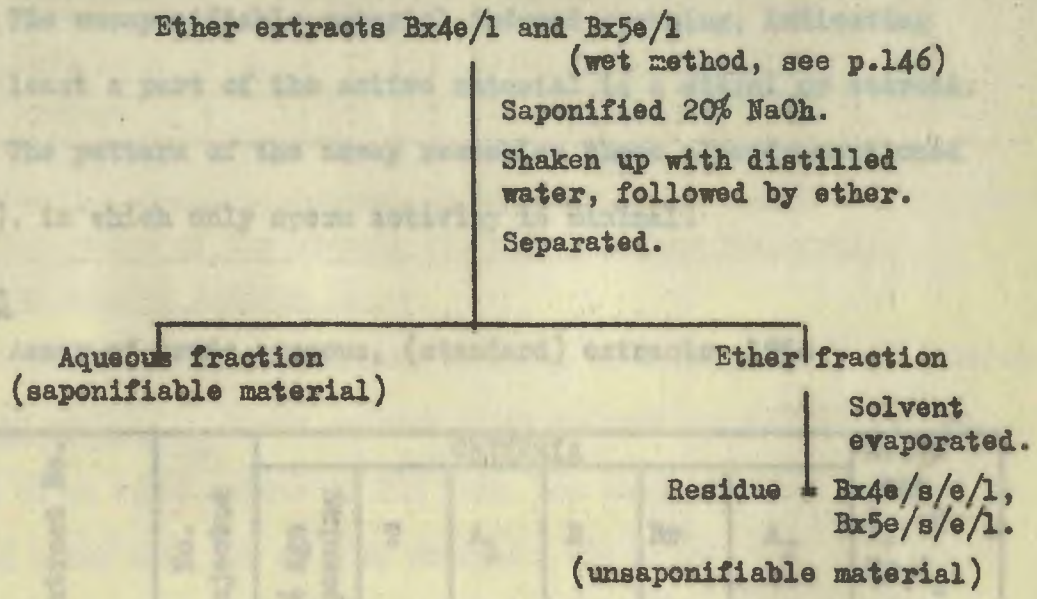


Fig. 22, Comparison of the assays of standard (paper filtered) aqueous extracts prepared in 1952 and 1954.

21. Separation of the sterols and steroids from the simple and complex lipides. I.

Extraction method: The sterol/steroid fraction was separated by saponification of an ether extract. An excess of 20% NaOH was added to the dry residue and this was immersed in a boiling water bath for 45 mins. Thereafter the saponifiable fraction was washed out with distilled water and the unsaponifiable material extracted with ether. A constant shaker was used to obtain more efficient mixing of solvent and emulsion.



N.B. Bx = 1953 extract, s = unsaponifiable material.

Experiments with the unsaponifiable material I.

5.11.53: 3 Worms received an injection of Bx4e/s/e/1.

12.11.53: 6 Worms " " " " Bx5e/s/e/1.

The results of the above assays are compared with those with normal ether extracts which were injected as follows:-

28.10.53: 4 Worms received an injection of Bx4e/1.

12.11.53: 4 " " " " " Bx5e/1.

Assay of unsaponifiable and normal ether extracts.

Spawning took place relatively rapidly after injection of both types of extract but particularly with the unsaponifiable material (table 44, fig. 23). The volumes of sperm spawned were practically identical. However, with the unsaponifiable material the activity of both the spawned and coelomic sperm was minimal (fig. 23) while the reaction assessed by criterion Br was relatively high.

Summary:

1. The unsaponifiable material induced spawning, indicating that at least a part of the active material is a sterol or steroid.

2. The pattern of the assay resembles those already mentioned (p. 158), in which only sperm activity is minimal.

Table 43

Assay of crude aqueous, (standard) extracts, 1954.

Experiment No.	Extract No.	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
			% age spawning	T	A_1	D	Br	A_2	
8/54	Cx4a/1	2	100	165	3.50	1.00	2.00	1.75	41%
8/54	Cx6a/1	2	0	-	-	-	3.25	3.25	33%
8/54	Cx7a/1	2	100	42	3.50	2.25	2.25	1.25	46%
8/54	Cx8a/1	2	100	62	2.25	-	1.87	0.50	23%
9/54	Cx7a/1	3	100	68	2.00	2.30	2.00	0.67	35%
	Average		82	84	2.25	1.11	2.27	1.48	36%

Table 44

Assay of the unsaponifiable material, I, with comparable normal ether extracts as a control.

Date	Extract No.	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
			% age spawning	T	A_1	D	Br	A_2	
5.11	Bx4e/s/e/1	3	100	21	1.50	1.33	1.66	0.33	24%
12.11	Bx5e/s/e/1	6	100	33	0.33	1.50	3.42	0.00	26%
	Average		100	27	0.72	1.44	2.77	0.11	25%
28.10	Bx4e/1	4	100	61	2.50	1.75	1.50	0.50	31%
12.11	Bx5e/1	4	75	53	2.00	1.50	1.75	1.75	35%
	Average		87	59	2.30	1.60	1.63	1.13	33%

22. Separation of the sterols and steroids from the simple and complex lipides II.

Experiments were continued in 1954, to verify the above results.

Extraction method: Crude aqueous extracts were saponified by the addition of an equal volume of 20% NaOH, the mixture being immersed in a boiling water bath for 45 - 60 minutes.

Crude aqueous extracts
Cx3b/1 and Cx7a/1.

Saponified boiling water, 45 minutes.

Saponified, boiling water, 60 minutes.

Unsaponifiable material

Extracted with ether 100 ml. X 4, on shaker.

Solvent separated.

Filtered No. 1 Whatman and distilled.

Residue = Cx3b/s/e/1, Cx7s/e/1.

Experiments with unsaponifiable material II.

Experiment 9/54: 3 Worms received an injection of Cx3b/s/e/l.

Experiment 10/54: 4 " " " " " Cx3b/s/e/l.

Experiment 10/54: 3 " " " " " Cx7s/e/l.

Experiment 11/54: 6 " " " " " Cx7s/e/l.

In these experiments 6 worms were injected with saline as controls.

Assay:

All the worms spawned. In other respects the Cx3 emulsion was more active than the Cx7 (table 45). A high degree of activity was recorded for the spawned sperm. The average value of the activity criteria was only slightly low in relation to the pattern of the standard (fig. 24).

Controls: Of the 6 controls, 3 spawned. In 2 of these the reaction was maintained (D = 2.0 and 2.5), however the activity of the spawned and coelomic sperm was nil and the breakdown of the latter was also low (table 45). In all, during the 1954 season, 17 controls were injected and only the 3 mentioned above spawned.

Discussion:

In this case although the activity criteria were relatively low, the drop was not significant. No fraction of the active material was therefore saponified. It is possible, however, that the method of saponification was inefficient as,

(a) the 20% NaOH was diluted by an equal volume of crude extract and

(b) under these conditions the period of immersion in boiling water was relatively short.

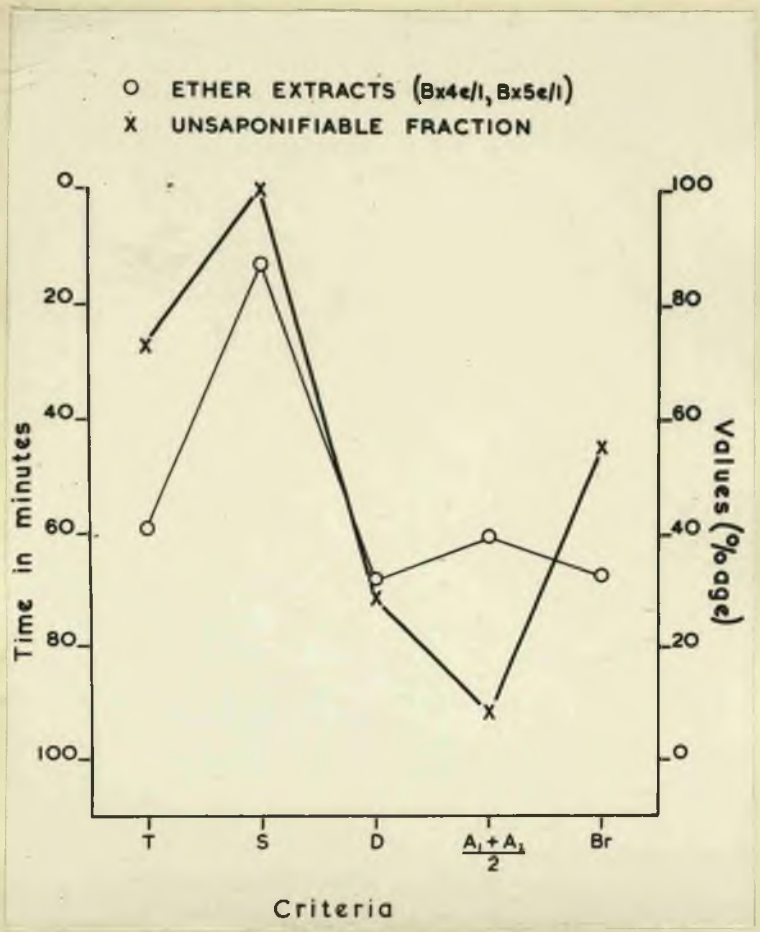


Fig. 23, Assay of the unsaponifiable fraction I. Also shown is an assay of the ether extracts from which this unsaponifiable material was derived.

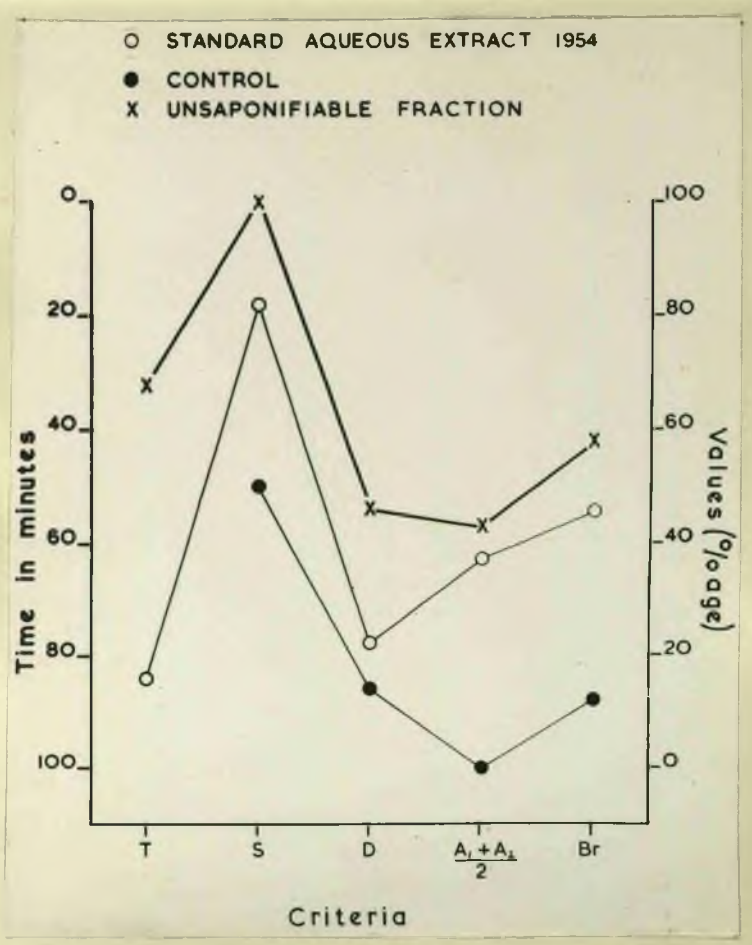


Fig. 24, Assay of the unsaponifiable fraction II.

Table 45

Assay of unsaponifiable material II.

Experiment No.	Extract No.	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
			% age spawning	T	A_1	D	Br	A_2	
9/54	Cx3b/s/e/1	3	100	39	4.70	3.70	3.50	3.00	74%
10/54	Cx3b/s/e/1	4	100	29	5.00	3.50	1.75	0.75	55%
10/54	Cx7s/e/1	3	100	34	0.83	1.00	3.83	0.33	30%
11/54	Cx7s/e/1	6	100	27	2.25	1.10	2.66	0.33	32%
	Average		100	32	3.2	2.3	2.9	1.1	47.5%
	Controls	6	50	16	0	0.7	0.6	0.2	7.5%

23. Separation of the sterols and steroids from the simple and complex lipides III.

A check extraction was carried out in which two ether extracts were combined and saponified using an excess of hot saturated alcoholic KOH over a period of 3 hours. The unsaponifiable material was extracted by ether in the usual way (Cx4.6e/s/e/1).

Experiment 12/54: 4 Worms received an injection of Cx4.6e/s/e/1.

For comparative purposes 4 worms were injected with the normal ether extract Cx4.6e/1.

Assay:

A striking reaction was obtained from the normal ether extract, particularly with regard to the activity criteria (table 46).

The magnitude of the reaction was less with the unsaponifiable material. The pattern of the reaction was, however, the same, except for sperm activity which dropped to a minimal amount (fig.25).

Conclusions:

This final result confirms the first attempt at saponification (p. 165 cf. figs. 23 & 25). It suggests that a factor inducing sperm activity is saponifiable, while that inducing the spawning reaction is unsaponifiable. A further tentative conclusion can be drawn from the above series of experiments, i.e. that the activity inducing substance is not readily saponified and may therefore have a high saponification number.

Table 46

Assay of normal and unsaponifiable ether extracts III.

Experiment No.	Extract No.	Date	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
				% age spawning	T	A_1	D	Br	A_2	
12/54	Cx4.6s/s/e/1	15.11	4	75	90	0.80	1.25	2.50	0.50	25%
"	Cx4.6s/1	15.11	4	100	55	5.00	2.00	3.10	1.60	59%
	Control	15.11		0	-	-	-	2.0	0	10%

24. Recovery of the activity inducing factor.

If the conclusions drawn above are correct, the saponifiable material should be rich in the activity inducing factor.

Extraction method: Re-extraction was carried out from the emulsions yielding -

(a) Cx6s/e/l: this was the unsaponifiable material after saponification of an aqueous extract by 40% NaOH for 3½ hours in a boiling water bath.

(b) Cx4.6e/s/e/l: extraction described above (p.168).

After extraction of the unsaponifiable material, the solution was acidified by adding conc. HCl till the pH was less than 3.6. The reconstituted fats were extracted by ether in the usual way. A light fat, dark brown in colour remained. These extracts were labelled Cx6Sa/e/l and Cx4.6e/Sa/e/l. It was almost impossible to suspend this material evenly in saline.

N.B. Sa = Saponifiable fraction.

Experiment 12/54: 2 Worms received an injection of Cx4.6e/Sa/e/l, and 3 worms received an injection of Cx6Sa/e/l.

Assay of the saponifiable extracts:

Results with Cx4.6e/Sa/e/l, were extremely poor. With Cx6Sa/e/l all 3 worms spawned; in 2 cases only a trace of sperm was exuded. However, this sperm was entirely free and intensely active (table 47). Coelomic sperm was less active but showed a high degree of breakdown.

Discussion:

The virtual absence of effect with Cx4.6e/Sa/e/1 was probably due to the dilution caused by repeated re-extraction from the same emulsion. Nevertheless, comparison of the average results given by the saponifiable fraction with those given by the unsaponifiable fraction (table 47, fig. 26) suggests that the saponifiable fraction is relatively rich in the activity inducing substance and weak in spawning stimulant, i.e. criteria A_1/A_2 are relatively high and criterion D relatively low when the saponifiable fraction is injected while the opposite is the case when the unsaponifiable fraction is injected.

Strictly speaking, if the saponifiable fraction contains only the activity factor, no worm should have spawned when this extract was injected, the spawning stimulant having already been extracted in the unsaponifiable fraction. It must be assumed that the unsaponifiable fraction was not exhaustively extracted.

Table 47

Assay of the saponifiable fraction.

Experiment No.	Extract No.	No. injected	CRITERIA						Av. % age A_1, D, Br, A_2.
			% age spawning	T	A_1	D	Br	A_2	
12/54	Cx4.6/Sa/e/1	2	50	48	0.50	0	0.25	0.25	5%
12/54	Cx6Sa/e/1	3	100	74	3.66	0.50	3.00	1.80	45%
	Average		80	66	2.4	0.3	1.9	1.2	29%
	Unsaponifiable *	13	92	43	0.73	1.23	2.73	0.23	25%

* Figures combined from tables 44 and 46.

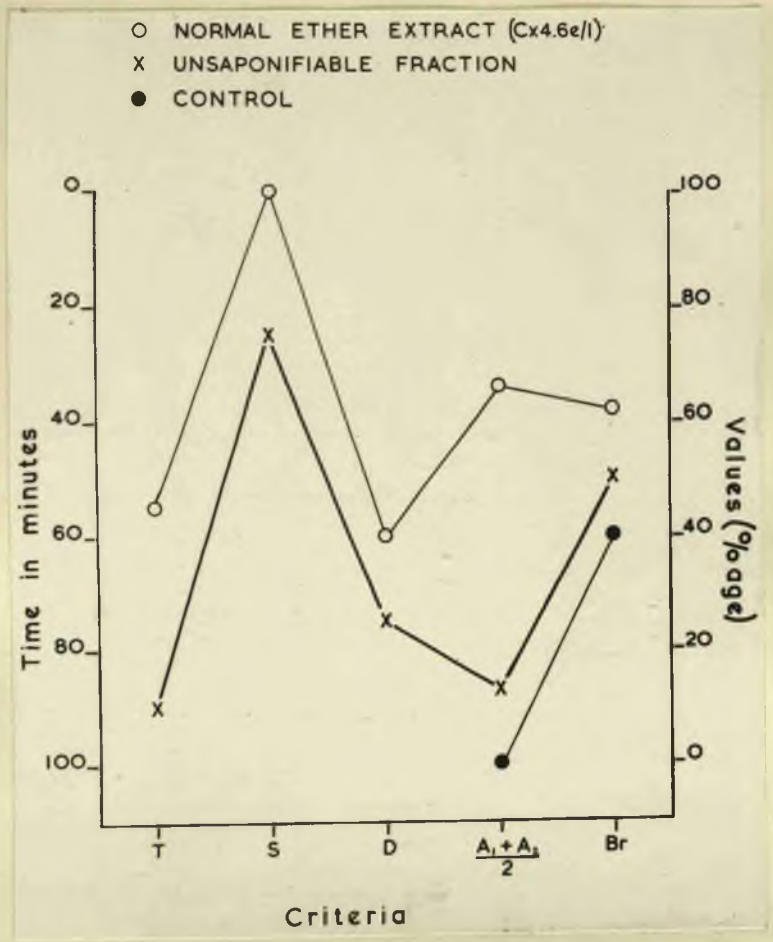


Fig. 25, Assay of the unsaponifiable fraction III. Also shown is an assay of the ether extract from which this unsaponifiable material was derived.

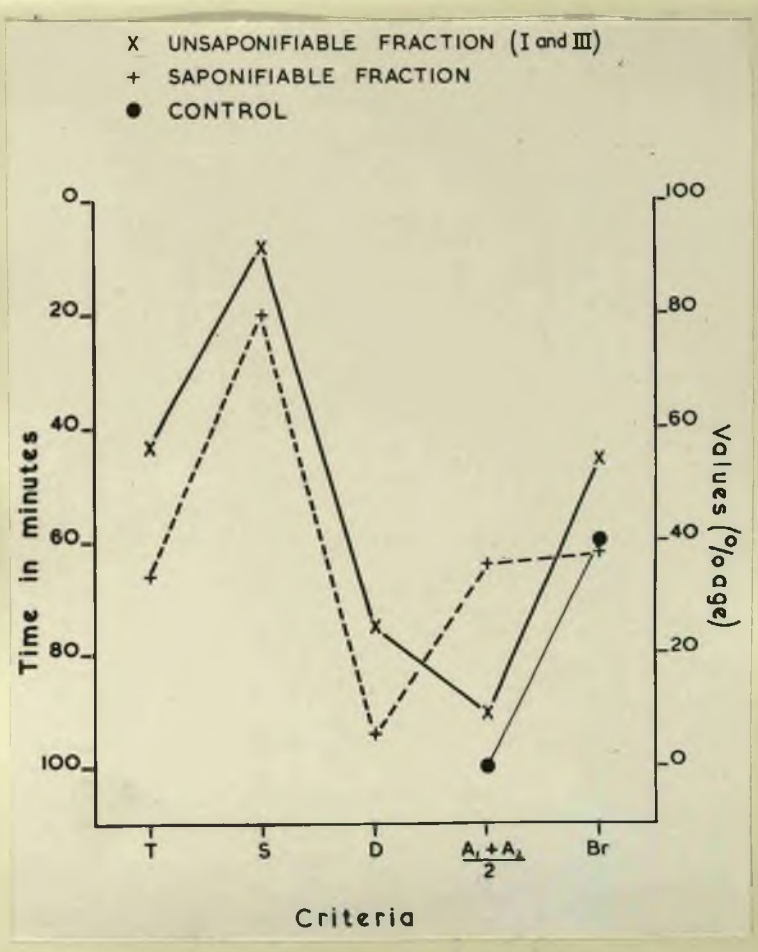


Fig. 26, Assay of the saponifiable fraction compared with the assay of the unsaponifiable fraction (average of I and III, figs. 23 and 25).

25. Summary of the physical and chemical properties of the active material.

The active material is a lipide. It has been shown that it is not a complex lipide. The spawning reaction is stimulated and a quantity of sperm exuded when the unsaponifiable fraction of the lipide is injected. The spawning stimulant is therefore a sterol or steroid.

It is possible to speculate that there are at least two substances in the active material, a spawning factor, already mentioned, and an activity inducing or ripening factor The latter is a saponifiable lipide which may have a high saponification number and is relatively insoluble in ether and acetone. The weight of evidence is against the possibility that sperm activity is merely less readily stimulated, or more easily inhibited, than the spawning reaction (see p. 158).

(d) Experiments with tissue extracts III

The following biological properties of the active substances were examined:-

1. The effect of dilution.
2. The reaction to separate extracts of male, female and 'empty' worms.
3. The anatomical distribution of the active material.

Experimental and assay techniques were the same as those previously described (pp. 120-122).

1. The effect of dilution:

Method:- A crude aqueous extract, Ax3a/1, was diluted with 0.6M saline, in the following proportions -

(a) Normal extract.
(b) 1 part extract : 1 part saline.
(c) " " " : 5 parts saline.
(d) " " " : 10 " "
(e) " " " :500 " "

6 Worms were injected with each dilution, 0.5ml being injected in each case.

Assay:

With increasing dilution there was a fairly rapid decline in the reactions assessed by all the criteria. The reaction was halved at a dilution of 1 part extract to 5 parts saline, (table 48). Minor irregularities occurred (fig. 27), e.g. in the dilution 1 : 5

where criterion D was relatively low. However, it is not suggested that this criterion drops relatively rapidly with dilution because at 1 : 10 although the sperm spawned was insufficient to form a pool (D = 0), there was enough to record its activity.

Discussion:

If the average value for all the criteria (expressed as a percentage) is plotted against dilution (fig. 28), the graph indicates a direct quantitative relationship between the degree of reaction and the concentration of extract injected. Trace quantities of active material produce no result. The results have a bearing on the problem of whether the active material is composed of one, or more than one substance (p.158). There is no indication that in dilute solution the active material might stimulate spawning, yet at the same time fail to maintain the reaction, and/or, fail to induce sperm activity proportionally.

Table 48

Assay of a crude aqueous extract (Ax3a/1), in various dilutions - Experiment 12, 1.10.52

Dilution Ext. : Sal.	No. injected	CRITERIA						Av. % age reaction	
		% age spawning	T	A_1	D	Br	A_2	ex. spg.	plus spg.
1 : 0	6	83	69	3.5	2.7	4.5	4.0	73%	75%
1 : 1	6	83	59	3.3	2.2	3.4	2.8	59%	64%
1 : 5	6	67	81	2.5	0.5	1.4	1.5	30%	37%
1 : 10	6	33	110	1.3	0	1.0	0.3	13%	17%
1 : 500	6	-	-	-	-	0.2	0.2	2%	1%
Control	4	0	-	-	-	0.5	0.2	3.5%	

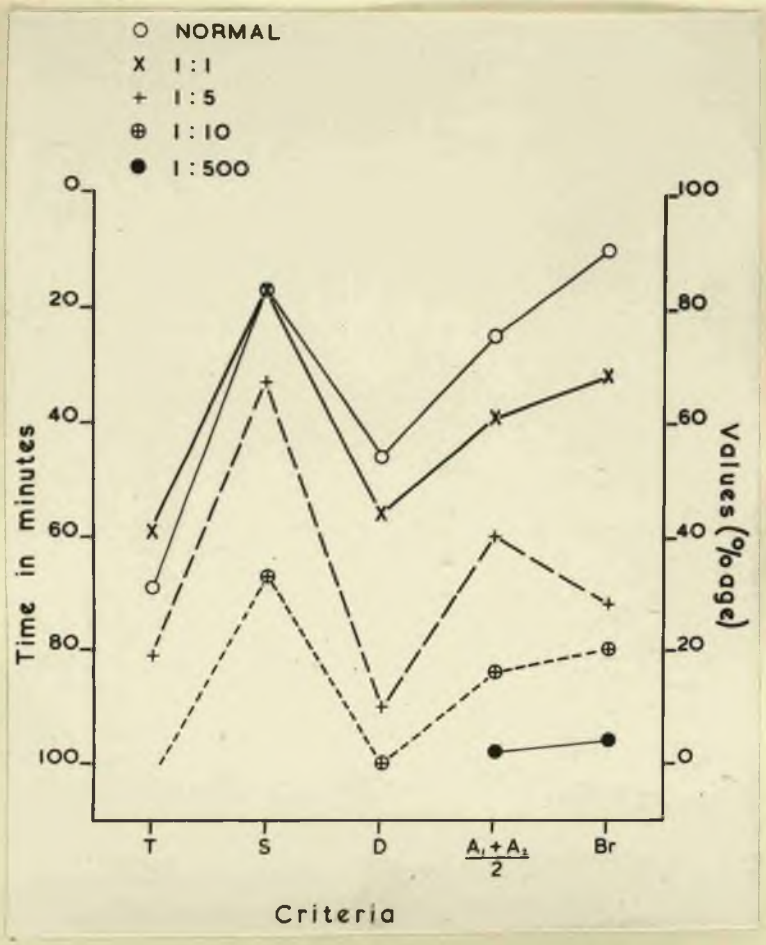


Fig. 27, The effect of dilution I. Assays of a paper filtered aqueous extract after dilution with various proportions of 0.6M saline.

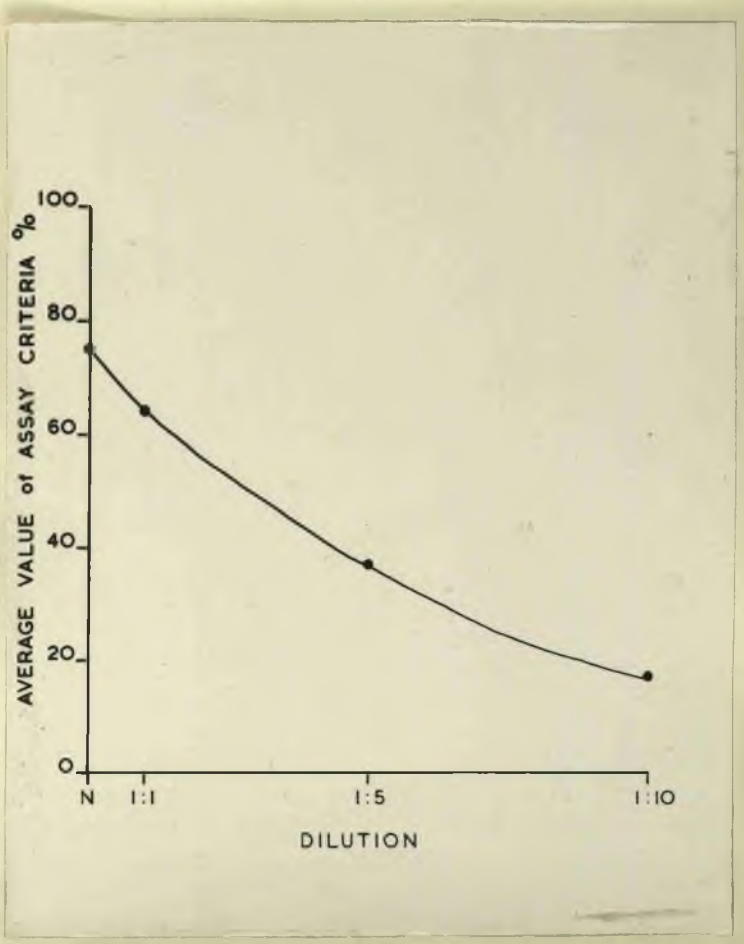


Fig. 28, The effect of dilution II. Points obtained by plotting the average value of all the criteria in each assay (shown in fig. 27) against the degree of dilution.

2. **The reaction to separate extracts of male, female and 'empty' worms:**

Extraction method: 2 Crude extracts were prepared, Ax2a/1 from females and Ax4a/1 from males. An ultra-filtered ether extract, Ax12e/2, was prepared from 'empty' worms, i.e. those which did not contain genital products during the Summer.

Assay:

Experiment 11: 6 Worms received an injection of Ax2a/1.
" " " " " " Ax4a/1.
Experiment 19: " " " " " " Ax12e/2.

The reactions given by Ax2a/1 and Ax4a/1 were exactly comparable with those given by the standard aqueous extract (table 49, fig. 29). The reaction to Ax12e/2 was very similar to the average reaction to normal ultra-filtered ether extracts, (table 49, fig. 30).

Table 49

Assay of aqueous extracts made from female (Ax2a/1) and male (Ax4a/1) worms and of an ether extract from 'empty' worms (Ax12e/2).

Experiment No.	Extract No.	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
			% age spawning	T	A_1	D	Br	A_2	
11	Ax2a/1	6	100	78	3.7	2.7	4.9	3.3	73%
11	Ax4a/1	6	83	72	3.3	2.5	3.8	3.3	65%
	Standard		92	83	3.9	2.6	4.1	3.7	72%
19	Ax12e/2	6	100	56	2.7	2.3	3.2	0.8	45%
	Av- e/2		91	76	2.5	2.5	4.1	1.6	53%
	Controls	6	33	(48)	0.7	0.2	1.6	1.0	17.5%

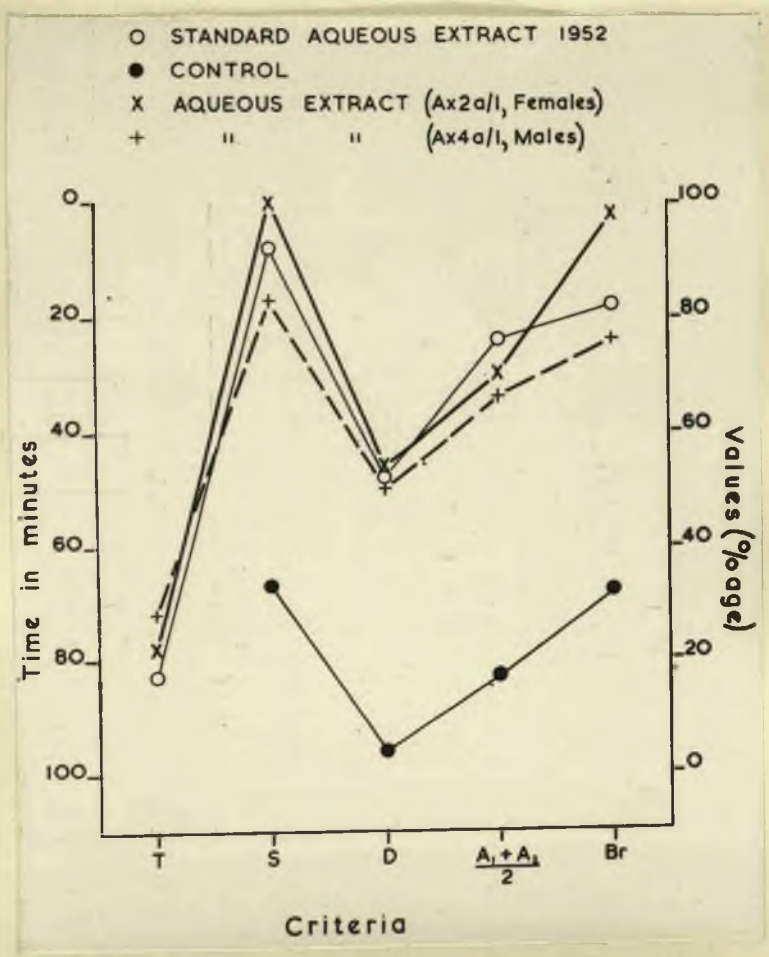


Fig. 29, Assays of aqueous extracts made from female worms only and from male worms only. These are compared to the assay of standard aqueous extracts.

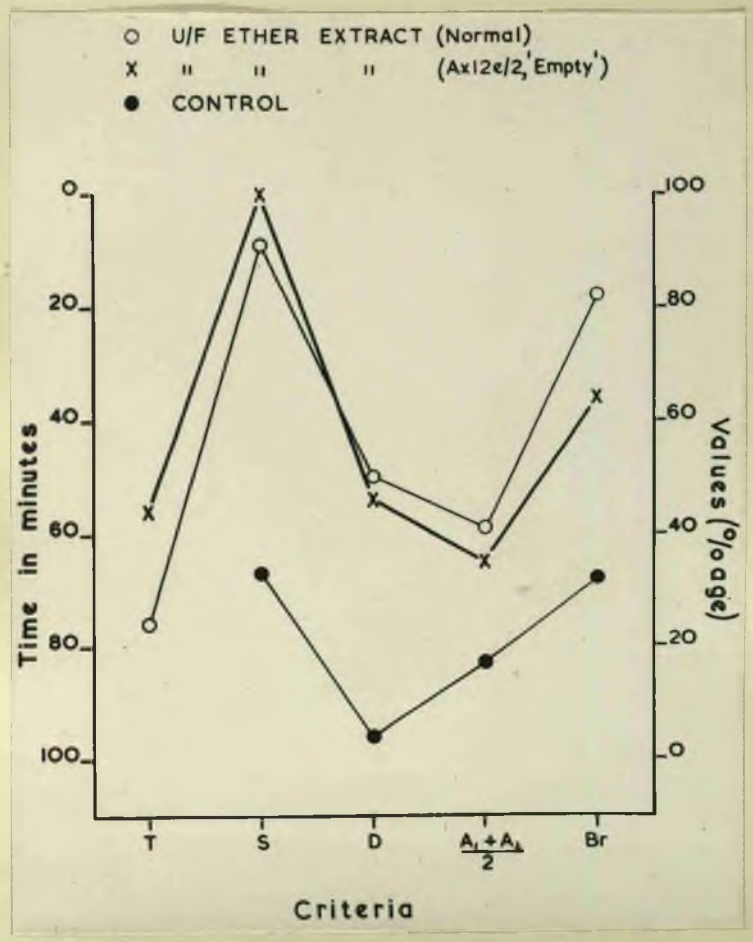


Fig. 30, Assay of an ultra-filtered ether extract made from 'empty' worms only. This assay is compared to the values given by a normal ultra-filtered ether extract.

Summary:

Extracts of male, female and empty worms prove equally potent in inducing spawning in male Arenicola.

3. The anatomical distribution of the active material:

Extraction methods: 21 Fresh worms were dissected. Ultra-filtered ether extracts were prepared as follows:-

Ax7e/2: The nephridia were dissected *, ground down in a mortar and extracted by ether for 3 days. The solvent was then ultra-filtered and evaporated in the usual way. The total fat yield was very small.

Ax8e/2: An extract of the gut (plus accessary glands), and

Ax10e/2: an extract of the body wall were prepared in the way outlined above for Ax7e/2.

Ax9e/2: The coelomic fluid (plus genital products) was extracted by ether after the material in suspension had been broken up by alternate freezing and thawing.

The residues were made up in equal volumes of 0.6M saline without regard to the variation in yield. Should the active material be concentrated in any one region, a full reaction should be obtained, irrespective of the total fat yield from that region.

Assay:

Experiment 16: Groups of 6 worms received an injection of each of the extracts noted above.

* The gonads were left attached to the nephridia.

The extracts induced spawning to a varying degree (table 50). Two of the six worms injected with extract of nephridia, Ax7e/2, spawned, but only a trace of sperm was exuded. Results with the body wall fraction were equally poor (Ax10e/2). In this case, the one worm which spawned, exuded a good (D = 3) volume of sperm. The gut and coelomic fluid fractions, Ax8e/2 and Ax9e/2, each stimulated spawning in 5 worms. Of the two, the gut fraction gave the poorer result. This was largely due to inactivity of both the spawned and coelomic sperm (fig. 31). The result given by the coelomic fluid fraction, approximated to that normally obtained with ultra-filtered ether extracts (table 50).

Table 50

Assay of extracts made from various body parts: Nephridia (Ax7e/2); Gut (Ax8e/2); Coelomic fluid (Ax9e/2) and Body wall (Ax10e/2).

Extract No.	No. injected	CRITERIA						Av. % age. A_1, D, Br, A_2.
		% age spawning	T	A_1	D	Br	A_2	
Ax7e/2	6	33	134	1.0	0	1.9	0.8	19%
Ax8e/2	6	83	58	1.0	1.8	2.7	0.3	29%
Ax9e/2	6	83	73	3.0	2.0	2.6	1.7	46%
Ax10e/2	6	17	36*	0.5	0.5	1.5	1.0	18%
Controls		0	-	-	-	1.5	1.0	12.5%

* Only one worm.

Discussion:

Certain immediate conclusions can be drawn from the results.

(a) The active material is not restricted to any one region of the worms but is present in varying degrees in all the regions assayed.

(b) The gut fraction gives the typical pattern of an extract weak in the activity inducing factor (fig. 31).

Although fat yields and dilutions of the injections were not equalised, tentative suggestions can be put forward with regard to the site where the active material is mainly secreted. Of the parts giving similar fat yields, the concentration is low in the body wall and high in the coelomic fluid and gut: either of the latter may be the site of secretion.

In view of the fact that extracts of 'empty' worms give results equivalent to those from gravid worms, it would appear unlikely that the genital products secrete the active substances. While the possibility has not been excluded that corpuscles in the coelomic fluid may produce the active substance, it seems more likely that the gut is the main site of secretion. If it is assumed that this is so, then the active material secreted by the gut may accumulate in the coelomic fluid or be taken up by the genital products. This would explain the presence of a high concentration of the active material in the coelomic fluid and genital products late in the season. The yield from the nephridia (and gonad) and its effect was so small that it is unlikely that this

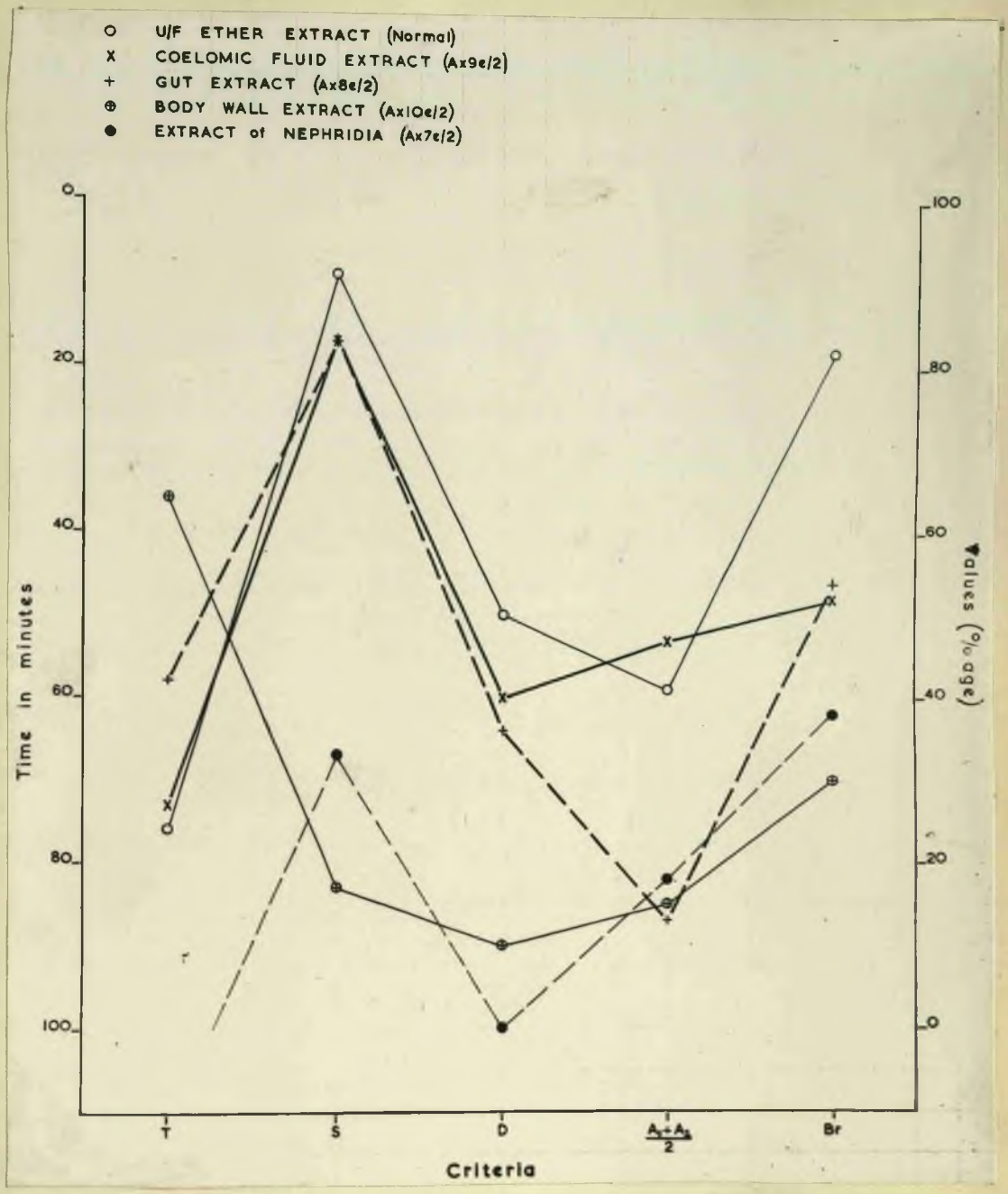


Fig. 31, Assays of ultra-filtered ether extracts made in each case from a single part of the worm. These assays are compared to the values given by normal ultra-filtered ether extracts.

region makes a significant contribution to the active material.

4. Summary of the biological properties:

(a) There is a direct quantitative relationship between the degree of reaction and the amount of active material injected. The material is ineffective in trace quantities.

(b) The active material is present in male, female and 'empty' worms equally.

(c) The active material is present in all parts of the worms but the main site of secretion is probably the gut or its accessary glands.

(e) Experiments with tissue extracts IV - injection of females.

1. Experimental method: The experimental technique was the same as when males were injected (pp. 114 & 120). Special care was taken to keep the dishes containing the worms free from sand as this might obscure spawned eggs. The extracts used had been assayed by injection of males. Controls (see p. 186) were treated with 0.6M saline, as before. The following observations were recorded:-

(a) the number of worms spawning;

(b) the time of spawning;

(c) results of fertilisations with spawned eggs, and/or,

(d) artificial fertilisations with coelomic eggs.

It has been shown in this work that fertilisations with spawned eggs can be brought about without difficulty (pp.48--57) but that with the exception of residual eggs from spent females coelomic eggs cannot be fertilised (pp. 60-86). Thus (c) and (d), above, are criteria for the normality of any spawning reaction induced by extracts and for any ripening effect the extracts may have on coelomic eggs. Except in a few cases artificial fertilisations were not attempted until there was a reasonable chance of success, i.e. during the breeding season.

2. Method for fertilisations:

Coelomic eggs were extracted from the body cavity by hypodermic syringe, generally between 5½ and 7 hours after injection. Thereafter, spawned and coelomic eggs were treated similarly. Small samples were washed by decanting and placed in either watch glasses or 100ml beakers. The sperm used was spawned in the laboratory, either as a result of injection, or naturally. As both sperm and eggs were spawned into laboratory circulation water, fertilisations were made in that medium. Within 17 hours the eggs were decanted and transferred to filtered outside sea water. Thereafter the water was changed daily. Eggs exhibiting advanced divisions were usually separated into fresh containers to avoid contamination by cytolising eggs. Cultures were kept at 15°C.

3. Trial Experiments:

The first of these has been described (p. 117), there was no reaction to injection. A second trial experiment was carried out on 18.9.52. 12 Worms were injected using Axla/1, again without result.

Third trial experiment - 24.9.52:

6 Worms received an injection of Axla/1, (aqueous).

6 " " " " " Axlc/1, (alcoholic).

Tail fragmentation occurred in two worms with the aqueous extract and in three with the alcohol extract.

Results: After 45 mins. small puffs of whitish fluid exuded from the nephridiopores of one of the worms injected with Axlc/1. This material consisted of concretions of cells from the coelomic fluid. In time all the worms injected with Axlc/1 exhibited this 'puffing reaction', which continued intermittently for up to 5 hrs. One worm treated with Axla/1 showed the reaction.

No eggs were shed. After 6 hours coelomic eggs were examined and they appeared to be unaffected by the extract. The average diameter was 174u, i.e. considerably smaller than eggs usually encountered during spawning.

Discussion: The puffing reaction from the nephridia was thought at first to be due to active excretion of nitrogenous waste (a description of excretion in Arenicola marina is given by Strunk, 1930). However, the fact that puffing took place over a prolonged

period might be interpreted as a sign that the spawning reaction had been stimulated. That no eggs were shed may be due to a selective effect of the nephridia, e.g. as in Amphitrite ornata where they reject all but ripe eggs (Scott, 1911).

4. Further experiments with aqueous extracts:

Experiment 10: 6 Worms received an injection of Ax3a/1. (25.9.52)

Experiment 20: " " " " " " " (16.10.52)

In neither case was a reaction of any description recorded.

(f) Injection of females II.

The main effort was delayed until just prior to the breeding season in order that the eggs might achieve the maximum size. Alcohol and ether extracts were used. The former as they had already promoted the puffing reaction and the latter because they had proved most effective with the males.

1. Experiments with alcohol extracts:

Extracts by both wet and dry methods were used (pp. 138-139).

Paper filtered extracts:-

Experiment 10: 6 Worms received an injection of Ax1c/1 (25.9.52)

Experiment 16: " " " " " " Ax5d/1 (9.10.52)

Experiment 25: " " " " " " Ax16d/1 (19.10.52)

Ultra-filtered extracts:-

Experiment 14: 8 Worms received an injection of Ax1c/2 (2.10.52)

Experiment 18: 2 " " " " " Ax5d/2 (14.10.52)

N.B. Spawning commenced on 16.10.52.

Results - paper filtered extracts:

The puffing reaction: This was shown by all 6 worms in experiment 10 and 3 in experiment 16. None of the worms in experiment 25 exhibited the reaction (table 51).

Spawning: In experiment 10, four worms shed a few eggs after 2 - 4¼ hours. In no case were sufficient eggs emitted to create a recognisable pool. No eggs were shed in the other experiments.

Fertilisations with spawned eggs: Sperm was added to 3 small samples of eggs from the worms in experiment 10. In two of these about 40% of the eggs assumed the appearance of those shed naturally i.e. they became spherical and the nuclear vesicle disappeared. There was no segmentation.

Artificial fertilisations: Sperm was added to samples of coelomic eggs from all the worms in experiment 25. In one, there was 13% segmentation, and in another, 14%. These eggs developed beyond the 32-cell stage. In the former the embryos died after about 40 hours due to a bacterial infection. In the latter they were still alive after 3½ days. No ciliated or hatching stages were observed and by 5½ days the embryos had died.

Results - ultra-filtered extracts:

10 Worms were injected, 1 showed the puffing reaction.

No other reaction was observed.

2. Experiments with ether extracts:

Again extracts by both wet and dry methods were employed.

Paper filtered extracts:-

Experiment 16: 6 Worms received an injection of Ax5e/1 (9.10.52).

Experiment 25: 4 " " " " " Ax3e/1 (19.10.52)

Ultra-filtered extracts:-

Experiment 18: 6 Worms received an injection of Ax13e/2 (14.10.52).

Experiment 20: 6 " " " " " Ax3e/2 (16.10.52).

Experiment 20: 6 " " " " " Ax13e/2 (16.10.52).

Experiment 23: 6 " " " " " Ax15e/2 (18.10.52).

12 " " " " " Ax15e/2* "

Experiment 25: 6 " " " " " Ax13e/2 (19.10.52).

* Volume of injection reduced to 0.1ml in 6 and to 0.2ml in 6. The fat content remained the same as in the normal 0.5ml injection.

Results with paper-filtered extracts:

Puffing reaction: 5 of the 6 worms in experiment 16, and 1 out of 4 in experiment 25 exhibited the puffing reaction.

Spawning: After 30 to 85 mins. a small number of eggs were shed by 4 worms in experiment 16 (table 52). There was no reaction in experiment 25.

Fertilisations with spawned eggs: No development was obtained.

Artificial fertilisations: These were attempted with samples from all the worms injected with this type of extract. In one case

Table 51

Reaction of females to the injection of paper filtered alcohol extracts and the results of fertilisations with spawned and coelomic eggs.

Extract	Date	Experiment No.	No. injected	P	S	T	Fert. sp. eggs		Fert. coelomic eggs		
							No.	Dev. in.	No.	Dev. in.	% age dev.
Ax1c/1	24.9	8	6	6	0	-	-	-	-	-	-
Ax1c/1	25.9	10	6	6	4	162	3	0	-	-	-
Ax5d/1	9.10	16	6	3	0	-	-	-	-	-	-
Ax16d/1	19.10	25	6	0	0	-	-	-	6	2	14% 13%

P = number showing the puffing reaction.
S = number spawning,
T = time after injection, (in minutes).

Table 52

Reaction of females to the injection of paper filtered ether extracts and results of fertilisations with spawned and coelomic eggs.

Extract	Date	Experiment No.	No. injected	P	S	T	Fert. sp eggs		Fert. coelomic eggs		
							No.	Dev. in.	No.	Dev. in	%age dev.
Ax5e/1	9.10	16	6	5	4	64	4	0	4	0	-
Ax3e/1	18.10	25	4	1	0	-	-	-	4	0	-

polar bodies were extruded but no further development occurred.

Results with ultra-filtered extracts:

Puffing reaction: 42 Worms were injected, 2 showed the puffing reaction, 1 each in experiments 18 and 20.

Spawning: Eggs were shed by 3 worms in experiment 18 (average time 107 mins.), 3 in experiment 20, and 2 in experiment 23 (table 53). Again the number of eggs shed was small.

Fertilisations with spawned eggs: Sperm was added to eggs from 5 of the spawners, mentioned above. Development occurred in one sample (table 53), 17% showed cell division. The embryos developed to ciliated stages but failed to hatch.

Artificial fertilisations: Fertilisations of coelomic eggs were attempted with samples from the 3 spawners in experiment 20 - without success. Of samples taken from all the worms in experiments 23 and 25, 20 were totally unsuccessful. In the remaining 4, two from each experiment, the percentage development was 7%; 23% and 18% (both in excess of 32 cells) and 14% (in excess of 16 cells). There was no hatch in the first or last of these samples, in the second, 3 larvae hatched and in the third, 8 larvae hatched. The maximum survival was to H + 11.

3. Controls:

During these experiments 23 worms were injected with saline, none of these spawned. During the spawning season, samples were taken from 7 of these worms. Artificial fertilisations were

attempted without success.

Table 53

Reaction of females to the injection of ultra-filtered ether extracts and results of fertilisations with spawned and coelomic eggs.

Extract No	Date	Experiment No.	No. injected	P	S	T	Fert. spawned eggs			Fert. coelomic eggs			
							No.	Dev. in.	%age dev.	No.	Dev. in.	%age dev.	No. hatch.
Ax13e/2	14.10	18	6	1	3	107	3	0	-	-	-	-	-
Ax3e/2	16.10	20	6	1	2	72	-	-	-	2	0	-	-
Ax13e/2	16.10	20	6	0	1	66	-	-	-	1	0	-	-
Ax15e/2	18.10	23	6	0	1	?	1	0	-	6	0	-	-
Ax15e/2 (.1ml)	18.10	23	6	0	1	92	1	1	17%	6	1	7%	-
Ax15e/2 (.2ml)	18.10	23	6	0	0	-	-	-	-	6	1	23%	3
Ax13e/2	19.10	25	6	0	0	-	-	-	-	6	2	18% 14%	8

4. The spawning reaction - summary and discussion:

84 Females were injected with alcohol or ether extracts and 16 spawned. In case case the number of eggs emitted was small.

The puffing reaction was exhibited by 24 worms. When this was first encountered, it was interpreted as a sign that the spawning mechanism had been stimulated (p.181). That this was the case was illustrated by the next experiment (experiment 10, 25.9.52), in which puffing was followed by spawning. The hope that more eggs would be

shed in later experiments as the eggs increased in size and ripeness, proved unfounded. In fact both the puffing and spawning reactions decreased in frequency with the onset of the breeding season. 22 of the 24 worms exhibiting the puffing reaction and 11 of the 16 spawners were injected prior to the breeding season.

An explanation was sought for these results. It was noted that either Ax16d/1, Ax13e/2 or Ax15e/2 were used for 36 of the 46 injections made during the breeding season. All of these were prepared at or about the breeding season. The assays on male worms show that Ax16d/1 was totally inactive (table 33, p. 142) while Ax13e/2 and Ax15e/2 gave an average reaction (assessed by criteria A_1, D, Br and A_2) of 44% as compared to 91% for Ax5e/2 and 61% for Ax19e/2. They were therefore relatively inactive. This was not evident at the time.

If this is the explanation for the decreasing results, extracts of known activity, prepared earlier in the season, should have induced better reactions. In fact, Ax3e/1, one of the most active extracts (table 36), and the corresponding Ax3e/2, stimulated spawning in only 2 worms out of 10. There is as yet no logical explanation for the failure of the spawning reaction during the breeding season.

5. <u>Fertilisation experiments - summary and discussion:</u>

<u>With spawned eggs:</u> Fertilisations with eggs spawned as a result of injection were successful on only one occasion, 17% of the eggs

developing. They survived to the ciliated stage.

With coelomic eggs: Fertilisations were attempted with 37 samples during the breeding season. Development took place in six. The percentage of dividing eggs varied between 7% and 23%. 11 Larvae hatched; these survived up to H + 11.

Little significance can be attached to the small number of fertilisations with spawned eggs. However, the fertilisations of coelomic eggs have undoubted significance, despite the paucity of result. The percentages of dividing eggs, given above, in 4 cases refer to eggs beyond the 32-cell stage and in 2 fertilisations a few larvae hatched. Other methods of bringing about fertilisations of coelomic eggs which have been mentioned in this work (pp. 66-86), the controls, and methods described by previous authors (Pirlot, 1933; Newell, 1948), were either totally unsuccessful or at most gave 8-cell stages. Thus, the extracts must have exercised some ripening effect on coelomic eggs. However, the results leave some doubt as to the effectiveness of the technique. In this connection certain points are worth noting. First, the samples were always small. A larger hatch would have been obtained in the two most successful fertilisations had an attempt been made to fertilise all the available eggs. Secondly, the fact that relatively inactive extracts were used may again be significant (p. 188). Finally, it is reasonable to suppose that the ripening of eggs might take longer than sperm activation. It is suggested that results could be improved by 'priming' the females

over a period of 2 or 3 days, with occasional small doses of extract. This would necessitate developing a method for keeping the worms in a healthy condition for this period of time. Reference has been made in this work (pp. 27 & 56) to difficulties associated with keeping Arenicola in the laboratory.

CONCLUSIONS.

1. The nature of the active material:

Tissue extracts of Arenicola marina contain a spawning stimulant whose physical, chemical and biological properties have been examined. The active material is probably a sterol, mainly derived from the wall of the gut. The effect on the spawning reaction brought about by various fractions of the crude material, show that an agent inducing sperm activity can be separated from the spawning stimulant. This second material may have a ripening effect on the genital products. This is borne out by the fact that coelomic sperm, normally inactive in sea water, gives fully active suspensions when the worm has been injected with extract, while similar treatment produces a considerable improvement in the results of fertilisations of coelomic eggs.

2. The role of the active material as compared with other organic spawning stimulants:

Several points must be borne in mind when attempting to make comparisons with previous work on this subject (see pp 105-113).

(a) Previous authors have stated that their experimental animals were physiologically ripe without defining their criteria of ripeness.

(b) The spawning mechanism could in each case be set in motion by straightforward muscular stimulation. Thus it was only necessary to apply a trigger impulse to the nervous system (e.g. Townsend,1939), or direct to the muscles of the gonad wall (e.g. Palmer, 1937), to

initiate the shedding reaction.

(c) The various stimuli, discussed above (pp. 105-110), play no essential part in the processes of ripening and spawning in the individuals stimulated e.g. in cases where spawning can be induced by genital products - some individuals in a population must first spawn without this stimulus.

Although the mode of action of the spawning stimulant in Arenicola was not investigated in the present work, certain deductions can be made from the evidence available. Spawning in Arenicola involves the acceptance of the genital products by the ciliated funnel of the nephridium which normally rejects them. Thus some change must occur either in the genital products or in the ciliary currents of the nephridial funnel. This suggests that a non specific muscle stimulant in the extracts could not, in itself, induce shedding. In any case, the chemical nature of the active agent is unrelated to any of the normal organic muscle stimulants.

It might be argued that the spawning stimulant in these extracts merely provides an artificial trigger to the shedding reaction. There is, however, considerable evidence that this is not so. The active agent will induce spawning up to 5 weeks prior to the normal breeding season when the worms are by no means 'physiologically ripe'. In addition, the reaction to extract is not an 'all or none' reaction but can be varied both in nature and extent by injecting various fractions of the crude material.

It is suggested, therefore, that when tissue extracts of Arenicola marina are injected, the active materials play their normal role in the physiological processes of ripening and spawning. The positive evidence with regard to the mode of action of these materials is included in a general hypothesis given below.

The role of the spawning stimulants described by previous authors obviously differs fundamentally from the role of the material in extracts of Arenicola. In consequence any attempt to link the properties and chemical composition of the spawning stimulant in Arenicola with the properties of spawning stimulants described by previous authors is unlikely to have any significance. With regard to methods, however, a general criticism can be made of this literature. The non-specificity of immediate or trigger spawning stimuli has been demonstrated on numerous occasions. Attempts to link natural stimulants with known substances capable of causing the same reaction, on the basis of a few ill-defined common properties, do not inspire confidence. It would appear that the only means of arriving at the true nature of these substances and their biological significance are the admittedly laborious fractionations and assays described here.

Bearing in mind the evidence already accumulated for the close linkage between final ripeness of the genital products and the shedding reaction (p. 81), it seems possible to put forward a working hypothesis for the control and order of events leading to spawning in Arenicola marina.

Hypothesis:- Closely linked substances, capable of influencing the ripening of the genital products and inducing spawning are secreted during the Summer. These are mainly derived from the wall of the gut or its accessary glands and are taken up by the coelomic fluid or the genital products. Their concentration gradually increases until a threshold value is attained at which they become effective. The spawning stimulant induces a slight state of tension in the body wall, placing the body contents under pressure. Meanwhile the genital products ripen under the influence of the ripening agent and this may result in a change of their shape, rigidity or specific gravity so that, aided by the pressure of the body wall, they are now taken up by the funnel of the nephridium and shed. The effect of injection is to increase the concentration of the active materials above the threshold value. Once the reaction commences the active material may be rapidly metabolised in the manner of vertebrate hormones. This would account for its absence in worms extracted during the breeding season.

The annual cycle of reproduction and restricted breeding season displayed by Arenicola, together with the lack of obvious environmental control, has already suggested that breeding may be controlled by some form of physiological rhythm (p. 23). It may be that this takes the form of a rhythm of secretion, as described above. This could, in turn, be controlled by seasonal changes in environmental conditions. Such a rhythm of secretion might have a wide applicability among invertebrates displaying sexual periodicity.

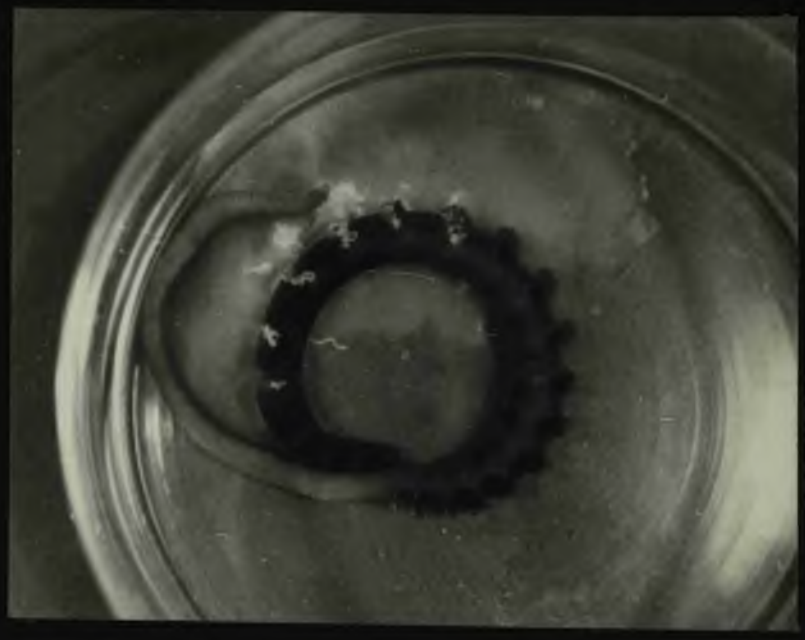
PLATES - SECTION III

Plate 6

A male spawning as a result of the injection of an extract made from whole Arenicola. Sperm is exuding from all but the first pair of nephridioporos.

Plate 7

Shadows on a photomicrograph produced by the 'clumping' of spermatozoa (agglutination) in an intensely active suspension.



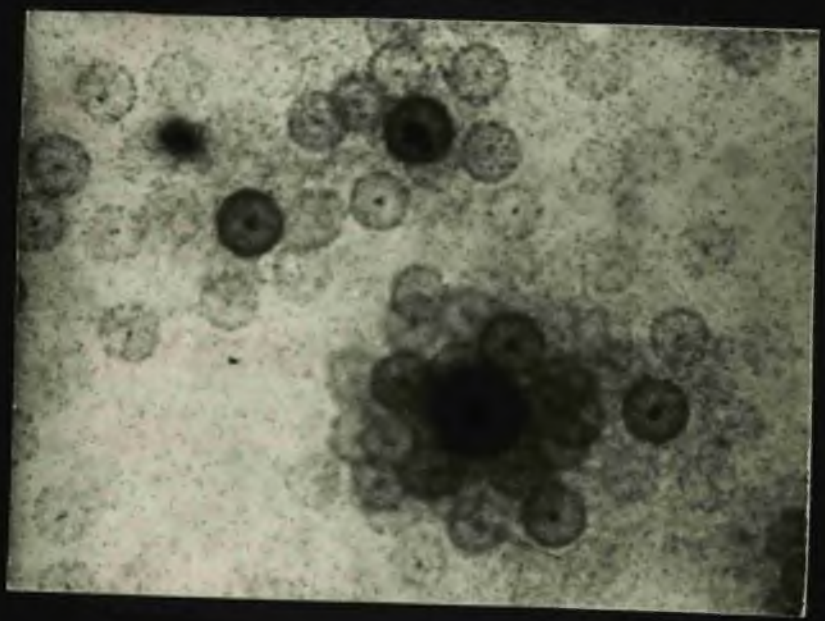


Plate 8

The circulation bench at the Gatty Marine laboratory, St. Andrews, with an assay experiment set up.

CRITERION D: The quantity of sperm spawned.

Plate 9

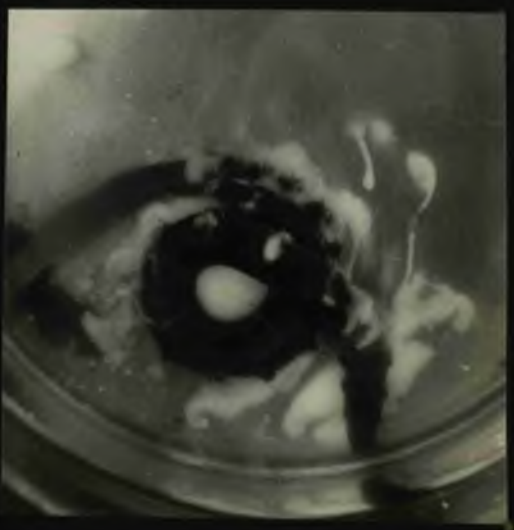
Poor pool (2).

Plate 10

Good pool (3)

Plate 11

Very good pool (4)



CRITERION Br: The degree of breakdown from sperm morulae to free spermatozoa in the coelom. Samples of coelomic sperm showing various degrees of breakdown.

Plate 12

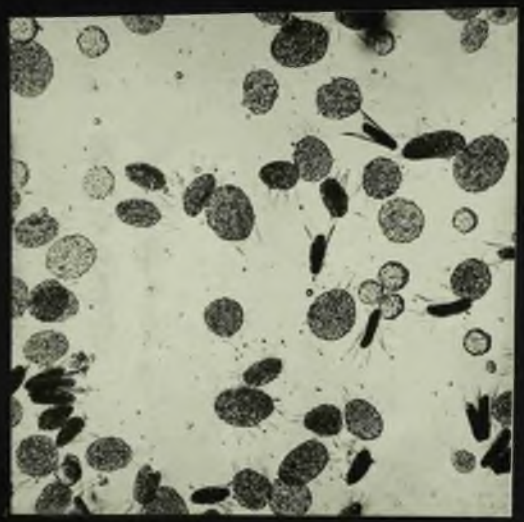
No breakdown (0).

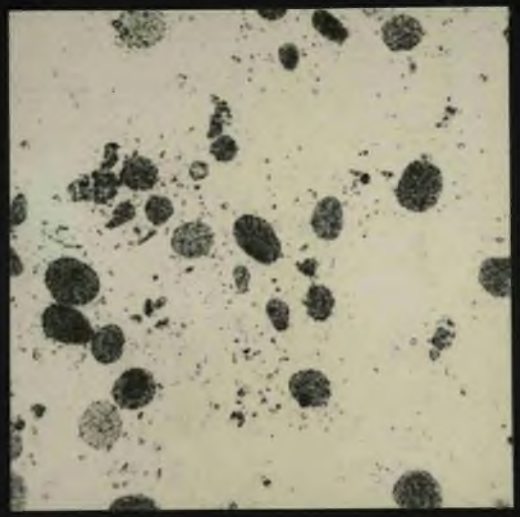
Plate 13

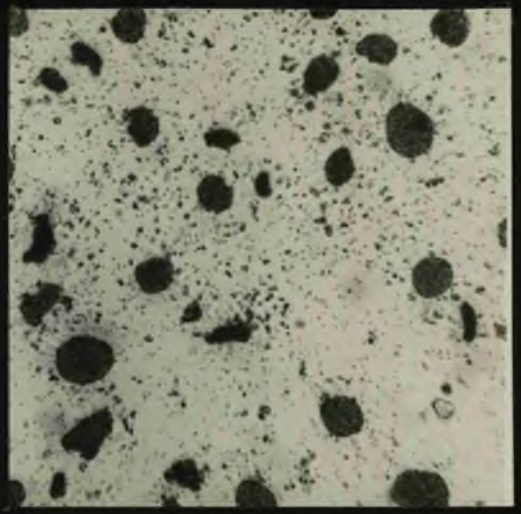
Small amount of breakdown (2).

Plate 14

Increased amount of breakdown (3).







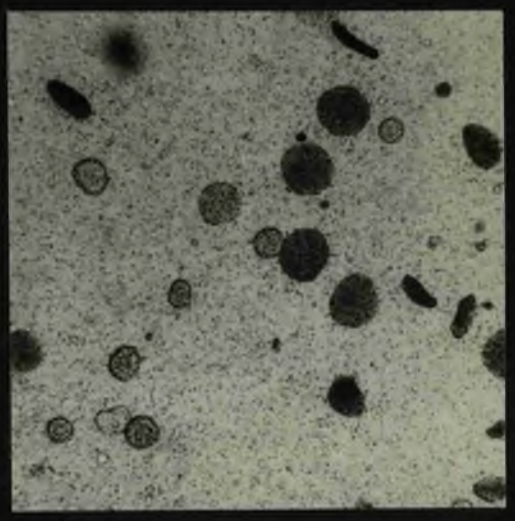
CRITERION Br: continued.

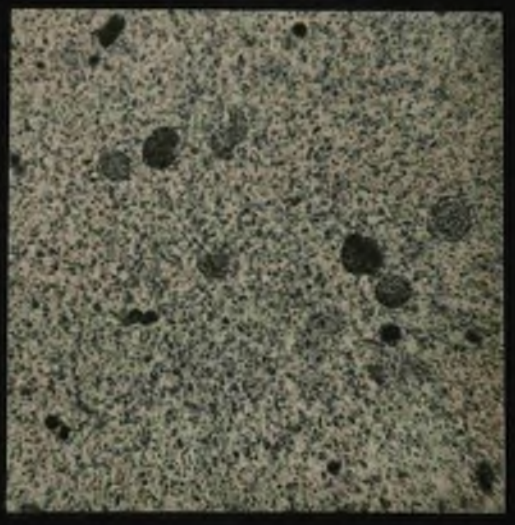
Plate 15

Large amount of breakdown (4).

Plate 16

Vast degree of breakdown (5).





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concluded that copper salts in sea water play an essential role in the metabolism of the spermatozoa. It was noted, however, that the inhibitory effect of copper on fertilisation is due to its effect on the eggs of Arbacia (Lillie, 1921). Further, Rothschild and Tuft state that the "limits within which Cu is physiologically active (but not of course lethally active) may be surprisingly small."

In view of the observations noted above, workers making artificial fertilisations have - (a) washed the gametes carefully and (b) used filtered outside sea water as a medium for their experiments.

Dodd (1951; in the press) suggested that difficulties encountered by previous authors in making artificial fertilisations in Patella vulgata were due to lack of criteria for the recognition of fully ripe genital products. Various refinements to the basic fertilisation technique were introduced. The genital products were never handled with metal instruments. Eggs and sperm were released by teasing the gonad with pointed glass rods. Cultures were kept at a constant temperature and it was found that rapid stirring of the fertilised eggs reduced the chances of polyspermy.

3. The activation and agglutination of sperm suspensions.

Pirlot (1933) found that coelomic sperm morulae of Arenicola marina, normally inactive in sea water, can be broken down and activated by adding magnesium hydroxide or by violent shaking; the greatest activity was obtained in alkalised sea