REPRODUCTIVE AND GENETIC VARIATION IN 'ARENICOLA MARINA' (L.) AND COMPARISON WITH 'ARENICOLA DEFODIENS' (CADMAN & NELSON-SMITH, 1993) (ANNELIDA : POLYCHAETA)

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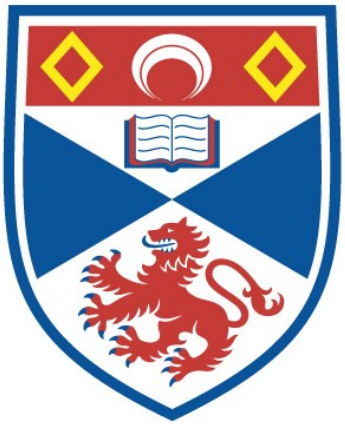
A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Reproductive and Genetic Variation in *Arenicola marina* (L.) and Comparison with *Arenicola defodiens* (Cadman & Nelson-Smith, 1993) (Annelida: Polychaeta)

by Mary Fraser Auckland

Submitted for the Degree of Doctor of Philosophy
in the University of St. Andrews.

School of Biological & Medical Sciences

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Abstract

Populations of *Arenicola marina* from different localities have been reported to show variation in certain characteristics. There are morphological differences such as colour, size and annulation patterns, and there is also variation in the duration and timing of the spawning seasons on different beaches. An extensive study of gametogenesis in individuals from three selected study sites was carried out by regular sampling throughout the year. Each site shows a different spawning pattern, either epidemic such as at the East Sands, St. Andrews, or an extended season, such as at the Eden Estuary, St. Andrews. These two sites are less than three kilometres apart and yet there are significant differences in the size of the gravid adults. The third site, Fairlie Sands on the west coast of Scotland shows a very unusual type of spawning season in that a small percentage of the population are gravid throughout most of the year.

Genetic variation was assessed using the technique of starch gel electrophoresis of allozymes. There is a degree of genetic differentiation between the three sites. Allele frequencies at the *GOT* locus are different between east and west coast sites and a third rare allele is present at Fairlie Sands. It is suggested that gene flow mediated by larval transport may be limited and this would cause inbreeding in some subpopulations.

The hormones involved in spawning have been investigated with particular reference to a putative female oocyte maturation hormone. Comparative work between *Arenicola marina* and *A. defodiens* has demonstrated the presence of an oocyte maturation factor in the prostomia of both species. While this factor acts directly on *A. defodiens* oocytes *in vitro,* the presence of a cofactor or secondary product produced *in vivo* is required in order to reinitiate meiosis in *A. marina* oocytes . Preliminary studies on the nature of reproductive endocrine substances present in the prostomia of female *A. marina* have been carried out.

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BSA	Bovine serum albumin
cAMP	Adenosine 3' : 5' - cyclic monophosphate (cyclic AMP)
CF	Coelomic fluid
Conc.	Concentration
ELH	Egg laying hormone
F	Fast (allele)
GSS	Gonad stimulating substance
GVBD	Germinal vesicle breakdown
HDP	Hormone dependent period
HPLC	High performance liquid chromatography
M	Medium (allele)
MIS	Meiosis inducing substance
MPF	Maturation promoting factor
pr.	Prostomium
S	Slow (allele)
sem	Standard error of the mean
SMF	Sperm maturation factor

Chapter 1

Introduction to the Biology of *Arenicola marina* and Aspects of Invertebrate Reproductive Endocrinology

Introduction

Different levels of variation within and between populations of *Arenicola marina* from a number of localities have been investigated during the course of this study. Particular emphasis has been placed on reproductive aspects of the life cycle. This first chapter introduces the thesis with a general literature review about the biology of *Arenicola marina* and the control of reproduction in polychaetes and other invertebrates. Inter-population differences in the timing and patterns of reproduction have been studied by monitoring gametogenesis in individuals from three different sites in Scotland. Genetic variation has been examined between these sites, using starch gel electrophoresis. Aspects of the reproductive biology have been studied in individuals from various sites on the east and west coasts of northern Britain and from France. The control of oocyte maturation in females has been investigated *in vitro* and *in vivo* and comparative work between two species of *Arenicola* has also been carried out.

1.1 Biology of Arenicolids

Arenicolidae are a widely distributed family of polychaetes with species represented in sandy shores worldwide (Ashworth, 1904). They are generally burrow dwelling, sedentary in habit and occupy intertidal areas. The family Arenicolidae consists of four genera: *Arenicola* Lamark (1801); *Abarenicola* Wells (1959); *Arenicolides* Mesnil; (1899) and *Branchiomaldane* Langerhans (1881). The first two groups are caudate species, they have an achaetous tail region at the posterior and are commonly known as lugworms (Wells, 1959). In Britain the most common species is *Arenicola marina* which has a wide distribution around North Europe (Wells, 1963). *Arenicolides branchialis* and *Arenicolides ecaudata* can also be found, although both these species tend to be more local in distribution (Eve & Southward, 1958).

A. ecaudata is known to occur from Iceland to northern Spain, and thus has a more boreal distribution than *A. branchialis* which ranges from the west coast of Scotland to Morocco, the Mediterranean and the Black Sea (Ashworth, 1912; McIntosh, 1915). During the course of this study a new species of lugworm has been described and named *Arenicola defodiens* (Cadman & Nelson-Smith, 1993). This species was previously considered to be a variety of *Arenicola marina* and this undoubtedly accounts for some confusion concerning aspects of the control of reproduction which is to be found in the literature and is examined further during this study. *Arenicola defodiens* has not been described from any beaches in Scotland, although two varieties of lugworm were originally described by Gamble and Ashworth in 1898 from the Firth of Forth. They were then named the laminarian and littoral lugworm due to the position each occupied on the shore. *A. defodiens* is reported to occur much lower on the shore extending into the sub-littoral zone and is now thought to be the laminarian variety (Cadman, 1992). It occupies a deep burrow, 0.5m to 1m deep, which is generally vertical or J-shaped.

A. marina lives in a characteristic U-shaped burrow consisting of a vertical head shaft, a vertical tail shaft and a horizontal gallery along the bottom where the worm is normally found (Wells, 1945). The head shaft is often full of soft, oxygenated sand. The worm feeds at the bottom of the head shaft by ingesting sediment causing a characteristic head depression on the sediment surface. There is also evidence to suggest that the worms sometimes move up into the head shaft to bring sand from the surface down into the burrow. Although the exact diet of *Arenicola* remains undetermined it is generally believed to be a deposit feeder, digesting the diatoms, bacteria and organic matter by injestion of the sediment (Hunt, 1925). Kruger (1959) suggested that the amount of food contained in the surface sand was inadequate and demonstrated a filtering action in the sand of the head shaft, suggesting a mode of suspension feeding to be taking place. Further studies by

Jacobsen (1967) examined the amount of organic matter present in the sand and in the gut of *A. marina* . He concluded that sufficient food was available to provide adequate nutrition if the worms were able to utilise most of the organic matter and that they are true deposit feeders. Wells (1945) also suggested that *Arenicola* must have a highly efficient digestive sytem. Eberhardt (1992) has demonstrated the presence of a high level of proteolytic activity and has isolated and characterized five serine proteases from the alimentary system. This suggests that proteases are important in utilising this type of food. A short distance away from the feeding depression, perhaps between 10 and 20cm , the sand cast made by defaecation may also be seen. Water flow through the burrow is maintained by waves of contraction passing anteriorly along the body, bringing water down through the tail shaft. The worms show a cyclic activity consisting of defaecating, feeding and irrigating the burrow (Wells, 1949a, b). The water current serves to oxygenate the burrow and may also help in keeping the sand in the head shaft soft. It has been suggested that oxygenation of the head shaft may stimulate the growth of microorganisms which are then ingested (Hylleberg, 1975).

Lugworms have a very important role in soft sediment communities where they are responsible largely for bioturbation. The volume of sediment that is reworked annually by *Arenicola marina* in the Wadden Sea has been measured using the amount of faeces produced (Cadée, 1976). The volume was dependent on the densities of *Arenicola* present and in areas with average densities of 85 per m^2, which is approaching the maximum density for this area of the Wadden Sea, the annual amount of faeces produced corresponded to a sediment layer 33cm deep. Bioturbation may account for a three to five fold increase in nutrient turnover mediated by bacteria (Hines & Jones, 1985). Asmus (1986) provided evidence to show that the presence of *Arenicola marina* significantly increases the remobilation of nutrients from sediments. Lugworms are also a very important food resource for wading birds and fish. In the Balgzand tidal flat area of the Wadden Sea, it has been estimated that lugworms have

the end of their tail nipped off by predators about once a week on average during the summer season (de Vlas, 1979a) and between 10% and 30% of the annual production of *A. marina* is removed in this way (de Vlas, 1979b; Kuipers *et al.*, 1986). It has also been estimated that *Arenicola marina* forms from 20% to 30 % of the total benthic biomass in the Balzand area of the western Wadden Sea (Beukema, 1974).

The Arenicolidae show a number of different reproductive strategies. *A. brasiliensis* and *A. cristata* both deposit fertilised eggs in a mucous cocoon attached to the burrows (Bailey-Brock, 1984). *A. ecaudata* has been observed spawning freely inside its transparent tube in the laboratory (Hentschel, 1930). This is characteristic of most other species of *Arenicola,* including *A. marina* which spawns the eggs inside the burrow. The eggs are retained there until fertilisation takes place (Farke & Berghuis, 1979).

1.2 Gametogenesis in *Arenicola marina*

One of the earliest descriptions of gametes in *Arenicola marina* was by Cunningham and Ramage (1888) in animals from the Firth of Forth. At this time the exact whereabouts of the gonads was unknown, although gametes had been found in the coelomic cavity. Ashworth (1904) descibed the gonads in *Arenicola marina* as simple masses of epithelial tissue situated on the nephridial blood vessel. There are five pairs of gonads present in all of the pairs of nephridia except for the first. Development of the gametocytes typically takes place in the coelomic cavity.

1.2.1 Spermatogenesis

Spermatogonia are released from the testis into the coelomic cavity as spermatocytes (Ashworth, 1904). These are balls of eight cells which divide and

eventually undergo spermatogenesis in the coelomic cavity (Olive 1983b, Bentley & Pacey 1989). The regulation and kinetics of spermatogonial proliferation has been studied by Olive (1972a, b) and it is suggested that the testis of *A. marina* is an exponentially growing cell population until the time when the oocytes first appear in the coelom (Olive, 1972b). Spermatogenesis results in the production of sperm plates or morulae (Howie, 1961a). These are clusters of spermatozoa with the heads remaining attached to a central mass of cytoplasm, the cytophore (see Sawada, 1984) and the flagella attached to each other at the distal end (Meijer, 1979b; Olive, 1983b; Bentley & Pacey, 1989). They remain arrested in this condition until spawning occurs (Howie, 1961a). As the worm approaches maturity all spermatozoa will be present as morulae (Newell, 1948). Spermatozoa in *Arenicola marina* are of the primitive type and are typically radially symmetrical with three distinct regions, the head, the middlepiece and the tail (Bentley & Pacey, 1989).

1.2.2 Oogenesis

In females oogonial divisions take place within the ovary and primary oocytes are released into the coelomic cavity at an early stage of development (Ashworth, 1904; Newell, 1948). The oocytes at this stage are about 15μm in diameter and they are transparent in appearance. The growth from 25μm in diameter to 60μm is initially slow, and during this phase the plasma membrane appears smooth but the egg envelope can be seen to cover the egg surface for the first time (Rashan, 1980). After a diameter of about 70 μm is reached, a rapid growth phase is entered. Extensive yolk granule and lipid drop deposition occurs. The plasma membrane becomes double layered, and at about 100μm in diameter pinocytotic or coated pits appear on the membrane (Rashan, 1980). The oocytes take on an orange brown appearance and the germinal vesicle is clearly visible (Rashan & Howie, 1982). The oocytes have a final diameter of about 180μm (Newell, 1948) and when they are fully developed yolk granules and lipid droplets are distributed throughout the ooplasm. The oocytes remain

in prophase of the first maturation division (meiosis I) and at this stage are unfertilisable (Pirlot, 1933; Meijer & Durchon, 1977). Both sperm morulae in males and the oocytes of females must undergo a final stage of ripening or maturation to initiate spawning. The maturation process is discussed below.

1.3 Spawning in *Arenicola marina*

In *A. marina* the final maturation stage of the oocytes (Pirlot, 1933; Howie, 1961c) and spermatozoa (Howie, 1961a) takes place in the in the coelomic cavity just before spawning. In the males this stage consists initially of dissociation of the morulae to free swimming spermatozoa (Howie, 1961a). In the females the maturation of the oocyte, resulting in meiosis reinitiation, is the final stage before spawning (Howie, 1961b; Meijer & Durchon, 1977). The endocrine control of these processes is discussed in Section 1.7 and oocyte maturation is discussed further in Section 1.11. The mature gametes are shed through the nephromixia, which are specialised nephridia present in the body wall (Newell, 1948). Ciliated funnels lead from the coelom through to an opening to the exterior (Kyle, 1896). When samples of activated sperm and mature eggs were injected into the coelomic cavities of animals that were not spawning themselves, they were shed automatically implying that only mature gametes are accepted by the nephromixia (Howie, 1961b).

Observations on males spawning in the laboratory have shown that muscular contractions of the body wall cause ejaculation of the sperm through the nephromixia (Bentley & Pacey, 1992). The sperm is ejected from the burrows of the spawning males onto the surface of the sand at low water in intertidal animals. The spawning of the females is a less active process (Pacey & Bentley, 1992) and the eggs are spawned into the horizontal shaft of the burrow (Farke & Berghuis, 1979 ; Howie, 1984). Sperm puddles can be observed on the beach and are formed by an agglutination

response of the sperm (Bentley, 1985). This is probably an energy saving response which takes place when the density of spermatozoa is very high. Sperm have only a limited energy resource and there is evidence to suggest that when the oxygen tension drops, for example at high densities, swimming is arrested (Pacey, 1991). When the sperm is diluted by the waters of the incoming tide swimming commences. The sperm are then drawn down into the burrows of the females as irrigation of her burrow takes place and fertilisation ensues.

1.4 Larval Ecology of *Arenicola marina*

Newell (1948) reported formation of a fertilisation membrane and two polar bodies lying inside the egg membrane. The eggs became more spherical in shape and delicate threads of cytoplasm could be seen crossing the perivitelline space. The first cleavage began about three hours after fertilisation and resulted in formation of two blastomeres, unequal in size. Cleavage is of a spiral type, similar to that already descibed for *A. cristata* (Child, 1900; Okada, 1941).

Blegvad (1923) observed fertilised eggs in an aquarium in August and followed development through to larvae. He found that the eggs hatched after four days into telotroch larvae with two eyes and long sensory hairs on the front part of the body. Early reports of larval development expressed conflicting views on the amount of time that the larvae spend in the water column. Blegvad (1923) suggested that the larvae of *A. marina* had a long pelagic larval life. This idea was reinforced by Thamdrup (1935) who found the spawning time to be restricted to a few days at the end of the summer in Denmark. He thought that the larvae were pelagic and over-wintered in the plankton, settling out in April and June of the following year. Post-larval stages had previously been descibed by Benham (1893) as the end of a pelagic developmental series of unknown duration. Thorson (1946) was the first to suggest that the larvae were

actually bottom dwelling. He carried out extensive plankton hauls but never found any evidence of pelagic larvae of *Arenicola marina*, even after the breeding season. Newell (1948) found fourteen day old larvae above the adult population on the shore after spawning. He suggested that they over-winter in the top two centimetres of the sand. This observation reinforced Thorsons' opinon that the larvae are in fact benthonic. Newell (1949) described a post-larval stage found in the surface layers of sediments from the upper shore at Whitstable Flats. The larva was essentially adult in form, although there were no gills and the segments were not divided into annuli. It was enclosed in a thick mucous tube, longer than the worm, implying that the mode of life may be different to that of the adults. Newell (1949) concludes that the post-larva originally described by Benham (1893) was not in fact from *Arenicola marina* and that the lugworm does not possess a pelagic larval stage in its life history.

More recently Farke and Berghuis (1979a, b), working on a population from the Dutch Wadden Sea, have established that fertilisation, embryogenesis and early larval development all take place within the burrow of the female. Observations made both in the laboratory and in the field have shown that the female stops feeding for 3 to 4 weeks after spawning to avoid ingesting the larvae although she continues to irrigate the burrow during this time. By the time the larvae have reached a stage with three chaetigerous segments they have migrated to the upper sediment layers and are carried to sheltered inshore areas. Larvae which have more than three chaetigerous segments are present in these areas throughout the winter where they grow to post larvae. At a length of approximately 6mm they undergo a secondary migratory phase, enclosed in a transparent gelatinpus tube, to adopt the adult burrowing habit. They will often go further up the shore to areas that appear only to be colonised by juveniles. This implies that there will be a subsequent migration down the shore. This life-cycle is summarised in Figure 1.1.

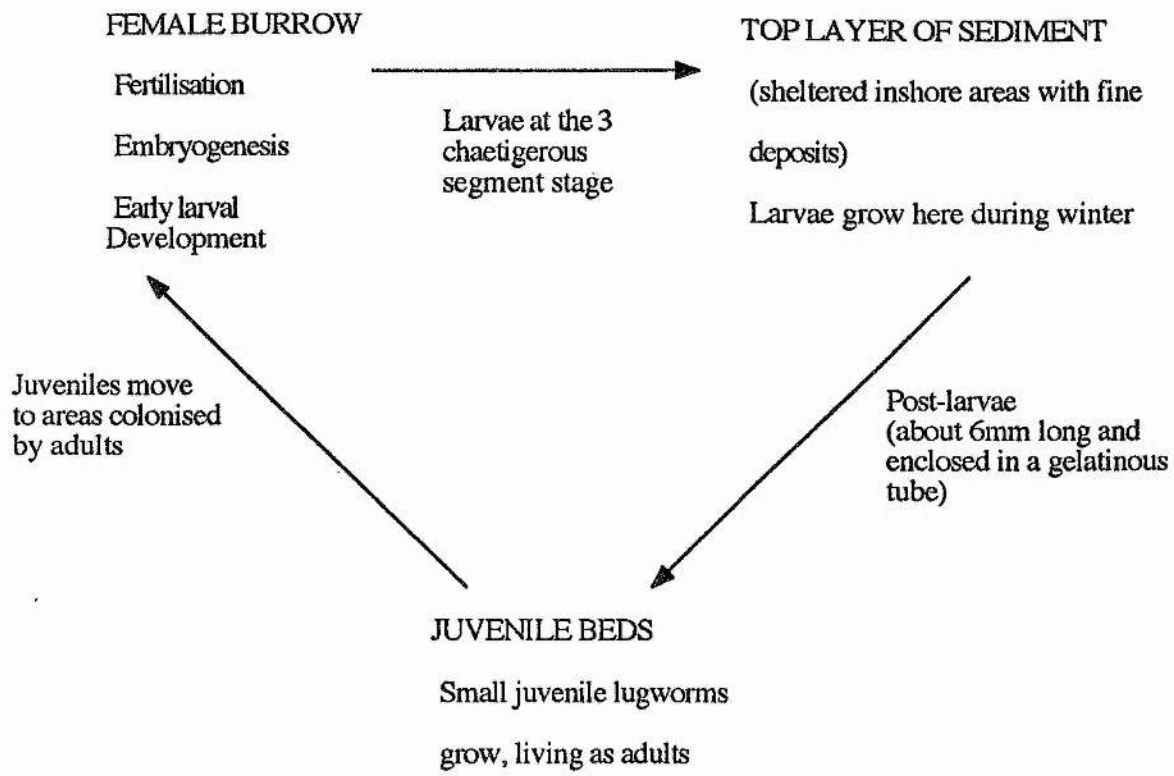


Fig. 1.1 Diagram representing the life-cycle of *Arenicola marina* (after Farke & Berghuis, 1979a)

Shahid (1982) studied developing larvae present in sediment from the field. He reported that at 100 days old the larvae had 19 pairs of chaetae with distinct tail and head although no gills or nephridia were visable. He suggested that after this stage the larvae left the beach and entered a pelagic phase for 3 to 4 weeks returning as minature adults. This suggestion is speculative because it is based on the fact that no larvae were found during this time.

Although the adult lugworm is described as a sedentary animal, there is evidence to suggest that it can actually move around during periods of high tide. They have often been observed swimming in the laboratory (Newell, 1948) and observations of them swimming in the sea have also been reported. Meek and Storrow (1924), noticed lugworms swimming at the surface of the sea in Northumberland at the time of full moon. This could, however, have been a post-larval migration. Lugworms have been reported to be found frequently in the prawn nets of fishermen seining off the beach (Pirlot, 1933). They have the ability to burrow very rapidly and may be completely buried in 3 to 4 minutes. (Chapman & Newell, 1947). The hydrostatic mechanisms and the co-ordination of burrowing activity in *Arenicola* has been investigated by Trueman (1965).

1.5 Phenotypic Variation between Populations of *Arenicola marina*

As described in Section 1.6 below, one of the principal differences between populations is in the length and timing of the spawning season. There are several other differences worth noting which are also reported in the literature. It has been noted that worms from different sites may vary enormously in physical characteristics such as size and colour. Kyle (1896) describes worms from St. Andrews as being of different colours according to their habitat. Worms found in clean sand are described as golden

in colour and exuded a clear gelatinous substance, whereas the worms from the muddy areas were dark brown and exuded a green slime. Duncan (1959) examined populations from sixteen differnt locations and reports differences in colour, size and diameter of gametes before spawning. The mean size of spawning adults from Cullercoats Bay, Northumberland was eight times smaller than that of a population from Llanfairfechan in Wales. For many years anglers and bait diggers have recognised that worms from one site are more suitable as bait than worms from another site. A very large population of *A. marina* in Budle Bay, Northumberland was dug extensively during the miners strike in 1986. The population was badly depleted and a local bylaw passed to prevent bait digging activities in this area. This was largely to protect populations of native and migratory wildfowl which used the bay as a feeding area. A site a few kilometres further north, Fenham Flats by Lindisfarne, where large populations of lugworm were also present was allocated for the use of the bait diggers. This area was never fully exploited however due to the supposed inferior quality of the worms as bait. The worms from this area were found to be much smaller and darker (Dr. M. G. Bentley, personal communication).

Wells (1957) found there to be a considerable amount of variation in the number of chaetigerous segments in the trunk region. There are normally nineteen but this can vary from seventeen to twenty one, within and between populations. He also looked at the chaetigerous annuli which in *Arenicola* are separated by smaller ordinary annuli. Normally two consecutive chaetigerous annuli are separated by four ordinary ones, although this number is reduced in the anterior trunk segments. The first three chaetigerous annuli are separated by only two ordinary annuli in *A. marina*. Some individuals of *A. marina* have very reduced or even absent annuli in front of a chaetigerous segment. Wells (1957) found that twenty percent of a population at Musselborough had this reduced annulation pattern, compared to only five percent for worms from Millport in the Firth of Clyde. The population from St. Andrews, thirty

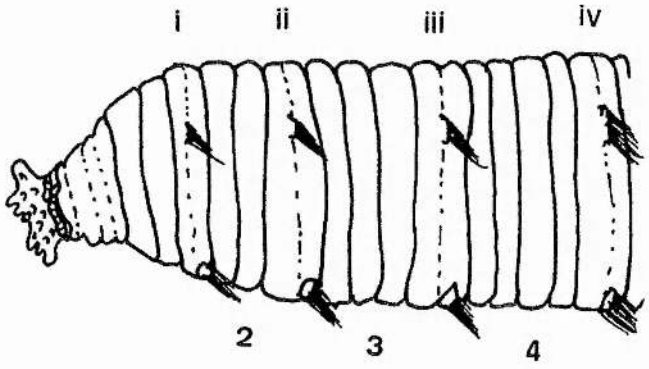
miles up the coast from Musselborough, had only four percent of the population with the reduced pattern. This is the first report that *A. marina* tends to separate into much smaller local populations with possibly some degree of genetic differentiation. The annulation pattern is also one of the principal morphological differences between *Arenicola defodiens* and *A. marina* (Cadman & Nelson-Smith, 1993). In *A. defodiens* there are three ordinary annuli present between the second and third chaetigerous annuli (see Fig 1.2).

Ashworth (1904) states that the size of a mature oocyte is 150μm in diameter, whereas Newell (1948) clearly says that the diameter of a mature oocyte in the population he was working on was 190μm. Newell (1948) also found a difference in the numbers of males and females present. He reports the sex ratio to be more than three females to one male. Conversely Cazaux (1966) found more males than females present during the spawning season at Arcachon in France.

1.6 Reported Variation in the Reproductive Cycle of *Arenicola marina*

The early reports about the reproductive cycle of *A. marina* are very confusing because there are many conflicting observations on the timing and duration of the spawning season. Cunningham and Ramage (1888) reported that there were no worms sexually mature in February or March in the Firth of Forth, and that the population spawns in August or September. A slightly later description by Kyle (1896) on worms from St. Andrews Bay reports spawning to take place during a period extending from January to September, though there is a cessation during April, May and the first part of June. This implies two spawning seasons, one in spring and one in autumn. The first report of a two day spawning crisis at either full or new moon was by Pirlot (1933) who studied worms at Blankenberg in Belgium. Newell (1948)

(a)



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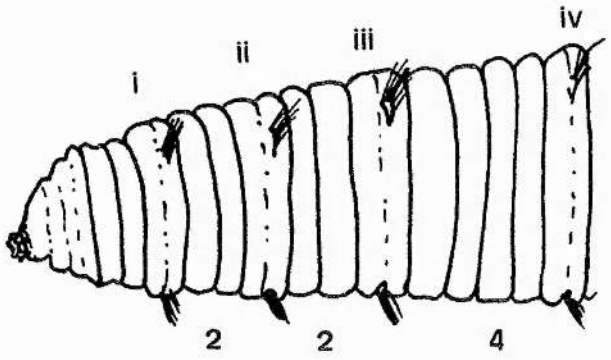


Fig. 1.2 *Arenicola* anterior ends showing the annulation patterns. The Roman numerals refer to the chaetigerous body segment number and Arabic numerals refer to the number of achaetigerous annulations between successive chaetigerous annulations. (a) *Arenicola defodiens* (b) *Arenicola marina* (redrawn from Cadman & Nelson Smith, 1993).

reviewed carried out a detailed study at Whitstable Flats. The spawning period for the *Arenicola* present at this beach was described as extended, lasting for two weeks in October. A few individuals spawned at the spring tides, with an increasing number spawning as neap tides approached. Sperm puddles were observed on the sand surface of the beach, released just before absolute low tide. Newell also recorded a significant degree of mortality after spawning, and suggested that these animals were those which were at the end of their natural life and had spawned for the last time. No eggs were detected on the sand but it was assumed that they were passed out of the burrow.

Duncan (1960) studied two populations of *A. marina* from the Isle of Man and also collated data on the time of the spawning season from nine other populations around the British Isles. Spawning occurred between October and December in all of these populations and the length of the breeding season was either very short as at St. Andrews and Whitstable (2 days) or extended over a two week period as at the rest of the sites studied. All of these populations, except for St.Andrews, showed the maximum number of individuls spawning during the neap period of the tidal phase. There have also been reports of populations spawning in the spring (von Brenning 1965) and another population at Fairlie Sands is reported to contain both spring and autumn breeders (Howie, 1959). De Wilde and Berghuis (1979a, b) studied a population in the Dutch Wadden Sea for four years. They reported that there were two distinct spawning seasons at this site. Between 60 and 80% of the ripe animals spawned between August and September and the rest spawned in early November. They suggest that the second group of individuals belong to a different, perhaps genetically determined group. This is of particular interest, because it is one of the few reports suggesting the existence of distinct sub-populations present on the same beach. Howie (1984) summarised the reported spawning periods in Europe and the British Isles (see Table 1.1).

Table 1.1 Spawning periods of *Arenicola marina* in Europe and the British Isles (modified from Howie, 1984).

Reference	Location	Spawning period / Duration	Notes
Spring or spring and autumn			
Kyle (1896)	St. Andrews	(i) January - March (ii) July & Sept.	Same population?
Gamble & Ashworth (1898)	Lancashire	(i) February - April (ii) Late summer	Laminarian variety Littoral variety
Ashworth (1904)	Lancashire	By end 2nd week in April	Laminarian and Littoral varities
Howie (1959)	Millport	(i) April - May (ii) Again later?	Same population
Duncan (1960)	Belfast	Spring?	
von Brenning (1965)	Wismar-Bucht	(i) April (ii) Late summer-early autumn (iii) Oct. - Nov.	Different populations
Late summer or autumn (non-epidemic)			
Cunningham & Ramage (1888)	Firth of Forth	August-September	
Ashworth (1904)	Firth of Forth	August-September	
Blegvad (1923)	Denmark	1st week August	Duration?
Storrow (1925)	Cullercoats	End of August	Duration? Sperm puddles reported
Thamdrup (1935)	Skallingen	End summer-early autumn	
Smidt (1951)	Esbjerg	(i) July /August (ii) End September	Same population
Howie (1959)	Dublin	(i) Oct.- early Nov. (ii) November -	Different populations
Duncan (1960)	Wales (2 stations)	Several weeks	
Cazaux (1966)	Arcachon	End Aug.- Sept.	Small nos. in October

Table 1.1 (continued)

Late summer or autumn (non-epidemic)			
de Wilde & Berghuis (1979b)	Wadden Sea	(i) August-Sept. (1974-1978)	
Farke & Berghuis (1979)		(ii) November	Second spawning epidemic
Pollack (1979)	Roscoff	(i) Oct.-Nov. (1975) (ii) October	
Autumn (epidemic)			
Pirlot (1933)	Belgium	1st fortnight October 1928, 1932, 1933	Sperm puddles observed
Newell (1948)	Whitstable	2nd fortnight October	Sperm puddles observed
Howie (1959)	St. Andrews	1-3 weeks mid Oct.-mid Nov. (1949-1952)	Sperm puddles observed
Bentley & Pacey (1992)	St. Andrews	2 days, mid-October spring tides	Sperm puddles observed
Duncan (1960)	Isle of Man (6 stations)	+ fortnights mid Oct. to mid Nov.	Sperm puddles observed

1.7 Control of Reproduction in *Arenicola marina*

The first stage in reproductive development is the proliferation of gametocytes by the gonads. It has been noted that there is an increase in the testis size between February and June in autumn breeding animals (Olive, 1972b). The production of spermatocytes, measured by the mean mitotic index of the testis of *A. marina* is low during the winter. There is a steady increase in the mitotic index during spring, leading to a high rate of proliferation in early summer (Olive, 1972a). As the time of spawning approaches the production of spermatocytes slows down, although there is a second minor increase just before spawning (Howie & McClenaghan, 1965; Olive, 1972a). Stripping a major proportion of the developing spermatocytes from the coelom of male worms, produces an outburst of mitosis in the testis (Howie & McClenaghan, 1965;

Olive, 1972a). Howie and McClenaghan (1965) report that this outburst of meiosis does not take place if the worms have been decerebrated previously and that mitotic activity may be restored by an injection of prostomial homogenate. They propose that accumulating gametes in the body may exert a negative feedback mechanism on a prostomial substance stimulating gonadial mitosis. Experiments carried out by Olive (1972b) on preparations of the testis *in vitro* confirmed that the coelomic spermatocytes do inhibit mitotic divisions. There was no evidence however to show that the prostomia were involved in the regulation of the rate of cell division in the testis.

In most polychaetes the development of the gametes has been shown to be dependent on hormonal control (see Olive & Clark, 1978; Franke & Pfannenstiel, 1984; Bentley & Pacey, 1992) but there is no direct evidence for this in *Arenicola marina* as yet. Decerebration of animals at the onset of vitellogenesis did not yield any conclusive results because the gametes continued to develop in a similar way to controls kept in the laboratory, but this was not comparable to natural development in the field (Rashan, 1980; Rashan & Howie, 1982). It has been proved that final maturation of the gametes and spawning is unable to take place in decerebrated animals (Howie 1963). The control of the final stages of reproduction (gamete maturation and spawning) is described below and oocyte maturation is discussed further in Section 1.11. The mechanism of spawning in *Arenicola marina* was investigated originally by Howie (1961a,b,c). He showed that spawning in gravid males could be induced by injection of the lipid fraction of tissue extracts from whole worms, into the coelomic cavity. This lipid extract was also found to act *in vitro* on the sperm morulae causing dissociation to a suspension of free swimmimg spermatozoa. He suggested that the stimulus for spawning brought about changes associated with ripening of the gametes. These changes enabled the gametes to pass through the nephromixia.

Howie (1963, 1966) produced evidence to suggest that the site of secretion of this gamete maturation factor was the prostomium. He worked on a population from Booterstown Strand, Dublin, which spawn over a three week period starting in mid-November. During the breeding season he found that decerebrated worms would not spawn. Administration by injection of homogenate of both male and female prostomia induced shedding of ripe sperm and eggs. In addition to this he tried to locate the area of the prostomium which caused this response. In sections of prostomia, although there was no evidence of particular secretory cells, the cells that most often contained secretory granules were more commonly found associated with the mid-brain and posterior lobes (Howie, 1966). He found that if the anterior half of the prostomium was removed and the posterior left intact, the worms still spawned. Conversely, in worms which had the posterior prostomium ablated and the anterior left intact there was no spawning.

Howie (1963) carried out an initial analysis of the chemical nature of the maturation hormone present in *Arenicola* prostomia. He suggested that it was a lipid and that it was present in both sexes. Injection of female worms with this substance however, failed to induce spawning, indicating that the male and female maturation hormones may be of a different nature. Bentley (1985) developed an *in vitro* bioassay, using the sperm activation response which meant that significant progress in the chemical identification of this sperm maturation factor (SMF) could be made. He reported that the SMF is only present in the animals during the breeding season and is undetectable after spawning. Using thin layer and gas-liquid chromatography the substance has recently been isolated and identified as the 20-carbon fatty acid, 8,11,14-eicosatrienoic acid (Bentley *et al.*, 1990; Pacey & Bentley, 1992). Injection of 8,11,14-eicosatrienoic acid into the coelomic cavity of sexually mature males induces a dramatic spawning response 50 to 60 minutes after injection. The response observed is identical to that induced in animals injected with the prostomial homogenate (Pacey & Bentley,

1992). Injection of the the fatty acid isomer 11,14,17-eicosatrienoic acid induced no spawning response. Females injected with the active fatty acid also did not spawn. This provides direct evidence for the differences in the reproductive endocrinology of the male and female lugworm.

Howie (1961a) noted that individual worms had a violent reaction when injected with emulsified whole body homogenates. He suggested that this was caused by the presence of a muscle stimulant which was different to the spawning stimulant, being present in the aqueous fraction as opposed to the organic fraction. Pacey & Bentley (1992) observed muscular contractions and writhing behaviour taking place when the gravid males were injected with prostomial homogenates and the sperm maturation factor (8,11,14-eicosatrienoic acid). They suggest that this spawning behaviour is induced either as a direct response to the SMF, or via secondary factors released from other endocrine organs or from the morulae themselves. When mature spermatozoa are injected into the coelom of gravid males they are ejected passively (Howie,1961b). Injection of 8,11,14-eicosatrienoic acid into non-gravid individuals will initiate spawning behaviour without gamete release, which implies that the gametes are not necessary for this to occur (Pacey & Bentley, 1992). These observations provide evidence that 8,11,14-eicosatrienoic acid acts as a true spawning hormone, although the possibilty of the involvement of more than one substance is not ruled out.

1.8 Control of Reproduction in Other Polychaete Species.

Polychaete reproduction has been reviewed extensively by Schroeder and Hermans (1975), Olive (1979, 1981a, 1984), Franke & Pfannenstiel (1984), and recently by Bentley and Pacey (1992). Polychaetes exhibit a number of different types of reproductive cycle (see Olive & Clark, 1978 for terminology). Four strategies can

be distinguished and they are described below. Monotelic polychaetes breed only once in their lifetime. The gametes are generally released in one large batch and the spent animals die after reproduction. Synchronisation of gametogenesis and spawning behaviour, between individuals is a prerequisite for synchronous spawning. Similarly gametogenesis must also be regulated within the individual, so that the maximum number of mature gametes may be released on spawning. The classic example of this type of reproduction is shown by the Nereids (reviewed by Clark, 1961), but this pattern of monotelic reproduction is generally atypical of polychaetes. Most species are polytelic, reproducing more than once in their lifetimes. Polytelic species may be divided into two categories on the basis of the degree of synchrony exhibited within the population. In a highly synchronised population, all the sexually mature individuals spawn at the same time, as in monotelic species. *Arenicola marina* is an example of a species with this type of strategy (Howie, 1984). Polytelic polychaetes may also show no synchronisation within the population but for the gametes to all reach maturity at the same time requires synchronisation of gametogenesis within the individual. Individual females of *Cirratulus cirratus* spawn yearly and asynchronously (Olive, 1970), consequently egg masses can be found on the shore all year round. The final type of reproduction is called semi-continuous reproduction and is typical of the smaller polychaetes such as Spirorbids (de Silva, 1967). Individuals release small numbers of gametes at intervals throughout an extended breeding season and then die. Some degree of synchronisation may also be achieved here. Members of the Syllidae such as *Typosyllis prolifera* reproduce by stolonization where the caudal part of the worm becomes transformed into a stolon which is full of sperm or eggs and developes specialised parapodia for swimming. At certain times this part then breaks off from the rest of the worm, (the stock) and swims to the surface where swarming and spawning takes place (this is discussed further below). The stolon then dies but the stock section regenerates and another stolon is produced.

In polychaetes, vitellogenesis and the greater part of spermatogenesis may take place in the coelom after the gametocytes have been shed from the germinal epithelium, for example in *Cirratulus cirratus* (Olive, 1971), *Arenicola marina* (Dales, 1957) and *Eulalia viridis* (Olive, 1975b). The gametes may also be retained in the ovaries and testes until just before spawning as shown by *Streblospio benedicti* (Eckelbarger, 1980), *Nephtys caeca* (Olive, 1978) and *Harmothoe imbricata* (Garwood, 1978). Oogenesis in polychaetes is reviewed by Eckelbarger (1984). *Arenicola ecaudata* also shows a delay in the release of the gametes into the coelom, with development taking place mainly in the gonads (Gamble & Ashworth, 1900).

Although there is a large variety in the types of reproduction within Polychaeta, most of these do require some degree of synchronisation of reproductive events at some stage during reproductive development. Regulation of gametogenesis and spawning may occur at any developmental stage of reproduction from the proliferation of the germinal epithelium to the maturation of the gametes (Clark, 1965). The mechanisms by which this is achieved is poorly understood for most species.

1.8.1 Environmental Control of Reproduction in Polychaetes.

Environmental control of polychaete reproduction has been reviewed a number of times, incuding reviews by Clark (1979), Olive (1981a,1984) and more recently by Bentley and Pacey (1992). In some species specific environmental conditions are required for successful gametogenesis and spawning to take place. These conditions may either act as a trigger to initiate specific gametogenic responses (Clark, 1979) or inversely could be inhibiting the same responses (Olive, 1981a). For example, in Welsh populations of the serpulid *Spirorbis spirorbis* breeding only becomes possible in the early summer, when the sea temperature rises above a certain threshold level. During the following several months the worms spawn synchronously every two weeks using the tidal cycle as a specific environmental cue (Knight-Jones, 1951; de

Silva,1967 & Gee, 1967). The control of spawning, however, is not as simple as it at first appears because Daly, (1977) found a population on the coast of Northumberland with synchronised repetetive spawning unrelated to sea temperature or tidal cycles.

Fong (1991) studied the effects of salinity, temperature and photoperiod on the frequency of epitokal metamorphosis in an estuarine population of *Neanthes succinea.* He found that summer temperatures were critical for metamorphosis to take place and that the frequency of metamorphosis was greatly increased at salinities of 20 ‰ compared to 5 ‰. In the field, increasing salinities corresponds to late summer and it is suggested that there is a selective advantage in metamorphosis at this time because the eggs and larvae cannnot osmoregulate as well as the adults (Dean & Mazurkiewicz, 1975).

There is evidence that swarming in Nereidae is dependent on minimum temperatures (Goerke, 1984). Field observations and laboratory experiments revealed a correlation between the geographical distribution and the minimum temperatures of swarming for three species of *Nereis* from the southern North Sea. The further the geographical range of the species extends, from the boreal to the tropical regions then the higher this minimum temperature will be. Swarming actually occurs within a temperature range which implies that other environmental factors may be effective also. In *Nereis diversicolor* from Jadebusen in the North Sea, maturation is induced by temperatures above 6°C. Spawning in the early spring is synchronised by raising temperatures after a period of low winter temperature. Spawning occurs mainly during periods of new and full moons (Bartels-Hardege & Zeeck, 1990).

Environmental stimuli may be active over a longer period of time than simply switching on or inhibiting gametogenic or spawning responses. Endogenous rhythms may be established and maintained by responses to external cycles acting as a zeitgeber (Hauenschild,1955, 1956, 1960). Hauenschild established that the lunar spawning

cycle observed in *Platynereis dumerilii* was controlled in this way. The end of a moonlit phase acts as a cue for gametes close to maturation to enter a rapid phase of development. This takes 18 days and after this time the gametes are spawned, coinciding with a particular phase of the moon (Olive, 1984). There are many examples of polychaete species which are known to use lunar periodicity as a cue to synchronise gametogenesis and spawning. One of the best known examples of this phenomenon is probably that of the spawning of the palolo worm *Eunice viridis*. The time of emergence of the epitokous segments of the palolo worm has been well documented, and coincides with the third quarter of the moon in October and November (Caspers, 1961; Hauenschild *et al.*, 1968). The natives of the Samoan Islands have collected the swarming worms for food for centuries (Caspers, 1984).

Populations of *Typosyllis prolifera* naturally reproduce synchronously during the second quarter phase of the moon. The caudal part of the worm is transformed into a stolon which detaches from the rest of the worm and swarms at the surface. This part then dies but the anterior stock of the animal remains benthonic and will survive to breed again. Individuals stolonise up to 18 times each at circalunar intervals of about 31 days (Franke, 1985). Asynchronous laboratory populations can be brought into synchrony by exposing them to artificial lunar cycles. Every 30 days the daily 8-hour dark period was illuminated by artificial moonlight. The actual cue for this entrainment is provided by the transition from illuminated to dark nights (Franke, 1986). This is an example where the environmental cue causes a direct endocrinological response, which is discussed further below.

In *Eulalia viridis*, vitellogenesis starts in most individuals in May (Olive 1977). Oocytes at early stages of development begin to accumulate in the coelom a long time before a phase of rapid growth is entered. Oocyte growth is not synchronised within individuals during vitellogenesis but the number of fully developed oocytes steadily increases until spawning in mid-summer. Garwood and Olive (1978) have shown that

sexual development is controlled progressively by increasing environmental temperature rather than by a sudden trigger mechanism. Their experiments suggested that low environmental temperature (5°C) directly limited gametogenesis and daylength played no significant role.

In *Kefersteinia cirrata* from Whitley Bay in Northumberland, previtellogenic oocytes and undifferentiated spermatogonia accumulate in the coelom from September through to December (Olive & Pillai, 1983). Gametogenesis continues until June when spawning occurs. Laboratory experiments maintaining the animals in different environmental conditions have shown that the rate of oocyte growth is increased by exposure to an elevated temperature and by a longer daylength (Olive & Pillai, 1983). The rate of growth was faster in animals maintained at 5° C, 16L: 8D compared with 15° C, 8L: 16D, implying that the daylength is the more important factor. As the environmental conditions change so vitellogenesis occurs more rapidly. Increasing temperature and lengthening days will result in the population becoming reproductively synchronised.

Harmothoe imbricata is an interesting species because it has two periods of vitellogenesis. The first starts in mid-December and spawning occurs in March. A second period of vitellogenesis then commences immediately and the oocytes grow rapidly with spawning taking place in April (Daly 1972, 1974). Garwood & Olive (1978) established a strong correlation between the onset of the first phase of vitellogenesis and the annual minimum daylength. Experimental conditions showed that oocytes grew faster at 5°C, compared to 10°C. At 15°C there was no growth and some of the larger oocytes started to degenerate. The growth rate of the oocytes was also accelerated when the photoperiod was altered to provide more light hours than were present at ambient conditions. Light and temperature therefore initially have a considerable influence on vitellogenesis and help to synchronise the reproductive development within the population. Conversely the oocytes from the second phase of

vitellogenesis grew fastest at 15°C. The influence of daylength and temperature was significantly reduced. This implies that endogenous factors may also be involved. Exposure to low temperatures in the laboratory can stimulate the start of gametogenesis prematurely, although in these animals the rate of vitellogenesis at 5°C and 10° C is much slower than that measured for female *H. imbricata* reproducing normally (Garwood, 1980). There is a mechanism present which can prevent premature maturation and spawning which might otherwise occur in response to a particularly cold winter. The growth rate of the oocytes of females that have been induced to enter vitellogenesis early, does not increase when the animals are exposed to daylengths longer than the critical daylength of between 10 and 11 hours (Garwood & Olive, 1982). Clark (1988) has demonstrated that for successful completion of the first period of vitellogenesis a certain number of days with winter conditions are required. The worms must be exposed to a maximum of 13 hours of daylight for a continous period of between 42 and 55 days. If this photoperiod is interrupted the oocytes start to degenerate and are resorbed.

1.8.2 Endocrine Control of Reproduction in Polychaetes.

As annelids do not possess any epithelial endocrine glands, any hormonal control of development is likely to be due to neurosecretions acting directly on peripheral targets (Highnam & Hill, 1977). The first evidence for the exsistence of neurosecretory cells was provided by Scharrer (1936). She discovered elements that had the cytological characteristics of neurosecretory cells in the supra-oesophageal ganglion or brain of *Nereis virens*. Since then neurosecretory cells have been found to be abundant in the brains of polychaetes. Golding and Whittle (1977) reviewed neurosecretion in annelids. They show that several types of neurosecretory cell are found in most annelid species. Some of these are capable of secreting peptide hormones via small vesicles. It is now widely accepted that growth and reproduction

can be controlled by hormones from the brain. The endocrine control of polychaete reproduction has been reviewed in the past by Clarke (1965), Olive (1979), Franke and Pfannenstiel (1984), and recently by Bentley and Pacey (1992).

Early studies on polychaete endocrinology were carried out on the Nereids (reviews by Durchon, 1967, 1976; Hauenschild, 1974a, 1975 & Baskin, 1976). The juvenile worms go through a period of somatic growth and when a certain size is reached reproductive development commences. The age at which this occurs depends on the growth rate of the individuals (Olive *et al* , 1986). After this initial and essential period of somatic growth, the control of reproduction becomes critical. Within the individual gamete development must be regulated so that all gametes are mature at the time of spawning. Synchronisation of gamete development must extend to all the members of the population in order for synchronous spawning to take place. The nature of the endocrine control has been established mainly by studies on oogenesis and has been reviewed more recently by Porchet (1984) and Fischer (1984). During somatic growth the immature worms can regenerate posterior segments, however, removal of the brain stops growth and inhibits regeneration (Durchon, 1956; Clark & Bonney, 1960). A brain implanted from a normal worm restores growth and the power of regeneration (Clark & Evans, 1961), implying that a hormone from the brain is involved. Hauenschild (1964) implanted the prostomium from a post-larval *Platynereis dumerilii* into an isolated parapodium of a maturing worm. He found that further development of the oocytes was inhibited. In worms which have entered the reproductive phase, removal of the brain accelerates vitellogenesis, but abnormal growth ensues (Hauenschild, 1966). In *Nereis diversicolor* for example, oocytes are shed into the coelom when they are between 15 and 20μm in diameter. They grow fairly slowly up to a diameter of about 70μm and they then enter a rapid growth phase, quickly reaching a diameter of about twice this size. The rest of the growth occurs at a slower rate and the final diameter of the oocyte when it is spawned is around 200μm.

Removal of the brain has no effect initially but the oocytes enter the rapid growth phase much earlier, when they are only 30μm in diameter. Vitellogenesis of these oocytes however does not proceed as normal and the oocytes although fertilisable will not undergo cleavage (Clark & Ruston, 1963a). This implies that the brain has some sort of inhibitory effect on the growth rate of the oocytes. Durchon and Porchet (1971) developed an *in vitro* assay to measure the endocrine activity of the brains of *Nereis diversicolor* and *Perinereis cultrifera.* They demonstrated that the endocrine activity of the brain diminishes during growth in both species. These and other experimental observations have led to the suggestion that the cerebral hormone is present at high concentrations in juveniles, when regeneration can take place. A decline in the concentration initiates growth and vitellogenesis. The hormone activity decreases as the gametocytes develop and the worm finally becomes sexually mature. The chemical nature of the cerebral hormone is yet to be determined but the hormone has been partially isolated by HPLC and the active fraction contained a small peptide of about 2000 daltons (Cardon *et al.,* 1980).

The developing oocytes may also play a role in regulating the endocrine activity of the brain. The implantation of sub-mature oocytes into the coelom of juvenile *Perinereis cultrifera* induces complete maturation and metamorphosis (Porchet, 1967). A substance can be extracted from the maturing oocytes which affects all the known endocrine activities of the brain (Porchet & Cardon, 1976). Two fractions (B_1 and B_2), separated from coelomic fluid by chromatography on a Sephadex column, have been shown to stimulate oocyte growth when injected into young females during vitellogenesis (Porchet *et al.,* 1979). It has been proposed that the purpose of this substance may be to reinforce the affects of the declining level of cerebral hormone in maturing animals. This positive feedback mechanism may lead to total suppression of endocrine activity. Some sort of feedback control of cerebral endocrine activity may

also occur in male *Platynereis dumerilii* but the origin of the substance has not been established (Hofmann & Schiedges, 1984).

In the Syllidae it has been suggested that a muscular part of the foregut, the proventriculus, is responsible for endocrine activity because removal of the proventriculus results in precocious formation of mature stolons (Durchon, 1959; Wissocq, 1966). In *Typosyllis prolifera* high endocrine activity of the proventriculus inhibits reproduction during winter conditions but during the reproductive period in the summer the activity of the proventriculus has been shown to be cyclical, being more active in worms in the caudal regeneration phase than in the stolonization phase (Franke, 1980). However, if winter conditions are changed artificially to summer conditions for two weeks and then changed back to winter, the response is different in animals with and without the prostomia. Reproduction was not prevented in the control animals but the decapitated worms failed to stolonize. Decapitated worms do however undergo stolonization if they are deprived of the proventriculus. This implies that a substance from the prostomium does have an affect on the initiatiation of the stolonization process, and may in fact be regulating the activity of the proventricular endocrine system (Franke, 1981; 1983a). There are therefore two distinct endocrine factors, a stolonization promoting hormone released from the prostomium and a stolonization inhibiting hormone released from the proventriculus. Under winter conditions the proventriculus shows a high endocrine activity and prevents reproduction from occurring whereas under summer conditions the prostomium becomes endocrinologically active, inhibiting proventricular activity and initiating reproduction. It has been proposed that during the summer the prostomial hormone is released cyclically in the final stage of the regeneration phase to allow stolonization to take place. When the level of prostomial hormone drops after stolonization, the proventriculus becomes active to allow a period of regeneration (Franke, 1983b).

Eulalia viridis spawns in mid-summer after an extended period of accumulation of previtellogenic eggs followed by rapid vitellogenesis (Olive, 1975a). Experimental studies have shown that decerebration of females before the onset of vitellogenesis, inhibits vitellogenesis completely (Olive, 1975b) indicating the presence of a vitellogenesis promoting hormone. In females with oocytes which have already entered vitellogenesis, decerebration caused arrest of development and some of the oocytes were resorbed. The ability of the oocytes to undergo vitellogenesis can be restored by the implantation of the cerebral ganglia into the coelom (Olive, 1976a). These observations provide evidence for the exsistence of a vitellogenesis promoting hormone which is present in the prostomium. The final stages of oocyte development may be hormone independent, as some of the oocytes were retained in decerebrated females with the majority of oocytes greater than 80μm (Olive, 1975b).

Two cerebral hormones are involved in the control of reproduction in *Nephtys hombergii*, a gonadotrophic hormone and a spawning hormone (Olive & Bentley, 1980; Bentley & Olive, 1982). Freeze dried brain homogenate obtained at different times of the year have been assayed *in vivo* (Olive & Bentley, 1980) and *in vitro* (Bentley & Olive, 1982) for gonadotrophic and spawning inducing activity. The gonadotrophic hormone can be detected in the female brain between September and March, and is necessary for continued oogenesis. The presence of the gonadotrophic hormone maintains gonad integrity, prevents premature ovulation and the onset of oocyte resorbtion (Olive & Bentley, 1980). Vitellogenesis is also promoted by the presence of the gonadotrophic hormone (Olive & Lawrence, 1990). The presence of a spawning hormone in the supra-oesophageal ganglion of *Nephtys hombergii* was initially demonstrated by Olive (1976b). Injection of supra-oesophageal ganglion homogenate from gravid *N. hombergi* into other gravid animals induced spawning. The spawning hormone seems to be present in both males and females for a short time before spawning takes place (Olive & Bentley, 1980). The spawning hormone in

Nephtys hombergii stimulates muscular contractions and gametes are released through the anus (Bentley *et al.*, 1984). An opening in the ventral gut wall, the pre-pygidial rectal cleft, develops as the time of spawning approaches. This opening allows the oocytes and spermatozoa to pass from the coelomic cavity into the posterior region of the gut for spawning to take place (Bentley *et. al.*, 1984). The action of the spawning hormone, which is to stimulate muscle contractions, in some respects resembles that of the proposed muscle stimulant in *Arenicola marina* (Howie, 1961a; Pacey & Bentley, 1992, see Section 1.7). There is no evidence of the presence of a maturation factor in the nephtyids however, because germinal vesicle breakdown and oocyte maturation occur a few minutes after release into sea water in *Nephtys* (Olive, 1976a).

A factor present in the suboesophageal ganglion-cement gland complex has been shown to induce spawning in gravid individuals of *Pectinaria gouldii* (Tweedal, 1980). The substance causes the movement of fully developed oocytes and sperm morulae into the nephromixia, where gamete maturation takes place. Mature oocytes, which have undergone germinal vesicle breakdown and free spermatozoa dissociated from the morulae are then shed. The substance is not sex specific as homogenates of both male and female ganglion-cement gland complex induce maturation in gametes of the opposite sex. Identification of this substance has not yet been possible but it has been shown to be inactivated by heating and is lost following dialysis, implying that it is a molecule larger than 12 000 daltons (Tweedal, 1980). The action of this substance is very similar to the maturation factors which have been described in *Arenicola marina* (see Section 1.7).

The ampharetid *Melinna cristata* (Sars) has a similar gametogenic cycle to *Arenicola marina.* The gametes are released into the coelom as secondary spermatogonia or previtellogenic oocytes during a proliferative phase of the gonads. The oocytes gradually increase in diameter and when they reach a critical size they enter a rapid phase of vitellogenesis. It has been reported that completion of the maturation

divisions occurs after the oocytes have been spawned (Nyholm, 1951). In animals from Newbiggin Point on the Northumberland coast this proliferative phase extends from January until September (Hutchings,1973). The release of gametes into the coelom is inhibited from September until the breeding season, which occurs at the end of December and beginning of January. The conditions which initiate the change from proliferative to a non-proliferative phase are not known but it is suggested that there could be some feedback from coelomic gametes which initiates the change (Hutchings, 1973), although these observations are speculative.

1.9 Control of Spawning in Other Invertebrates

In simple animals such as hydrozoans the environmental input into the control of spawning can be direct and straightforward. Darkness will induce spawning in *Hydractinia epiconcha* (Yoshida, 1959), *Eutima mira* and *Gonionemus murbachii* (Costello & Henley, 1971). There is evidence for the presence of a spawning inducing substance which is produced in the jellyfish ovary. *Spirocodon saltatrix* normally spawns after nightfall or when placed in the dark (Yoshida, 1952). Oocytes from the ovary are immature and have a germinal vesicle present whereas the spawned oocytes are fully mature (Dan, 1950). Ikegemi *et al.* (1978) found that isolated ovaries that had been exposed to darkness for a period of 30 minutes produced a peptide of low molecular weight which acted as a spawning inducing substance. Centrifuged filtrate of the seawater this *in vitro* response had taken place in, induced maturation and spawning in other ovaries that were in continuous light. Changes from light to dark has also been shown to induce spawning in other groups of invertebrates. Synchronous spawning takes place in the ascidian *Ciona intestinalis* . Individuals are hermaphrodite and both sperm and eggs reach maturation simultaneously (Berrill, 1947). Spawning naturally occurs at dawn but can be artificially induced by

manipulation of light-dark cycles. Exposure to light after a minimum of a 1-hour period in the dark will induce spawning but in constant illumination no gametes are released (Lambert & Brandt, 1967).

It has been established that spawning can be induced in molluscs by raising the temperature of the seawater (Galstoff, 1938a, Kinishota *et al.*, 1943, Yamamoto, 1951). Spawning of gravid female oysters, *Ostrea virginica* can be induced by exposure to a constant temperature a few degrees higher than that of its environment (Galtsoff,1938b). Temperature, however is not the only stimulus because gravid female oysters in a constant temperature also spawn on addition of a sperm suspension. The sperm suspension contains a spawning inducing substance. The effect of a temperature raise could stimulate production of a neurotransmitter which initiates the spawning response.

Lunar periodicity also affects spawning of inter-tidal molluscs. The chiton, *Acanthopleura japonica* releases gametes just before high tide from July to October (Yoshioka, 1987). Release occurs primarily in the morning but also in the evening during spring tides. The timing mechanism of synchronous spawning is entrained by a diurnal light-dark cycle and the semi-diurnal tidal immersion cycle (Yoshioka, 1989).

Other substances have also been found to be effective in inducing spawning. For example, Hirai *et al.* (1988) working on *Spisula* the surfclam found that injection of 2mM 5-hydroxytryptamine (5-HT) into male and female gonads induced spawning. Shedding of the gametes occurred 2 to 3 minutes after the injection and produced immature oocytes with the germinal vesicle intact. When oocytes were incubated *in vitro* with 20μM 5-HT oocyte maturation occured. Perhaps it is not surprising that general neurotransmitters such as 5-HT will induce spawning as they must short-circuit the maturation response and the action of any endocrine substance remains unspecified. Addition of hydrogen peroxide to seawater causes gravid male and female abalones

(*Haliotis rufescens*) to spawn (Morse *et al* ., 1977). The spawning response was blocked by the addition of aspirin to the sea water 25 minutes before adding the H_2O_2. The conversion of arachidonic acid to prostaglandin endoperoxidase is catalysed by the enzyme cyclo-oxegenase, which is inhibited in the presence of aspirin. This implies that prostaglandin endoperoxidase synthesis may be required for the induction of spawning. The H_2O_2 provides a specific radical which can activate the prostaglandin endoperoxidase synthesis and hence artificially induce spawning.

An egg laying hormone (ELH) has been isolated and identified from the nudibranch *Aplysia califonica.* (Nagle *et al.*, 1989). The hormone is a peptide, which induces ovulation and acts on the central neurons causing egg laying behaviour. The hormone is synthesised in neurosecretory bag cells which are located in the abdominal ganglion (Coggeshall, 1967). The peptide was purified by passing bag cell extract through a Sephadex G-50 column and then carrying out reversed-phase HPLC on the fractions (Chiu *et al.*, 1979). The 19-residue peptide was then sequenced and has subsequently been synthesised (Strumwasser *et al.*, 1987).

An enormous amount of research has been carried out on the reproductive mechanisms of echinoderms. The animals generally tend to be widely distributed and easy to collect. They have a high fecundity and egg development is synchronised. These factors make the animals an ideal model in which to study cellular activation. There are two gonads in each arm of the starfish located in the interambulacral area. When the starfish spawn the ripe gametes are shed into the sea from all gonads simultaneously, suggesting some regulatory mechanism. An endocrine system controls oocyte maturation and release. Meiosis is reinitiated, inducing maturation so that fertilisation may occur. Successful fertilisation can be maximised by ensuring that gametes mature synchronously. This means of synchronisation has also been observed to be the mode of development for many other marine invertebrates for example, the brittle star *Amphiopholis kochii* (Yamashita, 1988), the surf clam *Spisula*

solidissima (Hirai *et al.*, 1984) and several species of hydrozoans (Freeman & Ridgway, 1988).

The main difference between the oocytes in the starfish and those of *Arenicola* are that the starfish have a layer of follicle cells surrounding the oocytes. These follicle cells play a role in the endocrine control of oocyte maturation and spawning. The follicle cells have a central nucleus and the cell body contains contractile elements (Schroeder, 1971). Each cell has a single flagellum and they are linked together to form an envelope around the oocyte which is prevented from moving by cellular adhesion. The distance between the follicle cells and the oocyte varies according to the species, it has been recorded as 2 to 16μm in *Patiria* and 10 to 25μm in *Pisaster*. This sub-follicular space is filled with flocculent material which corresponds to the oocyte jelly coat (Schroeder, 1981). The follicle cell has cytoplasmic extensions which penetrate this jelly layer and the oocyte vitelline layer to make contact with the oocyte plasma membrane (Meijer & Guerrier, 1984).

The hormonal mechanism has been studied in detail and has most recently been reviewed by Kanatani (1979), and Meijer and Guerrier (1984). Characteristically starfish remain stationary and raise the central disc when spawning. The arms can be seen to undergo repeated circular contractions from the tips to the gonopores. The responses observed in the animals can be broken down into three phases which make up spawning.

1.) Oocyte maturation, characterised by germinal vesicle breakdown (GVBD)

2.) Separation of the follicle cells from oocytes

3.) Behavioural changes and gamete release through contraction of the ovary.

Reinitiation of meiosis depends on at least three substances, each responsible for inducing the subsequent endocrine step (Meijer & Guerrier, 1984). First a neurosecretory product known as gonad stimulating substance (GSS) stimulates the

follicle cells to produce and release a second hormone. One of the initial discoveries demonstrating the hormonal nature of the system was that aqueous extract of radial nerve injected into the perivisceral cavity of one arm will elicit a spawning response (Chaet & McConnaughy, 1959). This response is not sex specific or species specific (Noumura & Kanatani, 1962; Chaet, 1966a, b; Kanatani, 1967b). Chaet (1966b) reported that high concentrations of the radial nerve extract inhibited the shedding of gametes. He explained this by the hypothesis that there was an inhibiting substance, shedhibin, present at a sub-threshold concentration. When radial nerves are assayed throughout the year, the inhibiting hormone is found only in animals with ripe gonads, while the shedding hormone is found continuously. This implies that the gamete shedding is primarily controlled by fluctuation in the concentration of the inhibiting substance (Chaet, 1966a). The time when a premature spawning is most likely, occurs as the gonads approach maturity. The high level of inhibitory substance would prevent spawning during this period. When the concentration of inhibiting substance begins to decline, the shedding hormone becomes increasingly effective. An anti-mitotic substance, the amino acid L-glutamic acid, (which prevents oogonial proliferation and oocyte growth) has been shown to be active earlier on in the breeding season (Ikegami *et al.*, 1967). This hormone would allow the gametes to become synchronised in development by arresting the release of new oocytes from the ovary.

A small quantity of the GSS from *Asterias forbesi* was partially purified by extracting dried nerves in 50% acetone, fractionation over CM and G-25 Sephadex columns and finally chromatography in butanol-H_2O-acetic acid. Between ten and eleven different amino acids were detected in the active fraction (Chaet, 1966b). It has also been partially purified from radial nerves of *Asterias amurensis* and found to be a peptide with a molecular weight of around 2000Da (Kanatani *et al* 1971). More recently its amino acid sequence has been partially established on a sample purified by an improved method (Shirai *et al.*, 1986).

Isolated oocytes without follicle cells do not undergo GVBD and complete maturation in the presence of GSS. They will however undergo GVBD and complete maturation in a medium in which ovaries had been incubated with GSS. This pointed to production of a secondary hormone released by the follicle cells. This was named meiosis inducing substance (MIS) and has been purified and identified as 1-methyladenine (Kanatani *et al.*, 1969). 1-methylyadenine has multiple sites of action in both male and female starfish of many species. It is believed to be responsible for synchronised spawning of mature gametes.

There is a certain amount of evidence that sexual pheromones are involved in coordination of spawning between males and females of the same species. Female echinoderms produce sperm attractants from the ovaries and eggs which are probably released into the water during spawning, (Miller, 1985 ; Ward *et al.*, 1985). Direct demonstration of a sex specific release of sperm attractant has been made for *Orthasterias koehleri* (Miller, 1989). The release of these sperm attracting substances with oocytes could induce animals downstream to start spawning, increasing the chance of successful fertilisations. This type of sperm attractant is not necessarily a stimulus for prespawning activity since aggregation is a slow process which precedes the initiation of spawning behaviour. It has been reported that individuals of *Marthasterias glacialis* aggregate several days prior to spawning (Minchin, 1987). The animals are dispersed when they are immature. The substance could however be effective over a wide distance if it was released over a long period of time, had a long half-life and was not subject to too much dilution by turbulent water flow. Miller (1989) found that the attractant is indeed long lived loosing about 50% of activity within two to four days and suggests that there is a possibility that another as yet unidentified substance could be released prior to spawning.

1.10 Oocyte Maturation in Starfish

During oocyte maturation the germinal vesicle breaks down, meiosis is reinitiated and a number of changes occur that are necessary for later stages of embryogenesis. Oocyte maturation and its control in starfish has provided a model for the study of maturational events and their endocrine control. Oocytes are arrested at the first prophase stage of meiosis and remain like this until immediately prior to spawning. When they are in this prophase of the first maturation division a clear germinal vesicle is visible in the centre of the oocyte as a light coloured circle under the light microscope. After maturation the germinal vesicle is broken down and the eggs assume a homogenous appearance . This happens in the ovary before the eggs are released (Meijer & Guerrier, 1984).

1-methyladenine is not retained in the follicle cells in advance of GSS stimulation which indicates that the action of GSS is not to stimulate release of stored 1-methyladenine. 1-methyl adenosine which is composed of the purine condensed with a pentose sugar, will produce effects similar to those of 1-methyladenine upon oocytes in the ovary, but has no effect at all on the maturation of isolated oocytes. 1-methyl adenosine is split into 1-methyladenine and ribose by an enzyme present in the ovarian tissue, 1-methyladenosine ribohydralase (Kanatai & Shirai, 1971). This suggests that the shedding hormone does not induce the formation of the enzyme, but instead may control the production of 1-methyladenosine by follicle cells, which is then split by the enzyme already present. This would also explain why when exogenous 1-methyladenine is applied to the ovaries it does not accumulate on the oocyte surface or within follicle cells (Toole *et al.*, 1974).

Incubation of isolated oocytes in 1-methyladenine will induce GVBD whereas microinjection of 1-methyladenine into an oocyte will not. Kanatani and Hiramoto (1970) concluded that 1-methyladenine therefore acts on the oocyte surface and not

directly on the germinal vesicle. Guerrier and Dorée (1975) have shown that oocytes need to be exposed to 1-methyladenine in the medium for a minimum contact time to ensure that GVBD takes place. This time was 4 minutes 30 seconds under experimental conditions. They assume that the 1-methyadenine binds instantly on to the receptor sites at the cell membrane but that it must remain bound for this critical period for GVBD to take place. After this initial time period the mechanism is triggered and nuclear maturation will occur without further requirement for 1-methyladenine. This period is called the hormone dependent period (HDP) and it is highly dependent on temperature but seems to be independent of the concentration of 1-methyladenine (Schuetz, 1969a; Guerrier *et al.*, 1976). 100% GVBD normally occurs at concentrations of 10^{-7}M and above.

The mechanism underlying the action of 1-methyladenine has been widely explored and many different changes occuring in the oocytes have been investigated. The only active structural analogues of 1-methyladenine are N1-substituted adenines. Methylglyoxal-bis(guanylhydrazone) diHCl (MGBG) a reversible inhibitor of S-adenosylmethionine decarboxylase has also been shown to induce meiosis reinitiation (Meijer and Guerrier, 1983). Experiments such as these help to describe the nature of the receptor sites for 1-methyladenine. Disulphide-reducing agents such as DTT and 2,3-dimercapto-1-propanol (BAL) will induce oocyte maturation (Kishimoto & Kanatani, 1973). These agents are believed to act in a similar way to 1-methyladenine because microinjection of DTT into the oocyte will not induce maturation. When cytoplasm taken from DTT treated oocytes undergoing maturation was injected into immature eggs they underwent GVBD. The sulphhydryl content of oocyte-cortex protein was found to have increased 10 minutes after 1-methyladenine administration and then it decreased before GVBD occured (Kishimoto *et al.*, 1976). The effect of 1-methyladenine in inducing oocyte maturation has therefore been ascribed to a disulphide reducing action.

The concentration of free calcium ions has a well established role in activation of cellular mechanisms. Dorée *et al.* (1978) found that 1-methyladenine, its active analogues and DTT triggered the release of free calcium ions from plasma membrane rich vesicles obtained from isolated cortices of intact oocytes *in vitro*. There was no significant release of calcium ions from either the endoplasmic reticulum or mitochondria. This agrees with previous studies which demonstrated the absence of any intracellular receptors for 1-methyladenine. Moreau *et al.* (1978a) managed to demonstrate the action of the hormone *in vivo* in *Marthasterias glacialis*. Release of free calcium ions takes place 1-2 seconds after application of 1-methyladenine to intact oocytes (Moreau *et al.*, 1978a). These findings support the view that the release of calcium from the plasma membrane into the cortical region of the oocyte is the first step in the sequence of events leading to metabolic activation and meiosis reinitiation.

A well established biochemical consequence of hormonal treatment well correlated with meiosis reinitiation is protein phosphorylation. Phosphate was incorporated into oocyte proteins of *Marthasterias glacialis* oocytes increased considerably, before GVBD on addition of 1-methyladenine (Guerrier *et al.* 1977). Protein phosphorylation was stimulated less than 2 minutes after hormone addition and much more extensively in the cortex of the oocyte than in the endoplasm, suggesting that phosphorylation of some cortical proteins might be a major event in meiosis reinitiation (Guerrier *et al.*, 1977).

In some species oocyte maturation can be induced by micromolar concentrations of a few fatty acids such as arachidonic and eicosapentaenoic acids or by nanomolar concentrations of hydroxyeicosatetraenoic acid (Meijer *et al.*, 1986). Arachidonic acid maturation has all characteristics of 1-methyladenine induced maturation (i.e. oocytes undergo GVBD, emit two polar bodies, become fertilisable) and perfectly normal larvae develop from the fertlised oocytes (Meijer *et al.*, 1984). The fatty acids could either mimic an event naturally occuring during 1-methyladenine-

induced maturation or act by a completely different mechanism such as an enzyme activator or an ionophore. The presence of bovine serum albumin (BSA) will inhibit the arachidonic induced but not 1-methyladenine induced maturation (Meijer *et al.*, 1986). This suggests that the arachidonic acid is not actually directly involved in 1-methyladenine action. Arachidonic acid also plays a significant role in sea urchin egg fertilisation (Perry, 1979; Epel *et al.*, 1982).

It has been observed also that there is a 10 to 30% decrease in the concentration of cyclic AMP (cAMP) after stimulation with 1-methyladenine. The drop in cAMP seems to be necessary for maturation induction but, unlike vertebrate oocytes, does not seem to be sufficient to trigger maturation (Meijer & Zarutski, 1987). Freeman and Ridgway, (1988) found that injection of cAMP into oocytes induced maturation in several species of hydrozoans.

In addition 1-methyladenine may have a role in ovarian contraction and other spawning behaviour. These responses can be induced by an injection of 1-methyladenine even if the starfish is spent. This implies that the hormone may have a wider effect than the one of oocyte maturation. It could act much more generally as a transmitter in the whole animal. Strictly speaking 1-methyladenine should not really be referred to as a hormone since it is not produced by a discrete organ and is not carried any distance in the body fluids. In effect 1-methyladenine is the means of interaction between the follicle cells and the oocytes, and acts as a link between intercellular and hormonal communication systems. Shirai *et al.* (1981) examined the direct effect of 1-methyladenine on ovarian contraction of *Asterias pectinifera* by setting up samples of the ovary wall on an isometric tentiometer. They found that administration of 1-methyladenine did not stimulate contraction. However when follicle cells and oocytes were present contraction was measured. Presence of the jelly components surrounding matured oocytes also caused contraction. It is therefore suggested that

confirm that the germinal vesicle is involved in the production of MPF in starfish oocytes. Recent work by Hoshi *et al.* (1992) has shown that a specific protein, called the G protein is present in the starfish oocyte plasma membrane. They have concluded that this protein is functionally coupled with the 1-methyladenine receptor sites and acts as a tranducer of the signal for MPF to be produced.

1.11 Oocyte Maturation in *Arenicola marina*

Pirlot (1933) stated definitely that the coelomic gametes are not viable until they have passed through the nephridia. He tried, unsuccessfully, to set up artificial fertilisations with the coelomic gametes. These observations were confirmed by Howie (1961b), who examined the relationship between the maturation and the spawning of the oocytes. By experimenting with coelomic oocytes of gravid, spent and partially spent worms he established that maturation takes place in the coelom, immediately prior to spawning. Howie (1961b) identified changes in the shape of the oocyte and in the location of the nuclear area that occurred during oocyte maturation. He also describes the appearance of metaphase figures in serial sections of the spawned oocytes which shows that meiosis had been reinitiated. The oocytes are arrested in the first prophase stage of meiosis in coelomic cavity until oocyte maturation takes place (Meijer & Durchon, 1977). Meijer (1979a) also carried out a morphological study of the changes that take place in the oocyte during maturation. He described morphological changes at four levels: the oocyte becomes more spherical in shape and the prodruding microvilli withdraw, leaving the external surface of the vitelline membrane smooth (the withdrawal of the microvilli enables the plasma membrane to separate from the vitelline membrane which is essential for the formation of a fertilisation membrane); the distribution of the cortical granules is also altered during maturation (they move away from the nuclear regions and the plasma membrane); the

nuclear envelope breaks down; the formation of a spindle can be seen after about 45 minutes.

Meijer and Durchon (1977) investigated oocyte maturation *in vitro* by incubating immature eggs from the coelomic cavity of gravid female worms with extract of prostomia from host animals in a similar reproductive condition. They found that at a concentration of 0.05 pr.ml^{-1}, all of the oocytes became mature after 30 minutes incubation. The minimum concentration at which maturation was induced was between 0.005 and 0.0025 pr.ml^{-1} , although at these lower doses the response took longer to occur (see Section 5.1). Simple microscopic observation was used to determine the percentage of germinal vesicle breakdown (%GVBD). Meijer (1980) induced maturation artificially by using drugs known to act on the calcium permeability of the membrane and modify the concentration of intracellular free calcium. Disulphide reducing agents, dithiothreitol and 2,3-dimercaptopropanol were also shown to induce oocyte maturation and it is suggested that -SH groups are important in meiosis reinitiation. There are similarities between the proposed mechanism of the oocyte maturation factor in *Arenicola* and the action of 1-methyladenine in starfish. The release of free calcium ions has been demonstrated to take place after application of 1-methyladenine to oocytes both *in vitro* (Dorée *et al.*, 1978) and *in vivo* (Moreau *et al.*, 1978). Disulphide reducing agents also induce oocyte maturation in starfish (Kishimoto & Kanatani, 1973). It is possible that the oocyte maturation factor in *Arenicola* acts in a similar way as 1-methyladenine does on starfish oocytes possibly by producing a maturation promoting factor which acts intracellularly on the germinal vesicle (Kanatani, 1970).

1.12 Aims of the Thesis

Arenicola is a genus which is very widely distributed and has members present on sandy shores worldwide (Ashworth, 1904). In Britain the most abundant species is *Arenicola marina* (Wells, 1957). Estimates from certain areas of the Wadden Sea suggest that as much as 20 to 30% of the whole benthic biomass may be made up of *Arenicola marina* (Beukema, 1974). Relatively little information is available on the general biology of the genus, for example a new species of *Arenicola, A. defodiens* has been described only this year (Cadman & Nelson-Smith, 1993). Numerous questions relating to the biology of *Arenicola* still remain unanswered. Differences in size, annulation pattern and colour of individuals have been observed to exsist between populations of *Arenicola marina* from different locations (see Sections 1.5). The timing and patterns of the spawning season also show considerable variation between populations (see Section 1.6). These differences may be caused by the effect of variable environmental factors present at each site or they may have a genetic origin.

Progress has been made in the study of the endocrine control of gamete maturation and spawning in male *A. marina* (Howie, 1961a,c, 1963, 1966; Bentley, 1985; Bentley *et al.*, 1990; Pacey & Bentley, 1992). There is evidence that the reproductive endocrine substances are not the same in males and females (Howie, 1963; Pacey & Bentley, 1992) but comparatively little is known about endocrine control of gamete maturation and spawning in female *A. marina.* A certain amount of confusion has arisen in the literature regarding the control of reproduction in female *Arenicola marina.* Meijer & Durchon (1977) describe oocyte maturation taking place *in vitro* in the presence of prostomial homogenate of *A. marina.* Meijer (1978) reported that this *in vitro* response of the oocytes did not occur in all populations. This implies that either, differences between populations extend to the physiological level or that Meijer was looking at more than one species. This thesis, therefore, contributes in

several ways to the information available on *Arenicola marina*. The principal objectives of this thesis, therefore, were:

1) To carry out an extensive study of the reproductive cycle at the level of the individual, in populations of *A. marina* from different locations. This would be carried out by collecting samples of *Arenicola* from specific locations on a regular basis. The information gained by this would not only establish spawning patterns for the population at each site but also describe gametogenesis in individuals from each population throughout the year.

2) To test for a degree of genetic variation within and beween populations of *Arenicola marina* from different localities, using starch gel electrophoresis. The results would provide information concerning the origin of observed differences between populations at different sites.

3) To establish whether physiological differences do exsist between populations of *Arenicola marina* and also to investigate the mechanism for controlling oocyte maturation in *A. marina*. This would be investigated by comparing oocyte maturation in *A. defodiens* and in *A. marina*.

4) To investigate the nature of reproductive endocrine substances present in the female prostomium.

Chapter 2

Materials and Methods

Materials and Methods

This chapter describes the definitive experimental procedures carried out during this research. The development of particular methodologies is described in the relevant experimental chapters.

2.1 Collection and Maintenance of *Arenicola marina*

Animals were removed from the sand at low water of spring tides by digging between the head hole and tail cast with a garden fork. The worms were normally found at a depth of 20 to 30cm. The animals were transported from the beach in a bucket containing a little sea water. When samples were collected from further afield the animals were transferred to large shallow plastic trays half full of wet sand for transport by car. In the summer months two freezer blocks were put at either end of the tray to maintain the temperature below ambient.

The animals were kept in large flat plastic trays with sand and filtered seawater. These were maintained in a cold room with conditions of constant illumination and constant temperature (7°C). The seawater and sand were changed twice weekly and the trays were washed at the same time. The water was changed daily for the first four days initially because of mortality. If the animals were required for electrophoresis they were left in seawater overnight to allow the gut contents to be expelled. During the period when the animals were mature, sexes were segregated to avoid possible inter-sexual pheromonal signals.

2.2 Assessment of the State of Maturity of the Animals

Samples of coelomic fluid were removed routinely from the worms with a 1ml disposable syringe and a 25g hypodermic needle. The needle was inserted into the

coelomic cavity on the dorsal side of the last 'trunk' segment and a volume of about 50μl of coelomic fluid was slowly withdrawn.

2.2.1 Males

Samples of coelomic fluid were examined under the light microscope and the number of spermatocyte clusters and morulae was counted from five fields of view. The mean percentage of sperm present as morulae was then calculated.

2.2.2 Females

The diameters of forty oocytes were measured using a graduated eye piece, calibrated with the use of a micrometer slide.

2.3 Preparation of Prostomial Homogenate

The animals were held down with forceps on either side of the nuchal groove which forces the prostomium to protrude. Excision was carried out using iridectomy scissors. Providing that the operation is carried out quickly and none of the pharynx is removed, there is a high survival rate. The prostomia were then placed in a volume of 1ml of distilled water and kept on ice. The tissue was homogenised by sonication in a MSE Soniprep 150 ultrasonic disintegrator.

2.4 Organic Extraction of Prostomial Homogenate

The organic and aqueous fractions of the prostomial homogenate were separated using a modified Folch method (Christie, 1982; Bentley *et.al.*, 1990). The volume of the homogenate was adjusted to between 1ml and 5ml with distilled water.

An equal amount of petroleum ether was added and the tube was shaken using a Whirlimixer. The upper organic layer was carefully removed using a pipette. This step was then repeated and the fractions were pooled. The organic fraction was then washed, by the addition of 0.88% potassium chloride and the mixture was shaken, then left to settle. The potassium chloride serves to increase the polarity of the aqueous fraction. This ensures complete partition of the less hydrophilic molecules. The aqueous layer was removed and added to the other aqueous fractions, this step was then repeated. The organic layer was dried by the addition of anhydrous sodium sulphite. Both fractions were dried down using a Genevac SF50 spin freezer as a centrifugal concentrator. The contents of the tubes was then resuspended in TFSW, and each tube was placed in an ultrasonicator at 9 x 10^4 c.p.s. for 10 seconds to remove any material which may have adhered to the tube. Each fraction could then be assayed. If further purification was to take place in the aqueous phase then the extract was resuspended in distilled water.

2.5 Partial Purification of the Aqueous Phase of the Homogenate

The homogenate was passed through a sterile Millex-GS filter unit (0.22μm). A Sep-pak C-18 cartridge was used for solid phase extraction. The cartridge was 'wet' with 20 ml of methanol and then washed with 20ml of distilled water. The filtrate of the prostomial homogenate was added. The cartridge was then washed through with 1ml each of water, 30% methanol, 70% methanol, petroleum ether and finally 100% methanol. Each washing was collected in disposable fraction collecting tubes and dried down using the Genevac SF50 spin freezer. Substances present in the tubes were resuspended and ultrasonicated as in Section 2.6. The solutions were then assayed for biological activity as described in the following section.

2.6 Spawning Induction

Gravid worms were placed in 200ml of filtered seawater in individual plastic pots. A volume of 250μl of the substance to be assayed was injected into the coelomic cavity of each worm. The worms were placed in a room with a constant temperature of 7°C. The worms were then examined at hourly intervals for the presence of gametes in the seawater. The number of gametes spawned per ml of seawater was then estimated. For the females the number of eggs present was estimated from three 1ml sub-samples that were taken from the seawater in the plastic pots after the contents had been stirred. The eggs were placed in a streak on a slide and counted using a handcounter and light microsope. The mean of the three counts was taken.

2.7 Bioassay for Sperm Activating Substances

The bioassay for sperm maturation factor originally used by Bentley (1985) has been modified for use here. It was carried out in round bottom multi-well plates. 5μl of sperm were incubated with 200μl of TFSW or an asssay sample resuspended in TFSW. Plates were either examined under the light microscope or read using a Dynatech MR5000 plate reader. The agglutination programme was modified in an attempt to standardise the response. In order to determine the range of concentrations over which the substance is active the assay is carried out in a series of double dilutions.

2.8 Oocyte Maturation Assay

The *in vitro* assay for testing for oocyte maturation inducing factors has previously been described by Meijer and Durchon (1977). The incubations were

carried out in multi well plates with a total volume of 1ml in each well. A range of concentrations of each treatment were assayed. The plates were incubated for 1hour at a temperature of 10°C. 200μl from each well were then examined under a light microscope under a coverslip. The percentage of GVBD was then estimated from three fields of view.

2.9 High Performance Liquid Chromatography (HPLC)

HPLC was carried out on a Perkin-Elmer analytical system which consisted of a pump (Series 410 LC), a UV spectrophotometric detector (LC-90) and a laboratory computing integrator (LCI-100). The column used was a C-18 reversed phase column (750 x 4.2 mm, particle size 10μm). The filtered aqueous phase of the prostomial extract was concentrated down to a volume of 200μl to load into the HPLC column. The solvent used was methanol with 0.1% TFA. Absorbance was measured at a wavelength of 214nm.

2.10 Starch Gel Electrophoresis

2.10.1 Preparation of the Gel

Gels were prepared with 11.5% starch (Sigma S-4051) in a 9.6mM tris-citrate gel buffer. The mixture was heated in a stoppered Buchner flask until it became thick and a few bubbles appeared in the bottom of the flask. The flask was then immediately degassed using a vacuum pump until all the large bubbles had disappeared. It was poured into a former between two glass plates and allowed to cool. Towards the end of the cooling it was placed in a refrigerator.

2.10.2 Preparation of the Sample.

The gut was dissected from *Arenicola* which had been maintained in seawater overnight and a piece of the gut wall about 5 x 10mm was removed. This was placed in 50μl of homogenising buffer in a grinding tray held on ice. The tissue was then homogenised by manual grinding with a glass rod. A small amount of sand was added to aid in this process. The homogenising buffer is that used by Cadman (1990). Small circles of sterile tissue paper were placed over the top of each well and then the wicks were placed on top of these. The tissue acts as a filter and stops the absorption of too much mucus by the wicks. Wicks (5mm wide) were cut from Whatman no.3 filter paper and left in the wells until soaked in the sample.

2.10.3 Loading and Running the Gel

The wicks were blotted once on each side with sterile tissue and loaded into small slots that had been made in the gel. The gels were run using a Shandon apparatus with constant voltage (180mV) D.C. power supplies (Raven E443 & Heathkit IP-2717). A constant current of 40mA for 5 hours was supplied giving a total of about 36Wh. The gels were run at a constant temperature of 4°C in a refrigerator.

2.10.4 Enzyme Assay

Following electrophoresis, the gel was sliced horizontally into 1.5mm thick slices. The slices were separated by floating them in water which was kept at a temperature of 4°C. Enzyme staining methods were based upon those of Shaw and

Prasad (1970), and Harris and Hopkinson (1976). A number of different enzyme systems were stained for and further details will be given in Chapter 4.

Chapter 3

Studies on Gametogenesis at three Different Localities

Studies on Gametogenesis at three Different Localities

3.1 Introduction

Populations of *Arenicola marina* at different geographic locations have varying lengths and timings of the spawning season. This is apparent from the many conflicting reports in the literature (see Section 1.6). Howie (1984) identified two different ways in which spawning may take place. Some populations have been shown to have an epidemic type spawning which lasts for two or three days (Pirlot, 1933; Howie 1959), whereas in other populations the spawning season may be extended or non-epidemic, lasting for two or three weeks (see Page 14). It appears that the majority of populations spawn during autumn although there have been some reports of spring spawnings (Gamble & Ashworth, 1898; Howie, 1959; Brenning, 1965). Gametogenesis is linked directly to the timing of the spawning season and synchronised spawning can only be achieved if individuals within that population reach maturity at the same time. The role each individual plays is therefore important because the environmental and endocrinological factors must initially act at the level of the individual. The aim of this study was to examine gamete development in relation to spawning activity in individual worms throughout the year at three different geographic locations: Fairlie Sands, Firth of Clyde; The Eden Estuary; and the East Sands, St. Andrews, Fife (see Fig. 3.1).

Fairlie Sands was selected because it it has been reported previously to contain a spring breeding contingent (Howie, 1959). Although there have been several reports in the literature of other spring breeding populations such as at Belfast (Duncan, 1960) and in the Baltic (Brenning, 1965) these populations are exceptional as most other reports are of an autumn breeding season (Cunningham & Ramage, 1888; Pirlott, 1933; Newell, 1948; Howie, 1959; Duncan, 1960; Cazaux, 1966; Farke & Berghuis, 1979). The Eden Estuary has been used previously for obtaining

Arenicola for studies at the Gatty Marine Laboratory but there are no records of the timing of the reproductive season or spawning in the literature. There are however several reports in the literature relating to the East Sands and they do do not all agree on the timing of the spawning season. Kyle (1896) believed it to occur between January and March and again between June and September. This site was also studied by Howie (1959) and he reports that spawning occurs over a period of two or three days only in the second week in October. Duncan (1960) notes St. Andrews as having a November spawning. Bentley and Pacey (1992) have observed in recent years that the worms show a highly synchronised breeding season, and spawn over a three day crisis period which coincides with the the second set of spring tides in October. These three sites are described further below. Continuous sampling of gametes from animals from these three populations throughout the year should enable a clear picture of gametogenesis to be built up and confirm any differences in the timing or length of the spawning season.

3.1.1 Description of the Study Sites

Site 1 : Fairlie Sands, Strathclyde.

Fairlie Sands is a beach just south of Largs on the Firth of Clyde, Grid reference, Latitude 55° 45.5', Longitude 5° 50.2' (OS - NS547217). The Islands of Great Cumbrae and Arran offer some shelter from prevailing westerly winds. The beach can be regarded as two halves separated at low tide by a physical barrier of a fresh water stream running down the middle (see Fig. 3.2). There is also a sewage pipe close to the stream. The substratum consists mainly of pebbles and shale interspersed with sandy patches where the *Arenicola* occur. Large areas of the beach are covered with algal mats and *Fucus* spp. Considerable changes have occured to the beach over the three years of this study. A much larger area is now covered by

mussels (*Mytilus edulis*) and at low tide less of the beach is exposed. The beach has also been under considerable pressure from bait diggers. This activity has brought shell debris to the surface which has offered attachment points for macroalgae and mussels.

Site 2 Eden Estuary, St. Andrews, Fife

The estuary is situated just to the north of St. Andrews, Grid reference, Latitude 56° 22', Longitude 2° 49.5' (OS - NO194493). It is an ecologically important area due to the large populations of wild ducks, geese and waders which visit and overwinter in the area. It has been declared a Site of Special Scientific Interest by the Nature Conservancy Council (now Scottish Natural Heritage) and has also been designated a local Nature Reserve, managed by Fife Regional Council. Several surveys have been carried out in the area (Johnston *et al.*, 1978; Johnston *et al.*, 1979) which describe the invertebrate fauna present and the feeding distribution and movements of shorebirds.

There is a paper mill at Guardbridge two miles upstream from the mouth of the estuary and around this area the sediment is a very fine mud. Further down towards the mouth the sediment becomes more sandy. Only a few areas are suitable for colonisation by *Arenicola marina* which cannot build burrows in very fine sediments (Longbottom 1970a). The study site is located on the south side of the estuary close to the mouth where *Arenicola* are found in large numbers (see Fig. 3.3). The site is very sheltered and worms occur up the shore to above the level of low water neap tides.

Site 3 East Sands, St. Andrews, Fife.

This is an east facing beach immediately adjacent to the Gatty Marine Laboratory, Grid reference, Latitude 56° 20.5', Longitude 2° 46.5' (OS - NO191525). There is some protection from north easterly gales due to a harbour wall at the northern end but the beach is very exposed to easterly winds. The substratum consists mainly of sand particles and although it is stable in the summer, it is subject to considerable movement in the winter months. At the southern end of the beach there is a rocky outcrop. *Arenicola marina* occur at the low water of spring tides level half way along the beach (see Fig. 3.4). There is also a bed of juvenile worms further up the shore. It is less than 5km distant from the Eden Estuary.

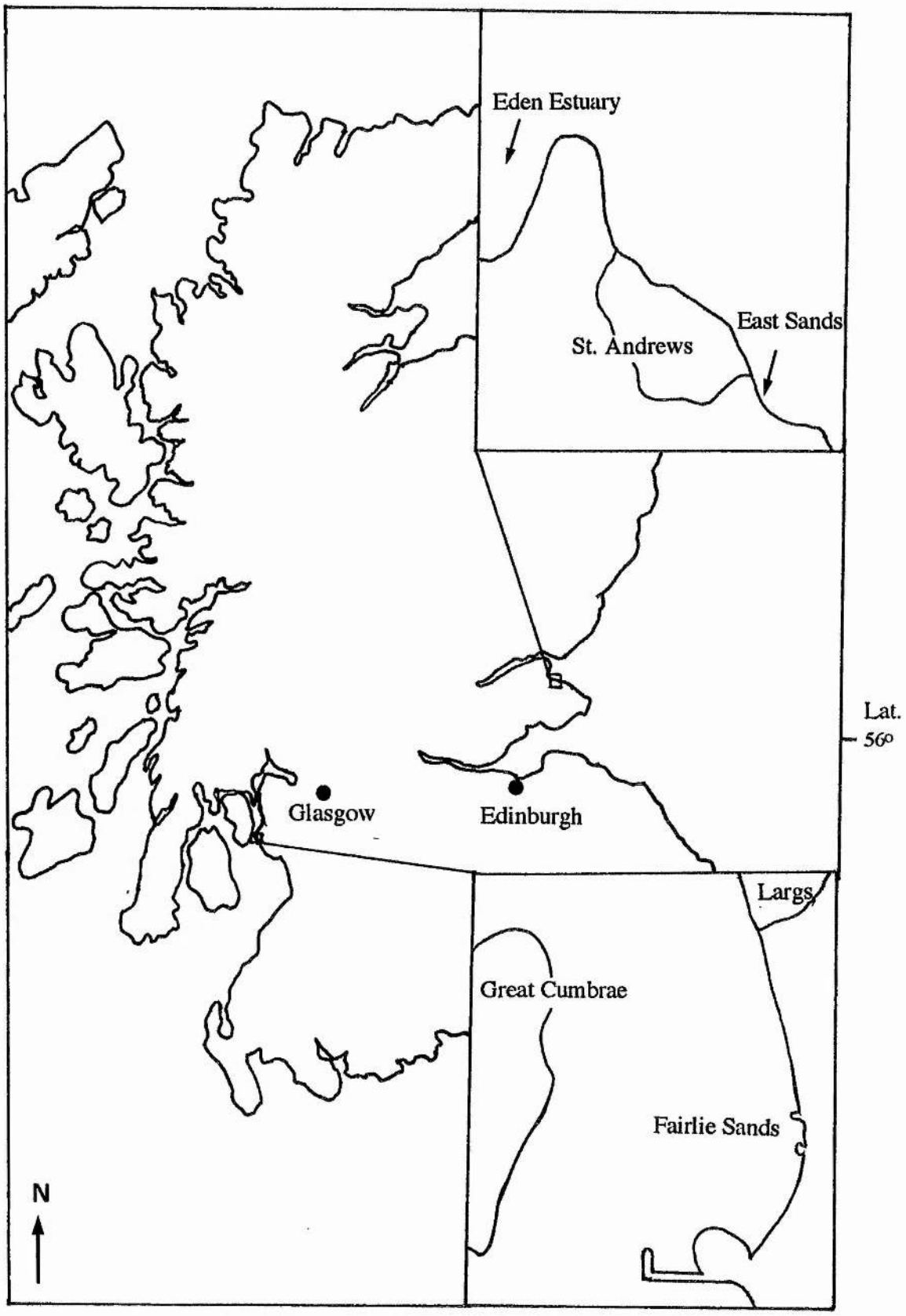


Fig. 3.1 Map of Scotland to show the location of the three sites selected for study in this investigation

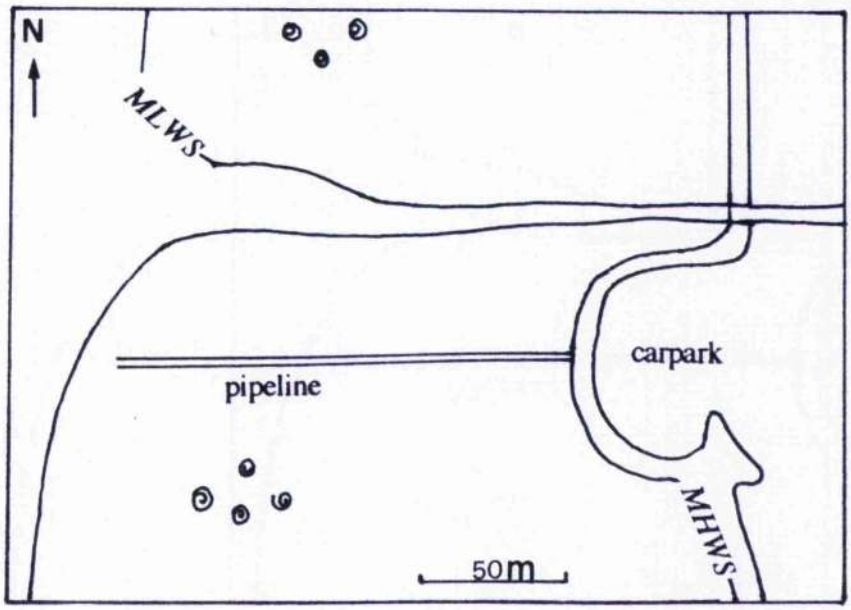


Fig. 3.2 Map of the beach at Fairlie Sands to show the area where *Arenicola* (ᘯ) were collected.

Fig. 3.2.1 Photograph of the beach at Fairlie Sands.

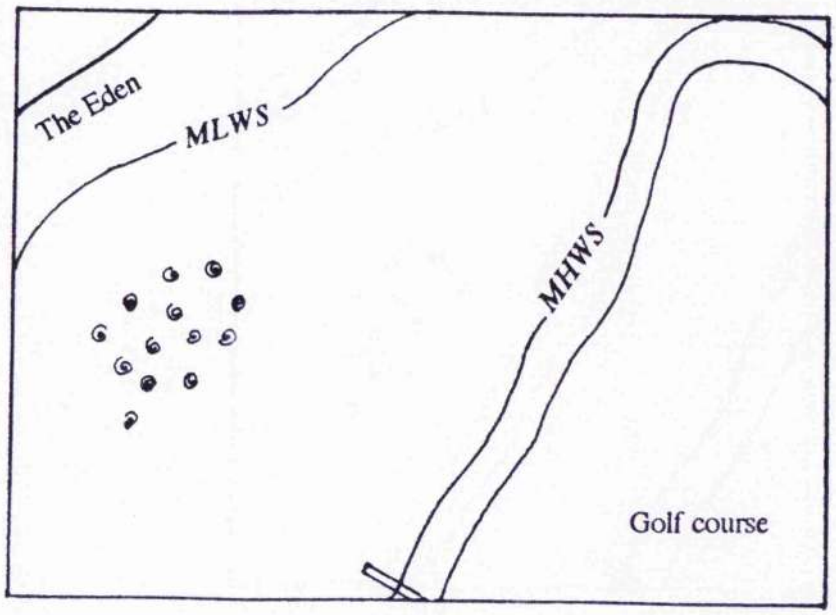


Fig. 3.3 Map of the beach at the Eden Estuary to show the area where *Arenicola* (●) were collected.

Fig.3.3.1 Photograph of the beach at the Eden Estuary.

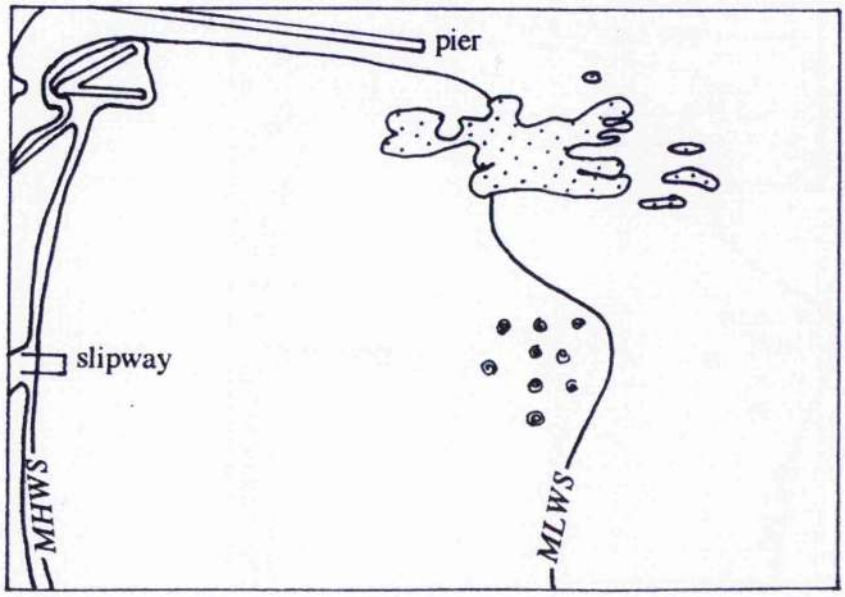


Fig. 3.4 Map of the beach at the East Sands to show the area where *Arenicola* (●) were collected. The shaded area represents rocks.

Fig. 3.4.1 Photograph of the beach at the East Sands.

3.2 Materials and Methods

3.2.1 Collection and Examination of Samples

About fifty individuals were collected at low water of the sping tides each month from each of the three study sites. 50 μl samples of coelomic fluid were removed from each worm and examined under a light microscope (see Section 2.4).

3.2.2 Data Presentation and Analysis

The mean percentage of sperm present as spermatocytes and morulae was calculated from the five fields of view for each individual. The mean percentage for the whole population was then calculated. Forty oocytes were measured from each female in the sample to provide the mean oocyte diameter, and maximum and minimum diameters. From this data the percentage of oocytes present in previtellogenic and vitellogenic stages were calculated for each individual. The oocytes start to become vitellogenic when they reach the size of about 70μm in diameter (Howie, 1984). The mean percentage of oocytes present in the vitellogenic state for the population was then calculated from this. Where two sets of data, for the same month, from different years had been obtained they were tested using the Kolmogorov-Smirnov two sample test, which tests differences in the distribution of two samples of continuous observations. If the observed critical value was less than the expected critical value the data sets were pooled.

3.3 Results

3.3.1 The Timing of Gametogenesis at the Three Different Study Sites.

January (1991)

In January at Fairlie Sands it can be seen from the pie chart of pooled data for the proportions of types of gametes present in males and females that about 9% of all the gametes are in the final stages of development (Fig. 3.5.1). There is one male and one female approaching maturity which represents approximately 10% of the population (Figs. 3.5.2. & 3.5.3) Most individual females have a small range of oocyte diameters with the mean between 20μm and 30μm and the rest of the males have 100% of gametes as spermatocyte clusters.

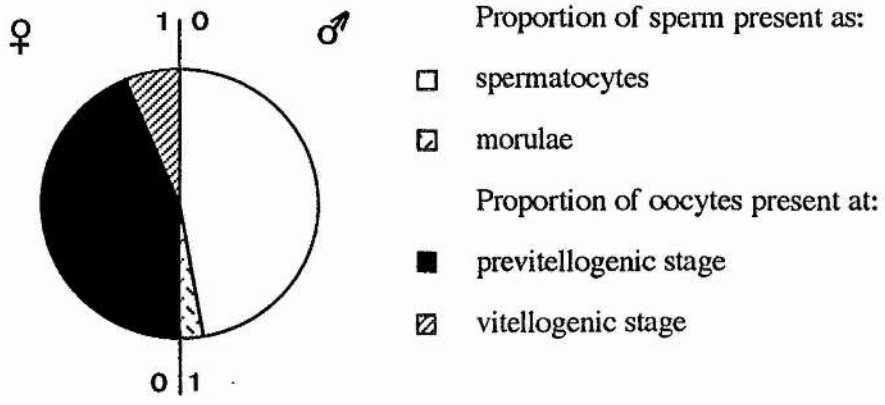
The pie chart for the Eden Estuary in January shows that all of the gametes are in very early stages of development with no vitellogenic oocytes or morulae present (Fig. 3.6.1). The means oocyte diameter and ranges of oocyte diameter in individual females are small and all of the males have 100% spematocytes present (Figs. 3.6.2 & 3.6.3).

No data are presented for the East Sands in January as the worms were found not to contain any gametes in their coelomic fluid. This suggests that production of gametes is initiated later in animals from this site.

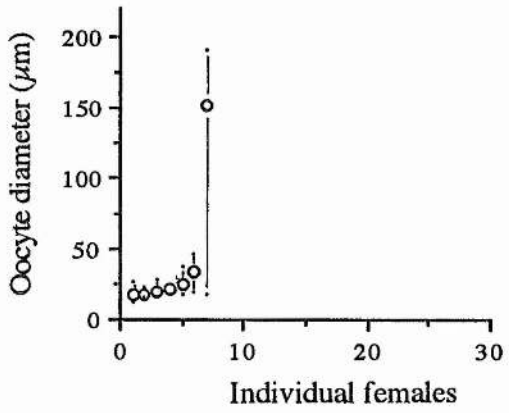
Fig. 3.5 The reproductive state of *Arenicola marina* at Fairlie Sands in January.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

January - Fairlie Sands (1991)

a)



b)



c)

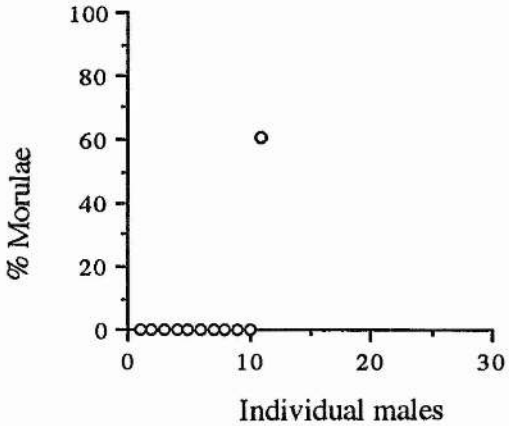
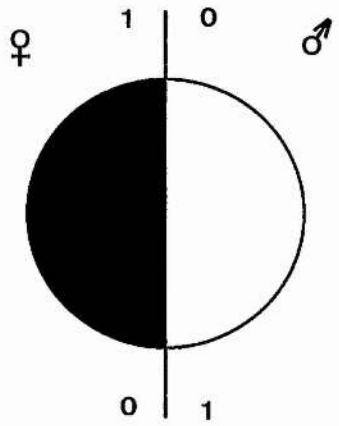


Fig. 3.6 The reproductive state of *Arenicola marina* at Eden Estuary in January.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

January - Eden Estuary (1991)

a)



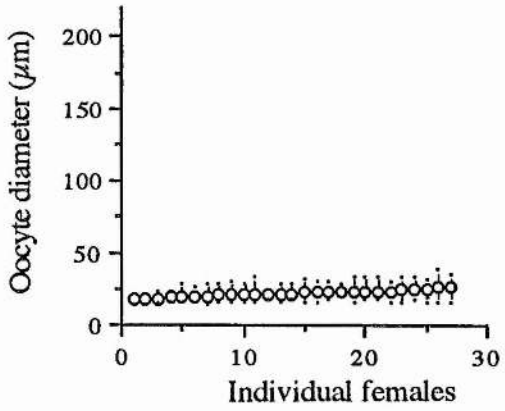
Proportion of sperm present as:

- spermatocytes
- morulae

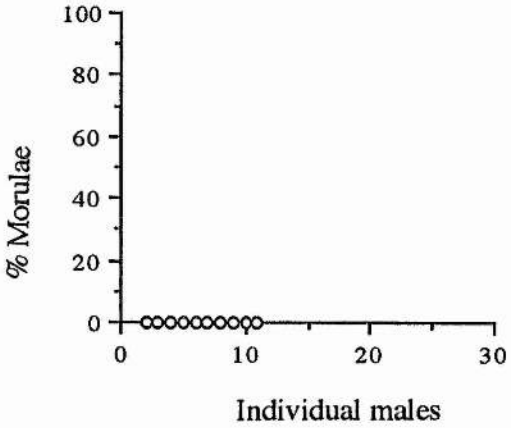
Proportion of oocytes present at:

- previtellogenic stage
- vitellogenic stage

b)



c)



February (Fairlie Sands, 1991; Eden Estuary, 1992)

In February at Fairlie Sands the percentage of gametes present in the final developmental stage has increased to 20% (Fig. 3.7.1). There are slightly more oocytes in the vitellogenic phase than sperm present as morulae but the rates of development of the oocytes and spermatocytes are not directly comparable. Figure 3.7.2 shows that there are females at all stages of reproductive development present. This pattern is also seen in Figure 3.7.3 which shows a range of percentages of sperm present as morulae.

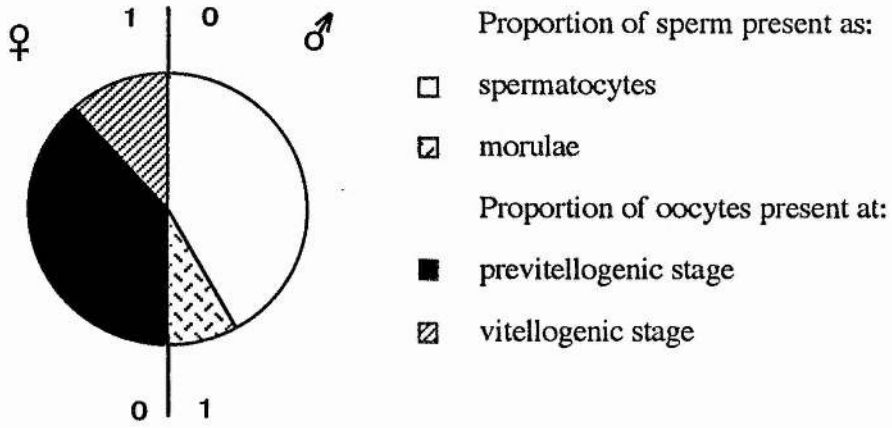
At the Eden Estuary in February a few of the oocytes have entered vitellogenesis but most of the gametes remain in early stages of development (Fig. 3.8.1). The most noticeable difference between February and January is that the upper range of oocyte diameters has slightly increased in individuals (Fig. 3.8.2). The sperm are still all present as spermatocytes (Fig. 3.8.3).

Again no data are presented from the East Sands for this month because only nine individuals out of twenty four worms contained any gametes. The oocytes in the females were few in number and all definitely previtellogenic. Similarly in the males there were only a few very small spermatocytes present in the coelomic fluid.

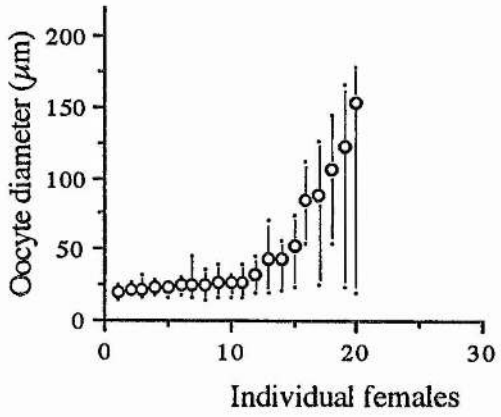
Fig. 3.7 The reproductive state of *Arenicola marina* at Fairlie Sands in February.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

February - Fairlie Sands (1991)

a)



b)



c)

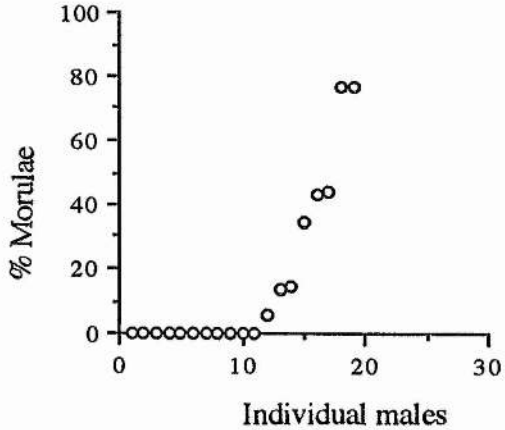
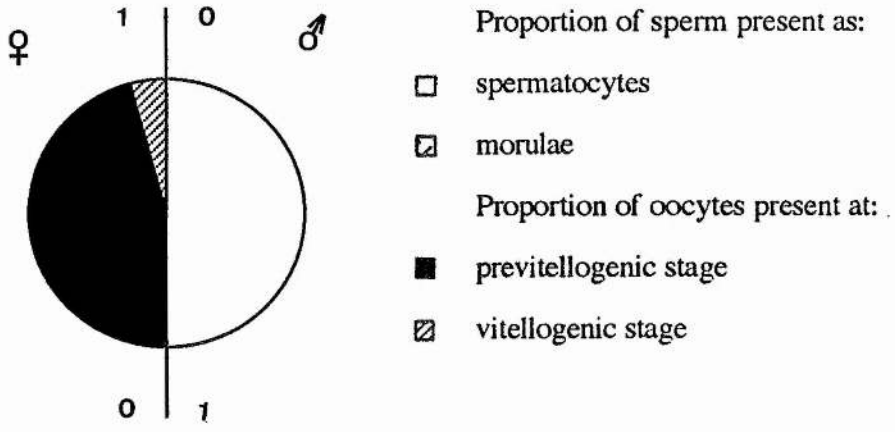


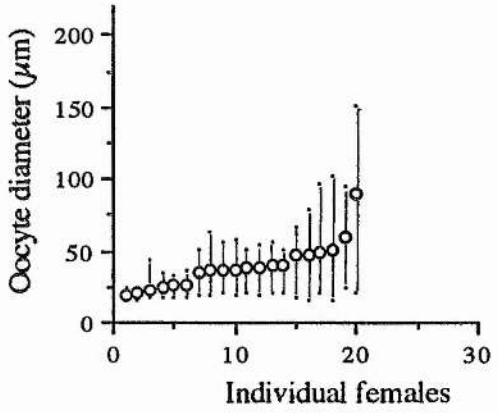
Fig. 3.8 The reproductive state of *Arenicola marina* at Eden Estuary in February.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

February - Eden Estuary (1992)

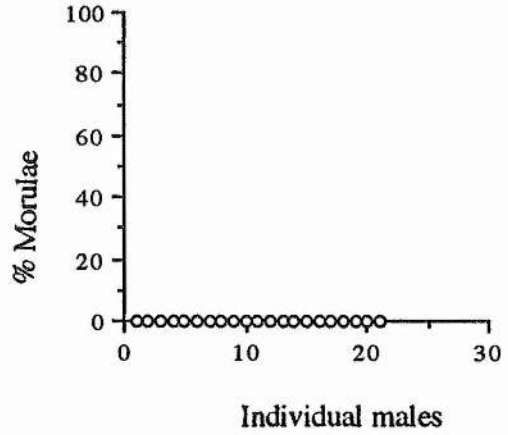
a)



b)



c)



March (1992)

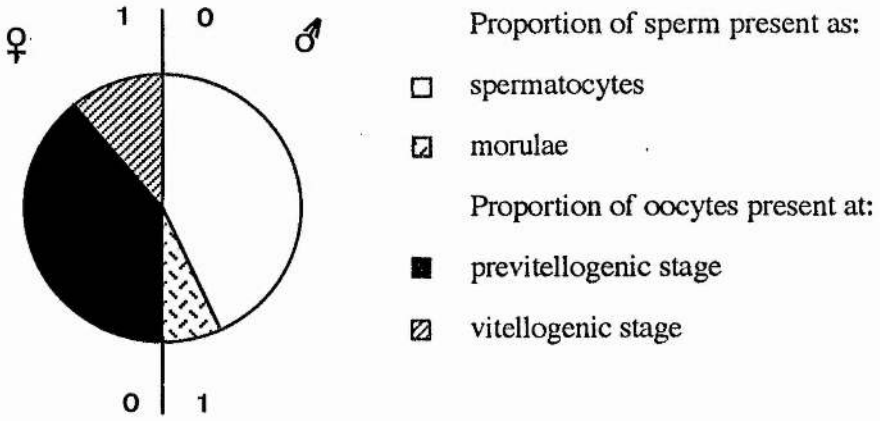
The pie chart for Fairlie Sands in March shows that a proportion of the gametes within the population are approaching maturity (Fig. 3.9.1). This results from six females and nine males which are present in the sample and which are nearly ready for spawning. The graphs showing the mean and ranges of oocyte diameter and the percentage of sperm present as morulae in individuals are similar to those shown for February with a wide range of developmental stages of gametes present in the population (Figs. 3.9.2 & 3.9.3). A greater proportion of the females collected are approaching maturity compared to the proportion of males with sperm present as morulae.

The pie chart showing the state of development of the gametes in worms from the Eden Estuary in March has not changed much since February (Fig. 3.10.1). Some of the oocytes are starting to grow but the majority remain previtellogenic (Fig. 3.10.2). The sperm are still all present as spermatocytes (Fig. 3.10.3). Data from the East Sands are presented and they show that all gametes are at very early stages of development, that is 100% immature (Fig. 3.11.1). The distribution of means and ranges of oocyte diameters and of the percentage of sperm present as morulae at the East Sands resembles that of the Eden Estuary in January (Figs. 3.11.2 & 3.11.3).

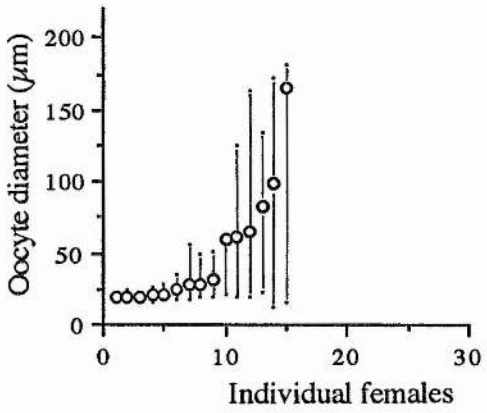
Fig. 3.9 The reproductive state of *Arenicola marina* at Fairlie Sands in March.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

March - Fairlie Sands (1992)

a)



b)



c)

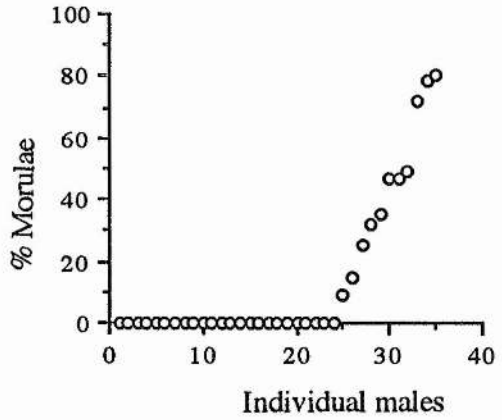
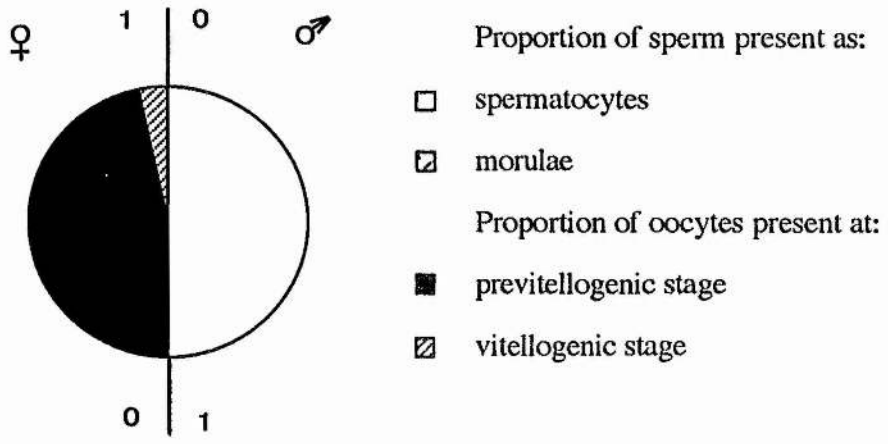


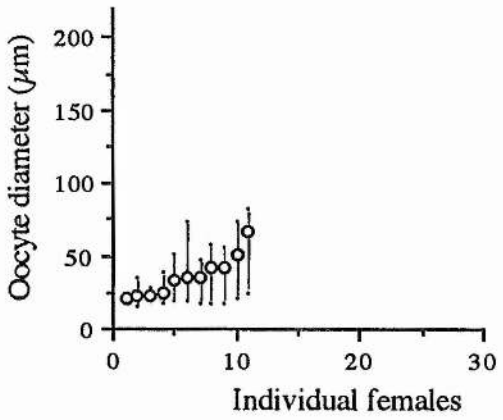
Fig. 3.10 The reproductive state of *Arenicola marina* at Eden Estuary in March.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

March - Eden Estuary (1992)

a)



b)



c)

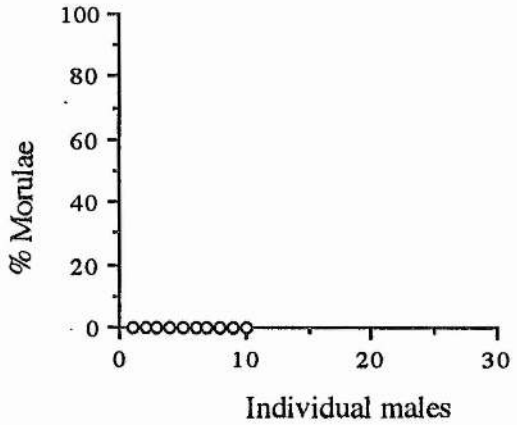
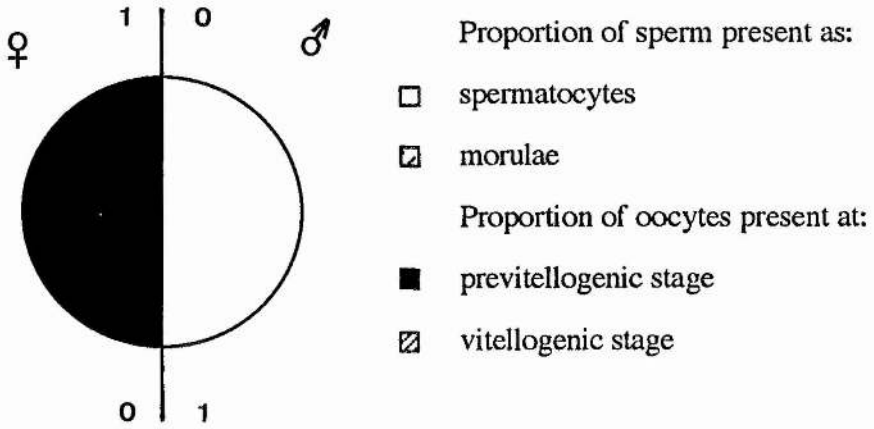


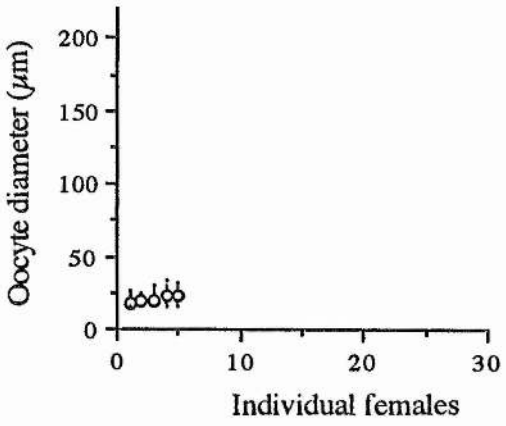
Fig. 3.11 The reproductive state of *Arenicola marina* at East Sands in March.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

March - East Sands (1992)

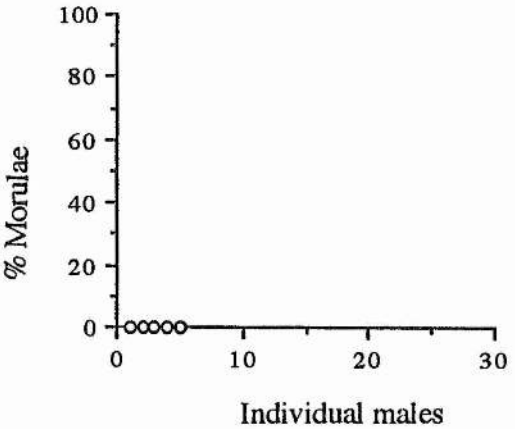
a)



b)



c)



April (Fairlie Sands, 1991 & 1992; Eden Estuary & East Sands, 1991)

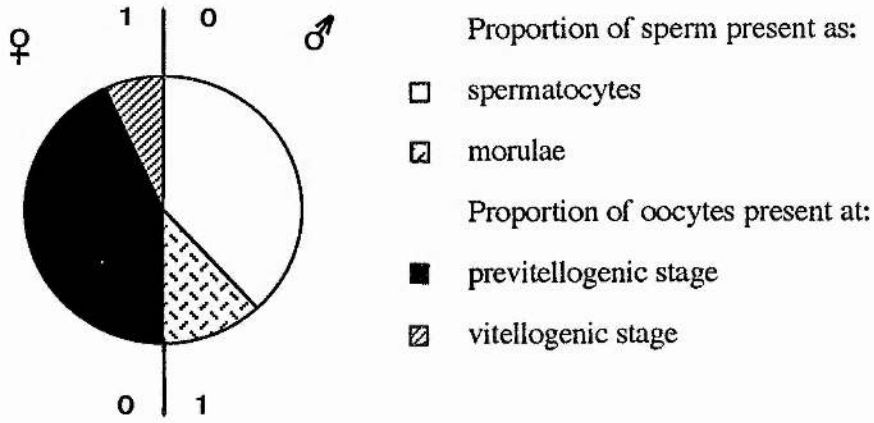
The overall proportion of gametes present in the final stages of development at Fairlie Sands remains about the same as that observed in March (Fig. 3.12.1). On the pie chart it looks as if the greatest percentage of mature gametes are from the males which is opposite to the situation in March. This may have resulted from spawning by some of the females as it was noted that two of the females examined were spent. A similar number of males and females are approaching spawning and this represents about 7% of the total population (Figs. 3.12.2 & 3.12.3). At this stage the two cohorts are easily distinguishable, particularly in Figure 3.12.3 where the mature males can be seen to have 100% sperm present as morulae. This implies that spawning in some individuals may be imminent.

The pie chart presenting data about the development of the gametes in April at the Eden Estuary shows that the proportion of oocytes that have entered vitellogenesis has nearly doubled since last month (Fig. 3.13.1). The males, however, remain in a similar stage of development to last month, with all sperm present as spermatocytes (Fig. 3.13.3). The proportion of the population that was observed last month to be developing gametes faster than the rest are continuing to follow the same trend (Fig. 3.13.2). The remaining proportion of the population continue to have a small mean oocyte diameter, with the range of oocyte diameters being close to the mean. Figure 3.14.1 shows that all of the male and female gametes examined during this month from the East Sands were still in early stages of development. Mean oocyte diameters are small (Fig. 3.14.2) and no spermatocytes have developed into morulae yet (Fig. 3.14.3).

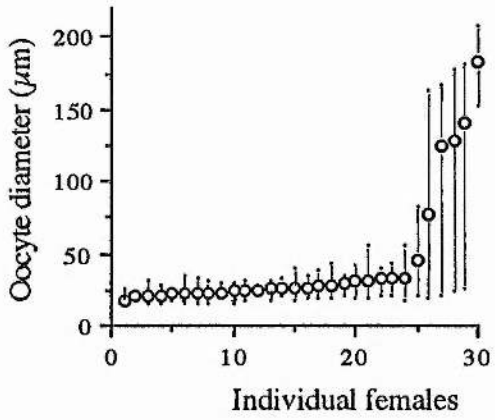
Fig. 3.12 The reproductive state of *Arenicola marina* at Fairlie Sands in April.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

April - Fairlie Sands (1991 & 1992)

a)



b)



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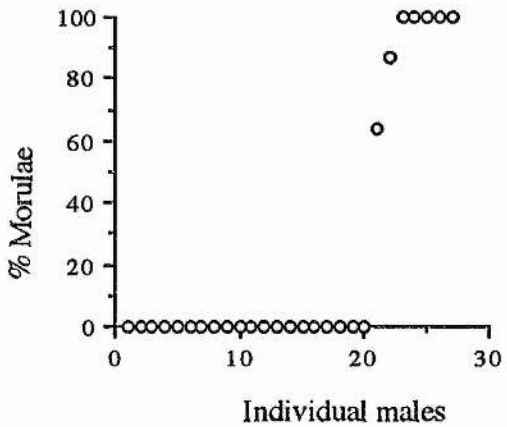
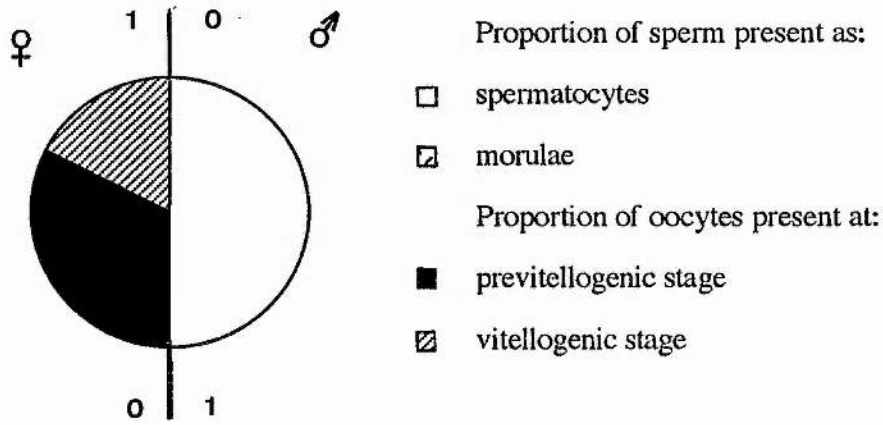


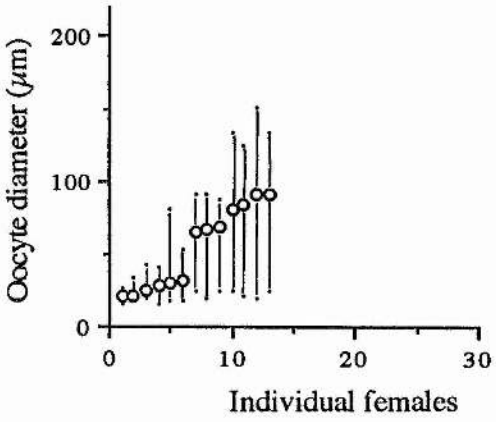
Fig. 3.13 The reproductive state of *Arenicola marina* at Eden Estuary in April.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

April - Eden Estuary (1991)

a)



b)



c)

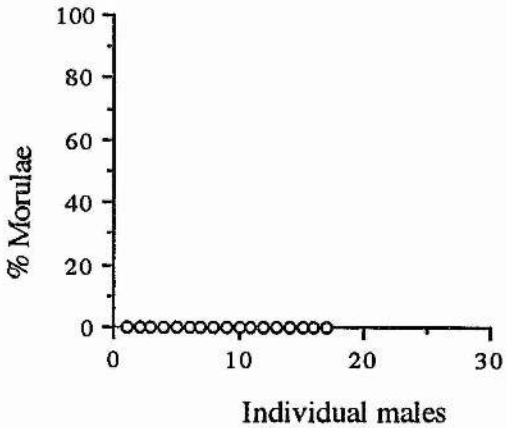
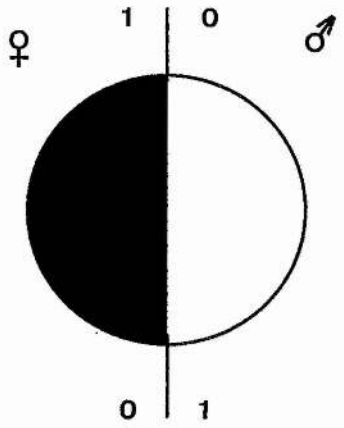


Fig. 3.14 The reproductive state of *Arenicola marina* at East Sands in April.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

April - East Sands (1991)

a)



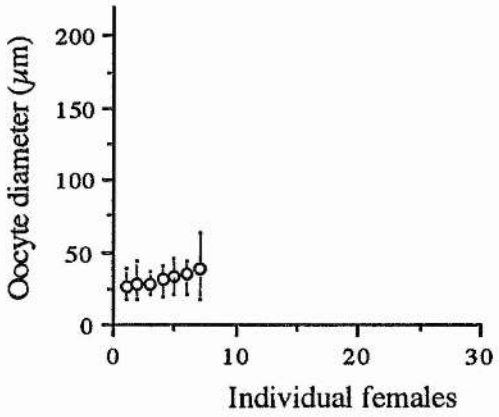
Proportion of sperm present as:

- spermatocytes
- morulae

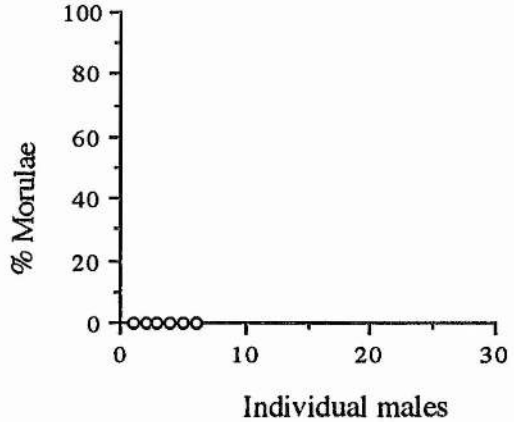
Proportion of oocytes present at:

- previtellogenic stage
- vitellogenic stage

b)



c)



May (Fairlie Sands, 1991; Eden Estuary & East Sands, 1992)

At Fairlie Sands in May 9% of gametes are present in the mature form (Fig. 3.15.1) . On examination of the means and ranges of oocyte diameters of individual worms, there is only one female approaching readiness for spawning (Fig. 3.15.2). Similarly there are two males which are close to spawning (Fig. 3.15.3). This implies that the majority of the animals that were close to spawning in last months sample have already spawned. The spawning season is non-epidemic because there are still a few left which have not yet spawned. At this time of year more than 90% of the worms only have gametes which are in early stages of development present in the coelomic fluid.

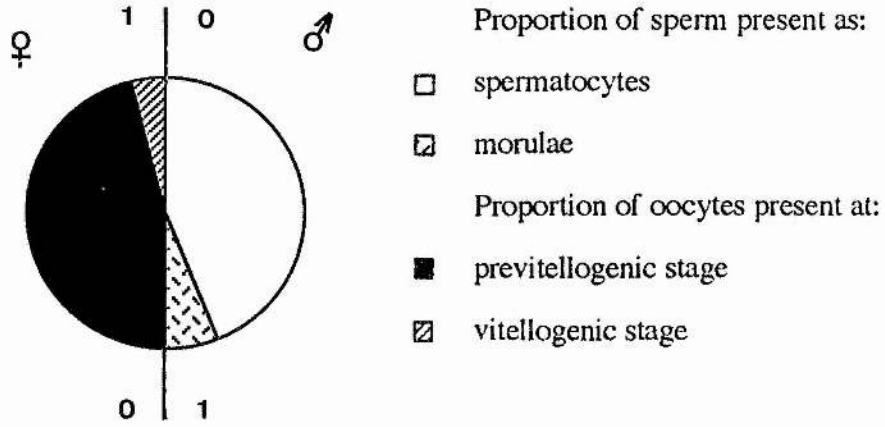
The developmental stages of the gametes from worms from the Eden Estuary in May are represented in Figure 3.16.1. About 16 % of the oocytes measured had entered vitellogenesis. The spermatocytes have also started to develop into morulae. The group of females which were more advanced in the reproductive development have become less well synchronised. This is because some of the oocytes enter the vitellogenic phase and small immature oocytes are probably still being released into the coelom from the ovaries. There is a wide range of oocyte diameters within some individuals (Fig. 3.16.2). There are three males that have some sperm present as morulae (Fig. 3.16.3).

A small percentage of oocytes from females from the East Sands are also starting to enter vitellogenesis but male gametes remain as 100% spermatocytes (Fig. 3.17.1). A similar pattern to that seen at the Eden Estuary a month ago is emerging for the East Sands (Figs. 3.17.2 & 3.17.3) although the sample size for this month was smaller.

Fig. 3.15 The reproductive state of *Arenicola marina* at Fairlie Sands in May.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

May - Fairlie Sands (1991)

a)



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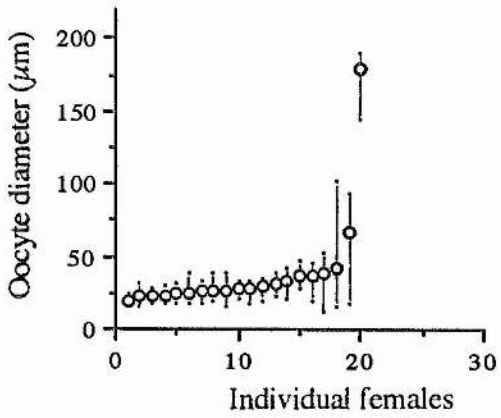


c)

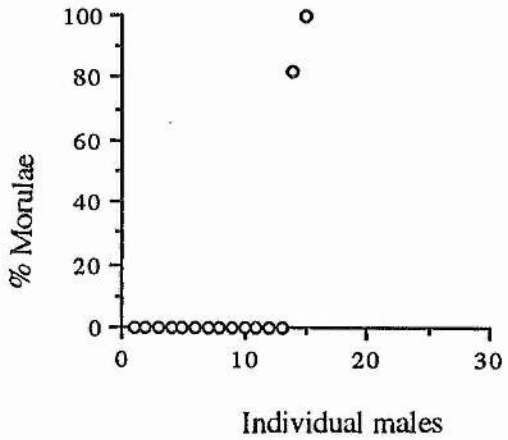
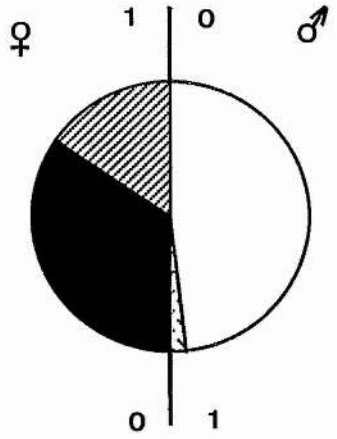


Fig. 3.16 The reproductive state of *Arenicola marina* at Eden Estuary in May.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

May - Eden Estuary (1992)

a)



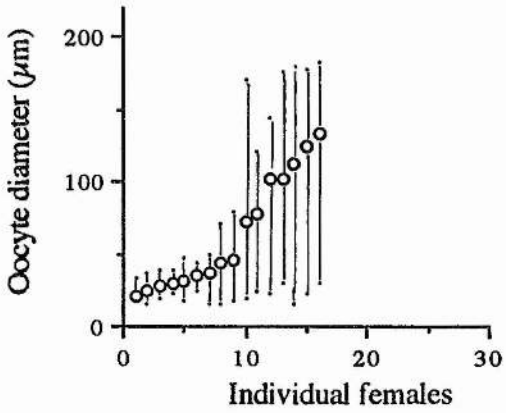
Proportion of sperm present as:

- spermatocytes
- morulae

Proportion of oocytes present at:

- previtellogenic stage
- vitellogenic stage

b)



c)

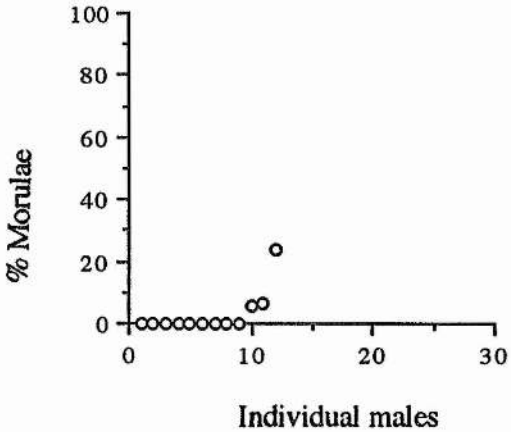
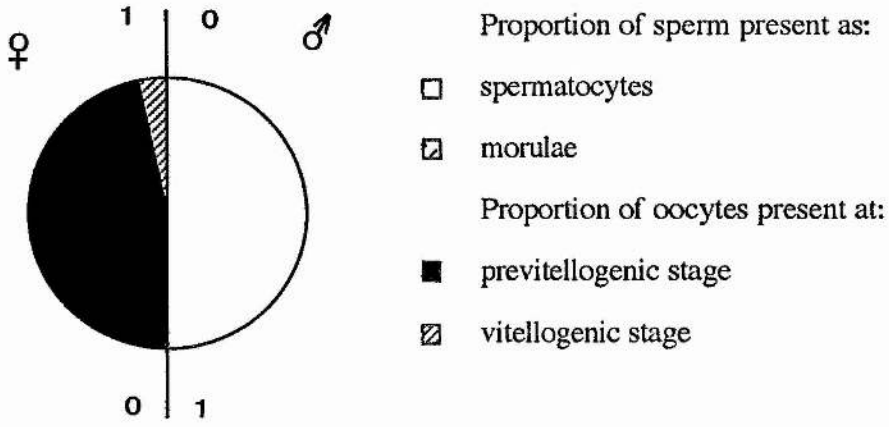


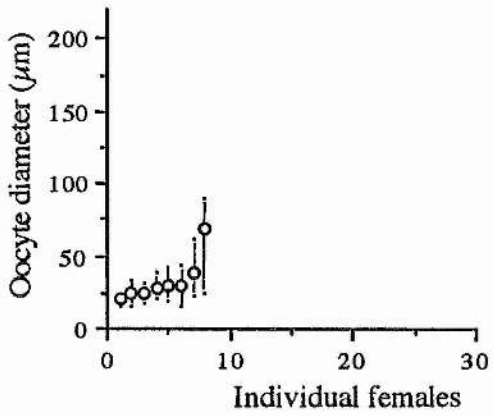
Fig. 3.17 The reproductive state of *Arenicola marina* at East Sands in May.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

May - East Sands (1992)

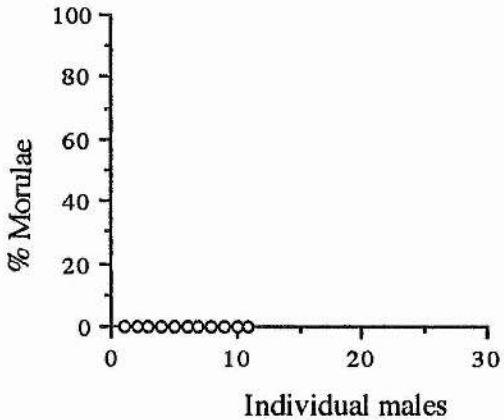
a)



b)



c)



June (1992)

In June the rise in the percentage of vitellogenic oocytes at Fairlie Sands is quite marked, having increased by a factor of eight since the previous month (Fig. 3.18.1). Nearly all the sperm is present as spermatocytes. There are, however, a wide range of oocyte diameters present in most individual females from Fairlie Sands (Fig. 3.18.2). There are animals present with oocytes at all stages of development. Nearly all of the males have no sperm present as morulae, although two out of the thirty examined are approaching maturity (Fig. 3.18.3).

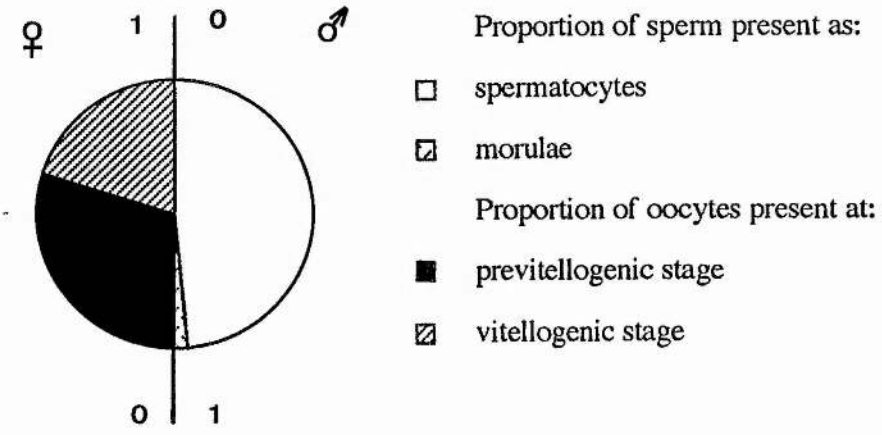
The pie chart of the proportion of gametes that are mature in the Eden Estuary population in June remains similar to that from last month (Fig. 3.19.1). The main difference is that slightly more sperm are present as morulae. In both males and females there is a group of individuals that are starting to become sexually mature while others in the population have gametes that remain in early stages of development (Figs. 3.19.2 & 3.19.3).

At the East Sands the percentage of oocytes in the vitellogenic phase has had a large increase, up to 10%, while the sperm remain present as spermatocytes (Fig. 3.20.1). The distribution of oocyte diameters has become very widely dispersed and oocytes have started to grow in all of the individual females except one (Fig. 3.20.2). None of the males have started to develop any morulae yet (Fig. 3.20.3). Reproductively the worms appear to be at much earlier stages of development than at either the Eden Estuary or Fairlie Sands.

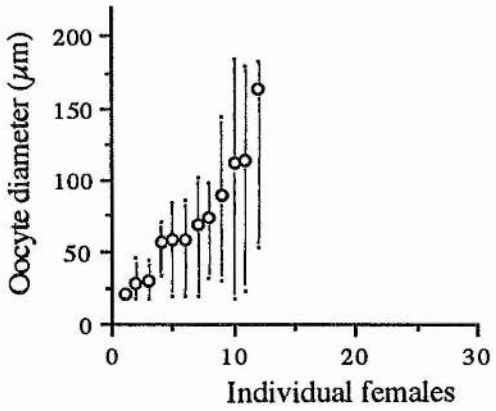
Fig. 3.18 The reproductive state of *Arenicola marina* at Fairlie Sands Sands in June.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

June - Fairlie Sands (1992)

a)



b)



c)

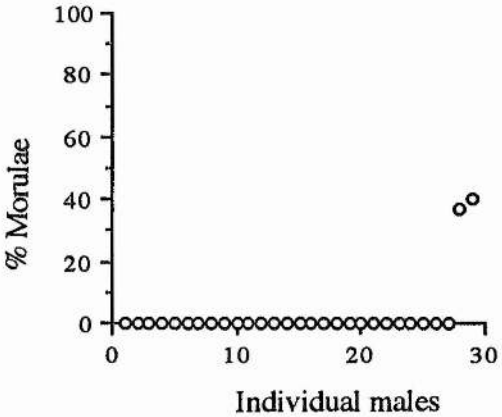
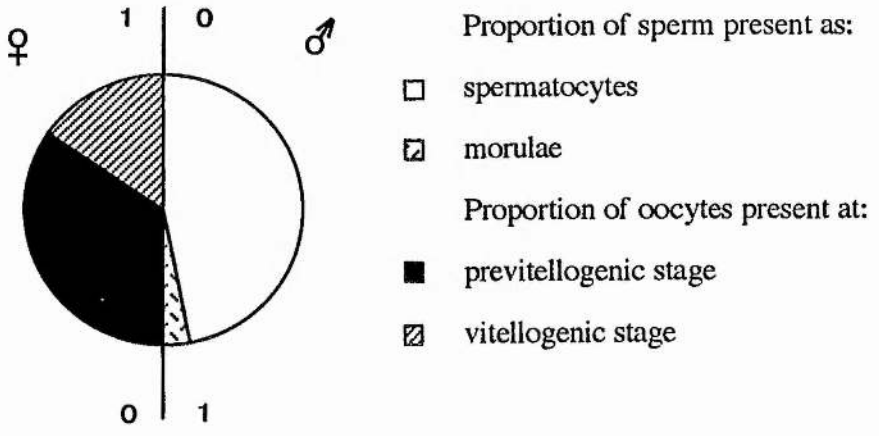


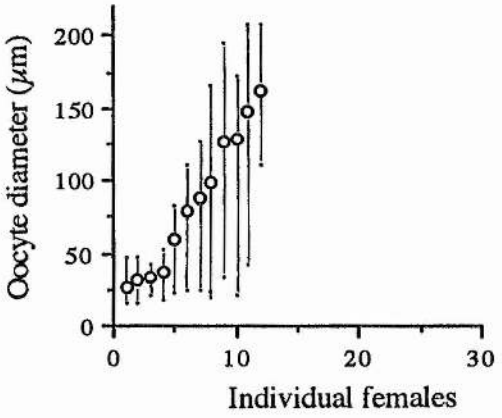
Fig. 3.19 The reproductive state of *Arenicola marina* at Eden Estuary in June.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

June - Eden Estuary (1992)

a)



b)



c)

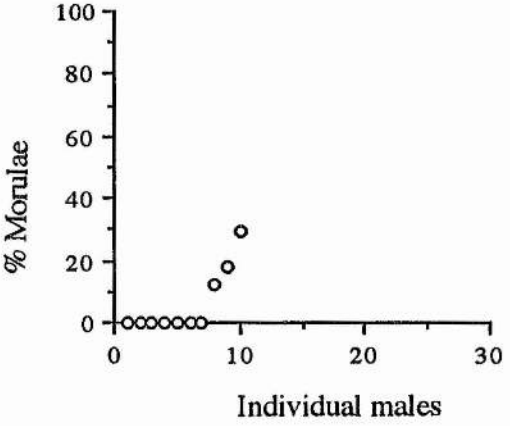
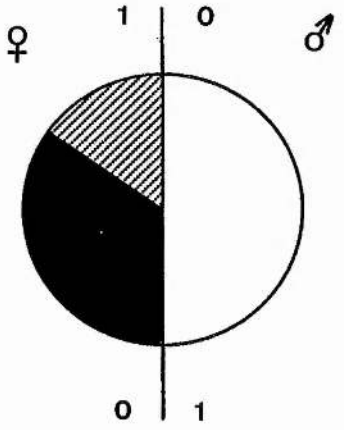


Fig. 3.20 The reproductive state of *Arenicola marina* at East Sands in June.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

June - East Sands (1992)

a)



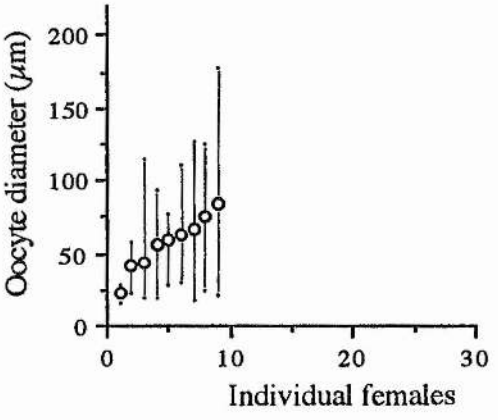
Proportion of sperm present as:

- spermatocytes
- morulae

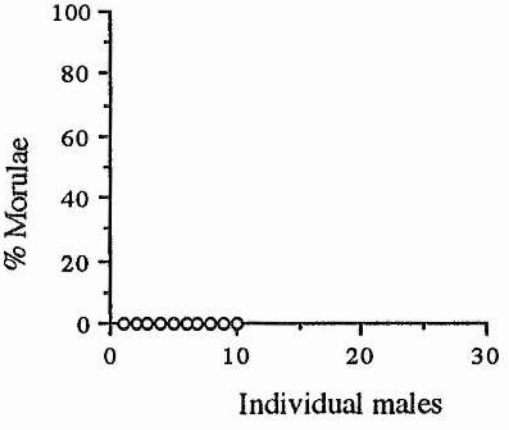
Proportion of oocytes present at:

- previtellogenic stage
- vitellogenic stage

b)



c)



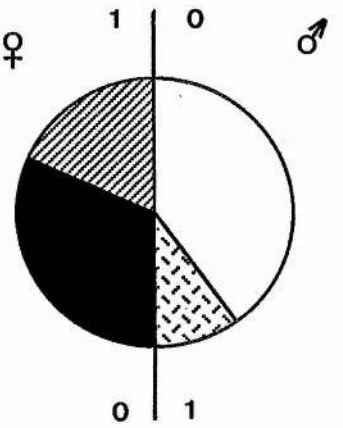
July (1991)

By July the spermatocytes have started to develop into morulae at all three sites. Large numbers of vitellogenic eggs are also present. Fairlie Sands shows the smallest percentage of mature gametes present (Fig. 3.21.1). The percentage of sperm present as morulae is higher at the Eden Estuary (Fig. 3.22.1) than at the East Sands (Fig.3.23.1). This means that the individual males are at a more advanced reproductive stage at the Eden Estuary compared to at the East Sands. This is the same trend that was noticed earlier for the oocyte development in the females. At the Eden and the East Sands over half the oocytes present have now entered the vitellogenic phase. Figures 3.21.2, 3.22.2 and 3.23.2 show that at all three sites there are a few individuals present that still have oocyte diameters with small means and ranges. These are in the minority because most individuals have oocytes which have entered vitellogenesis and hence have larger mean oocyte diameters with a wide range of oocyte sizes present. The graphs which present the percentage of sperm present as morulae (Figs. 3.21.3, 3.22.3 & 3.23.3) show that there are two or three individual males at each site which are starting to develop morulae from the spermatocytes.

Fig. 3.21 The reproductive state of *Arenicola marina* at Fairlie Sands in July.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

July - Fairlie Sands (1991)

a)



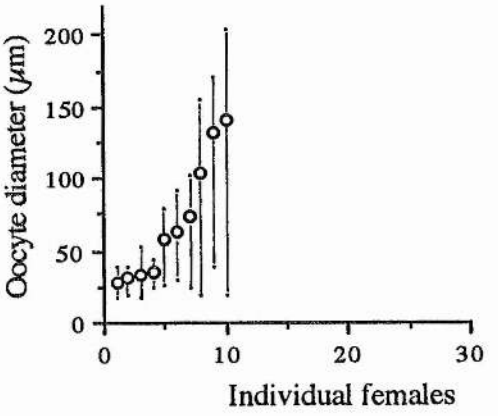
Proportion of sperm present as:

- spermatocytes
- morulae

Proportion of oocytes present at:

- previtellogenic stage
- vitellogenic stage

b)



c)

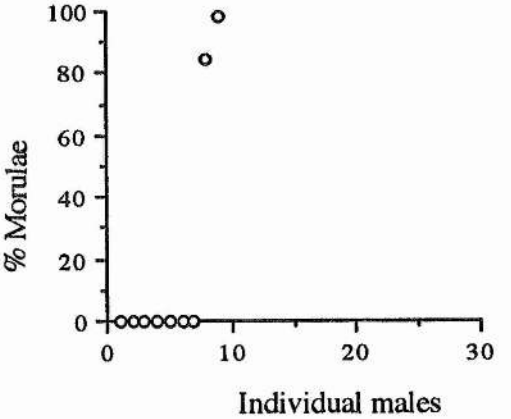
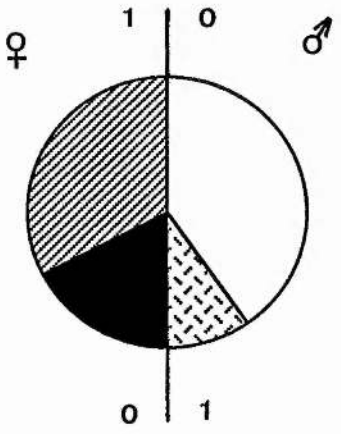


Fig. 3.22 The reproductive state of *Arenicola marina* at Eden Estuary in July.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

July - Eden Estuary (1991)

a)



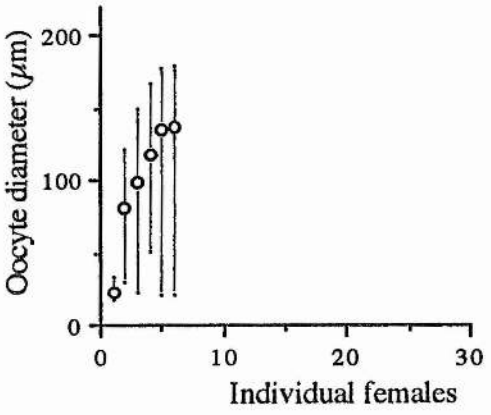
Proportion of sperm present as:

- spermatocytes
- morulae

Proportion of oocytes present at:

- previtellogenic stage
- vitellogenic stage

b)



c)

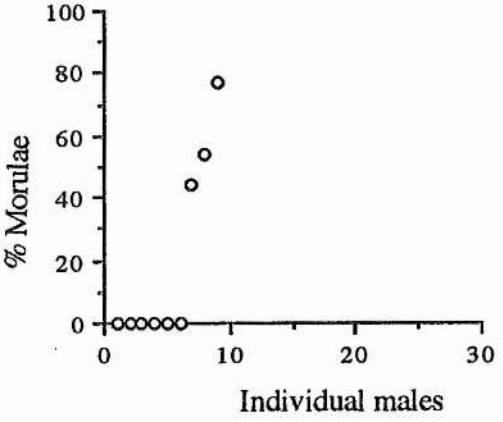
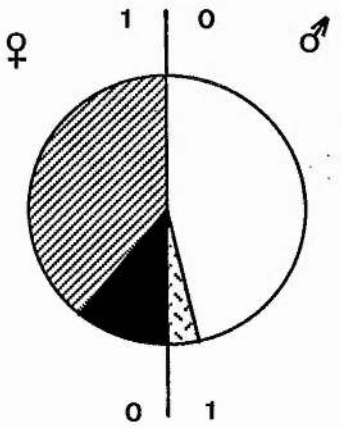


Fig. 3.23 The reproductive state of *Arenicola marina* at East Sands in July.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

July - East Sands (1991)

a)



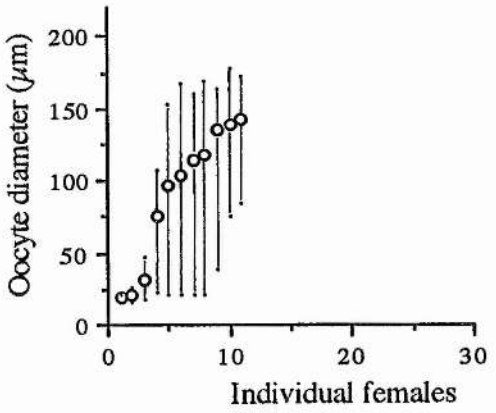
Proportion of sperm present as:

- spermatocytes
- morulae

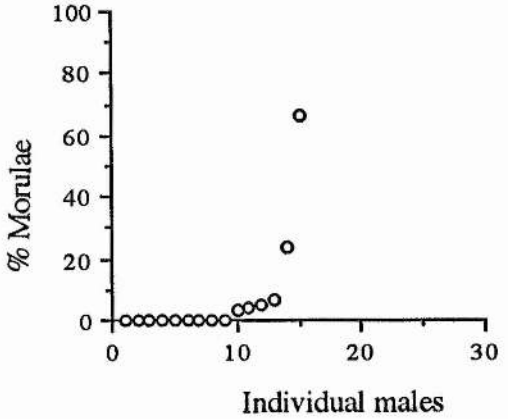
Proportion of oocytes present at:

- previtellogenic stage
- vitellogenic stage

b)



c)



August (1992)

Data for August from Fairlie Sands show a decline in the percentage of gametes present in the mature state (Fig. 3.24.1). This may be due to spawning since July. The oocytes of most females are starting to grow but there is really only one female that is more reproductively advanced than the others (Fig. 3.24.2). Similarly Figure 3.24.3 shows that all the males except for one have all their sperm still present as spermatocytes.

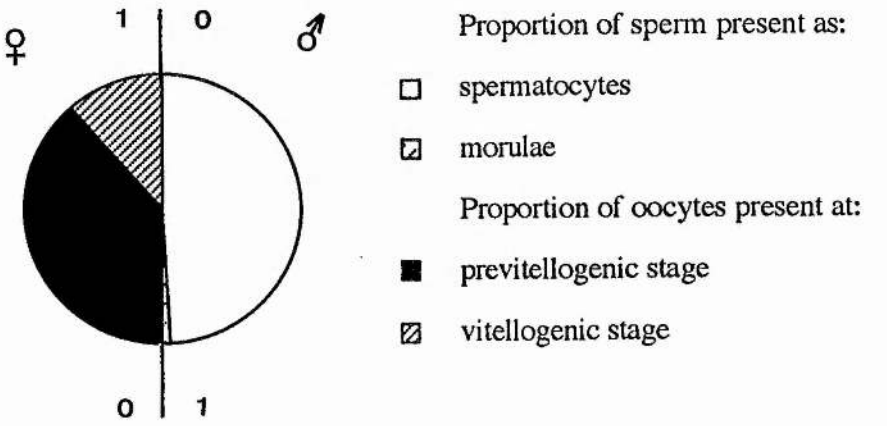
The pie chart for the Eden Estuary in August shows the situation to be similar to that shown for July with a growing proportion of male and female gametes present in the mature state (Fig. 3.25.1). There is a component of the population which have gametes present in the coelomic cavity still in early stages of development (Figs. 3.25.1 & 3.25.2). Just under 50% of the individuals at this site are approaching maturity.

At the East Sands there has been a large increase in the percentage of sperm present as morulae since last month (Fig. 3.26.1). The population appears to be developing more synchronously than at either of the other sites which is apparent from Figures 3.26.2 & 3.26.3. The means oocyte diameters in the individual worms are very large but there is also a very wide range of oocyte sizes present in all of the females examined. All of the males had some sperm present as morulae.

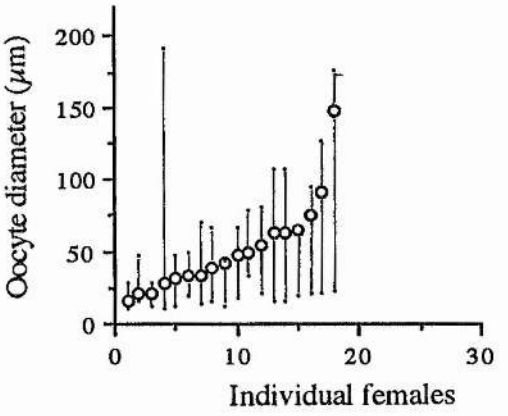
Fig. 3.24 The reproductive state of *Arenicola marina* at Fairlie Sands in August.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

August - Fairlie Sands (1992)

a)



b)



c)

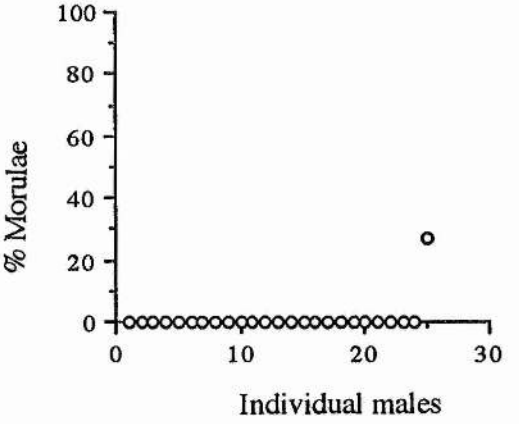
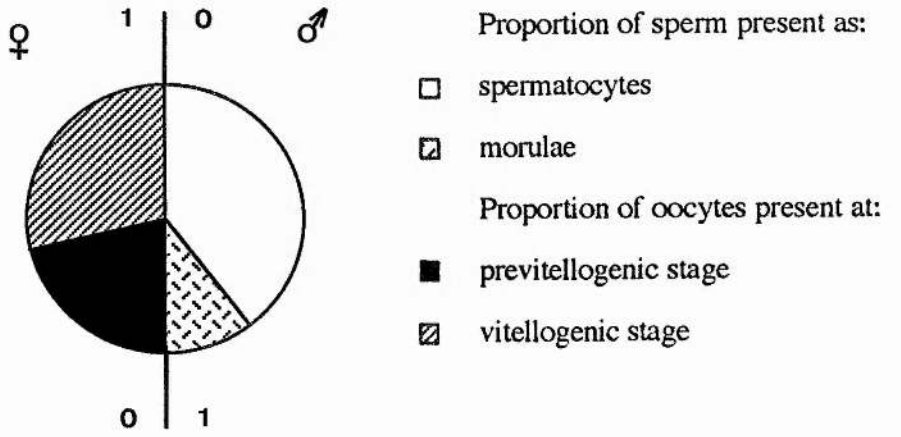


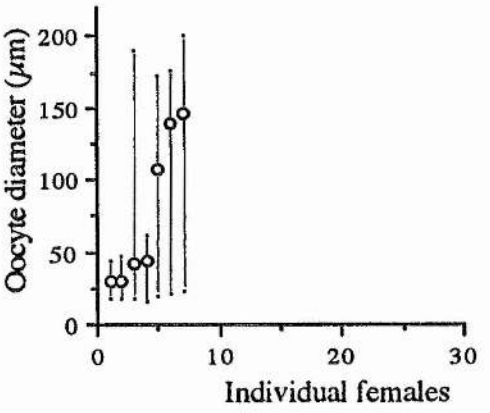
Fig. 3.25 The reproductive state of *Arenicola marina* at Eden Estuary in August.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

August - Eden Estuary (1992)

a)



b)



c)

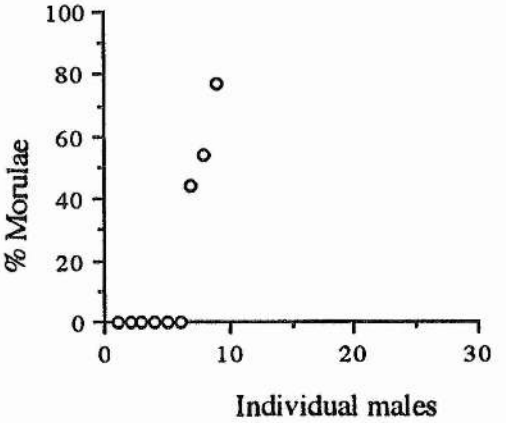
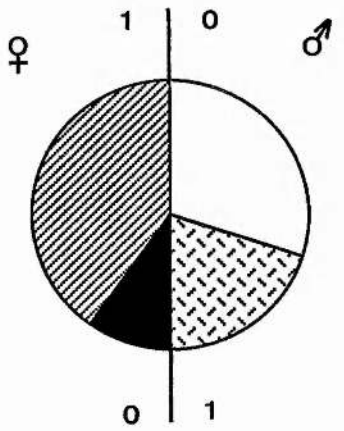


Fig. 3.26 The reproductive state of *Arenicola marina* at East Sands in August.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

August - East Sands (1992)

a)



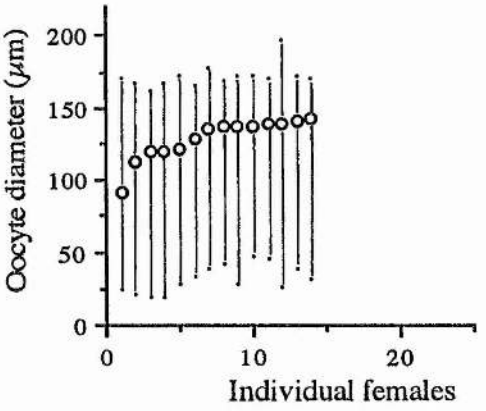
Proportion of sperm present as:

- spermatocytes
- morulae

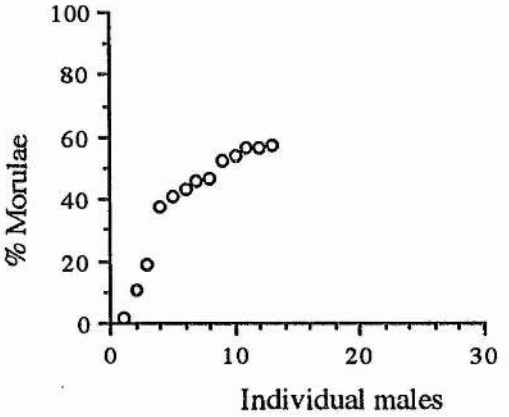
Proportion of oocytes present at:

- previtellogenic stage
- vitellogenic stage

b)



c)



September (Eden Estuary, 1991; Fairlie Sands & East Sands, 1992)

About 10% of the gametes are mature at Fairlie Sands in September (Fig. 3.27.1). Oocytes in about half of the females examined are just entering vitellogenesis (Fig. 3.27.2). Four of the eighteen males collected had some sperm present as morulae. (Fig. 3.27.3).

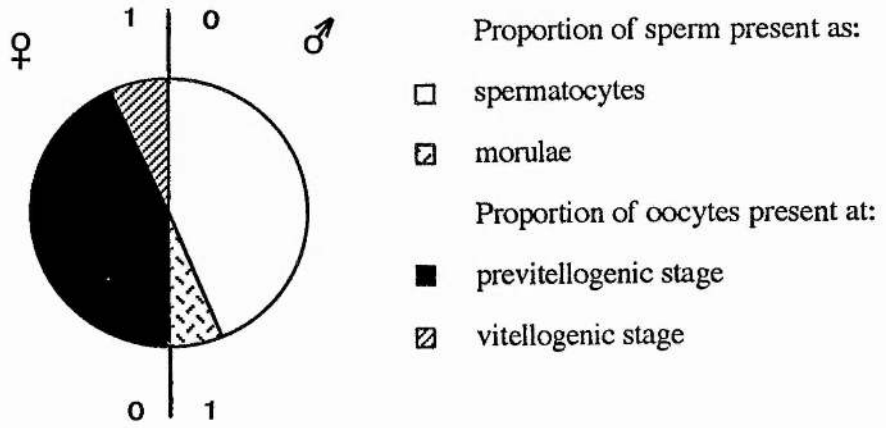
The Eden Estuary population show an increase in the percentage of gametes which are mature (Fig. 3.28.1). The means and ranges of oocyte diameters are scattered but clearly show those individuals which are approaching maturity (Fig. 3.28.2). There is also a clear division in the males between those that have sperm present as morulae and those which remain with 100% spermatocytes in the coelomic cavity (Fig. 3.28.3).

The proportion of gametes present in the mature state is highest at the East Sands (Fig. 3.29.1). Nearly all of the oocytes are vitellogenic and just under 70% of the sperm are present as morulae. The graph of means and ranges of the oocytes show that fourteen out of fifteen individuals have mean oocyte diameters greater than 150μm (Fig. 3.29.2). There are still a few oocytes in each individual that remain previtellogenic. All of the males have morulae present but there is a wide range of the percentage of sperm present as morulae between tne individual males (Fig. 3.29.3).

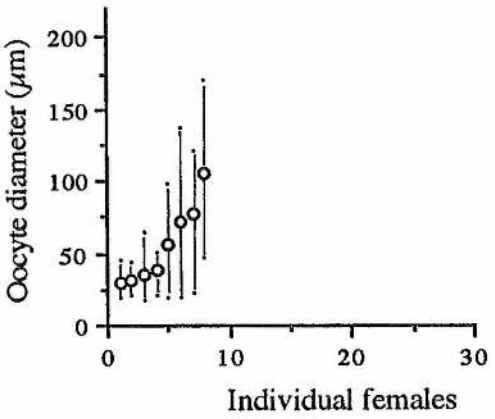
Fig. 3.27 The reproductive state of *Arenicola marina* at Fairlie Sands in September.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

September - Fairlie Sands (1992)

a)



b)



c)

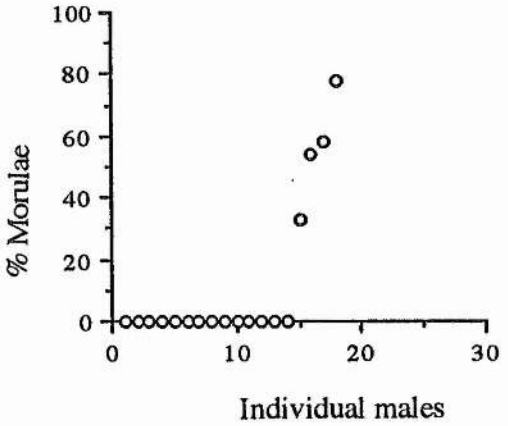
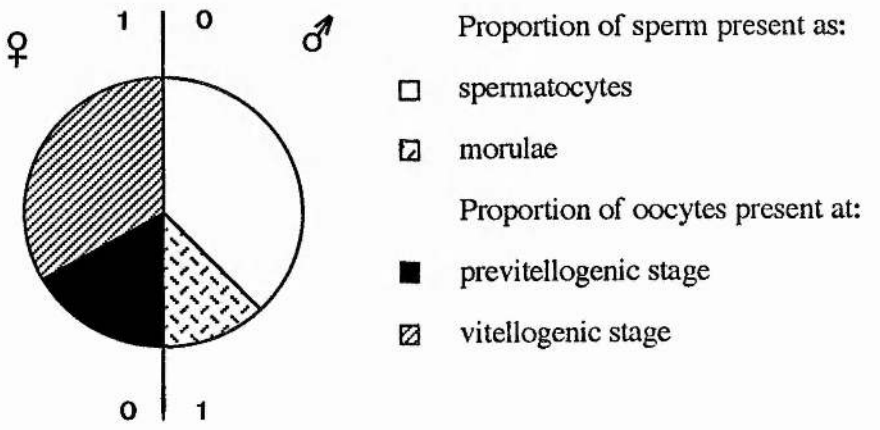


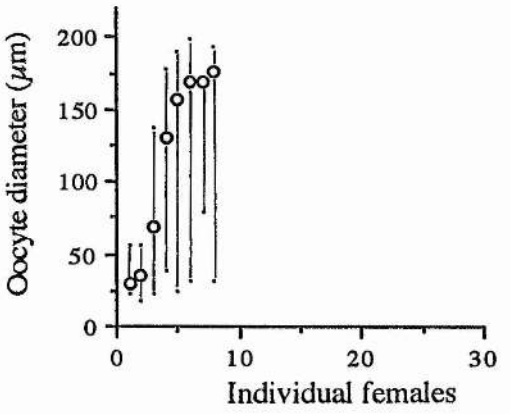
Fig. 3.28 The reproductive state of *Arenicola marina* at Eden Estuary in September.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

September - Eden Estuary (1991)

a)



b)



c)

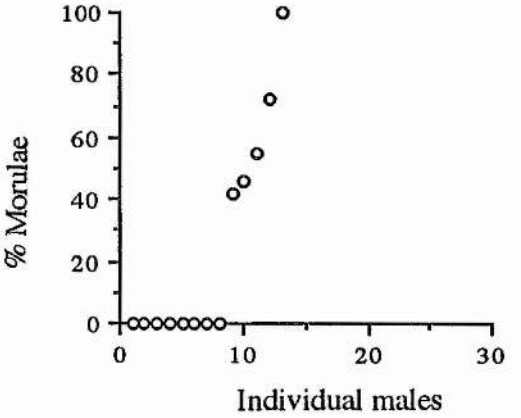
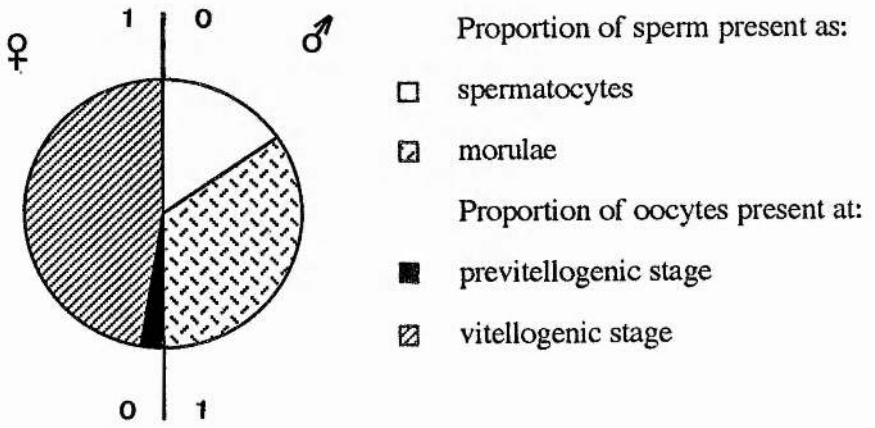


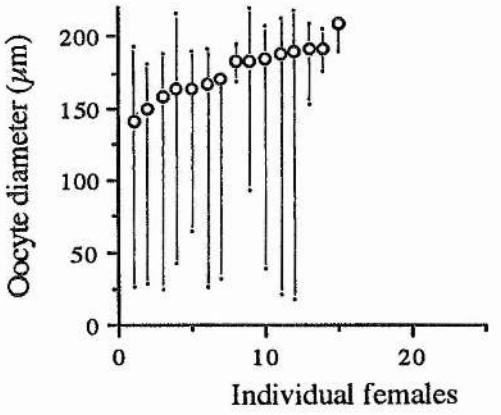
Fig. 3.29 The reproductive state of *Arenicola marina* at East Sands in September.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

September - East Sands (1992)

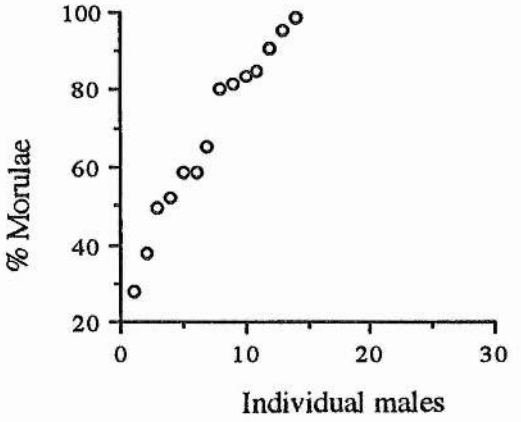
a)



b)



c)



October (Fairlie Sands & Eden Estuary, 1990 & 1991, East Sands, 1992)

A higher percentage of developing gametes were present in October than in September in animals collected from Fairlie Sands (Fig. 3.30.1). Figures. 3.30.2 and 3.30.3 show that there are two distinct groups of worms which are different reproductive stages.. The group of five individual females which have large mean and small ranges of oocyte diameters are approaching spawning. The rest of the animals sampled had gametes at early developmental stages present. Similarly there are also five males which have sperm present as morulae while the rest have 100% spermatocytes.

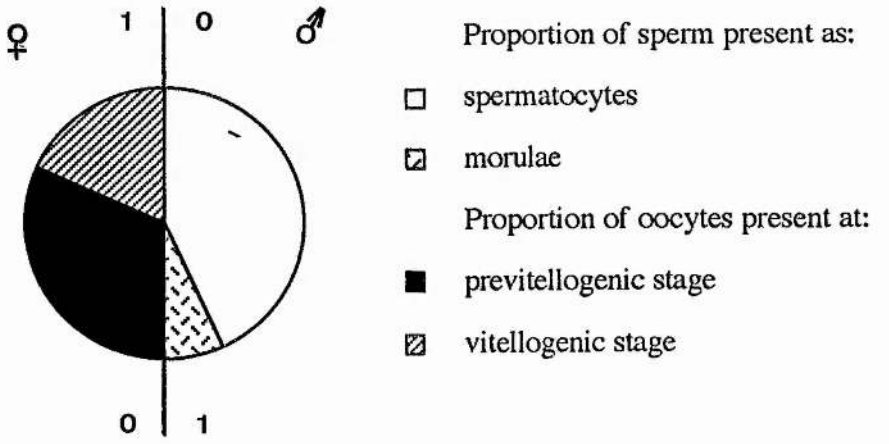
There is a slight decrease in the proportion of mature gametes at the Eden Estuary (Fig. 3.31.1). This is probably due to some spawning having taken place as three females were found to be spent. The coelomic fluid of the spent worms was very granular in appearance due to the presence of large numbers of coelomocytes and a few gametes that were breaking down were visable. The mature worms have mean oocyte diameters of around 170μm and the range of sizes has become much smaller (Fig. 3.31.2). The two cohorts are most noticeable on this graph, which clearly shows the group of worms ready to spawn and the group of worms which do not have vitellogenic oocytes. Figure 3.31.3 reflects a similar situation with the males. There is a clear division between the breeding and non-breeding cohorts.

By October at the East Sands all the gametes were fully developed and were presumably ready to be spawned (Fig. 3.32.1). The means and ranges of oocyte diameters indicate that all of the smaller previtellogenic oocytes that were present in September are no longer present and the ranges of oocyte diameters within individuals are small (Fig. 3.32.2). The individual males nearly all have 100% of the sperm present as morulae and are ready to spawn (Fig. 3.32.3).

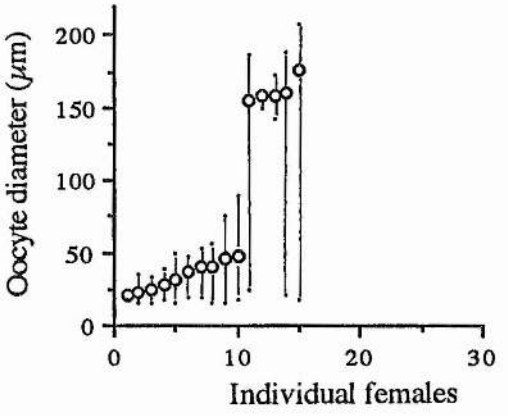
Fig. 3.30 The reproductive state of *Arenicola marina* at Fairlie Sands in October.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

October - Fairlie Sands (1990 & 1991)

a)



b)



c)

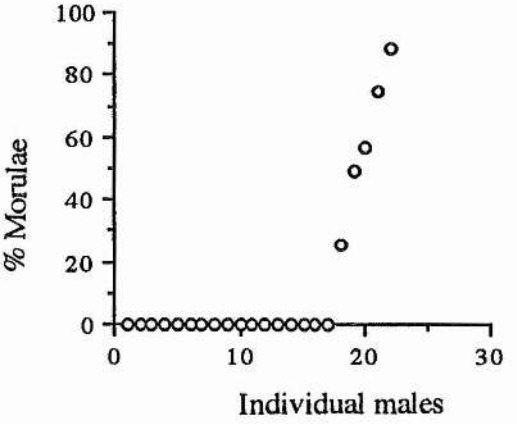
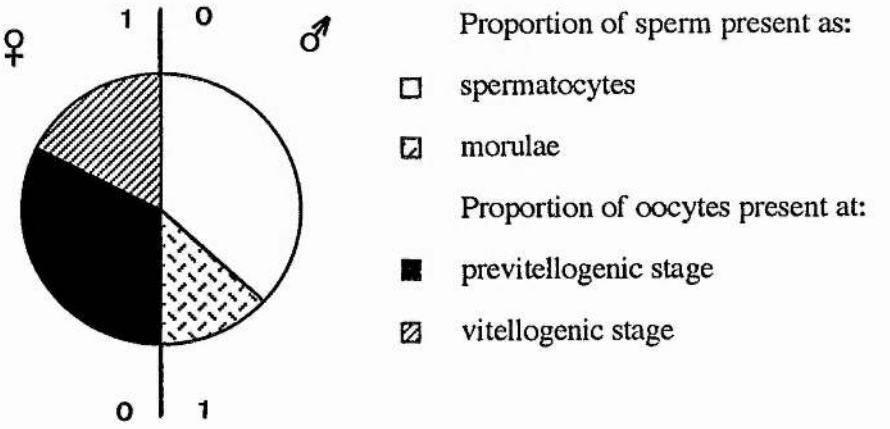


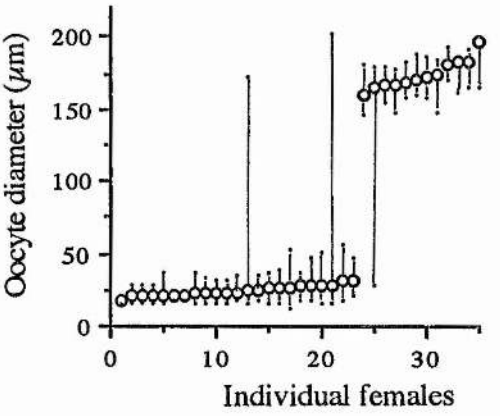
Fig. 3.31 The reproductive state of *Arenicola marina* at Eden Estuary in October.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

October - Eden Estuary (1990 & 1991)

a)



b)



c)

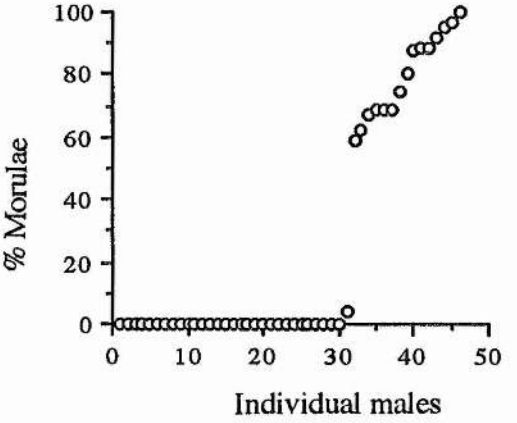
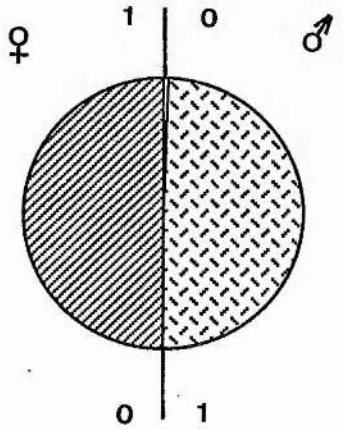


Fig. 3.32 The reproductive state of *Arenicola marina* at East Sands in October.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

October - East Sands (1992)

a)



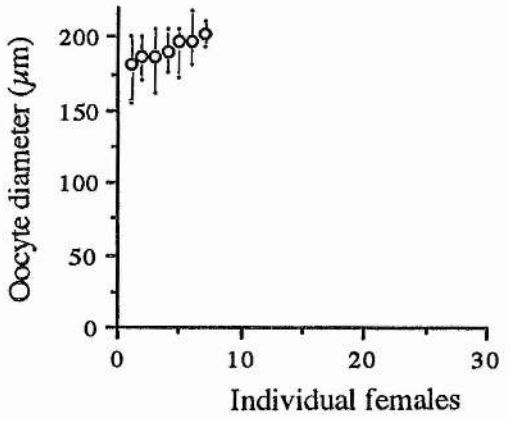
Proportion of sperm present as:

- spermatocytes
- morulae

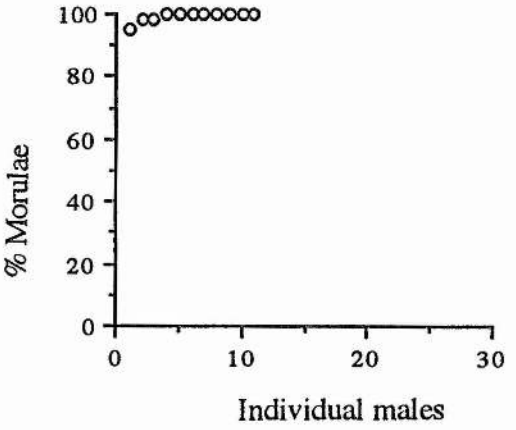
Proportion of oocytes present at:

- previtellogenic stage
- vitellogenic stage

b)



c)



November (1990)

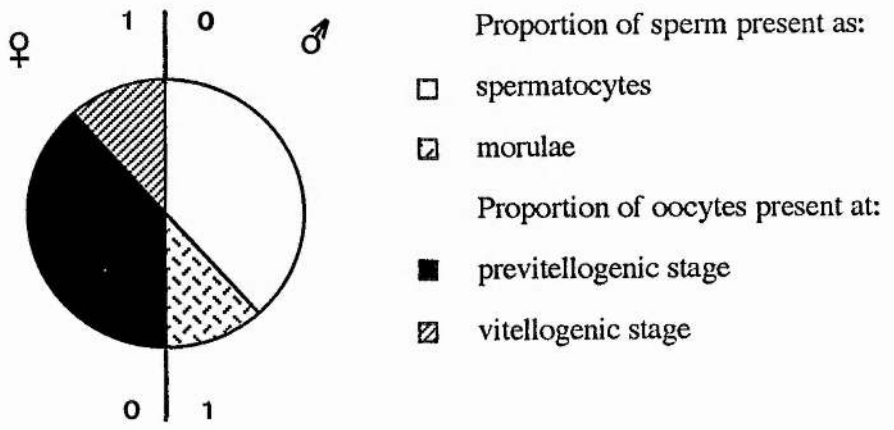
The proportion of maturing gametes in November at Fairlie Sands remains similar to that found in October (Fig. 3.33.1). There are five individual females that are approaching spawning but they are all at different stages of development. More than half of the females have only previtellogenic oocytes present (Fig. 3.33.2). Half of the males examined had morulae present implying that they are approachimg the time of spawning (Fig. 3.33.3).

At the Eden Estuary in November most of the individuals have spawned although a small proportion of developed gametes remain (Fig. 3.34.1). It can be seen from Figures 3.34.2 and 3.34.3 that there is only one gravid male and one gravid female present in this sample of worms from the Eden Estuary. This does imply that the spawning season is not completely finished here. By November all the worms from the East Sands have spawned and so no data are presented. A few remaining oocytes and morulae were found to be present in some individuals along with high densities of coelomocytes. Sperm puddles were observed on the beach on October 13 and 14.

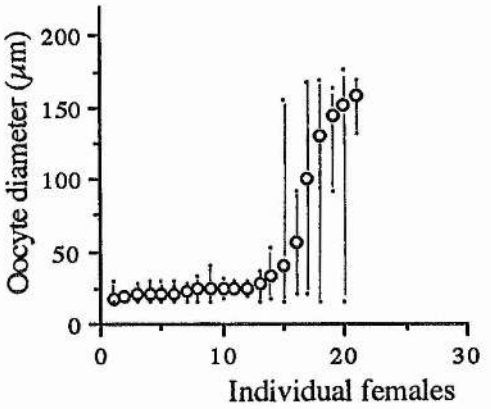
Fig. 3.33 The reproductive state of *Arenicola marina* at Fairlie Sands in November.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

November - Fairlie Sands (1990)

a)



b)



c)

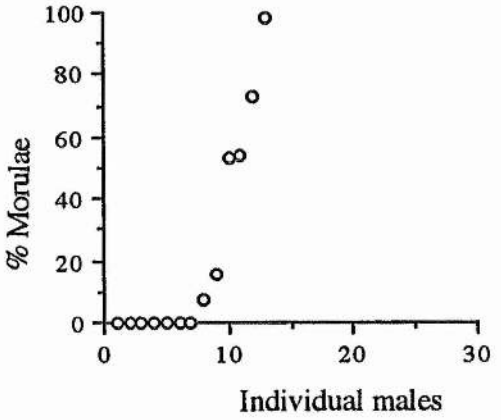
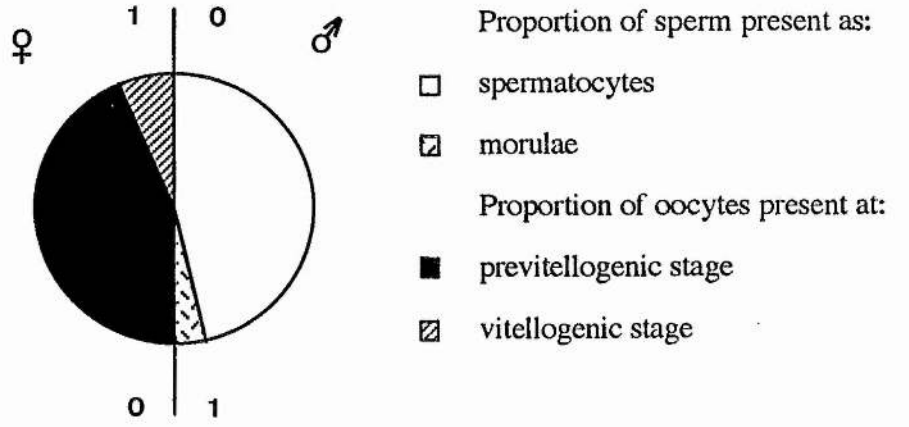


Fig. 3.34 The reproductive state of *Arenicola marina* at Eden Estuary in November.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

November - Eden Estuary (1990)

a)



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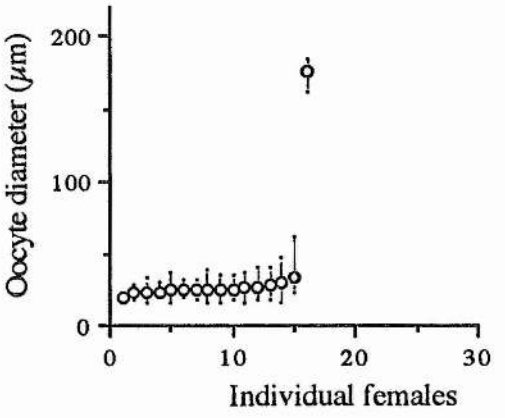
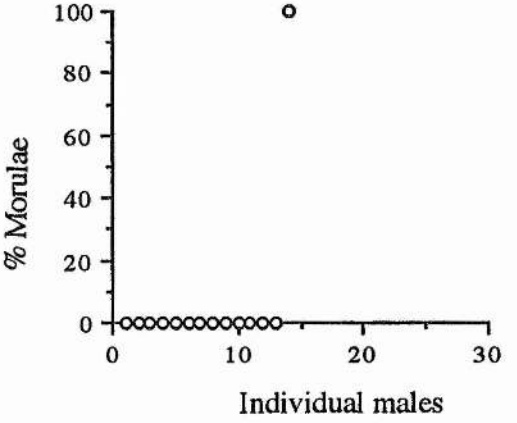


c)



December (1990)

In December at Fairlie Sands there was a decrease in the percentage of developing gametes since the November sample was taken (Fig. 3.35a). This implies that some spawning had taken place. Figure 3.35b shows that there was a single female which had some vitellogenic oocytes in the coelom. There were however, still two males present with nearly 100% morulae (Fig. 3.35c). The Eden Estuary populations had no individuals containing mature gametes at this time (Fig. 3.36a), although a large proportion of individuals still had immature gametes present (Figs. 3.36b & 3.36c). No data are presented for the The East Sands because there were no developing gametes present in the coelom.

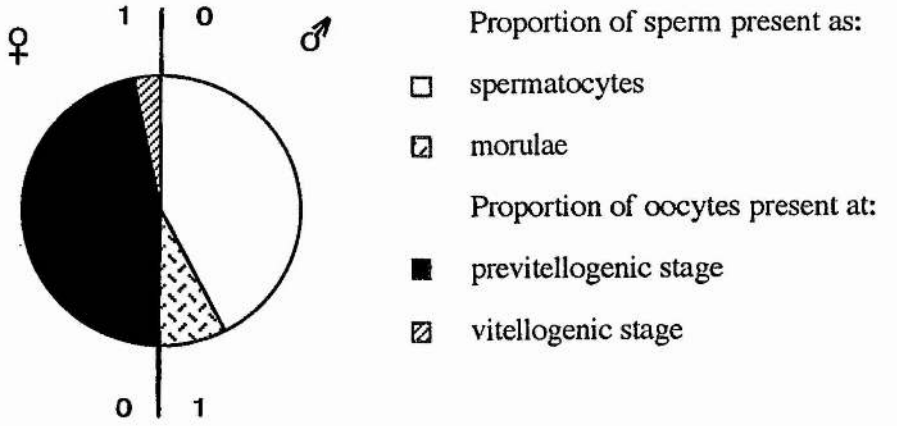
A Summary of the Annual Gametogenic Cycle at Fairlie Sands, Eden Estuary and East Sands.

Figures 3.37 and 3.38 sum up the annual gametogenic cycle at the three sites for females and males respectively. It is very clear from these two figures that the patterns of gametogenesis are different at each site. In the females at Fairlie Sands there are some vitellogenic oocytes present in a few individuals throughout the whole year, implying that spawning may occur in any month of the year. If we look at the months which have the smallest proportion of vitellogenic oocytes present, they are December, January and May. More individuals therefore spawn in spring or in autumn and the spawning season may loosely be described by two very extended seasons, one in spring and one in autumn, although in reality they are overlapping. This pattern is also observed in the percentage of sperm which are present as morulae throughout the year. The months when the population was furthest away from spawning were December, June and August. This is similar to the females, except for August where there are very few mature males present.

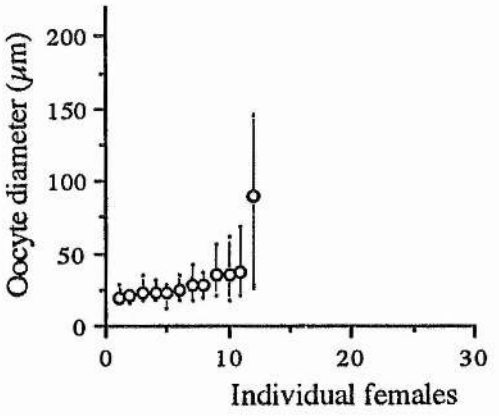
Fig. 3.35 The reproductive state of *Arenicola marina* at Fairlie Sands in December.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

December - Fairlie Sands (1990)

a)



b)



c)

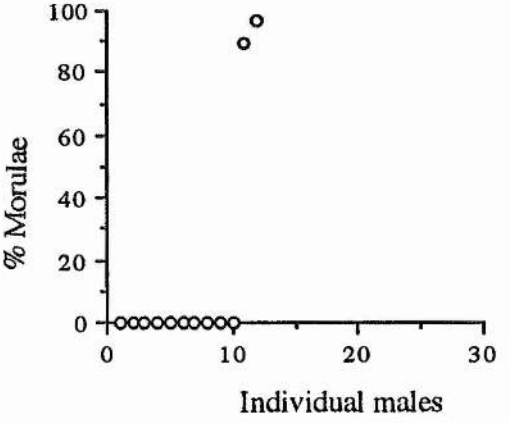
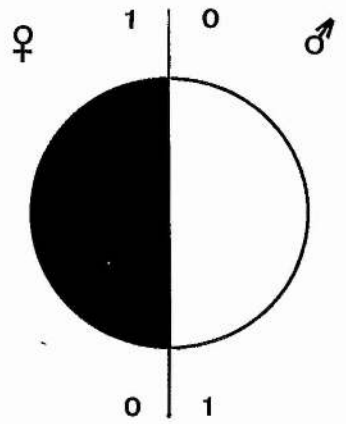


Fig. 3.36 The reproductive state of *Arenicola marina* at Eden Estuary in December.
a) The proportion of male gametes present as spermatocytes and morulae, and also the proportion of female gametes present as previtellogenic and vitellogenic oocytes.
b) The mean and ranges of oocyte diameter present in individual females. The open circles (o) represent the mean oocyte diameter for each worm and the dots (.) either side represent the largest and smallest oocyte diameter measured in that female.
c) The percentage of sperm present as morulae for each individual male, represented by open circles (o) on the graph.

December - Eden Estuary (1990)

a)



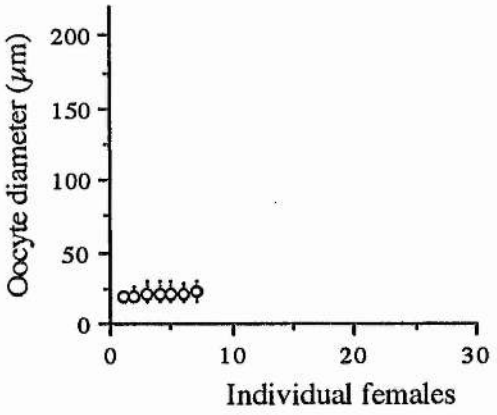
Proportion of sperm present as:

- spermatocytes
- morulae

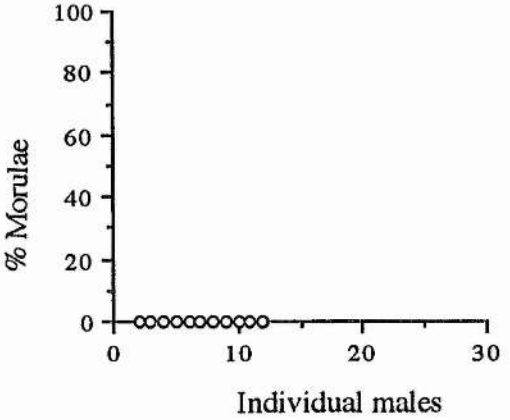
Proportion of oocytes present at:

- previtellogenic stage
- vitellogenic stage

b)



c)



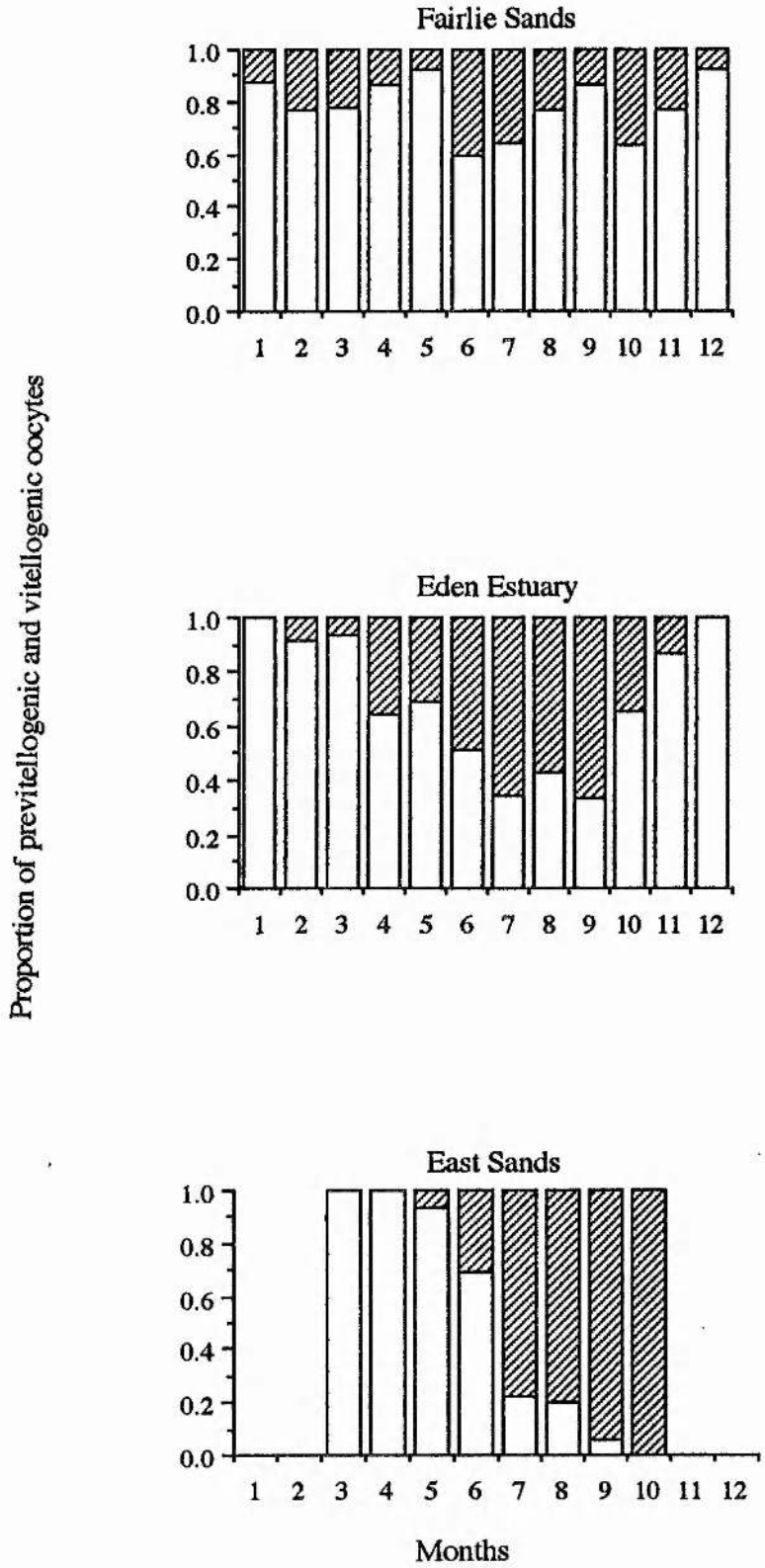


Fig. 3.37 The annual gametogenic cycle in female *Arenicola marina* at the three study sites. The white part of the graph represents the proportion of previtellogenic oocytes, and the striped part represents the the proportion of vitellogenic oocytes present in the population for each month.

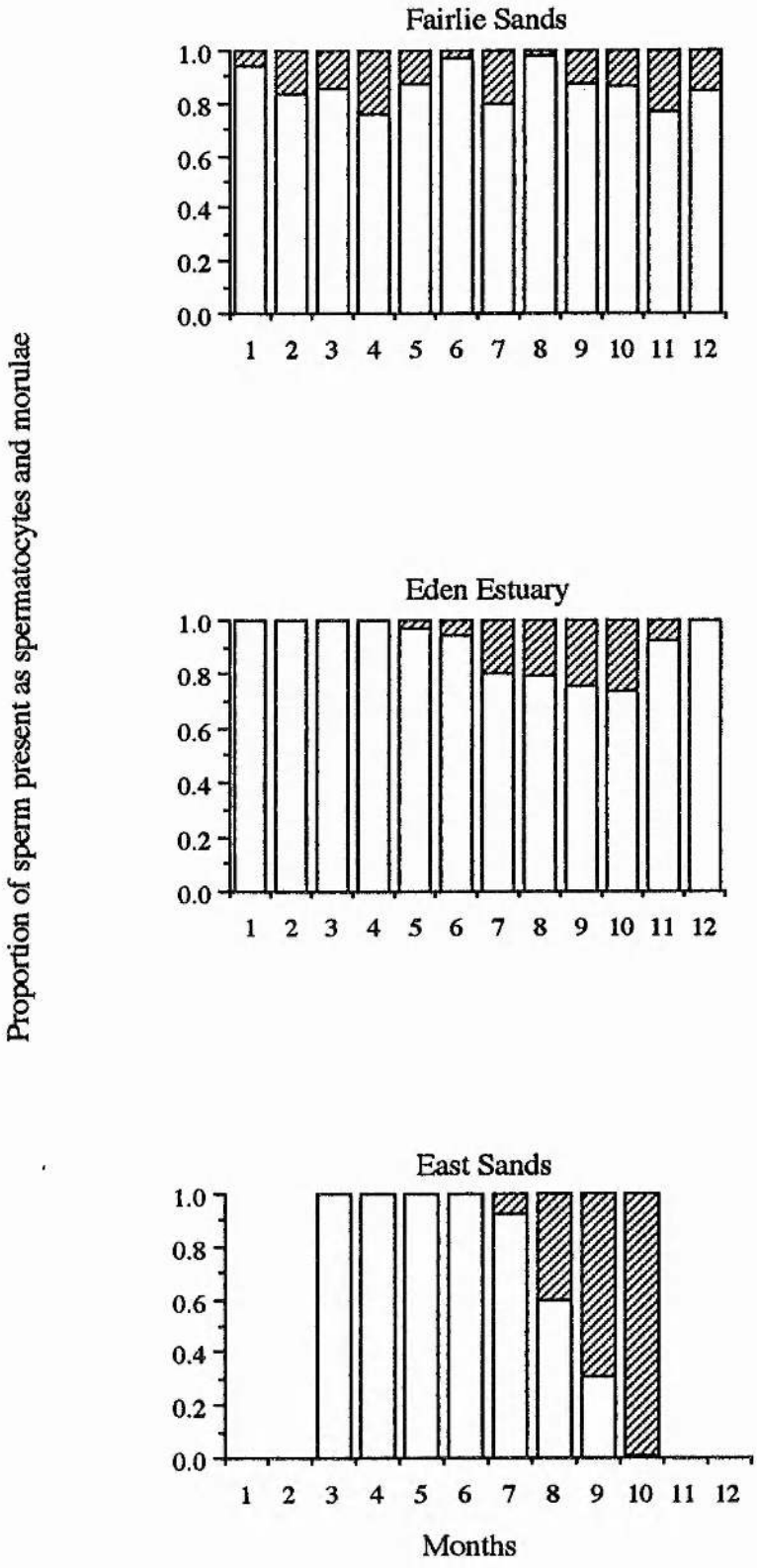


Fig. 3.38 The annual gametogenic cycle in male *Arenicola marina* at the three study sites. The white part of the graph represents the proportion of sperm present as spermatocytes and the striped part represents the proportion of sperm present as morulae in the population for each month.

The pattern of gametogenesis at the Eden Estuary clearly show the spawning season to take place in the autumn. The month with the highest proprtion of vitellogenic oocytes present is September. The proportion of vitellogenic oocytes then slowly decreases as more individuals spawn, until there are no mature individuals left in December. This means that the spawning season is extended and probably lasts for about four weeks in October and extending into November. Interestingly the month with the highest proportion of sperm present of morulae is October, which is later than for the females. There are always previtellogenic oocytes and spermatocytes present in certain individuals throughout the year.

East Sands shows a gametogenic pattern typical of organisms with a highly synchronised reproductive phase. The proportion of vitellogenic oocytes increases from May through to October where it can be seen that all of the oocytes in the population are vitellogenic. The majority of the oocytes enter vitellogenesis some time between June and July, and after this time they continue to develop in the coelom.. The proportion of sperm present as morulae increases steadily from July through to October. Spawning occured shortly after the October sample was dug and it is evident that the spawning was synchronous because in November the worms were all spent.

3.3.2 The Role of Size in Reproduction

To test if there is any correlation between the size of the worm and the stage of reproductive development, scatter plots were drawn of weight against the mean oocyte diameter of individual female worms. This was done in order to test whether there was a size difference between the two cohorts that were present at the Eden Estuary and at Fairlie Sands. This may be able to tell us whether one cohort is

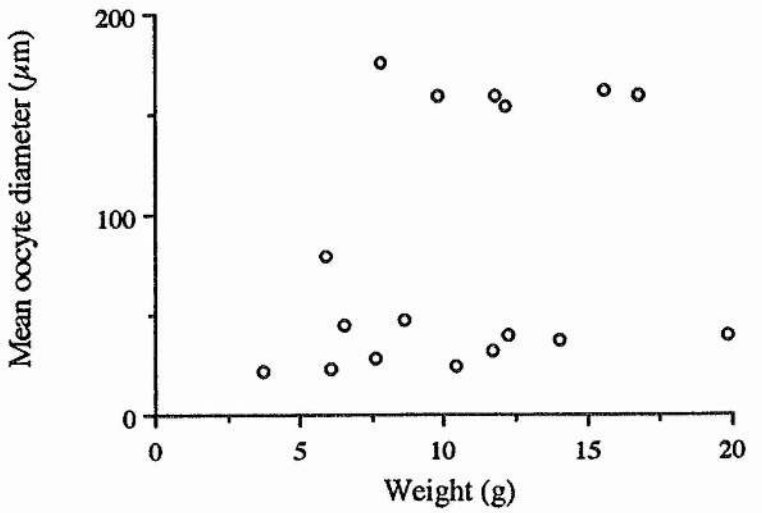


Fig. 3.39 Graph to show the mean oocyte diameter in relation to weight of individual female *A. marina* at Fairlie Sands in October. The open circles (o) represent the mean oocyte diameter in individual females.

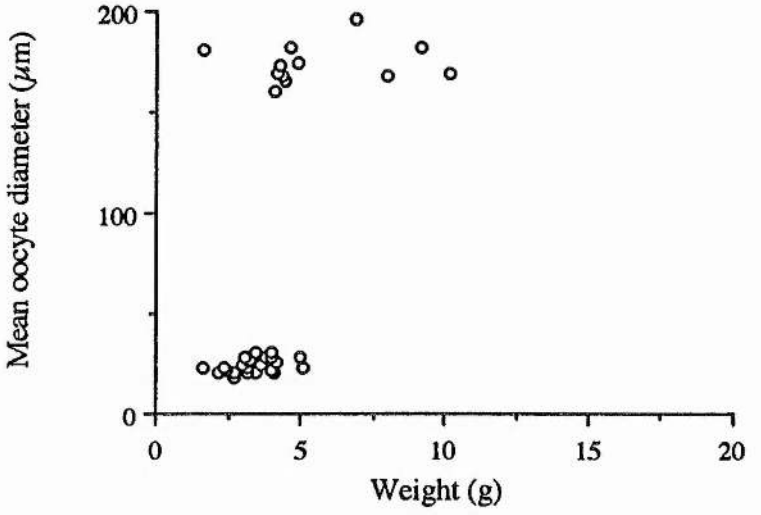


Fig. 3.40 Graph to show the mean oocyte diameter in relation to weight of individual female *A. marina* at the Eden Estuary in October. The open circles (o) represent the mean oocyte diameter in individual females.

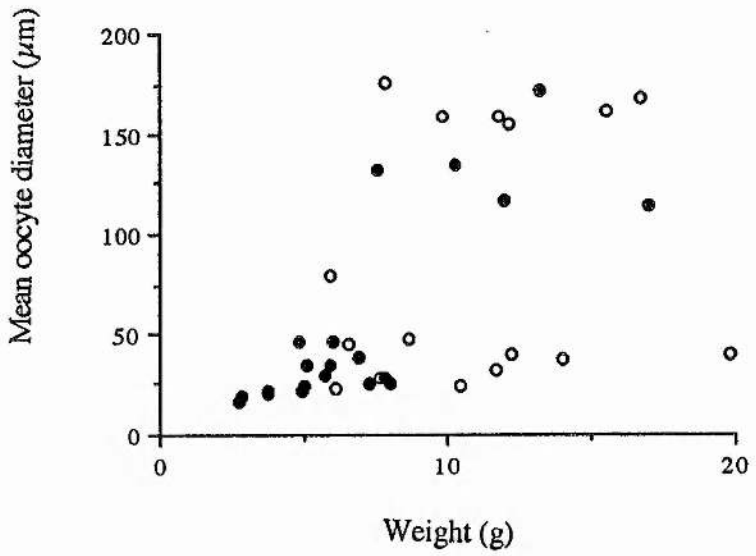


Fig. 3.41 Graph to show the mean oocyte diameter in relation to weight of individual female *A. marina* from different sides of the beach at Fairlie Sands in October. The open circles (o) represent the mean oocyte diameter in females from the north side of the beach and the closed circles (o) represent the mean oocyte diameter in females from the south side of the beach.

recruited from a different year class. The data used were from the October because the division of the cohorts is most obvious at this time (Figs. 3.30b & 3.31b).

Figure 3.39 shows that at Fairlie Sands the weight distribution is not closely related to the mean oocyte diameter. Figure 3.40 shows the distribution of the weight of worms against the mean oocyte diameter at the Eden Estuary in October. From the graph it is clear to see that most of the worms which are not reproductively active are smaller and this implies that they are younger than the ones approaching spawning. The weight distribution is more scattered in the breeding worms. This implies that the reproductive part of the population is not necessarily all from one year class.

3.3.3 Differences between the North and South Sides of the Beach at Fairlie Sands

Samples were dug from both sides of the beach in October, 1990 and worms were examined for the state of maturity and weighed. ANOVA was carried out on mean oocyte size in relation to the weight of the worm and this showed that there was no significant difference between sides ($F=2.31$, $P=0.138$). Figure 3.41 shows the mean oocyte diameter plotted against the weight of the worms from the two different ends of the beach. It appears from this graph that there are fewer large worms which are not mature at the south end of the beach.

3.4 Discussion

All of the *Arenicola* examined in this investigation were of the species *A. marina* and there was no evidence of *A. defodiens* at any of the study sites. At all three study sites individuals go through the same developmental process of

gametogenesis. At the beginning of the reproductive cycle the small oocytes and spermatocytes are released into the coelomic cavity. During this phase numbers of oocytes and spermatocytes increase in the coelomic cavity. The gametes then enter a fast growth phase a few months before spawning takes place. In the females it is assumed that the oocytes that were inititally released into the coelom will enter vitellogenesis before others released later on. When these oocytes first enter vitellogenesis the ovaries may still be releasing oocytes into the coelom. The females start to enter this developmental phase before the males from the same site do. This is probably because the time taken for the oocytes to develop is longer than for sperm production. During this phase the range of oocyte diameters measured was very wide. As spawning approaches the smaller oocytes catch up with the ones that are already fully developed and a uniform diameter of between 175 and 200μm is reached. The percentage of sperm present as morulae approaches a hundred. When individuals from the same and different sites are compared it can be seen that at the population level the situation is somewhat more complicated.

At Fairlie Sands as suggested by (Howie, 1959), there are gravid worms present on the beach in Spring. There are not really two distinct spawning seasons, however, because there appears to be a few worms which are approaching spawning present at most times of the year. This is very unusual because at all of the other sites where the spawning season has been noted, the length is generally restricted in some way. There are several reports of non-epidemic spawning seasons which extend over two months (see Howie, 1984), but there are no sites reported to have some mature individuals present at all times of the year. At Fairlie Sands the percentage of worms which are gravid has a marked increase in April and again in November suggesting these are the main spawning times. It could be that these two spawning seasons have become very extended. In nearly all months at Fairlie Sands more mature females than males are found. This could be because it takes less time for the males to

become mature and so there may be others that were missed out by monthly sampling. The sex ratio in October which is when the greatest part of the population were observed to spawn was observed to be about 1:1. Newell (1948) reports finding a sex ratio of 3.8 females to 1 male in September at Whitstable Flats. At the East Sands and the Eden Estuary the sex ratio immediately prior to spawning was also 1:1.

This asynchronous spawning at Fairlie Sands is very strange as it might be expected that the reproductive success of these few individuals would be reduced. It may be that the spawning in males is stimulated by the production of a pheromome by the female. If the female produced such a pheromone prior to or during spawning, any gravid males in the near vicinity would be induced to spawn. This would increase the chances of successful fertilisation considerably. This may also take into account the time delay observed by Pacey and Bentley (1992), when spawning was induced in male and female *Arenicola marina* in the laboratory. Males started spawning fifty minutes after being injected with crude prostomial homogenate whereas females did not start to spawn until five hours after injection. Spawning may be delayed in females in order to allow for males to respond to the release of a pheromone. *Cirratulus cirratus* have been shown to have an unsynchronised breeding cycle at the population level. Gametes mature synchronously within individuals which then spawn throughout the summer months (Olive, 1970). There is a possibility that pheromonal action takes place here also.

The lugworm population at Fairlie Sands is under intense pressure from bait diggers. One possibility that could explain some of the inconsistencies in these data is that the bait diggers are positively selecting the larger worms. Figure 3.37, however, suggests that the size of the worm is not related to its reproductive state. There were small and large worms present in all stages of reproduction in October 1990. If the beach is constantly being disturbed the individual worms will move around a lot more and the population will be generally less stable.

These results show that in the Eden Estuary the spawning season lasts for about a month with most of the spawning taking place during the last three weeks in October and being completely finished by mid-November. Although this is a protracted period, the population is synchronised in comparison to the population at Fairlie Sands. In a large percentage of the population maturation of gametes does not take place and the worms remain with the immature gametes in the coelom, probably until the following year. These animals increase the proportion of immature gametes present throughout the year. However the graphs of means and ranges for the individual worms shows the presence of two cohorts within the population that are easily distinguishable. One cohort consists of individuals which are approaching spawning. The other group contains individuals that have gametes present in the coelom but these gametes do not start to mature and the worms do not spawn that year. This group is different from juvenile worms which are easily distinguishable by being smaller, more transparent and they have no gametes present in the coelom. This non-spawning cohort is probably the worms that were recruited from the year before the juveniles.

The Eden Estuary is a very sheltered site and consequently the substratum is stable. The shore slopes fairly steeply so there is a small intertidal area and the worms can be found up to the mid-neap tide mark. Juveniles and first time breeders are, therefore, found in the same areas as the adult worms. Sampling included worms from different year classes. As the worms breed at a much smaller size here than at either of the other two sites, the differences in the cohorts are not immediately obvious. It is possible that these worms which have immature gametes present throughout the breeding season are in their first reproductive year. They may go on to breed next year. The cohort of breeding worms could be in their second year of reproduction, corresponding to the third year since recruitment to the population. In natural populations of *Nereis diversicolor* and *N. pelagica*, females are in their

second year of growth before oocytes start to appear in the coelom (Olive & Garwood, 1981). Oocyte development then takes eighteen months and the worms breed in their third year. It seems likely that the reproductive development in *A. marina* at the Eden Estuary follows a similar pattern, although age determination is a problem in *Arenicola*. Olive *et al.* (1986) have demonstrated that the three year reproductive cycle is not fixed and is in fact dependent on the growth rate of the individuals. Olive and Garwood (1981) suggest that in natural populations there are some individuals which become mature after two or four years. In this way genetic isolation between successive year groups would be prevented. It is possible that the age at which *Arenicola marina* spawn for the first time may also vary between two and four years depending on external conditions.

The density of lugworm is very high at the Eden Estuary with counts of up to 35 casts per m^{-2} in some places (Johnson *et al.*, 1978). It is possible that these worms are actually all of the same year class but the high density limits the spawning. Reproductive failure has been reported in *Nephtys hombergi* (Olive *et.al.*, 1981b) where the animals produce gametes but spawning does not occur. The unspawned gametes are then resorbed. It was suggested that this could be density dependent inhibition of breeding. If the population is near to the carrying capacity of the environment limitations on food resources may become critical. This could have some effect on the reproductive processes within the population.

At the East Sands there is no evidence of gametes in the coelomic fluid until March and these increase in number until April. The reproductive development is the same as in the animals from the Eden Estuary but it starts a month later. However in the later stages of the cycle the worms from the East Sands seem to become very synchronised and spawn simultaneously. Spawning took place over a two or three day period during the second set of spring tides in October as observed by Bentley and Pacey (1992). The worms appear to all be from the same cohort as they all have

developing gametes present. At the East Sands the juvenile population is completely separated from the adult population, being situated further south along the beach and higher up the shore. This means that juveniles were not regularly sampled with the adult population as happened at the Eden Estuary. The reason why the adults and juveniles are separated may have something to do with the fact that the East Sands is a much more exposed shore than that found at the Eden Estuary. This will lead to a gradation of particle sizes which affects the distribution of *Arenicola* (Longbottom, 1970). Certain areas for example, the patches of sand between rocks, will be a lot more stable and may be easier for the juveniles to colonise.

Routine sampling of the population during February of 1993 revealed a strange phenomonon. Twenty worms were collected and of these two males contained sperm present as morulae in the coelomic cavity. The worms were stimulated to spawn with 8,11,14-eicosatrienoic acid and a full spawning response was observed. During the last seven years of sampling from this population, there has never been any gravid worms present at this time of year before (M.G. Bentley, personal communication). There is a report in the literature of worms from St. Andrews spawning in the spring (Kyle, 1896). It may be that certain extreme conditions, for example, a particularly cold winter, could alter the spawning patterns of some of the population.

Variation in the timing of the spawning period with geographic location is not so unusual in invertebrate populations because the environmental factors, which vary from site to site may be responsible for controlling certain aspects of reproduction (see Section 1.8.1). What is unusual in these populations of *Arenicola marina* is that the spawning patterns are markedly different between the populations. Howie (1959) noticed a correlation between a fall in the environmental temperature in the Autumn and the onset of the breeding season in *Arenicola marina.* Spawning is often induced when very mature worms are brought into the laboratory. This could be because of

the change in temperature. However, when mature worms were kept at a constant temperature in the laboratory prior to spawning, they still spawned at the same time as the worms on the shore (Howie, 1963). The idea that *Arenicola* needs to be exposed to a critical temperature to induce spawning was however, firmly ruled out by Duncan (1960) on the basis of some of Newells results. He found that the worms at Whitstable Sands spawned at the same time every year, even in 1951 when the temperature of the sand was 5º colder. The low temperature theory was later accepted as invalid by Howie (1984). This sort of cue could not explain spawning in populations which have an extended breeding season, such as the one at Fairlie Sands. The Eden and the East Sands are also subject to the same temperature regime, yet experience different spawning periods.

An environmental factor that is often linked with temperature is photoperiod. For example, *Harmothoe imbricata* needs to be exposed to a critical photoperiod of 13hours for less for a period of between 42 and 55 days for gametogenesis to take place (Clark 1988). In the later stages of vitellogenesis low temperature and long day length are important (Garwood, 1980 ; Garwood & Olive, 1982). Photoperiod will be of less importance in a species such as *Arenicola marina* because they have no eyes or pigment spots present. *Harmothoe* living on rocky shores will be much more exposed to changes in light intensity. Some interstitial animals are affected by photoperiod, for example *Nephtys caeca* has photosensitive spots present in the prostomium and it is believed that temperature and photoperiod control gamete development (Bentley *et al.*, in press.).

Other environmental factors such as tidal cycle may also differ greatly in different areas. The importance of tidal influence on invertebrates spawning is well known. Several species which show a tidal periodicity of spawning in the North Sea and Atlantic show a lunar periodicity in the Mediterranean (Korringa, 1947). In other species showing variation with location the environmental differences are not so

obvious. For example on the Welsh coast *Spirorbis spirorbis,* show a tidal periodicity of spawning but Daly (1977) found populations on the Northumberland coast in which spawning was not related to the tidal cycle.

Previously reported spawning events in *Arenicola marina* occur in different tidal phases at different sites. The reports for one site tend to be consistent from year to year. Newell (1948) reports that the worms at Whitstable Flats spawned at neap tides for five consecutive years, whereas the worms at the East Sands in St. Andrews spawn very predictably on the second set of spring tides in October every year (Bentley & Pacey, 1992). Duncan (1960) claims that out of nine populations of lugworms examined in Britain, eight of them spawned over neap tides. Spawning on neap tides may increase the chances of successful fertilisation because there is less water movement compared to on a spring tide which may facilitate the sperm reaching the female burrow.

One of the principal differences between the Eden Estuary and the East Sands is the exposure of the shore. This affects the slope of the beach and hence tidal range. The volume of water moving in and out over the East Sands is much greater than that at the Eden Estuary. If the worms were using changes in pressure to detect the tidal cycle, it would be a lot easier to determine the state of the tide on a beach like the East Sands. It is therefore possible that the reduced tidal stimulus present at the Eden Estuary means that the spawning cannot be as synchronised as at the East Sands.

Lugworms must have a way of measuring the tidal cycle. It may be that they can detect changes in pressure between high and low tides, and between spring and neap tides. The pressure difference between spring and neap tides at St. Andrews is 0.2bar which is equivalent to 150mm Hg, about 20% more than normal atmospheric pressure. This could provide some kind of regulatory mechanism or stimulus for spawning or a particularly big spring tide may entrain the endogenous cycle.

When *Arenicola* is removed from these environmental factors such as light, temperature and tides, spawning has been observed, at least in some individuals to occur at the same time as in natural populations (Howie, 1963; Farke & Berghuis, 1979b). This does not necessarily mean that these factors have no effect. It may be that that there is some internal biological clock that is started early on in life by the tidal cycle and once this is established it cannot be changed. This has become known as a zeitgeber, when the biological clock is constantly being reset by response to external cues such as lunar periodicity. These cues may be more effective at certain sites than at others. This could explain the differences in the lengths and timing of the spawning seasons that have been observed. Worms from the East Sands may be able to reset their biological clock more effectively than those at the Eden Estuary. It seems that the breeding season at Fairlie Sands is very extended. This is unusual with regard to information about spawning at other locations (Howie, 1984).

Populations of *Pygospio elegans* from different localities have different modes of reproduction (Anger, 1984). Individuals from three different populations were maintained in the laboratory for fourteen months under different conditions of salinity and temperature. The mode of reproduction did not alter during this time. This could imply that the reproduction is influenced by genetic factors. If there is a lack of genetic exchange between different populations reproductively distinct populations could arise.

Populations of *Pecten maximus* in Brittany, France and Scalpay, Scotland are also known to have different spawning seasons. This could be due to differences in environmental factors and has been tested by transplant experiments. Scallops transplanted as post-settlement juveniles showed the features of the reproductive cycle characteristic of their sites of origin (Ansell *et al.*, 1988). This may mean that certain aspects of the reproductive cycle are under genetic control.

There is some evidence to suggest that this could also be the case for *Arenicola*. As described in Section 1.4 the eggs are spawned into and fertilised in the burrow of the female worm. It is unlikely that the eggs could be fertilised by spermatozoa from worms from another beach when there are male worms spawning in the same location, although the spermatozoa do survive for up to 24 hours. The larvae are benthonic and therefore do not travel great distances. The post-larval migration to juvenile beds and then the migration of the juveniles down the shore to the adult beds may account for further distribution. As the time spent in the water column during these migratory periods remains undetermined there is the potential for subpopulations to become reproductively isolated.

Chapter 4

Variation in Populations of *Arenicola marina* from Different Localities

Variation in Populations of *Arenicola marina* from Different Localities

4.1 Introduction

As described in Section 1.5 and 1.6 there are many observed differences between local populations of *Arenicola marina*. These involve principally body size, colour and the length and timing of the spawning season but other morphological differences have also been noted such as annulation pattern. These contrasts between populations could be phenotypic, arising through different environmental conditions or they could be genetic in origin. There are several ways to investigate this, including the possibility of transplant experiments. There are, however, obvious problems associated with transplant experiments on *Arenicola*. Being infaunal and intertidal creates problems in relocating the animals. Also, although they are described as sedentary there is evidence to suggest that they can swim out of their burrows at high tide (Cazaux, 1966; Meek and Storrow, 1924).

A more convenient method is to look for genetic variation between the different populations. Starch gel electrophoresis was chosen as a simple, relatively inexpensive and reliable way to estimate the degree of genetic variation within and between different populations. This technique concerns the quantification of variation in enzymes by visualisation of particular loci on a gel. Variation at given loci can produce protein products that are functionally identical but even a single amino acid substitution may lead to a difference in the overall ionic charge of the molecule. These ionically different forms of the same enzyme are known as isozymes. The isozymes can be separated by their different mobilities through a medium, in this case a starch gel. The rate of mobility will depend largely on the ratio of the number of positively charged to negatively charged amino acids present in the enzyme. If there is no allelic variation at

a particular locus, all individuals will have an enzyme with the same electrophoretic mobility and this is termed a monomorphic locus. To estimate the variation present in a population several polymorphic loci need to be visualised. The allele frequencies at loci can then be calculated and used as a measure of the genetic variation within and between populations.

Genetic variation, in the form of multiple alleles for many individual loci, exists in most natural populations. The amount of variation that exists within a species, and the nature of that variation, can vary considerably from one genetic locus to another. Nevo *et al* (1984) summarised the results of electrophoretic surveys of more than 100 genes in populations of 1111 species. He found that invertebrates have the highest average amount of variation, followed by plants with vertebrates showing the least variation. The validity of the extrapolation of the results of a few loci to the whole genome is questionable. The main problem is that the proteins most commonly used in electrophoretic surveys are not a random sample of all the proteins present in a living organism. There is evidence to suggest that there are locus specific effects on levels of variation; for example, certain groups of loci tend to be highly polymorphic whereas others are almost always monomorphic (see Gillespie, 1991). This may introduce a systematic bias if polymorphic loci are being chosen to estimate genetic variation. It has been shown that there is a highly significant negative correlation between estimates of average heterozygosity and the number of loci used in a study (Singh & Rhomberg, 1987). This implies that levels of genetic variation may have been overestimated by using only a few loci. Within a species however, the degree of genetic variation estimated from a few loci may give an insight into differences among subpopulations with respect to the extent of gene flow between different localities. There may be different degrees of genetic variation between different populations of *Arenicola marina*. Evidence for this lies in recorded morphological differences at different

geographic locations and the observed differences in duration and timing of the spawning season.

4.2 Materials and Methods

4.2.1 Phenotypic variation

Observations were made on the individuals collected from the study sites. The samples of individuals were collected from Fairlie Sands and the Eden Estuary in October, 1990 and from the East Sands in 1992. The worms were weighed and linear measures taken. The worms were blotted dry with tissue paper in a standardised manner and then weighed, to the nearest 10mg, on a top pan balance. For the contracted worm a measure of trunk length was also taken.

4.2.2 Genetic Variation

The animals used were collected from the three study sites described in Section 3.2. In addition some data were obtained from two other sites - notably Budle Bay, Northumberland, England, Grid reference, Latitude 55° 37', Longitude 1° 45' (OS - NU150365) and Loch Ryan, Dumfries and Galloway, Scotland, Grid reference Latitude 54° 55.5', Longitude 5° 05' (OS - NX683042); although the sample sizes from these two latter sites were much smaller, the data obtained are none the less of interest because of the geographical location of the sites.

The definitive electrophoretic protocol employed was essentially standard and is described in Section 2.11, however a number of adaptations to this method were necessary to optimise the visualisation of allozyme variation. Initially, there were problems with streaking and the clarity of resolution of the bands. A number of

different parameters were changed in the method to try and resolve these problems. Initially muscular tissue from the body wall of the tail only was used. This provided a very convenient source of tissue because it does not kill the animal and permitted repeated screening of the same individual if necessary. However this tissue was found to produce a lot of mucus, causing streaking and poor resolution of the bands. A number of methods were employed to try and reduce these affects.

1) The tissue homogenate was centrifuged before the wicks were soaked and placed in the gel.

2) The tissue was frozen to -20°C or to -70°C before homogenization.

3) The gels were run at lower power over an extended period of time.

4) Gels of different thickness were tried.

5) Glycerol was added to the homogenising buffer.

6) A number of buffer systems were tried also including phosphate / citrate, morpholine / citrate, tris-citrate (pH7.1) (Ahmed *et al.*, 1977), tris-borate buffer (William *et al.*, 1983) and a tris-citrate-EDTA (pH 6) (Cadman & Nelson-Smith, 1990).

7) Different body parts of the animal were tried, including the prostomium and parts of the gut.

4.3 Results

4.3.1 Difference in Size between Beaches

ANOVA was used to test if there was a significant difference between the weights of the worms from the different beaches. The worms from the Eden Estuary were significantly smaller than those from either of the other two sites ($F=15.39$, $P=<0.001$). Worms from the East Sands were comparable in size to worms from Fairlie Sands. Histograms to show the distribution of weights of worms from the three sites are shown in Figure 4.1.

4.3.2 Differences in the Degree of Genetic Variation

Table 4.1 summarises the results of all the staining systems that were tried during the initial phase of this investigation. The four enzymes that consistantly showed the best results are described below.

Got (Glutamate Oxaloacetate Transaminase - E.C.2.6.1.1)

This enzyme showed two zones of histochemical activity (see Figs. 4.2 and 4.2.1). The slower locus (i.e. that of the lesser electrophoretic mobility) was monomorphic but the faster locus was polymorphic. The enzyme has a dimeric subunit structure and proved to be highly variable. For individuals from the Eden Estuary and the East Sands two alleles could be visualised. A third allele was found to be present in a few individuals from Fairlie Sands.

Pgi (Phoso-Glucose Isomerase - E.C.5.3.1.9)

One zone of activity which was attributable to a single polymorphic locus (see Fig.4.3) was noted. The amount of variation observed at this locus was dependent on the site. The peptide has a dimeric quaternary structure and stained with the expected three banded heterozygotes.

α-Gpdh (α–Glycerophospate Dehydrogenase - E.C.1.1.1.8)

One zone of activity was recorded and which corresponded to a single highly variable polymorphic locus (see Fig.4.4). Two alleles were present in the population. The heterozygotes were of 3-banded type, as would be expected for a dimeric enzyme.

Acon (Aconitase - E.C.4.2.1.3)

There was one zone of activity on the gels for this enzyme (see Fig.4.5). This enzyme is a monomer with heterozygotes staining up with two bands. The results obtained with this enzyme are not very extensive due to problems with the activity of isocitric dehydrogenase, a critical part of the staining process. The product used originally (Sigma catalogue number, I-5882) was discontinued and replaced by the manufacturer with an alternative product (Sigma catalogue number, I-2002) with which no further results could be obtained.

The relative mobilities of the enzymes were assessed on the basis of the most common allele for each locus, considered here to be 100. A relative mobility of 50 therefore represents one-half the migration distance of the fastest allele. The fastest allele is represented by the letter F, the slowest by the letter S and the medium allele by the letter M. Relative mobility, genotype and allele frequencies for these four loci for *Arenicola marina* from the five study sites are shown in Tables 4.2 to 4.4. The genotype and allele frequency data presented for Fairlie Sands, Eden Estuary and East Sands are taken from pooled samples of lugworms collected monthly to increase the overall sample sizes (Section 3.1). This was done in order to eliminate any bias that may be introduced through small sample size, especially as the number of loci used was small. Pooling data gives an opportunity to compare the sites directly, which was considered to be more useful in conjunction with Chapter 3 than assessing the variation present at each site at different times of the year.

These results show that for *GOT* the allele frequencies for the sites on the east coast differ from those sites on the west coast. The East Sands, Eden Estuary and Budle Bay had allele frequencies for the fast allele ranging between 0.75 and 0.80, whereas for the two west coast sites, Fairlie Sands and Loch Ryan, the frequency of the same allele was approximately 0.50. A third allele was present also at Fairlie Sands, but this was not detected at any of the other sites. For *PGI* the slow allele was the more common at Fairlie Sands, and the fast allele was more common at the Eden Estuary and Loch Ryan. The medium allele was the most prevalent allele at all five sites. *αGPDH* showed similar allele frequencies at the three sites where it was measured.

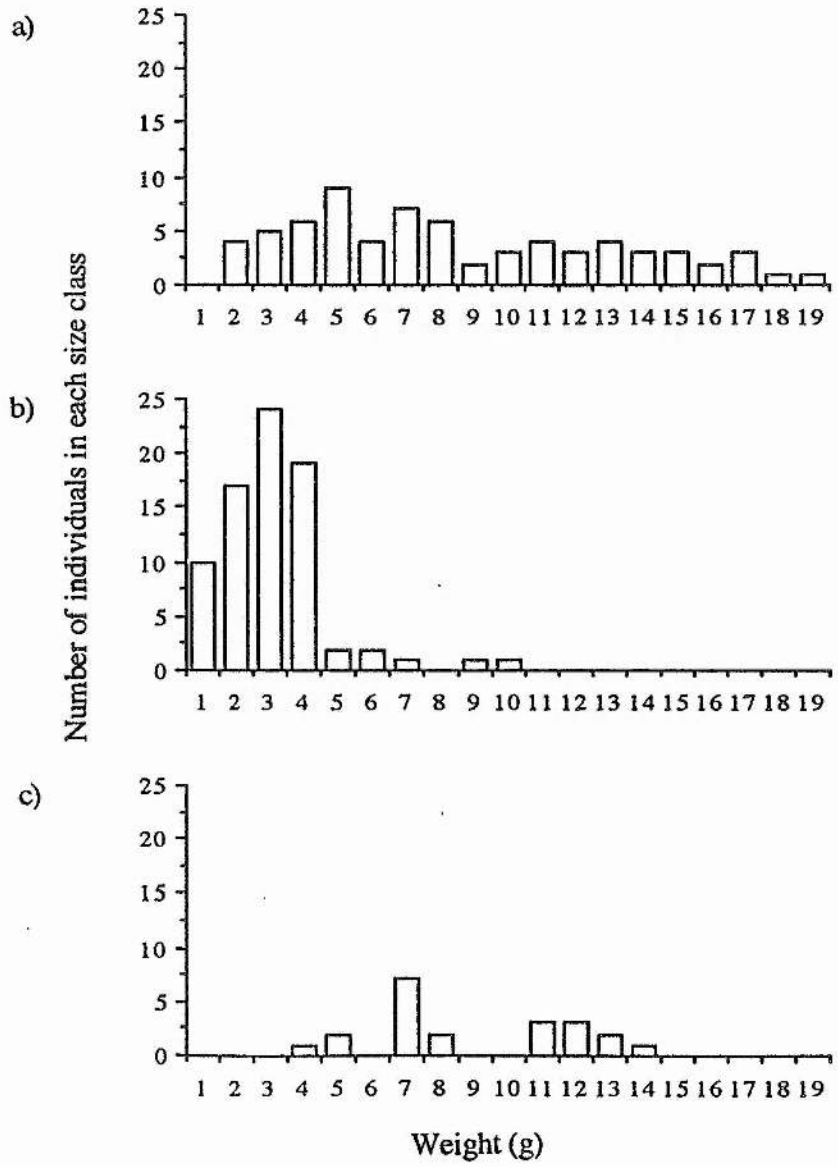


Fig. 4.1 The distribution of weight of *Arenicola marina* collected from three different sites in October. Weight is divided into size classes (ie. 0g-0.99g, 0.99g-1.99g etc.), and the histograms represent the number of individuals present in each size class. a) Fairlie Sands b) Eden Estuary c) East Sands

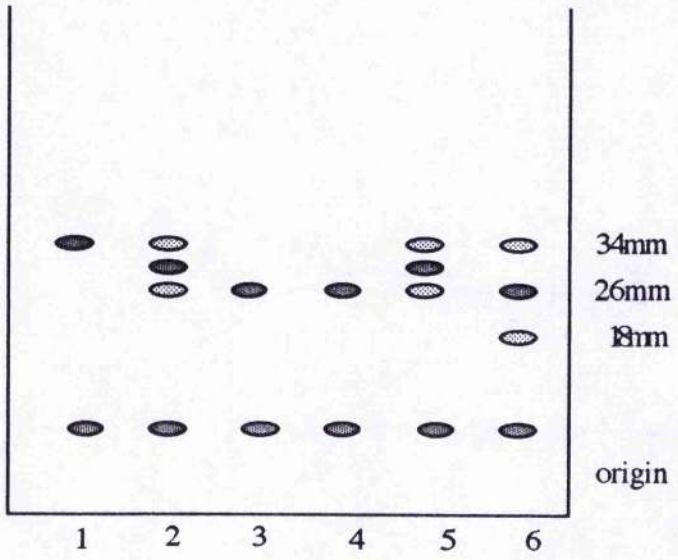


Fig. 4.2 Diagram of a gel to show the genotypes observed at the *GOT* locus Individual 1 is homozygous for the fast allele (FF); 3 and 4 are homozygous for the medium allele (MM); 2 and 5 are heterozygous for the fast and medium alleles (FM) and 6 is the rare fast and slow allele heterozygote that was only present at Fairlie Sands.

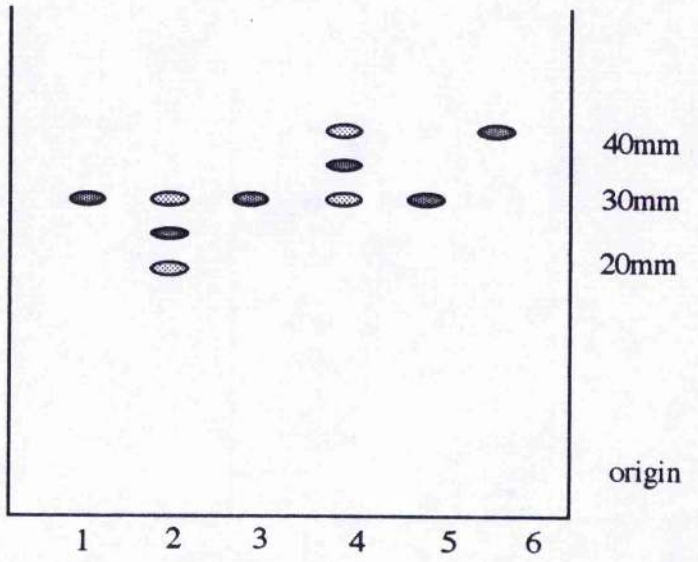


Fig. 4.3 Diagram of a gel to show the genotypes present at the *PGI* locus. Numbers 1, 3 and 5 are homozygous for the medium allele (MM); 6 is homozygous for the fast allele (FF); 2 is heterozygous combining the medium and slow alleles (MS) and 4 is heterozygous combing the fast and medium alleles (FM).

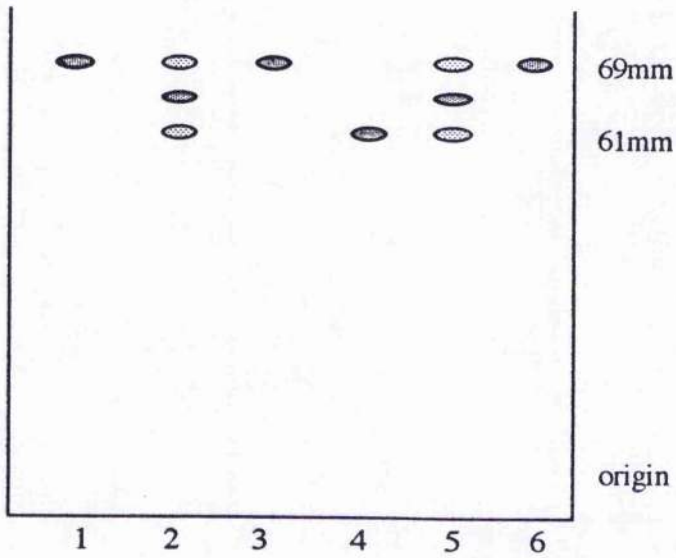


Fig. 4.4 Diagram of a gel to show the genotypes observed at the *αGPDH* locus. Individuals 1, 3 and 6 are homozygous for the fastest allele (FF); 4 is homozygous for the slowest allele (SS), whereas 2 and 5 are both heterozygous (FS)

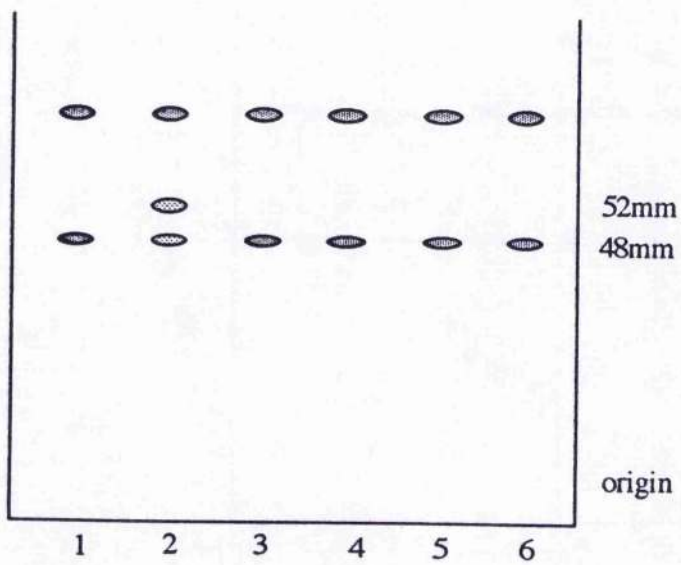


Fig. 4.5 Diagram of a gel to show the genotypes observed at the *ACON* locus. All of the individuals are homozygous for the slow allele (SS) except for 2 which is a heterozygote (FS).

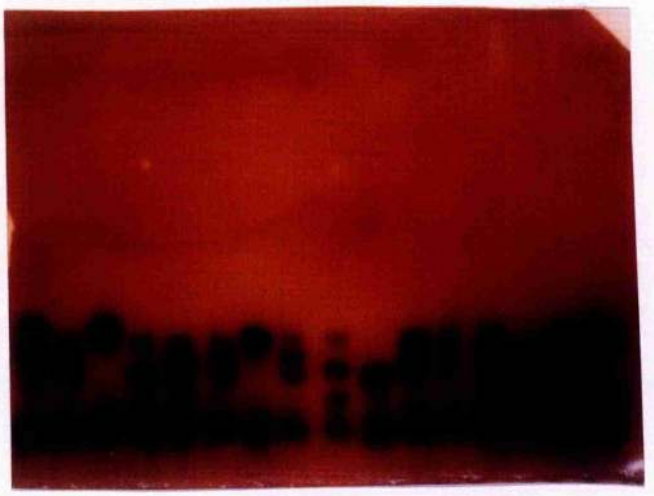


Fig. 4.6 Photograph of a gel to show the genotypes observed at the *GOT* locus. Individuals are *Arenicola marina* from Fairlie Sands. Numbers 3, 7 and 16 are homozygous for the fast allele (FF); 10 and 14 are homozygous for the medium allele (MM); 1,2,5,6,8,11,12,13 and 15 are all heterozygous combining the fast and medium alleles (FM); 4 and 9 are heterozygotes combing the the medium and rare slow allele, which was only observed at Fairlie Sands.

Table 4.1 Information on the range of enzymes that were stained for during initial trials. From these the results of four enzyme assays were used to estimate genetic variation within and between populations.

Enzyme	Variation	Staining Intensity	Degree of Streaking	Resolution
Acon	polymorphic	medium	negligible	good
Ak	polymorphic	medium	great	poor
Alcdh	monomorphic	medium	none	good
Ald	?	very poor	-	-
Alp	polymorphic	medium	variable	unreliable
Dia	?	very poor	-	-
Fum	?	very poor	-	-
Gcsdh	monomorphic	medium	none	good
G-6-pdh	?	good	great	poor
α Gpdh	polymorphic	generally poor	none	good
6-Gpdh	?	good	great	poor
G-3-pdh	polymorphic	medium	great	poor
Got	polymorphic	good	none	good
Hex	polymorphic	poor	-	very poor
Idh	polymorphic	good	none	poor
Lap	polymorphic	poor	-	very poor
Ldh	?	medium	great	poor
Me	polymorphic	poor		unreliable
Mdh	monomorphic	good	none	good
Mpi	?	very poor	-	-
Odh	polymorphic	medium	some	very poor
6-Pgdh	?	good	great	
Pgi	polymorphic	generally good	some	generally good
Pgm	monomorphic	medium	none	good
Pnp	?	very poor	-	-
Sordh	monomorphic	medium	none	good
Tpi	monomorphic	medium	none	good
Xod	?	very poor	-	-

Table 4.2 Relative mobilities of alleles of four loci from *Arenicola marina* .

Enzyme	Alleles	Relative mobility
Got	Fast (F)	100
	Medium (M)	76.5
	Slow (S)	52.9
Pgi	Fast (F)	133.3
	Medium (M)	100
	Slow (S)	66.6
αGpdh	Fast (F)	113.6
	Slow (S)	100
Acon	Fast (F)	108.3
	Slow (S)	100

Table 4.3 Genotype frequencies of *Arenicola marina* collected from five different locations in the northern British Isles.

Site		Fairlie Sands	Eden Estuary	East Sands	Loch Ryan	Budle Bay
Locus	Genotype					
GOT	FF	34	92	91	11	31
	FM	59	67	44	21	12
	MM	36	9	6	7	4
	FS	8	0	0	0	0
	n	137	168	141	39	47
PGI	FF	0	1	3	2	0
	FM	9	19	34	9	1
	MM	54	129	46	17	24
	MS	10	2	1	3	1
	n	73	151	84	31	26
αGPDH	FF	9	8	8		
	FS	28	17	30		
	SS	73	33	74		
	n	110	58	112		
ACON	FF		0			
	FS		3			
	SS		25			
	n		28			

Table 4.4 Allele frequencies of *Arenicola marina* collected at five different locations in the northern British Isles.

Site		Fairlie Sands	Eden Estuary	East Sands	Loch Ryan	Budle Bay
Locus	Allele					
GOT	F	0.493	0.747	0.801	0.551	0.787
	M	0.478	0.253	0.199	0.449	0.213
	S	0.029				
PGI	F	0.062	0.069	0.238	0.210	0.019
	M	0.870	0.924	0.756	0.742	0.962
	S	0.068	0.007	0.006	0.048	0.019
αGPDH	F	0.209	0.285	0.205		
	S	0.791	0.715	0.795		
ACON	F		0.054			
	S		0.946			

4.3.3 Analysis of the Genetic Data.

Testing for Hardy-Weinberg Equilibrium

The Hardy-Weinberg principle is a method of predicting genotype frequencies within populations. The model predicts homozygote and heterozygote frequencies as a function of the allele frequency assuming that certain criteria are being met by individuals within the population. These are that the individuals reproduce sexually, mating is random, the population is large with no migration taking place and natural selection is not affecting the genes under consideration. The analysis is therefore testing these conditions and deviation from Hardy-Weinberg equilibria means that one or more of the criteria are not being met.

The goodness-of-fit between the observed numbers and the numbers predicted by the Hardy-Weinberg principle are assessed quantitatively by the use of the chi squared statistic (χ^2). Chi squared values and the probabilities indicative of the conformance of the observed genotype frequencies with expected values under Hardy-Weinberg equilibrium were calculated. The results for these are shown in Table 4.5.

Genotype frequencies did not differ significantly from Hardy-Weinburg expectations at the *PGI* locus in any of the populations. However at the *GOT* locus there was an excess of heterozygotes present at Fairlie Sands. Genotype frequencies at the *GOT* locus for the other four sites conformed to Hardy-Weinburg expectations. At the *αGPDH* locus there was a significant departure from the Hardy-Weinburg equilibrium for Fairlie Sands, the Eden Estuary and the East Sands. This is due to a heterozygote deficiency at all sites.

Calculation of the F -Statistics

The BIOSYS-2 (Swofford & Selander, 1981) program was used for determining the F -statistics. The F -statistics are used for assessing population division which is attributable to random genetic drift. If the population is subdivided it may be assessed at three different levels, that of the individual, the subpopulation and the total population. The fixation index (F_{ST}) measures the variation in allele frequencies between subpopulations and provides a summary measure of differentiation among populations (Nei, 1977; Wright, 1978). In most natural populations the values of F_{ST} are typically close to zero. Hierarchical analysis is a method of analysing F -statistics and variance components of subpopulations and which assesses the amount of variation contributed by individual subpopulations to the overall genetic variation of the population (Hartl & Clark, 1989). F_{IS} is the inbreeding coefficient and is a convenient measure for the reduction in heterozygosity of an individual (I) due to non-random mating within its population. F_{IT} is the overall inbreeding coefficient and measures a reduction in heterozygosity of an individual relative to the total population (T). The three F -statistics are related:

$$(1-F_{IS})(1-F_{ST}) = (1-F_{IT})$$

The values obtained for Fairlie Sands, the Eden Estuary and the East Sands are given in Table 4.6 below. The inbreeding coefficient (F_{IS}) shows that the average measure of heterozygote deficiency across the three loci ranges from -0.0073 for *GOT* to 0.2812 for $\alpha GPDH$ indicating heterogeneity. Heterozygote deficiencies are noted as positive F_{IS} values. The value for $\alpha GPDH$ is high but this is not surprising because there is heterozygote deficiency at this locus which results in a significant departure from Hardy-Weinberg equilibrium for all three sites sampled. Possible explanations for this are discussed below. The mean F_{IS} (0.0895), represents a moderate level of variation which indicates that individuals within subpopulations (ie. different sites) do show some inbreeding and in this case the variation in F_{IT} is

attributable to variation in both F_{IS} and F_{ST}. the populations contain moderate levels of variation. F_{ST} values for individual loci ranged from -0.0061 for *αGPDH* to 0.0966 for *GOT*. The mean F_{ST} over all loci (0.0548) falls within the range recognised as moderate population differentiation (0.05 to 0.15) (Wright, 1978).

Testing for Linkage Disequilibrium

If there is a random gametic association beween alleles of different genetic loci it is called linkage equilibrium. The data were tested for linkage disequilibrium, using Weir's Fortran program for analysing genetic data (Weir, 1990). This tests for the probability that D =0, given that a pair of genes are in linkage disequilibrium (Hill, 1974). The value of D is the difference between an allelele frequency and its expected value when there is no association between genes (ie. a positive value of D implies a positive correlation of alleles). Linkage disequilibrium was estimated only for Fairlie Sands, the Eden Estuary and the East Sands (Table 4.7). There were insufficient data to estimate linkage disequilibrium for populations from Budle Bay or Loch Ryan. It would be inappropriate to measure linkage disequilibrium between *GOT* and *αGPDH* because one of the assumptions for measuring D is that the the population is in Hardy-Weinberg equilibrium at each locus (Hill, 1974). We know that this is not true for *αGPDH* and the results would therefore be meaningless. There is no significant occurrence of linkage disequilibrium between *GOT* and *PGI* and there were no trigenic or quadrigenic disequilibria.

Table 4.5 Chi-square values calculated by comparison of observed genotypes to the numbers expected under Hardy-Weinberg equilibrium. An asterisk indicates statistically significant departure from expected values.

Site		Fairlie Sands	Eden Estuary	East Sands	Loch Ryan	Budle Bay
Locus						
GOT	χ^2	9.296 *	1.073	0.049	0.305	2.655
	P	<0.003	>0.3	>0.99	>0.6	>0.1
PGI	χ^2	1.64	0.183	1.406	1.311	0.044
	P	>0.2	>0.99	>0.25	>0.25	>0.99
αGPDH	χ^2	5.971 *	4.556 *	8.278 *		
	P	>0.025	>0.034	<0.005		

Table 4.6 The *F* -statistics of loci from *Arenicola marina* collected from three different localities in Scotland.

	GOT	*PGI*	*αGPDH*	Over all loci
F_{IT}	0.0900	0.0279	0.2768	0.1395
F_{ST}	0.0966	0.0633	-0.0061	0.0548
F_{IS}	-0.0073	-0.0377	0.2812	0.0895

Table 4.7 Estimates of linkage disequilibrium comparing *GOT* with *PGI* at three different localities in Scotland. The number of degrees of freedom is 1.

Site	*D (GOT & PGI)*	χ^2
Fairlie Sands	0.0588	3.21
Eden Estuary	0.0149	2.08
East Sands	0.0199	0.64

4.4 Discussion

The variation that is most readily observed in natural populations does not have a simple genetic basis. Variations in size, shape and colour are nearly universal among individuals in populations. Typically there is no single gene that can account for the variation observed in phenotypic traits like these. We can however use these traits to a certain degree to compare the populations from different localities. *Arenicola marina* at the Eden Estuary are predominantly brown or black in colour, whereas worms from Fairlie Sands and the East Sands are more likely to be red or brown (personal observation). The worms from the Eden Estuary are also significantly smaller than the worms from either Fairlie Sands or the East Sands (see Section 4.3.1). Differences in size could have a genetic basis but other environmental factors cannot be ruled out. Size can vary considerably according to external parameters such as food supply and population density, which have not been assessed in this study. In a survey of the invertebrate fauna of the Eden Estuary, the distribution and density of species was recorded (Johnston *et al.*, 1978). For the location nearest to the area sampled in this study they report the number of faecal casts per square metre to be as many as 35 in the

summer. A considerable drop in numbers is reported to occur in October. It was observed during sampling that the density of lugworm is consistantly higher at the Eden Estuary than at either the East Sands or Fairlie Sands. At the Eden Estuary it was not uncommon to obtain two or three worms in one fork load of sand, whereas at the other two sites it was necessary to remove two or three forks loads of sand before the worm was reached.

It is possible that the Eden Estuary could support a higher population density due to an increased supply of organic matter. The paper mill at Guardbridge about two kilometres up the estuary from the study site releases organic waste into the water. This may lead directly to an increased food supply if particles settle onto the substratum. The food supply may also be increased indirectly due to a higher level of nutrients present from surface run off from surrounding fields into the estuary. If there are increased nutrients in the water, then primary productivity may increase leading to higher levels of organic matter being deposited on the shore. A consequence of this may be that the beach can support a higher density of worms than would otherwise be expected. This does not imply that nutrients are necessarily limited. If this were the case however, it would not be expected that the growth of the adults would be limited unless there was a competition-induced small size effect. More extensive data for the structure of the population would need to be collected before this could be ascertained. Levin (1986) studied the influence of organic enrichment on growth and reproduction in a population of *Streblospio benedicti* . She found that at concentrations of nutrients (N, P and Si) eight times and thirty two times the average aerial input resulted in an increase in body length and a doubling in brood size in *S. benedicti* females. This implies that an increased level of nutrients in the Eden Estuary would not necessarily have a size limiting effect on the *A. marina* population. Weight specific growth rates of different size classes of *Capitella capitata* have been measured in the laboratory under varying conditions of population density and the rate of food supply (Tenore &

Chesney, 1985). It was found that a high population density reduces worm production if the food supply is constant but if the food supply is not a limiting factor then a higher population density may be supported. As expected a decrease in the food supply and an increase in population density has been shown to have a negative effect on growth and reproduction in populations of the spionid *Polydora ligni* (Zajac, 1986).

Predation on invertebrates by shorebirds is an important ecological factor at the Eden Estuary. Large numbers of oystercatcher, knot, dunlin, godwits and shelduck were observed feeding on Kincaple Flat, which is a large mudflat close to the site sampled during the course of this study (Johnston *et al.*, 1979). It could be that predation is affecting the overall mean distribution of the size of lugworms. Predation by birds on lugworms normally occurrs through tail nipping, when the worm occupies a vertical tail up position in the vertical tail shaft of the burrow, for example when defaecating. The worm is not killed by the loss of any part of the tail owing to complete segmentation in this region. If *Arenicola marina* was subject to intense predation pressure at the Eden Estuary it would be expected that the mean tail length would be shorter. The tail length, expressed as a percentage of total body length to account for the different body sizes of the worms, was compared for a sample of worms from the East Sands and the Eden Estuary using ANOVA. The tail lengths were found to be comparable in size ($F=1.34$, $P=0.253$) which implies that the effect of predation is not increased at the Eden Estuary compared with the East Sands.

Cadman and Nelson-Smith (1990) used electrophoresis on *Arenicola* for taxonomic purposes. They looked at variation in the loci, *6PGDH, ESTD, αGPDH, SOD, PGI* and *LAP*. Results of this work showed that *A. marina* and *A. defodiens* are fixed for different alleles at three loci out of the six studied and there was little similarity at the variable loci which did have common alleles. Allele frequencies for *αGPDH* are comparable between the *A. marina* in this study and the *A.marina* from Wales (Cadman and Nelson-Smith, 1990). Cadman and Nelson-Smith report that they

found five alleles present at the *PGI* locus in a sample size of 47. The total number of individual *A. marina* examined at this locus in this study was 365, but only three alleles were observed. Shahid (1982) looked at variation at the *PGI* locus in populations of *Arenicola marina* from five different locations in Northumberland and the results showed the allele frequencies to be in Hardy-Weinberg equilibrium but comparisons of various populations with each other showed significant variation implying that they were reproductively isolated. Shahid (1982) and Cadman (1991) both report severe streaking on the gels and poor resolution problems. Similar difficulties were encountered in the investigations described in this chapter as well but the results clearly show that there is variation in the allele frequencies for each site. Departure from Hardy-Weinberg equilbrium was observed at the *GOT* locus in animals from Fairlie Sands and also at the *αGPDH* locus in animals from Fairlie Sands, Eden Estuary and East Sands in this study. The problems of making assumptions such as these have been pointed out by Lessios (1992). He says that an inability to reject a null hypothesis does not necessarily make it true, because the sample size may have been inadequate. Type I errors occur when a hypothesis that is true is rejected. On the other hand, if an inadequate statistical test is used Type II error may result, where the hypothesis will not be rejected, even if it is false. The significant results must be explained either by a source of error or by a departure from one of the prerequisites that must hold for Hardy-Weinberg equilibrium to exist.

The first level at which bias may have been introduced into the sample is sampling on the shore. If the larvae are not travelling great distances the distribution of individuals within a population could be discontinous. This is more likely to occur at a site like the Eden Estuary which is a very sheltered shore. When the worms were dug they were generally taken from a relatively small area. Pooling data from several months would help to eliminate this source of error as obviously the same precise spot would not be sampled again. A further source of error could have been in the

interpretation of the gels. *αGPDH* tended to have a very low activity on some of the gels making scoring of all individuals impossible. If the heterozygotes are particularly hard to visualise through being too faint and consequently were not scored, this would obviously bias the sample toward a lower level of heterozygotes. The number of individuals not scored for this locus on gels where others had been scored successfully was thirteen from Fairlie Sands, ten from the East Sands and five from the Eden Estuary.

If there are no apparent sources of error then one of the assumptions pertaining to Hardy-Weinberg equilibrium may not have been satisfied. The rare slow allele of *GOT* at Fairlie Sands is of relevance in this context. Both the fast and medium alleles were present in similar proportions and it is unusual that no medium / slow heterozygotes were observed, although the expected frequency would be very low. For a population conforming to Hardy-Weinburg expectations, it would be predicted that the rare allele should be present in heterozygous combinations both with the medium and fast alleles. It is possible that these individuals with the rare slow allele had originated from another site. This could happen during one of the migratory phases in the life-cycle (see Figure 1.1), particularly in the right conditions such as a strong current. There are many sites near to Fairlie Sands where there are large populations of *Arenicola* present. If there was a particularly bad storm a group of larvae from a different beach could have all arrived simultaneously. The adults have also been reported as swimming pelagically on occasion (Meek & Storrow, 1924; Cazaux, 1966). Juveniles migrating down the beach must also leave their burrows. It is possible that they could be taken along the coast by a current once in the water column. The literature on the larval dispersal potential for polychaetes has naturally focused on those species with a pelagic larval phase. These species are generally those which are not infaunal in habitat, for example the Serpulids. The majority of infaunal polychaete species do not have free swimming larvae; instead direct development takes place either

free within the sediment or in a cocoon (Westheide, 1984). The potential for larval dispersal amongst these species has not been discussed widely.

A heterozygote excess has been reported previously for some other populations of marine invertebrates. Marcus (1977), working on the sea urchin *Arbacia punctulata*, found an increase in heterozygosity at certain loci. Similarly Hess *et al.* (1988) noticed an increase in heterozygotes at at the hexokinase (*HK*) locus in a brooding holothuroid. These effects were attributed to a positive natural selection in favour of heterozygotes. This would then shift the genotype frequencies out of Hardy-Weinberg equilibrium. Positive selection may take place at the different sites, in the present study; for example, an increased tolerance to freshwater runoff or pollutants may have a selective advantage at some sites, such as the Eden Estuary, but may be irrelevant at other sites.

The deviations from Hardy-Weinberg equilibrium observed at the *αGPDH* locus are due to lower than expected numbers of heterozygotes. This condition typically is associated with inbreeding in a population. One of the criteria that must exist for a population to be in Hardy-Weinberg equilibrium is that there is random mating taking place. Where the breeding season is highly synchronous, such as for *Arenicola* at the East Sands, there will be a greater chance of random mating within that population than at a beach where the spawning season is extended. As described in Chapter 3, the spawning season at the Eden Estuary is extended over a period of three weeks, whereas at Fairlie Sands there are a few individuals mature at most times of the year. This situation could lead to a decrease in random mating.

The question of the extent of gene flow that occurs between populations of *Arenicola marina* still remains largely unanswered. Conclusions from models of gene flow seem to suggest that any regular and consistent migration between populations is enough to maintain uniform genetic diversity within the total population. If the larvae

are however, only migrating very short distances and very occasionally when conditions are favourable this may lead to a degree of reproductive isolation between certain sites. Larval migration must be restricted because the populations on the east and west coasts do have different allele freqencies present. Locally however, there may be some genetic exchange occurring. It will depend as much on the proximity of the next suitable habitat as the amount of gene flow that can take place. This means that there is reproductive isolation, between the east and west coasts. For example, the possibility of worms being able to interbreed naturally between the East Sands and Fairlie Sands is very unlikely. Allopatric speciation occurs when a species with a wide geographic range becomes subdivided into subpopulations between which gene flow is restricted. This type of allopatric speciation is referred to as Type Ia (Bush, 1975). The subpopulations may evolve in their own ways, adapting to specific local conditions.

Reproductive isolation need not necessarily be associated with a benthonic larvae. Todd *et al.* (1988) have shown that populations of *Adalaria proxima* show substantial differences in allele frequencies at different localities. The larvae of this species are pelagic and lecithotrophic. Chow and Fugio (1985) found two genetically different types of the fresh water shrimp, *Palaemon paucides* , inhabiting the same water system. It is suggested that this shrimp is in the process of diversifying into two species. The two types have different egg sizes and are believed to be reproductively isolated as no natural hybrids could be identified from expected allelic characteristics. The mechanism by which this could have occurred is not known but the evolution of reproductive isolation is one of the key elements in speciation.

Differences in genetic variation over geographical ranges have been observed to be present in other species of marine invertebrates, for example the horseshoe crab *Limulus polyphemus.* This species was found to be polymorphic at nine of fifteen loci studied. At all but two of these loci there were significant differences between populations from the Atlantic and Gulf coasts of America (Selander *et al.,* 1970).

Differences have also been noted for fish (Weinstein & Yerger, 1976; Johnson, 1974). The classic paper by Grassle and Grassle (1977) divides the polychaete species of *Capitella capitata* into a species complex consisting of at least six sibling species. These sibling species are morphologically so similar that their identities were ascertained only when their isozymes were examined electrophoretically. They have a very recent common ancestry, but have diversified to exploit new habitats. Differences in the reproductive biology between the sibling species were discovered. Decreasing egg size is correlated with increasing clutch size and with increasing duration of the pelagic phase. This species has been descibed as opportunistic because it will rapidly colonise any areas of disturbed habitat.

Other polychaete species have been subdivided into sibling species on the basis of electrophoretic data. There is confusion in the genus *Polydora* arising from high levels of morphological variation within and between different populations (Mustaquim, 1986). Rice and Simon (1980) analysed five populations of *Polydora ligni* from North America. The electrophoretic survey of 10 loci revealed some differences between the populations. Four out of the five populations did not show considerable genetic differences but the fifth population was significantly different from the others. The habitat at this site was bivalve shells as opposed to mud at the other sites. Mustaquim (1988) has shown that two forms of *Polydora ciliata* are significantly different from each other with respect to *PGI, Est* and *ACP*. He suggests that they may be in the first stages of speciation.

A more informative assessment of the genetic variation of these populations of *Arenicola* would be provided by molecular analyses such as PCR cloning. DNA fingerprinting would be a good technique because a large amount of information can be gained from a relatively small sample size. The process of peptide mapping involves comparing two proteins in two stages. Firstly the proteins are digested by an enzyme (e.g. trypsin) into short peptides. These are aligned using chromatography and then

separated by electrophoresis. The resolution achieved is much greater although the technique is more complex. Peptide mapping may also be carried out using 2-dimensional chromatography. Protein sequencing can detect all the amino acid substitutions but because of the involved technique the size of the sample to be examined is limited. This would give a more detailed study of the type of variation at each site and would be useful for the study of a number of geographically close sites. For example by sampling lugworms from Fairlie Sands and from some of the other beaches close by, we would be able to determine the extent of naturally occuring genetic exchange. As the sample sizes are smaller however, the amount of genetic variation present in the whole population cannot be determined very accurately.

Chapter 5

Observations on Experimentally Induced Oocyte Maturation and Spawning in *Arenicola marina* and *A. defodiens*

Observations on Experimentally Induced Oocyte Maturation and Spawning in *Arenicola marina* and *A. defodiens*

5.1 Introduction

The endocrine control of reproduction in *Arenicola marina* is described in detail in Section 1.7 and oocyte maturation has been discussed further in Section 1.11. It has been established that in *Arenicola marina,* the oocytes are not fertilisable until they have passed out of the coelom (Pirlot, 1933; Newell, 1948; Howie, 1961b). By examining oocytes from gravid, spawning and spent worms, Howie (1961b) demonstrated that maturation of the oocytes takes place in the coelom immediately prior to spawning. He also noted that if mature oocytes from a spawning female are injected into a gravid but non-spawning female, they will be shed through the nephromixia. This observation led to the suggestion that the matured ooctes are automatically taken up by the nephromixia. The stimulus for spawning in females was therefore believed to be the oocyte maturation factor (Howie, 1961b). Further investigations by Howie (1963) led to the idea that this oocyte maturation factor was present in the prostomium because decerebrated animals failed to spawn.

The immature oocytes are arrested in the first prophase stage of meiosis (Meijer & Durchon, 1977). There are certain morphological changes associated with the maturation process which are discussed in Section 1.11 and these have been described by Howie (1961b) and Meijer (1979a). Meijer and Durchon (1977) investigated oocyte maturation *in vitro* . Their results suggest that maturation of oocytes takes place *in vitro* in the presence of prostomial extract. Oocytes were incubated at different concentrations of prostomia. The percentage of oocytes undergoing maturation was monitored with time by observing the percentage GVBD at regular time intervals. At a

concentration of 0.1prostomia (pr) per ml, 100% GVBD had taken place after one hour. At 0.01pr per ml it took three hours for 100% GVBD and at 0.005pr per ml only 70% of the oocytes had matured after five hours. It is perhaps strange that the maturation response of the oocytes takes longer as the concentration of prostomia decreases. Guerrier and Dorée (1975) have shown that starfish oocytes need to be exposed to 1-methyladenine in the medium for a minimum contact time of 4 minutes 30 seconds to ensure that GVBD takes place. The 1-methyladenine must remain bound to the receptor sites at the cell membrane for this critical period. After this initial time period the mechanism is triggered and nuclear maturation will occur without any more 1-methyladenine being required. This implies that for meiosis to be reinitiated, which would be noticed by the breakdown of the germinal vesicle, the presence of a certain substance is required at the cell membrane. As long as there is some of this substance present, some GVBD will take place. The actual response of the oocytes to this substance would not be expected to be time dependent once the initial reaction has taken place. In starfish it was reported that this minimum contact period was independent of the concentration of 1-methyladenine (Schuetz, 1969a; Guerrier *et al.*, 1976), although below 10^{-7} M the percentage of GVBD would be less than 100. Similarly in *Arenicola* it would be expected that as long as there was enough oocyte maturation factor present, GVBD would take place and at low concentrations the reaction would still happen at the same speed but there would be a lower percentage of GVBD.

The processes of oocyte maturation and spawning in *Arenicola* and in the starfish show remarkable similarities in their endocrine control, although the hormones involved may be of a totally different nature. A popular experimental approach to understanding how a hormone works at the cellular level is to use specific chemicals with known functions on the cells to see if they can mimic the action of the hormone (agonists). Section 1.10 describes aspects of the artificial stimulation of meiosis

reinitiation in relation to spawning in starfish. Similar experiments have also been carried out with the oocytes of *Arenicola marina* (Meijer, 1980). Meijer used different drugs which are known to affect the permeability of the cell membrane to calcium and modify intracellular free calcium concentration. He incubated fully developed but immature oocytes *in vitro* with different concentrations of these drugs and then counted the percentage GVBD after 2 hours (Meijer, 1980). These experiments give some insight into a possible mode of action for the hormone and Meijer suggested that calcium ions may play a key role in the stimulation of oocyte maturation in *Arenicola.* It has been shown in starfish oocytes, using the calcium activated photoprotein aequorin, that 1-methyladenine (the meiosis initiating substance) produces a rapid calcium release in the cytoplasm when it is applied externally to aequorin injected oocytes (Guerrier *et al.*, 1978; Moreau *et al.*, 1978). Meijer (1980) suggests that a similar response may take place in *Arenicola* oocytes when they are stimulated by the oocyte maturation factor from the prostomium. Disulphide reducing agents have been shown to induce oocyte maturation in starfish (Kishimoto & Kanatani, 1973) and they are also effective on *Arenicola* oocytes (Meijer, 1980) which is further evidence that the mechanism may be similar.

Meijer used populations of *Arenicola* collected from beaches near Boulogne and Calais on the north coast of France, including Wimereux, during his investigations. He also did some work with populations collected from Fairlie Sands and Kames Bay, which is a beach near Millport on the island of Great Cumbrae, opposite Fairlie Sands. In his thesis Meijer (1978) accepts that oocyte maturation could not be induced *in vitro* in oocytes taken from individuals collected from Scottish populations. It is possible that this may be due to some physiological differences between populations of *A. marina* at different locations which was to be investigated in this study. A further possibility is that the two different responses to oocyte maturation *in vitro* are from the two different species of *Arenicola* that have recently been separated into *Arenicola*

marina and *A. defodiens* (Cadman & Nelson-Smith, 1993). This confusion provides the basis for this investigation, with a view to compare the control of oocyte maturation in both *Arenicola marina* and *A. defodiens*. In order to do this, lugworms were collected and identified from a number of different sites. Sites included Wimereux, on the north coast of France, because Meijer had worked previously on these animals and found that GVBD did occur *in vitro* (M.G. Bentley, personal communication); Swansea Bay in Wales, where both *Arenicola defodiens* and *A.marina* are present (Cadman & Nelson-Smith, 1990); East Sands and the Eden Estuary in St. Andrews, Fife where only *A. marina* has been reported previously (Howie, 1959; Bentley & Pacey, 1992).

5.2 Materials and Methods

5.2.1 Collection and Identification of Lugworms from the Different Sites

Lugworms were collected from the following locations: Wimereux on the north coast of France; Swansea Bay, Wales; East Sands and the Eden Estuary in St. Andrews, Fife. At Wimereux a small shovel with the edges bent inwards and a very long handle was used. A deep channel surrounding the tail cast was dug quickly and the core of sand from the middle then removed. The tail end of the worm was generally visible from about 60cm to 1m down. The worms from Swansea Bay were collected by P.S. Cadman using the Alvey bait pump which sucks the worm out of its burrow. It is very difficult to collect *Arenicola defodiens* by the traditional method of digging, described in Section 2.2, because their burrows are so deep (Cadman, 1992). The worms collected from Wimereux and from Swansea Bay were all positively identified as *Arenicola defodiens* commonly known as black lug (Cadman & Nelson-

Smith, 1993). The worms collected from East Sands and the Eden Estuary were collected using a fork and were all positively identified as *Arenicola marina* or blow lug.

5.2.2 Incubation of *A.marina* Oocytes with Prostomial Homogenate *in vitro*

Lugworms were collected from Fairlie Sands on 28th April 1992. Oocytes from mature females were examined to check that the animals were close to spawning. The *in vitro* assay is described in Section 2.8. Coelomic fluid was removed from female worms using the method described in Section 2.3. The volume removed from each worm was about 2ml. The coelomic fluid was then centrifuged for 1minute at a speed of 6500rpm to remove any coelomocytes and oocytes present.

5.2.3 Incubation of *A.marina* Oocytes with Various Calcium Agonists *in vitro*

Oocytes from the same animals used in 5.2.2 were incubated with three different concentrations (10^{-2}M, 5 x 10^{-3}M and 10^{-3}M) of the following drugs which had been made up in filtered seawater:

a) lanthanum chloride
b) oxprenolol
c) propranolol
d) tetracaine
d) procaine

The assay described in Section 2.8 was then carried out. To estimate the mean percentage of GVBD that had occurred in each well, three samples of 100 oocytes were examined with a light microscope under a coverslip. The mean and standard error were then estimated from these figures.

5.2.4 Cross-reactivity of Prostomial Homogenate on Gamete Maturation *in vitro* on Animals Collected from Three Different Sites

The prostomial homogenate were prepared from 2 prostomia from each experimental group of animals homogenised in 1ml of filtered seawater and made up to a volume of 10ml. The incubations were carried out in a volume of 200μl of filtered seawater or homogenate. Each well therefore contained the equivalent of 0.04 prostomia. Six different individuals from each experimental group were used and the incubations were carried out in triplicate. The sperm or eggs added from each worm was kept separate so that any variation caused by specific individuals could be detected.

The sperm from animals from all three sites was also incubated with the sperm maturation factor, 8,11,14,-eicosatrienoic acid. The stock solution was diluted a hundred fold in filtered seawater, and then by a half again, giving a final concentration of 5×10^{-5}M. This concentration is known to be effective on the sperm of *A. marina* (Bentley *et al.*, 1990).

5.2.5 The Response of Gravid Female *Arenicola marina* to Prostomial Homogenate *in vivo*

Prostomial homogenates of 4 prostomia in 1ml of filtered seawater were prepared as before from male and female worms from each of the three sites. 8 males and 8 females from the East Sands were then injected with 250μl of the homogenate (1 prostomium equivalent). 8 animals were also injected with 250μl of seawater as a control. The animals from the East Sands were used as an assay because they were the closest to their natural spawning time and the population is the most synchronised of those investigated.

5.3 Results

5.3.1. Incubation of *A. marina* Oocytes with Prostomial Homogenate *in vitro*

The results of this experiment are presented in Table 5.1 which clearly shows that no germinal vesicle breakdown was observed in any of the *in vitro* incubations of the oocytes. Diffferent concentrations of prostomial homogenate had no effect and neither did the addition of coelomic fluid. The oocytes were checked at hourly intervals for five hours after the start of the experiment.

5.3.2 Incubation of *A. marina* Oocytes with Various Calcium Agonists *in vitro*

a) Lanthanum chloride did induce germinal vesicle breakdown (Fig. 5.1). The greatest percentage of GVBD occurred at the highest concentration of lanthanum chloride (10^{-2}M) and the percentage decreased with the molarity of the lanthanum chloride. The presence of the coelomic fluid appears to increase the percentage of GVBD at the lower concentrations but has no effect at the higher concentration of lanthanum chloride10^{-2}M.

b) Oxprenolol induced GVBD artificially in this study (Fig. 5.2). At a concentration of 10^{-2}M, 100% GVBD was observed in all of the incubations with coelomic fluid. This implies that the coelomic fluid does have an enhancing influence on GVBD because in oocytes incubated at the same concentration without coelomic fluid there was less GVBD. This effect was not the same at all concentrations tested because at the middle concentration (5×10^{-3}M) there was a lower percentage of GVBD when coelomic fluid was present. At 10^{-3}M without coelomic fluid there was no GVBD.

c) Propranolol also induced GVBD (Fig. 5.3). The percentage of GVBD was generally much lower at all concentrations with the propranolol as compared to the oxprenolol. There was no GVBD at the lowest concentration of 10^{-3}M propanolol in the incubations without coelomic fluid. The coelomic fluid only had a noticeable effect at this concentration.

d) Tetracaine and e) procaine both had GVBD inducing properties (Figs. 5.4 & 5.5). Tetracaine appears to have an increase in activity at the lower concentrations whereas the procaine was more active at higher concentrations. The effect of the coelomic fluid was not noticeable, except at the lowest concentration of tetracaine where it had a decreasing effect on the percentage of GVBD.

Sperm was added to samples of all the artificially matured oocytes after the 1hour incubation period. A fertilisation membrane appeared in the oocytes which had undergone GVBD but no cleavage was observed in any them, even after 12 hours. After this time some of the oocytes started to degenerate, particularly those which were incubated in the higher concentrations of the drugs.

5.3.3 Cross-reactivity of Prostomial Homogenates on Gamete Maturation *in vitro* on Animals Collected from Three Different Sites

The results presented in Table 5.2 are taken from experiments carried out on the sperm of gravid male animals from Swansea and the Eden Estuary. Spermatozoa from the Swansea animals underwent maturation and the agglutination response when incubated with a prostomial homogenate from worms collected at both Swansea and the Eden Estuary. Two of the Swansea controls were scored as a positive / negative result which means that there is some visible breakdown in the structure of the morulae, but the sperm are not actively swimming. This sometimes occurs when the animals have been damaged in some way, which may easily have happened during

transport from Swansea. Spermatozoa from the Eden Estuary animals responded to prostomial homogenates from worms collected at Swansea. In table 5.3 similar results can be observed with sperm from the animals collected at Wimereux and the East Sands. Spermatozoa from both *A. defodiens* and *A. marina* are activated when incubated with 8,11,14- eicosatrienoic acid. There was no breakdown of morulae in any of the controls.

Table 5.4 shows the results of the cross reactivity experiments carried out with the oocytes of worms collected from Swansea and the Eden Estuary. The results of this experiment are particularly interesting. They show that the oocytes of *Arenicola defodiens* from Swansea can be induced to undergo GVBD *in vitro* when incubated with prostomial homogenate of gravid females from the same site. GVBD also occurs when the oocytes are incubated with prostomial homogenate from gravid *Arenicola marina* females from the Eden Estuary. Figure 5.6 shows oocytes from *A. defodiens* and *A. marina* which have been incubated in female *A. defodiens* prostomia. The oocytes from the Swansea individual are less well developed but GVBD can be seen to have taken place. Oocytes from *Arenicola marina* do not undergo GVBD *in vitro* . The results of the *in vivo* experiment show that *Arenicola marina* from the East Sands spawned when injected with prostomial homogenates from gravid females of both species (Table 5.5).

Table 5.1 Percentage of GVBD occurring after 1hr incubation in seawater, coelomic fluid and prostomial homogenate

Treatment	%GVBD
Seawater control	0
Coelomic fluid (CF)	0
Prost. homogenate ($1.ml^{-1}$)	0
Prost. homogenate ($0.5.ml^{-1}$)	0
Prost. homogenate ($0.25.ml^{-1}$)	0
Prost. homogenate ($0.125.ml^{-1}$)	0
Prost. homogenate ($1.ml^{-1}$)+ CF	0
Prost. homogenate ($0.5.ml^{-1}$)+ CF	0
Prost. homogenate ($0.25.ml^{-1}$)+ CF	0
Prost. homogenate ($0.125.ml^{-1}$)+ CF	0

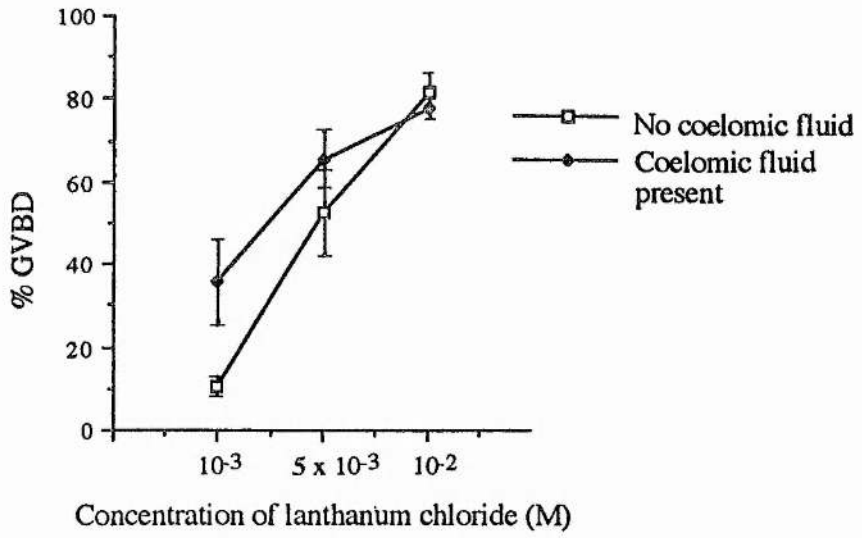


Fig. 5.1 Percentage GVBD (mean ± sem) in *A.marina* oocytes after incubation with lanthanum chloride, with and without coelomic fluid present.

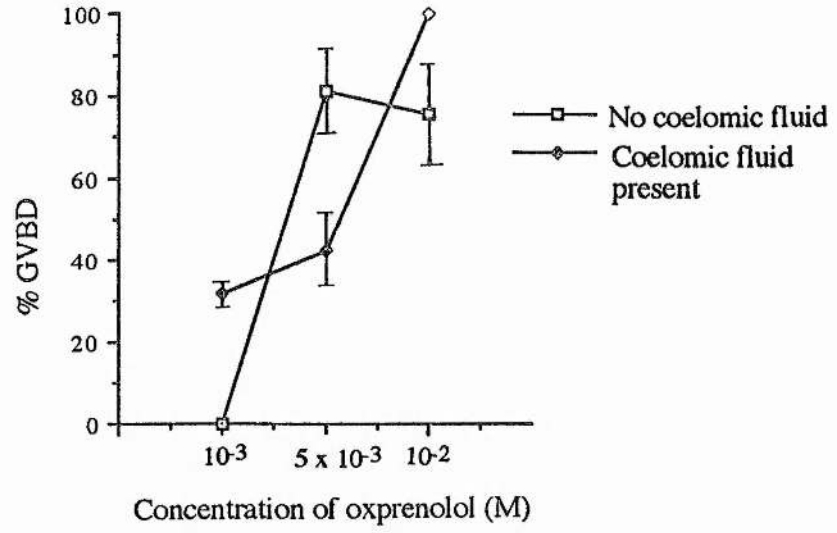


Fig. 5.2 Percentage GVBD (mean ± sem) in *A.marina* oocytes after incubation with oxprenolol, with and without coelomic fluid present.

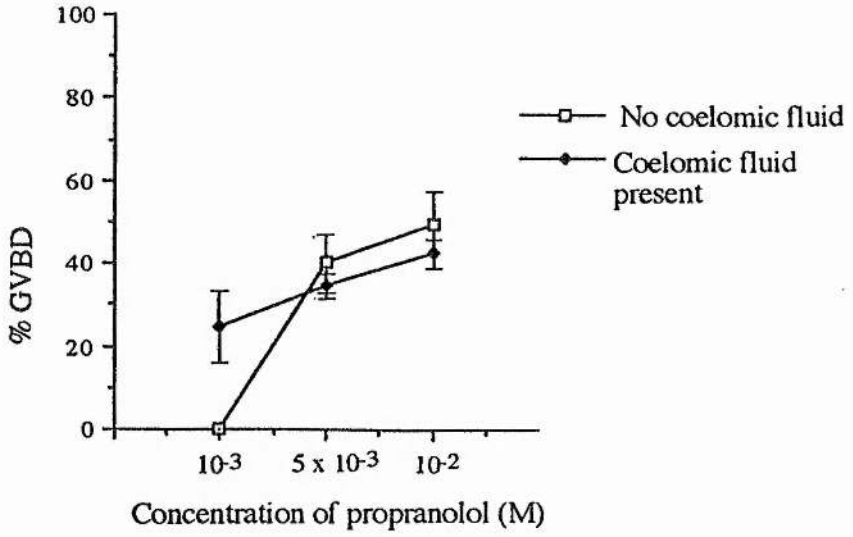


Fig. 5.3 Percentage GVBD (mean ± sem) in *A.marina* oocytes after incubation with propranolol, with and without coelomic fluid present.

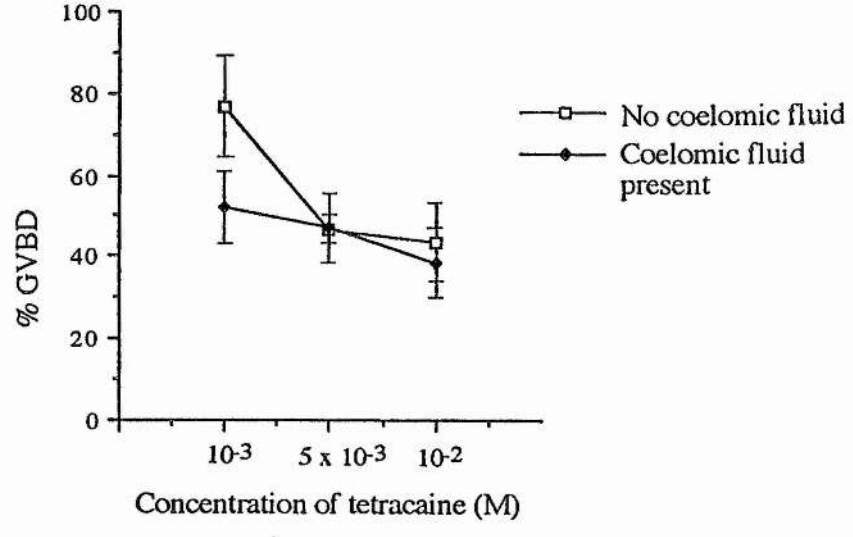


Fig. 5.4 Percentage GVBD (mean ± sem) in *A.marina* oocytes after incubation with tetracaine, with and without coelomic fluid present.

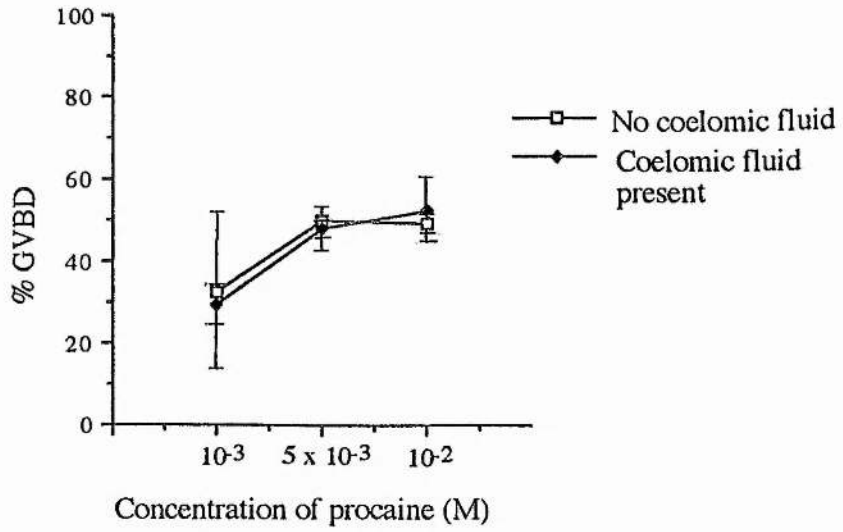
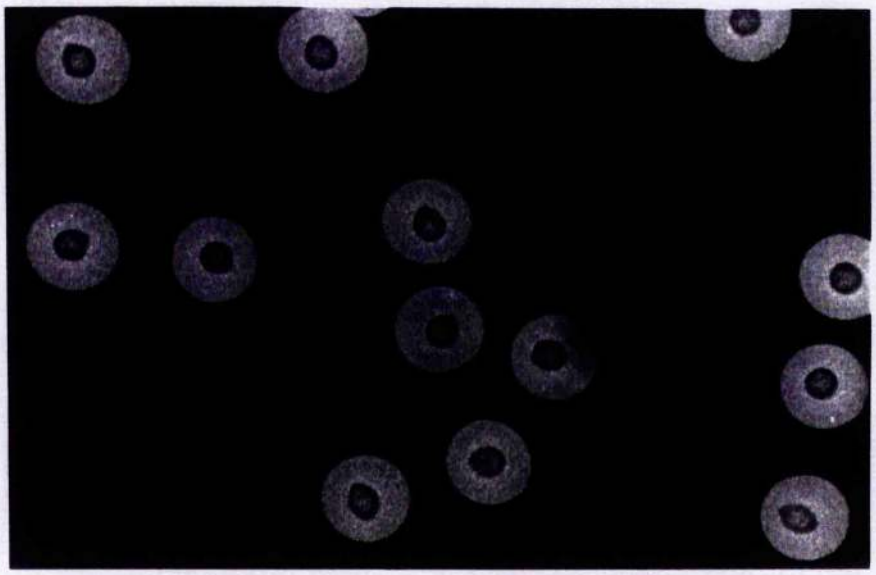


Fig. 5.5 Percentage GVBD (mean ± sem) in *A.marina* oocytes after incubation with procaine, with and without coelomic fluid present.

a)



b)

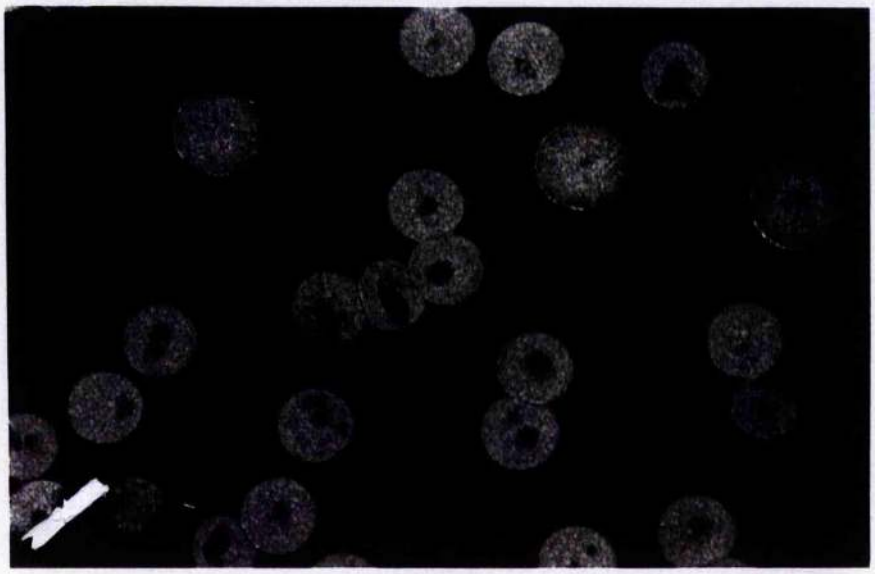


Fig. 5.6 *Arenicola* oocytes which have been incubated in female *A. defodiens* prostomial homogenate. a) *A. marina* oocytes showing the intact germinal vesicle b) *A. defodiens* oocytes in which the larger oocytes have undergone GVBD. The oocytes from *A. defodiens* are at an earlier stage of development than those of *A. marina.*

Table 5.2 The results of the *in vitro* assay using sperm and prostomial homogenate from the Eden Estuary *A. marina* and from Swansea *A. defodiens.*

Source of Donor Sperm	Treatment	Dose	Results
Swansea	Swansea prostomia	$0.2pr.ml^{-1}$	+
Swansea	seawater	-	-*
Eden Estuary	Eden prostomia	$0.2pr.ml^{-1}$	+
Eden Estuary	seawater	-	-
Swansea	Eden prostomia	$0.2pr.ml^{-1}$	+
Swansea	seawater	-	-*
Eden Estuary	Swansea prostomia	$0.2pr.ml^{-1}$	+
Eden Estuary	seawater	-	-
Swansea	8,11,14- eicosatrienoic acid	$5x10^{-5}M$	+
Eden Estuary	8,11,14- eicosatrienoic acid	$5x10^{-5}M$	+

* Some evidence of sperm morulae breakdown in two individuals but no evidence of sperm activation.

Table 5.3 The results of the *in vitro* assay using sperm and prostomial homogenate from East Sands *A. marina* and from Wimereux *A. defodiens.*

Source of Donor Sperm	Treatment	Dose	Results
Wimereux	Wimereux prostomia	$0.2pr.ml^{-1}$	+
Wimereux	seawater	-	-
East Sands	East Sands prostomia	$0.2pr.ml^{-1}$	+
East Sands	seawater	-	-
Wimereux	East Sands prostomia	$0.2pr.ml^{-1}$	+
Wimereux	seawater	-	-
East Sands	Wimereux prostomia	$0.2pr.ml^{-1}$	+
East Sands	seawater		-
Wimereux	8,11,14- eicosatrienoic acid	$5x10^{-5}M$	+
East Sands	8,11,14- eicosatrienoic acid	$5x10^{-5}M$	+

Table 5.4 The results of the *in vitro* assay using oocytes and prostomial homogenate from the Eden Estuary *A. marina* and from Swansea *A. defodiens*.

Source of Donor Oocytes	Treatment	Dose	Mean % GVBD	standard error
Swansea	Swansea prostomia	0.2pr.ml^{-1}	43.75	11.94
Swansea	seawater	-	0	
Eden Estuary	Eden prostomia	0.2pr.ml^{-1}	0	
Eden Estuary	seawater	-	0	
Swansea	Eden prostomia	0.2pr.ml^{-1}	42.92	9.72
Swansea	seawater	-	0	
Eden Estuary	Swansea prostomia	0.2pr.ml^{-1}	0	
Eden Estuary	seawater	-	0	

Table 5.5 The number of gravid females, collected from the East Sands which spawned after injection with prostomial homogenate from animals collected from different sites. The numbers in parentheses are the number of animals that were injected.

Animals Injected with :	Number spawning
filtered seawater	0 (8)
A. marina prostomial homogenate	6 (8)
A. defodiens prostomial homogenate	5 (8)

5.4 Discussion

The results observed in Section 5.3.1 indicate that maturation of the oocytes from individuals from populations of *Arenicola marina* investigated in this study cannot be induced *in vitro.* These results do not agree with Meijer and Durchon (1977) who observed induced oocyte maturation *in vitro. Arenicola* collected from Wimereux in France were identified as specimens of *Arenicola defodiens* in this study (see Section 5.3.3). Wimereux was also one of the collection sites used by Meijer. There is, therefore, every likelihood that the interpopulation differences reported by Meijer (1978) are due to differences between *A. defodiens* and *A. marina.* The mechanism of oocyte maturation has previously been suggested to involve an intracellular change in the concentration of calcium ions (Meijer, 1980). This mechanism has been well researched in starfish oocytes. Moreau *et al.* (1978) investigated oocyte maturation in *Marthasterias glacialis,* where they demonstrated the action of a hormone *in vivo* which acts at the plasma membrane, triggering the release of intracellular calcium ions (Ca^{2+}). This then reactivates the cell metabolism, reinitiating meiosis and the subsequent maturation of the oocyte. This has been shown to be the possible mechanism of meiosis reinitiation in *Arenicola* by the action of the calcium agonists (Meijer, 1980). If Meijer was in fact working on *A. defodiens* , then experiments to investigate the action of calcium agonists on oocyte maturation *in vitro* will reveal if the cellular mechanism for meiosis reinitiation is similar in *A. defodiens* and *A. marina.*

Lanthanum chloride is known to interact with the transport of calcium across the cellular membranes because the lanthanum ion, La^{3+} is a specific antagonist of Ca^{2+} in biological systems (Weiss, 1974). It can replace calcium ions at well defined tissue sites, which has the effect of augmenting the Ca^{2+} dependent reaction in the *Arenicola* oocyte. As the concentration of lanthanum chloride increases, more Ca^{2+} will be displaced and a higher percentage of GVBD will be observed in the incubations.

This is shown very clearly to be the case in Fig. 5.1 and the results are directly comparable to those reported by Meijer (1980). Oxprenolol and propranolol are known to increase the calcium permeability of the cell membrane by displacing calcium ions which are adsorped to phospholipid complexes at the membrane. The rate of Ca^{2+} uptake is increased and this is accompanied by the transport of potassium ions which causes the membrane to become hyperpolarised. This causes further uptake of calcium (Szasz, 1977). These two drugs would be expected therefore to cause an increase in the intracellular concentration of calcium ions. Tetracaine and procaine are local anaesthetic amines, they will readily adsorb to biomembranes, and compete with and displace the membrane bound calcium ions from the phospholipids in the cell membrane (Seeman, 1972). They are used as anaesthetics because they reduce the rate of increase in an action potential by altering the external sodium ion concentration. This then reduces the resting membrane potential (Seeman, 1972). They act in a similar way to oxprenolol and propranolol.

Results from these experiments show that GVBD is induced artificially by the presence of propranolol, oxprenolol, procaine, tetracaine and lanthanum chloride in *Arenicola marina*. These observations are important, assuming that Meijer was working on *Arenicola defodiens* because they imply that the cellular mechanism of meiosis reinitiation is the same in both species. The presence of coelomic fluid in the incubation generally had an augmenting effect on the percentage of GVBD observed. This implies that some factor associated with the coelomic fluid may play a role in the reinitiation of meiosis.

It appears from the cross reactivity experiments with the male *A. defodiens* and *A. marina*, that the sperm maturation process is the same in both species. The prostomial homogenates have the same effect on the sperm of each species *in vitro* . The hormonal control is similar because the sperm maturation factor, 8,11,14-eicosatrienoic acid, isolated from *Arenicola marina* (Bentley *et al.*, 1990) has the same

effect on sperm morulae from *A. defodiens.* These experiments show clearly that the sperm maturation factor present in the prostomia of male *Arenicola* is not species specific.

In the females the situation is somewhat more complex. The hormonal control of the oocyte maturation process is less well understood. It cannot be assumed that the hormonal control of gamete maturation is the same in both species because the oocytes of *Arenicola defodiens* can be induced to undergo GVBD *in vitro* whereas the oocytes of *A. marina* will not undergo GVBD *in vitro.* This implies that in *A. marina* the maturation response is not caused directly by a neurosecretory product from the prostomium. If it was we would have seen oocyte maturation taking place *in vitro.* The cross reactivity experiments prove that one essential part of the whole maturation process must be different in the two species.

The oocytes from *A. defodiens* will undergo GVBD in the presence of prostomial homogenate from *A. marina.* The oocyte maturation factor for *A. defodiens* must therefore also be present in *A. marina* prostomia but for some reason it does not cause the same response on *A. marina* oocytes *in vitro.* One obvious explanation of these observations would be the involvement of a secondary factor in the reinitiation of meiosis in *A. marina.* This factor may be something that can only be produced *in vivo.* It could be some cofactor associated with the coelomic fluid or it could be that the prostomial factor causes the release of a secondary factor which either acts directly on the oocyte surface or somehow enables the prostomial factor to become active. If there are two factors present, one of them must be similar to the *A. defodiens* factor because it can induce GVBD of *A. defodiens* oocytes *in vitro.* The oocyte maturation factor for *A. defodiens* could be a primary factor in *A. marina* which then acts in some way to stimulate production of another substance (see Fig. 5.6).

The results of the experiments using the calcium agonists implied that the cellular mechanism is similar in the oocytes of both species. If the oocyte maturation factor acts at the surface of the oocyte, as 1-methyladenine does in starfish oocytes, then there must be receptor sites on the oocyte surface. Receptor sites associated with a specific G protein have been identified on the starfish oocyte plasma membrane and it has been shown that binding at these receptor sites triggers the maturation response (Hoshi *et al.*, 1992). The results of the *in vivo* experiment are very interesting because they show that *A. marina* from the East Sands spawned fully mature oocytes when injected with *A. defodiens* prostomia (see Table 5.5). This is different from what occurred *in vitro*. Fig. 5.6 summarises the results of the effect of prostomial homogenate *in vivo* and *in vitro*. The oocyte maturation factor is present in the prostomia of both *A. defodiens* and *A. marina*. The oocytes of *A. defodiens* will undergo maturation *in vitro* , whereas the oocytes of *A. marina* will not. An internal cofactor or secondary product is therefore required to induce maturation in *A. marina* oocytes. This evidence points to the possibility of the receptor sites for the oocyte maturation factor at the oocyte membrane being different in the two species. The oocyte maturation factor present in the prostomia of *A. marina* and *A. defodiens* needs to be modified in some way, or requires the action of a cofactor to become active in *A. marina* (see Fig. 5.7).

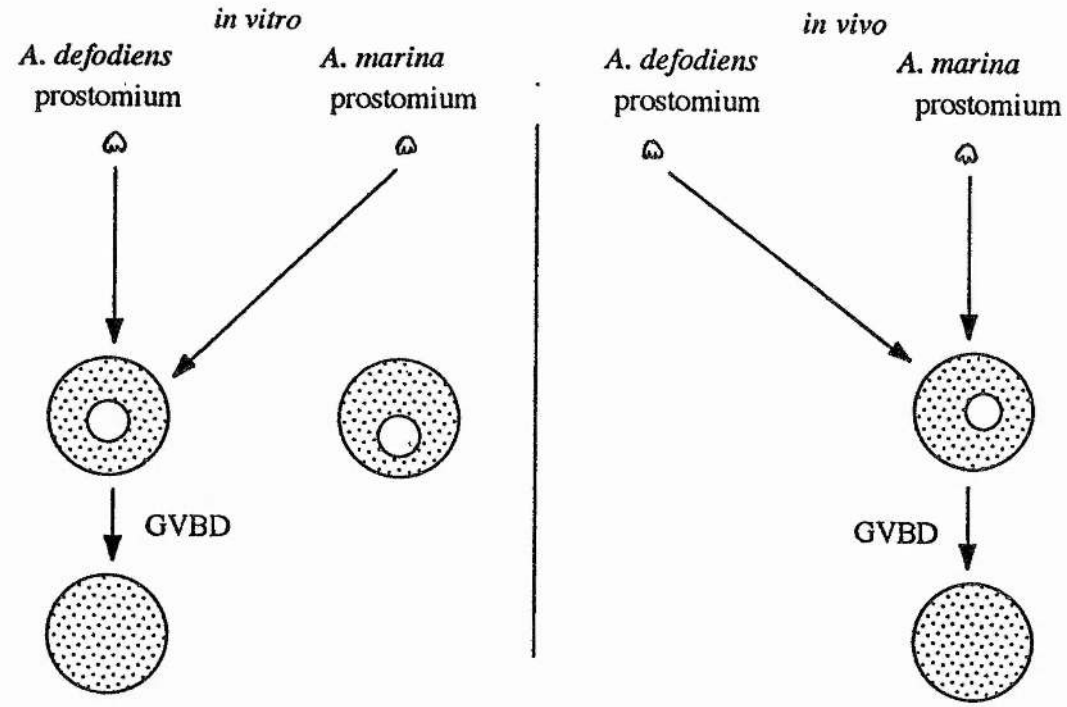


Fig. 5.7 A summary of the results of the *in vitro* and *in vivo* cross reactivity experiments on the endocrine control of oocyte maturation and spawning in *Arenicola marina* and *A. defodiens*.

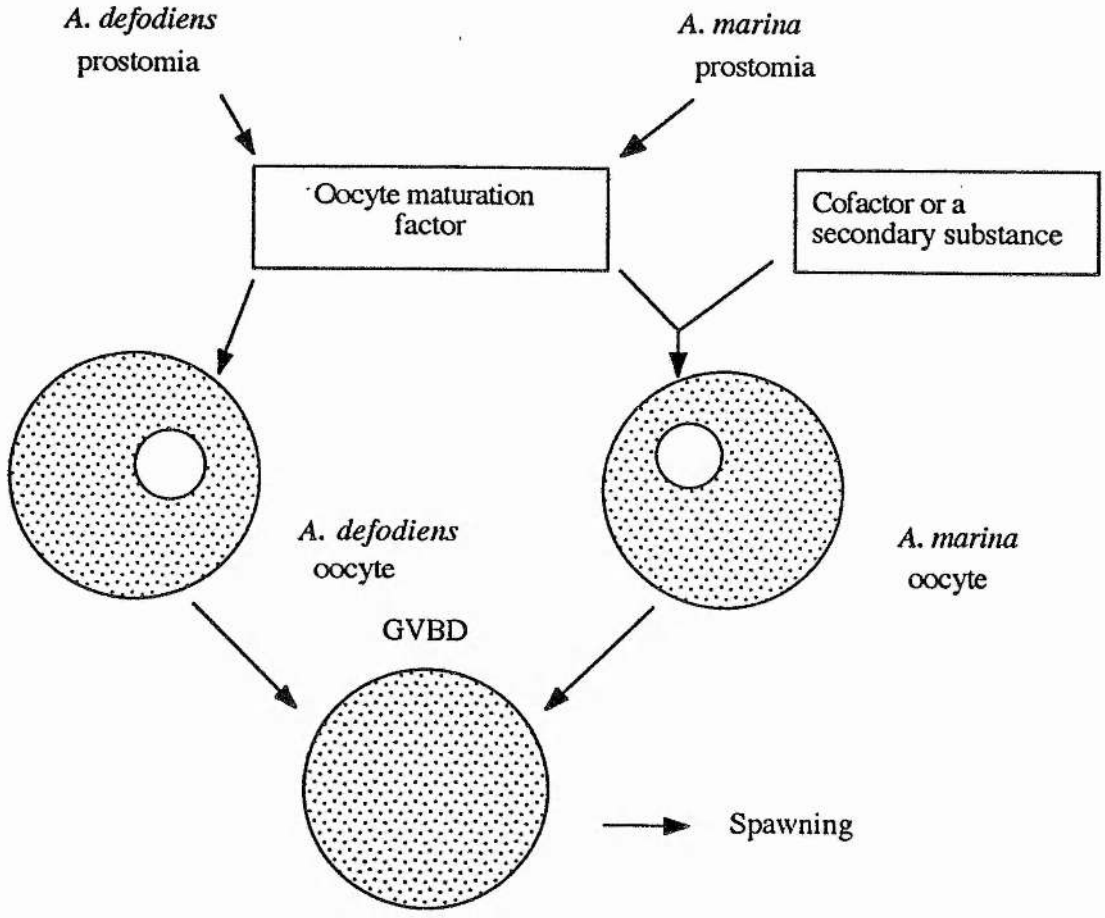


Fig. 5.8 A possible pathway for the endocrine control of oocyte maturation in female *Arenicola marina* and *A. defodiens*.

Chapter 6

Investigations in the Chemical Nature of Hormones Controlling Reproduction in *Arenicola marina*

Investigations into the Chemical Nature of Hormones Controlling Reproduction in *Arenicola marina*

6.1 Introduction

Preliminary evidence for a spawning inducing substance in *Arenicola* was introduced by Howie (1961a), who showed that male lugworms could be induced to spawn by administering the lipid fraction of whole worm homogenate (see Section 1.7). The same technique, however, failed to induce spawning in females. This observation is important because it is the first indication that the hormonal systems controlling reproduction differ in males and females. Further evidence for an endocrinological dichotomy between sexes is provided by Bentley *et al.* (1990), and Pacey and Bentley (1992) when they identified the sperm maturation factor, 8,11,14-eicosatrienoic acid. Injection of this substance into the coelomic cavity of males caused spawning but there was no response from similarly injected females (Pacey & Bentley, 1992).

There is little evidence of invertebrates which have sex specific reproductive hormones in the literature, but the subject has not been widely investigated. In starfish the gonad stimulating substance (GSS) isolated from the radial nerves is not sex specific (Chaet, 1966b). Although the end result of injection of this substance is spawning in both sexes, the mode of action of the hormone must be different in males and females. In the females the GSS causes the follicle cells in the ovaries to release 1-methyladenine (Kanatani *et al.*, 1969; see Section 1.9). Several non-sex specific spawning inducing substances have been discovered in molluscs (see Section 1.9) (eg. hydrogen peroxide in abalone, *Haliotis rufescens* (Morse *et al.*, 1977), and 5-hydroxytryptamine in the surf clam, *Spisula* (Hirai *et al.*, 1988)). Examples of invertebrate sex specific hormone are the egg laying hormones in *Aplysia* (Kupfermann, 1967) and the egg spawning hormone in *Gibbula umbilicalis* (Clare,

1986). *Aplysia* is an example which is not directly comparable to broadcast spawners because in *Aplysia* copulation takes place prior to egg laying. There is, however, evidence to suggest that an egg spawning hormone is present in the cerebral ganglia of the broadcast spawning gastropod *Gibbula umbilicalis* (Clare, 1986). This is interesting because it has been demonstrated to be present in both males and female ganglia but it will only induce spawning in the female.

Howies' early investigations also demonstrated that maturation of the gametes takes place in the coelom immediately prior to spawning (Howie,1961b,c). Howie suggested that this maturation process enabled the gametes to be accepted by the nephromixia and automatically spawned (See Section 1.3). Howie (1961b) injected gravid females with ether, alcohol and crude extracts of whole worm homogenate three weeks before and during the breeding season. He describes a puffing response, where small puffs of a whitish material, consisting of coelomocytes were emitted from the nephridiopores. In total four out of twenty eight worms given alcohol extract and twelve out of fifty two given ether extract released some oocytes. These were mainly immature and unfertilisable. Howie (1961b) suggested that the puffing response implies that spawning is not entirely passive and there may be a factor present responsible for the active uptake of cells from the body cavity.

Howie (1963) examined the effect of decerebration on gamete maturation and spawning in males and females. He established the neurosecretory role of the prostomium in *Arenicola marina* and also suggested the presence of maturation hormones which act on the immature gametes. The sperm maturation factor in *Arenicola marina* has been identified as 8,11,14-eicosatrienoic acid (Bentley *et al.*, 1990) and we know that this does not have any effect on gravid females. Therefore even taking the simplest scenario, where spawning is controlled by the maturation hormones there must still be a sperm maturation factor and an oocyte maturation factor involved. Some reported observations imply that the control of spawning may in fact

be more complex than this, with several endocrine substances involved as in starfish. For example Howie (1961a,b) noted that both male and female worms showed a violent reaction when injected with emulsified whole body homogenate (see Section 1.7). The presence of a possible muscle stimulant was proposed, which could act on the body wall to aid in the release of the gametes. Pacey and Bentley (1992) do not report a violent reaction from the worm on injection but do recognise distinctive spawning behaviour such as writhing and contractions. This behaviour may well be caused by the sperm maturation hormone, 8,11,14-eicosatrienoic acid, in which case it is suggested that it should be considered as a true spawning hormone (Bentley & Pacey, 1992). Alternatively there could be secondary factors involved which are released internally from other endocrine organs or maybe from the morulae themselves. Pacey and Bentley (1992) report that when females spawn the response is much less active than that observed from the males and there were no noticeable muscle contractions. In natural conditions this may be expected because the female spawns into the burrow whereas the male has to pump the sperm out onto the surface of the sand. It may also mean that the oocyte maturation hormone acts as a spawning hormone, with the oocytes being uptaken by the nephromixia as Howie (1961b) suggested. Several interesting observations raise questions about this simple theory. The first is the fact that oocyte maturation cannot be induced in *Arenicola marina in vitro*. Investigations carried out in Chapter 5 imply that oocyte maturation is not induced by a single endocrine substance stored in the prostomium and the involvement of a secondary factor is implicated. A second interesting observation is the fact that there is a time delay in the female spawning response after the female has been artificially stimulated to spawn. Pacey and Bentley (1992) report that when gravid male and female lugworm are injected with prostomial homogenate, the males spawn about fifty minutes after injection, whereas the females did not start to spawn until at least five hours after the time of injection. In the *in vitro* experiments the oocytes can

be seen to start GVBD after about thirty minutes incubation with prostomial extract (Meijer, 1979a). There is, as yet, no explanation for why this time delay exsists.

This chapter addresses some of the outstanding problems relating to the chemical nature and mode of action of sperm activating substances and oocyte maturation inducing factors present in the prostomia of female *Arenicola marina.* One of the major prerequisites for this type of investigation is a reliable assay technique. The possibilities for assaying these substances have been investigated and are discussed.

6.2 Materials and Methods

6.2.1 Injection of Aqueous and Organic Fractions from Female Prostomial Homogenate into Gravid Individuals

Gravid individuals were collected fron Budle Bay on 4 December 1990. Three prostomia were removed from mature females and homogenised in 1ml of filtered seawater. The aqueous and organic phases were then separated. The method is described in Section 2.7. In this experiment however, the solvent used was a chloroform / methanol (2:1 v/v) mixture although the protocol was the same. 200μl of each of the aqueous and organic fractions were injected into five males and five females, giving a dose of 0.6 prostomial equivalents per individual. Positive and negative controls were set up in which five males and five females were injected with either crude prostomial extract or filtered seawater respectively.

6.2.2 Injection of Male and Female Prostomial Homogenates into Gravid Individuals

Gravid individuals were dug from the East Sands at low tide on 14 October 1992. Twenty prostomia were removed from ten mature males and ten mature females. Each group of prostomia were then homogenised in 2ml of filtered seawater as described in Section 2.6. Forty mature individuals (twenty males and twenty females) were divided into the following experimental groups:

a) Five males - injected with male prostomial homogenate

b) Five males - injected with female prostomial homogenate

c) Ten males - injected with filtered seawater

d) Five females - injected with female prostomial homogenate

e) Five females - injected with male prostomial homogenate

f) Ten females - injected with filtered seawater

The animals were placed in individual pots with 200ml of filtered seawater. They then received an injection in 200μl volume with each worm receiving a dose equivalent to one prostomium. The worms were placed in the cold room at a temperature of 7°C and examined hourly for the next six hours for evidence of spawning.

6.2.3. Injection of Gravid Females with Fractions of Female Prostomial Homogenate Separated on a C_{18} Reverse Phase Cartridge

Gravid lugworms were collected from the East Sands at low tide on 2 October 1992. Eight prostomia from mature female worms were removed and homogenised. The sample was then passed through a C_{18} reverse phase cartridge (Sep-pak by Millipore) which had been pre-wet and prepared as described by the manufacturers. A pump was used so that a slow steady rate of $1ml.min^{-1}$ could be maintained. 5ml of the following solutions were then passed through the Sep-pak and collected.

a) 5ml distilled water

b) 5ml 30% acetonitrile

c) 5ml 70% acetonitrile

d) 5ml 100% acetonitrile

Reverse phase separation depends upon the hydrophobicity of the substances being separated. The more hydrophobic a substance is the longer it will stay bound onto the C_{18} silica. The samples were dried down in a centrifugal evaporator and resuspended in 2ml of filtered seawater. They were sonicated to ensure resuspension of all substances present. Eight gravid female worms were then injected with 250μl of each fraction. Two groups of controls were also injected, one with seawater and one with crude prostomial homogenate. A second separation was carried out in which the number of prostomia used initially, was doubled. The solvents were also passed through the Sep-pak at a much slower speed ($0.3ml.min^{-1}$).

6.2.4 Injection of Gravid Females with Fractions of the Aqueous Phase of Female Prostomial Homogenate, Separated using High Performance Liquid Chromatography (HPLC)

Gravid individuals were collected from Budle Bay in Northumberland at low water of spring tides on 24th November 1991. The procedure described in Section 2.9 was carried out. Initially six brains were homogenised. The flow rate was set at $1ml.min^{-1}$. Fractions were collected every minute in LP4 tubes, pooled in 3ml volumes and lyophilised. They were then resuspended in 1ml of filtered seawater. Four female worms were then injected with 250μl of the tube contents.

6.2.5 The Effect of Female Prostomial Homogenate on Sperm Activation *in vitro*

The method of assaying for sperm activating factors is described in 2.10. This assay was modified by using the Dynatech MR5000 plate reader, in an attempt to quantify the results. An agglutination programme was employed which measures the optical density of the sperm suspension. This provides an objective estimate of sperm activation in relation to the the agglutination response of the sperm suspension following activation used by Bentley (1985). This assay was used to monitor the populations to see when the males started to respond to the activation factor.

a) Male prostomial homogenate was assayed on sperm from a population of worms collected from the Eden Estuary on 29th July 1991.

b) Female prostomial homogenate was assayed on sperm from the same population.

c) The female prostomial homogenate was separated into aqueous and organic fractions as described in Section 2.7 and these fractions were assayed using sperm.

6.2.6 Incubation of the Organic Phase of Female Prostomial Homogenate with BSA

Bovine serum albumin (BSA) will form complexes with any free fatty acids that are present (Bentley *et al* , 1990). It may therefore be used to remove any lipid sperm activating factors from the prostomial homogenate (eg. 8,11,14-eicosatrienoic acid). It is also a double check for the results from the separation of the aqueous and organic phase. Bentley *et al.* (1990) found that sperm were still activated at concentrations of $1mg.ml^{-1}$ BSA using a standard prostomium concentration of $0.5.ml^{-1}$. A concentration of $10mg.ml^{-1}$ was therefore chosen initially.

6.2.7 Incubation of Female Prostomial Homogenate with Pronase and Trypsin

The female prostomial homogenate was separated as before and the aqueous extract was incubated with solutions of trypsin and pronase at concentrations of $1mg.ml^{-1}$. A positive control of prostomial homogenate was used. Three negative controls were used, seawater, denatured trypsin with seawater and denatured pronase with seawater. The peptidases were denatured by heating and then centrifuged. The supernatant was recovered for use in bioassay.

6.2.8 High-Performance Liquid Chromotography

This procedure was carried out as described in Section 2.9. Initially six brains were homogenised. The flow rate was set at $1ml.min^{-1}$. Fractions were collected every two minutes in LP4 tubes and lyophilised. They were then resuspended in sea water. The fractions were assayed with sperm to determine if there were any sperm activating factors present.

6.3 Results

6.3.1 Effect of Injection of Aqueous and Organic Fractions from the Female Prostomial Homogenate into Gravid Individuals

The organic fraction of the female prostomial homogenate did not cause spawning in any of the animals injected. The aqueous fraction did however, induce spawning in all of the males and two of the females. All of the males injected with the whole female prostomial homogenate spawned and so did two of the five females (see Table 6.1). The male spawning response was assumed to be complete as the water was completely white with spermatozoa. The mean number of oocytes released by trhe females is shown in Fig. 6.1.

6.3.2 Effect of Injection of Male and Female Prostomial Homogenates into Gravid Individuals

The males spawn on injection with both male and female prostomial homogenate. Although the sperm was not actually counted the water was totally cloudy and the sperm was actively swimming. It is assumed, therefore, that a full spawning response took place. Two out of the five females injected with female prostomial homogenate spawned and there was no spawning in any of the controls (see Table 6.2). The number of oocytes released were estimated to be 48,000 ($\pm$ 6099) and 43,600 ($\pm$ 1327) respectively. The oocytes were found to have undergone GVBD when examined under a light microscope. This spawning response is not a full spawning response, there were still some oocytes which had not undergone GVBD remaining in the coelom.

6.3.3 Injection of Partially Purified Fractions of the Aqueous Phase of Female Prostomial Homogenate into Gravid Female Worms.

Six out of the eight worms injected as a positive control spawned (see Table 6.3). The mean number of oocytes released per individual was $1.14 \times 10^5 \pm 0.315 \times 10^5$ and this data is presented in Fig. 6.2 below. This is equivalent to a full spawning response and all of the oocytes had undergone GVBD. There was still no spawning in two individuals. The fraction eluted with 30% acetonitrile induced spawning of fully mature oocytes in three out of eight individuals, with a mean number of $1.01 \times 10^5 \pm 0.149 \times 10^5$ oocytes released per individual. Spawning was also observed in one individual from the group injected with 70% acetonitrile.

6.3.4 Injection of Female Gravid Worms with Fractions of Aqueous Female Prostomial Homogenate Separated by HPLC

Spawning occured in two of the females that were injected with Sample 5 (see Table 6.4). The mean number of oocytes released by these two individuals was $1.47 \times 10^5 \pm 0.502 \times 10^5$. This is a full spawning response and the oocytes had undergone GVBD. Fig.6.3 shows the HPLC profile and identifies the region of possible biological activity which corresponds to the fraction with a retension time of 16 minutes. The profile of the concentration of the solvent indicates that at this time there was approximately 55% acetonitrile.

6.3.5 The *in vitro* Sperm Activation Assay

Fig. 6.4 shows that the sperm is activated by the male prostomial homogenate. The most agglutination takes place between the concentration range of 0.05 to 0.3 prostomia.ml^{-1}. There is less agglutination taking place with increasing concentration

of prostomia after this. At high concentrations of prostomia the response is inhibited (Bentley *et al.*, 1990). On the graph the optical density drops below that of the control at a concentration of about 0.5 prostomia.ml^{-1} and above. This is due to the fact that the morulae dissociate into spermatozoa but the agglutination response does not occur. This would then register as a less dense reading.

6.3.6 Testing the Female Prostomial Homogenate for Sperm Activating Factors

There is a sperm activating factor present in the female prostomium (see Fig. 6.5). It is active over a similar range of concentrations as the male sperm activating factor, although there is less activity at lower concentrations than with the male prostomial homogenate. The optimal activity occurs at around 0.15 prostomia.ml^{-1}.

6.3.7 Results of Testing the Aqueous and Organic Fractions of Female Prostomial Homogenate for Sperm Activating Factors

The organic and the aqueous fractions have some sperm activating properties. They are both not active at such low concentrations as the untreated female prostomial homogenate (see Table 6.5). The aqueous phase is active down to a concentration of 0.0625 pr.ml^{-1} whereas the organic phase is only active down to concentrations of 0.125 pr.ml^{-1}. Some of the results are scored as positive / negative because the activation did not seem to be complete in all of the wells. Agglutination did not occur and for this reason the assay plate had to be scored under the light microcope. Although the morulae had broken down the sperm were not swimming actively as observed in the controls. The movement observed was slow and only occurred in some of the sperm. This effect may be one caused by a neutral pH in the assay plate.

Swimming in the sperm is stimulated by a change in the pH which occurrs when the sperm is diluted in seawater (A. A. Pacey, personal communication).

6.3.8..Results of Incubation of Aqueous Female Prostomial Homogenate with Pronase and Trypsin

There was no activity in the negative seawater control or in either of the wells that contained deactivated pronase or trypsin. Any sperm activation effects could not therefore be due to the presence of the peptides themselves. Trypsin did not affect the activity of the aqueous fraction on sperm morulae. The activity of the aqueous phase was inhibited when pronase was present (see Table 6.6).

6.3.9 Incubation of the Organic Fraction with BSA

The presence of the BSA inhibits the activity associated with the organic fraction. As expected the prostomial homogenate is active at a much lower concentration than either the organic fraction or the prostomial homogenate with the BSA. The results of this experiment are presented in Table 6.7.

Table 6.1 The results of *in vivo* spawning experiments after injection with aqueous and organic fractions of female prostomial homogenate. Numbers in the columns are the numbers of worms which spawned and the numbers in parentheses are the number of animals that were injected.

Injected with :	Males	Females
Organic fraction of female pr.	0 (5)	0 (5)
Aqueous fraction of female pr.	5 (5)	2 (5)
female prostomial homogenate	5 (5)	2 (5)
Filtered seawater	0 (5)	0 (5)

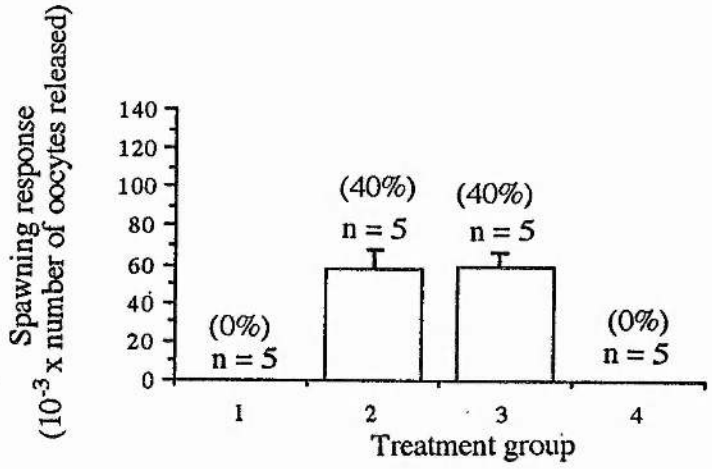


Fig. 6.1 The spawning response of female *Arenicola marina* to injection of: (1) organic fraction of female prostomial homogenate; (2) the aqueous fraction; (3) female prostomial homogenate; (4) filtered seawater. The spawning response is indicated as the mean number of oocytes spawned (+ sem) in each control group. The figures in brackets are the percentage of animals in that control group that underwent a spawning response; n is the number of individuals in each group.

Table 6.2 Results of *in vivo* spawning experiments after injection with male and female prostomial homogenates. Numbers in the columns are the number of worms that spawned and the numbers in parentheses are the numbers of worms which were injected.

Injected with :	Males	Females
Male prostomial extract	5 (5)	0 (5)
Female prostomial extract	5 (5)	2 (5)
Filtered seawater	0 (10)	0 (10)

Table 6.3. The number of worms in which spawning occured after injection with the various fractions. The number in parentheses is the total number of worms injected.

Injected with :	Females
FPH	6 (8)
Filtered seawater	0 (8)
Aqueous fraction	0 (8)
30% acetonitrile fraction	3 (8)
70% acetonitrile fraction	1 (8)
100% acetonitrile fraction	0 (8)

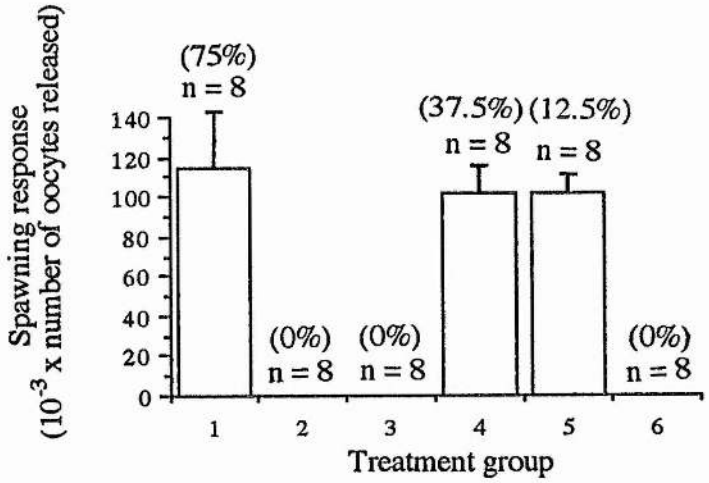


Fig. 6.2 The spawning response of female *Arenicola marina* to injection of: (1) female prostomial homogenate; (2) filtered seawater; (3) aqueous fraction; (4) 30% acetonitrile fraction; (5) 70% acetonitrile fraction; (6) 100% acetonitrile fraction. The spawning response is indicated as the mean number of oocytes spawned (+ sem) in each control group. The figures in parentheses are the percentage of animals in that control group that underwent a spawning response; n is the number of individuals in each group.

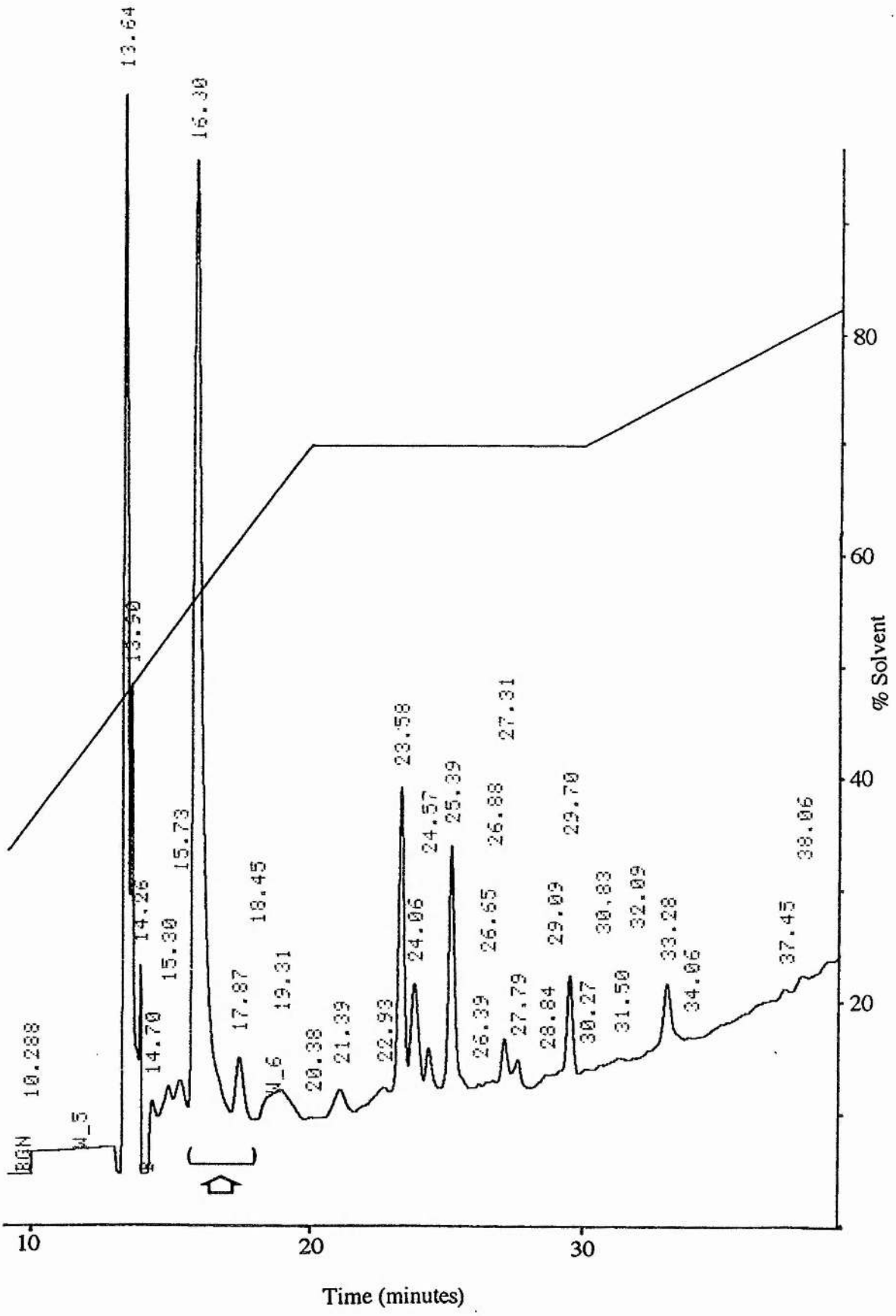


Fig. 6.3 Trace from HPLC of female prostomial homogenate. The solvent gradient is the line running across the top and the % solvent is marked on the right hand axis. The area which showed biological activity is marked with an arrow and includes two peaks.

Table 6.4 The number of animals spawning on injection of the different fractions obtained from HPLC of the aqueous fraction of FPH. The numbers shown in parentheses are the total number of animals that were injected.

Fractions	Sample	Nº. Females Spawning
1,2,3	1	0 (4)
4,5,6	2	0 (4)
7,8,9	3	0 (4)
10,11,12	4	0 (4)
13,14,15	5	0 (4)
16,17,18	6	2 (4)
19,20,21	7	0 (4)
22,23,24	8	0 (4)
25,26,27	9	0 (4)
28,29,30	10	0 (4)
31,32,33	11	0 (4)
34,35,36	12	0 (4)
37,38,39	13	0 (4)
40,41,42	14	0 (4)
Seawater		0 (4)

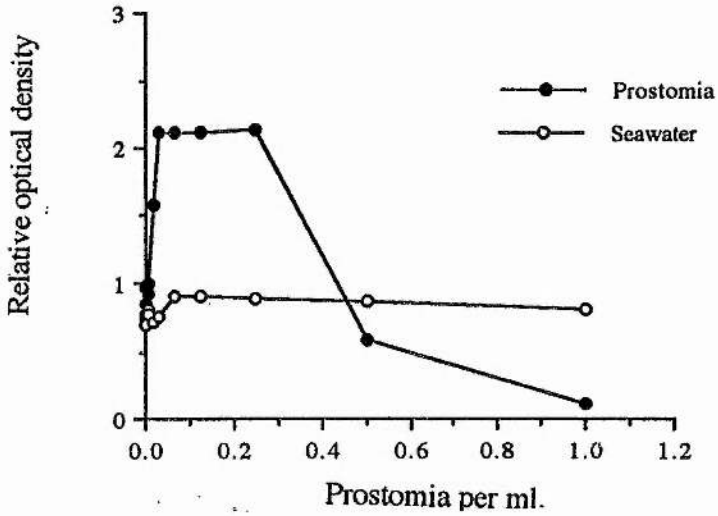


Fig. 6.4 Graph of the relative optical density of sperm, incubated with male prostomial homogenate *in vitro*.

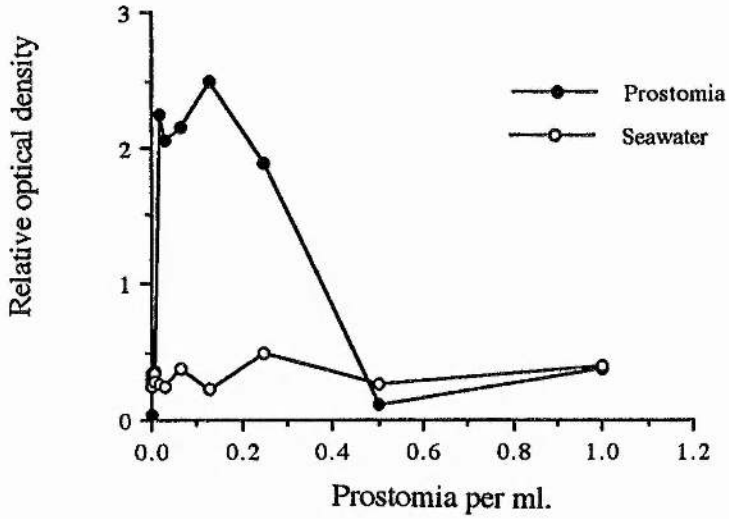


Fig. 6.5 Graph of the relative optical density of sperm, incubated with female prostomial homogenate *in vitro*.

Table 6.5 The maximum and minimum concentrations at which activation of the sperm occurred after incubation with fractions of female prostomial homogenate *in vitro*.

Treatments	Result	Minimum effective conc. for sperm activation ($pr.ml^{-1}$)	Maximum conc. at which sperm activation occurs ($pr.ml^{-1}$)
Seawater	-	-	-
Female pr.homogenate	+	1.6×10^{-2}	0.25
Organic fraction	±	0.125	1
Aqueous fraction	±	0.0625	1

Table 6.6 The maximum and minimum concentrations at which activation of the sperm occurred arter incubation with aqueous fractions of female prostomial homogenate *in vitro*.

Treatments	Sperm Activation (+/-)	Minimum effective conc. for sperm activation ($pr.ml^{-1}$)	Maximum conc. at which sperm activation occurs ($pr.ml^{-1}$)
Prostomial extract	+	6.3×10^{-2}	0.25
Aqueous fraction	+	6.3×10^{-2}	1
Denatured pronase	-	-	-
Prost.+ denat.pronase	+	6.3×10^{-2}	1
Prostomia + Pronase	+	0.125	1
Aqueous + Pronase	-	-	-
Denatured trypsin	-	-	-
Prost.+ deact.trypsin	+	6.3×10^{-2}	1
Prostomia + trypsin	+	6.3×10^{-2}	1
Aqueous + trypsin	+ / -	0.25	-

Table 6.7 The maximum and minimum concentrations at which sperm activation occurred after incubation with organic fraction of female prostomial homogenate and BSA *in vitro*.

Treatments	Results	Minimum effective conc. for sperm activation ($pr.ml^{-1}$)	Maximum conc. at which sperm activation occurs ($pr.ml^{-1}$)
Prostomia	+	1.6×10^{-2}	0.25
Seawater	-	-	-
Prost. + BSA	+	0.125	0.25
Organic fraction	+	0.125	1
BSA + Organic fraction	-	-	-

6.4 Discussion

The main aim of the investigations described in this chapter were to examine the nature of the endocrine substances present in the female prostomium which affect reproduction in *Arenicola marina*. As outlined in the introduction, the reproductive endocrinology of the males and females is different (Howie, 1961a; Pacey & Bentley, 1992). Considerable progress in the understanding of the endocrine control of the male reproductive system was made by Howie (1961a, b; 1963) using *in vivo* experiments. The development of the *in vitro* sperm maturation assay (Bentley, 1985) was however a significant development because it enabled the final stages in isolation and purification of the sperm maturation hormone to be carried out. Investigations described above

have employed some *in vivo* and also some *in vitro* experiments in order to determine the effects of endocrine substances present in the female prostomium.

The first experiment (6.2.1) shows that two out of five females spawned when injected with female prostomial homogenate and the aqueous fraction of female prostomial homogenate. The fact that not all of the females injected with female prostomial homogenate spawned is similar to Howies' (1963) observations. He reports in this paper that only 50% of the females injected with female prostomia could be stimulated to spawn. Pacey and Bentley (1992) however observed a 100% spawning response in a group of sixteen females, collected from the East Sands and from Budle Bay and injected with a mixed prostomial homogenate from males and females (0.1 prostomium per g body mass). Their experiments were carried out a few weeks before the natural spawning time of each population. The natural spawning time for animals from Budle Bay is two to three weeks in the middle of December (M. G. Bentley, personal communication). As the spawning season is extended or non-epidemic it is possible that the worms may still be at slightly different stages of development. This would mean that the individual responses to female prostomial homogenate would vary. There was no spawning in any of the females injected with either the seawater control or the organic fraction. The results of this experiment suggest the possibility that the putative oocyte maturation hormone is present in the aqueous fraction of female prostomial homogenate.

The results from the males that were injected are surprising because there is a full spawning response from all the males to the aqueous fraction and none of the males that were injected with the organic fraction spawned. It would be expected that they would spawn on injection with the organic fraction if eicosatrienoic acid was present in the female brain (Bentley & Pacey, 1992). These findings can be interpreted in two ways. One possibility is that the partitioning of the two fractions has been incomplete

and some of the organic phase is still remaining in the aqueous fraction. It is unlikely that this is the case here as there was no spawning in any of the worms injected with the organic phase. It is possible that most of the activity was lost from the organic phase or the concentration retrieved was too low to stimulate spawning. Whatever is present in the aqueous fraction could be active at much lower concentrations.

Howie (1963) also injected gravid males with various fractions of tissue homogenates at a dosage of four brain equivalents. He injected eight males with the phospholipid fraction and fourteen males with the lipid minus the phospholipid fraction. None of these animals spawned. He also injected seventeen males with the residue after a benzene extraction and of these nine spawned. He concluded that this fraction was active and that at least two spawning stimulants can be extracted from the tissues of the worm. Benzene, however, is not very good for organic extraction as too low a pH will cause the fatty acids to stay in the aqueous phase. This may explain why there was spawning in animals injected with this fraction in Howies' experiments.

The results obtained in the second part of this study (Experiment 6.2.2) confirm previous observations that the endocrine systems that control gamete maturation and spawning are sex specific in *A. marina* (Howie, 1963, Pacey & Bentley, 1992). Male prostomial homogenate is capable of inducing spawning in gravid males only . Female prostomial homogenates can induce spawning in gravid females and males. The spawning response of the females injected with female prostomia was not 100% (two spawned out of five injected). The number of oocytes spawned was only about a third of the number termed a 100% response by Pacey and Bentley (1992). This experiment was actually carried out on animals from the East Sands one month before the dates of spawning. The probable explanation is that this was too early and not all of the animals were fully responsive to the oocyte maturation factors. It could also be that levels of

the oocyte maturation factor present in the prostomia of the females at this time are much lower than when the spawning season is closer.

The fact that the female prostomia can initiate spawning in both males and females is very interesting. As we have established that there is a dichotomy in the endocrine substances controlling reproduction in males and females there must therefore be at least two of these substances present in the female brain. One of these could be a substance similar to the sperm maturation factor, 8,11,14-eicosatrienoic acid (Bentley *et al.*, 1990), although it has been established that when 8,11,14-eicosatrienoic acid is injected into gravid females there is no response (Pacey & Bentley, 1992). Alternatively it could be that the oocyte maturation hormone, or another neurosecretory product, may act in some way on the reproductive system of the males. This could be in the form of direct action on the sperm causing activation and hence spawning, or it may be to stimulate the release of the sperm maturation factor in the males. The fact that a crude homogenate of the female brain activates sperm *in vitro* makes it seem likely that 8,11,14-eicosatrienoic acid is also present in the female brain.

The results from Section 6.3.3 show that six out of eight females injected with prostomial homogenate spawned. The animals used in this experiments were collected from the highly synchronous population on The East Sands on 2 October 1992. Spawning at the East Sands in 1992 took place on 13 October, only eleven days after the animals were collected. They might all be expected to be responsive at the same time and to the same degree and there is no apparent reason why some would not respond to injection with prostomial homogenate. This makes interpretation of the results very difficult. The fact that three out of eight worms injected with the fraction eluted with 30% acetonitrile spawned cannot be ignored. Although the response is not as great as for the controls. However one worm spawning out of eight injected with the fraction eluted with 70% acetonitrile cannot be counted as a positive result. This

points tenuously to the presence of a spawning hormome which is soluble in around 30% acetonitrile. It may be fully adsorbed at a concentration somewhere between 30% and 70%. This could explain why none of the experimental groups show as much spawning as in the group injected with female prostomial homogenate.

The results obtained from the HPLC do agree with the results from the separation using the Sep-pak. The only group of animals to spawn was the group injected with fractions 16,17 and 18. Again, unfortunately only two individuals out of the four in this group spawned. There was, however, no spawning in any of the other groups of animals. This corresponds with a solvent concentration of 55% and if we look at Fig.6.3, which is a trace from the HPLC there is a peak which comes off at this time. The animals used in this experiment were collected from Budle Bay in Northumberland on 24th November 1991. The spawning season there lasts for about two weeks in mid-December (M.G. Bentley, personal communication), so if the period in which they can be stimulated is a fortnight or less it could too early for some of the worms.

It is interesting to note that in the *in vitro* assays the same response can be seen in the sperm when incubated with male or female prostomial homogenate. This does imply that eicosatrienoic acid or a similar fatty acid is also present in the female brain. The range of concentrations at which the male and female prostomia activate the sperm is very similar, although there is a greater inhibition of the reaction at higher concentrations with the female prostomia. When the female prostomial extract is split into the aqueous and organic fractions both fractions are active. The aqueous fraction is active over at lower concentrations than the organic fraction. This may explain why the animals only spawned to the aqueous fraction when they were injected. This raises several questions, the most obvious being, is the sperm activating factor which is present in the organic fraction eicosatrienoic acid or a similar substance? In order to test

this, the incubations were carried out with BSA. The organic fraction will not activate sperm when it is incubated with BSA which implies that the activating factor is in fact 8,11,14- eicosatrienoic acid or a similar fatty acid.

The fact that the aqueous fraction of female prostomial homogenate also activates the sperm *in vitro* is very strange. The most obvious explanation for this is that separation has been incomplete and there is still some of the fatty acid present in the aqueous phase. Alternatively there may another factor present in the aqueous phase that has sperm activating properties. If this substance is soluble in water there is the possibility that it could be a peptide. Incubation with pronase which is a general protease stops sperm activation. Trypsin is much more specific acting only at arginine-leucine bonds. Partial deactivation still occured in the presence of trypsin. It is possible that there is a peptide present that does not contain any, or only a few bonds of this nature.

A factor which is present in the aqueous phase and which is present in the female brains could be initiating the spawning response in the males through some non-specific action. This may be linked with the strange observation that there is a difference in the timing of the spawning response after injection. In the males the response takes fifty minutes to occur, and in the females there is a delay of five hours or more (Pacey & Bentley, 1992). The male spawning response could be initiated by the female releasing some pheromone into the sea before low tide, as the females spawning mechanism is triggered. The pheromone may then act on the males to initiate their own spawning mechanism.

Another difference between the sexes is that the males can be induced to spawn months in advance of the actual spawning period. Spawning in the females on the other hand cannot be induced until immediately prior to spawning. This could be due to low concentrations of the maturation factor in the prostomium until a few weeks

before spawning or there could be an inhibitor present throughout gametogenesis which prevents the maturation hormone from acting. It could be that the oocytes are unable to respond to the presence of the maturation factor. If the spawning in the female somehow induces the spawning in the male there would be less margin for an error in the mistimimg of spawning. The females not being able to repond until a few weeks before spawning would prevent a premature spawning response in the males.

We know that prostomial homogenate induces maturation in females (Howie, 1966). A substance stored in the prostomium could act directly on the oocyte which might then produce a secondary substance as in the starfish. This seems unlikely in regard to the experiments carried out in Chapter 5. If the neurosecretory product from the prostomia does act directly on the oocytes we would have expected to see oocyte maturation taking place *in vitro* . Alternatively the neurosecretory product from the prostomia might act at another site either in the coelomic fluid, for example on the coelomocytes or maybe at the wall of the coelom. This may then induce the secretion of a secondary product, the oocyte maturation factor, which then acts directly on the oocyte.

The results from Chapter 5 indicate that the oocytes from *Arenicola defodiens* undergo GVBD *in vitro* when incubated with prostomial homogenate from the same species. More significantly the *Arenicola defodiens* oocytes also undergo GVBD when incuated in prostomia homogenate of *A. marina* . There is therefore considerable potential for advancing this work using the *A. defodiens* oocytes as an *in vitro* assay for the identification of the *A. marina* spawning hormone. The fractions separated from the HPLC could be tested very easily on the oocytes of *A. defodiens.*

Chapter 7

General Discussion

General Discussion

This thesis has investigated morphological and genetic variation in *Arenicola marina* from different geographic locations. The morphological differences include the number of chaetigerous segments and the arrangement of the annuli which separate these chaetigerous segments (Wells, 1957), as well as the more noticeable phenotypic differences in size and colour (Kyle, 1896; Duncan, 1959). Populations of *Arenicola marina* at different geographic locations also have varying lengths and timings of spawning season (Howie, 1984; see Section 1.6). Howie (1984) identified two different ways in which spawning may take place. Some populations have been shown to have an epidemic type spawning which lasts for two or three days (Pirlot, 1933; Newell, 1948; Howie 1959), whereas in other populations the spawning season may be extended or non-epidemic, lasting for two or three weeks (Ashworth, 1904; Duncan; 1960; Cazaux, 1966; Pollack, 1979). Some of these reported differences may be explained by the the recent separation of *Arenicola marina* into two species, *A. marina* and *A. defodiens* (Cadman & Nelson-Smith, 1993), with confusion arising from the attribution of characteristics of *A. defodiens* to *A. marina.* This thesis has set out to investigate these inter-population, and likely inter-specific, differences for *A. marina* with particular emphasis on reproductive aspects of the life cycle.

Gametogenesis in *Arenicola marina* has been monitored at three study sites and is discussed in Chapter 3. Polytelic polychaetes use a wide range of strategies in order to produce a cohort of gametes that are all fully developed at the same time. The way in which the gametes develop in *A. marina* is in many respects similar to other polytelic polychaetes. During the main phase of vitellogenesis, which starts about three months before the actual time of spawning, there is a wide range of oocyte sizes present in the coelomic cavity. This also is the case in *Cirratulus cirratus* (Olive, 1970), *Lanice conchilega* (Smith, 1989), and both *Nephtys caeca* and

N. hombergii, although in case of the the Nephtyids the gametes are retained in the gonads during this period of development (Olive, 1978). While there is no evidence for endocrine control of gametogenesis in *A. marina* (Rashan & Howie, 1982), the presence of a gonadotrophic hormone has been demonstrated in *Nephtys caeca* and *N. hombergii.* The presence of this hormone is essential for oogenesis to proceed because it maintains the integrity of the ovary (Olive & Bentley, 1980) and also has a vitellogenesis promoting rôle (Olive & Lawrence, 1990). Endocrine control of gametogenesis is not limited to those species which have the gametes associated with distinct gonads because a vitellogenesis promoting hormone has been demonstrated in *Eulalia viridis* (Olive, 1976a, b, 1977). In *Eulalia viridis* gamete development takes place entirely in the coelomic cavity and is not well synchronised (Olive, 1975a).

Gametogenesis is linked directly to the timing of the spawning season. The data from Chapter 3 provide evidence for an epidemic spawning season at the East Sands in St. Andrews. This confirms Howies' observations (1959) although he reports that spawning at St. Andrews occurred during a one to three week period in mid-October to mid-November between 1949 and 1952. It would seem that the spawning period is actually very specific and predictably occurs over three days during the peak of the second set of spring tides in October. The data from Chapter 3 show that at the Eden Estuary spawning is non-epidemic and lasts for three weeks during October. It appears that the majority of populations from northern Europe that have been studied show an autumn spawning season, although there have been reports of spring spawnings (Howie, 1959; Duncan, 1960; von Brenning, 1965). At Fairlie Sands spawning was reported to take place in the spring and then again in the autumn (Howie, 1959). Data from Chapter 3 suggest that the population at Fairlie Sands is very unusual because a small percentage of the population were found to be gravid throughout the year. There was a peak in the number of gravid individuals found in April and then again in November. It could be that there were two distinct spawning

seasons which have become very extended and merged into each other. De Wilde & Berghuis (1979b) report that in a population of lugworms at the Wadden Sea there are two spawning seasons. There is an extended spawning season which occurs between August and September and there is then a second season which is epidemic and occurs in November. A possible explanation for this observation is that both *A. marina* and *A. defodiens* occur on this beach, as they do at the beach in Swansea Bay (Cadman & Nelson-Smith, 1990), and the two different species have different spawning seasons. There are, as yet, no direct observations on the timing of the spawning season of *Arenicola defodiens.* All of the populations investigated in Chapter 3 were *Arenicola marina* and it is clear that the populations at the three study locations have very different spawning patterns.

The majority of populations of *A. marina* spawn in autumn, from the beginning of September through to the end of November (Howie, 1984). This is unusual in comparison to other populations of polytelic polychaetes for which spawning seasons have been observed, such as *Kerfesteinia cirrata* (Olive & Pillai, 1983), *Nephtys caeca, N. hombergii* (Olive, 1978) and *Harmothoe imbricata* (Daly, 1972) all of which are reported to spawn in spring. Other species, such as *Lanice conchilega* (Smith, 1989) and *Eulalia viridis* (Olive, 1975a), are reported to spawn in early summer. Thorson (1946) reported that most temperate species of invertebrate reproduce in the spring when the ambient temperature is rising. The environmental temperature is very important because as temperatures rise in spring the rate of development of the larvae increases also (Todd & Doyle, 1981). By October, which is when *A. marina* from the East Sands spawn, the ambient temperature had fallen considerably from a maximum of about 14°C in July and August to about 8°C (Gatty Marine Laboratory data): local temperature minima in January, February are approximately 3-4°C. It might, therefore, be expected that the larvae of *Arenicola marina* from autumn spawnings would grow and develop relatively slowly.

The observed morphological differences and variation in the timing of the breeding season in populations of *A. marina* at different localities may be explained in two possible ways. The first is that the populations are genetically similar and the observed differences are due to the worms responding to the different environmental conditions which the animals experience at different sites. Secondly the populations are reproductively isolated from each other and this has caused a degree of diversification in the genotype within the species. Differences in the timing of reproduction between sites would serve to enhance effects of reproductive isolation. Studies on populations of *Pecten maximus* have shown that scallops transplanted from Scotland to France, and *vice versa*, as post-settlement juveniles showed spawning seasons characteristic of their sites of origin (Ansell *et al.*, 1988). This implies that genotype may be able to influence timing of reproduction.

There is evidence provided in Chapter 4 to suggest that there is a degree of genetic variation of *A. marina* between different sites. Allele frequencies at the *GOT* locus were different between east and west coast sites and a third rare allele was present in a few individuals from Fairlie Sands. Allele frequencies at the *PGI* locus also showed differences between some populations. Shahid (1982) reported significant variation at the *PGI* locus between five populations of *A. marina* from the Northumberland coast. Although the extent of gene flow which takes place through larval dispersal remains largly undetermined these preliminary results do suggest that it could be restricted. Analysis of the genetic data using the *F* -statistics showed that inbreeding in subpopulations may be affecting genetic variation in the total population.

In *Arenicola marina* developing gametes accumulate in the coelomic cavity during the period prior to spawning and in the females the oocytes are arrested in the last prophase division of meiosis (Meijer & Durchon, 1977). The final maturation

stage, which results in the reinitiation of meiosis, occurs in the coelom prior to spawning. The hormones involved in spawning have been investigated here with particular reference to a putative female oocyte maturation hormone (Chapters 5 & 6). Investigations have been carried out using a mixture of *in vitro* and *in vivo* techniques and some inter-specific endocrine investigations between *Arenicola marina* and *A. defodiens* have also proved useful in the investigation. Results have shown that the sperm maturation factor 8,11,14-eicosatrienoic acid (Bentley *et al.*, 1990) from *A. marina* acts in the same way on the morulae of *A. defodiens*. In females, endocrine control is more complex and there are differences between the two species. *In vitro* oocyte maturation in *A. marina* has been reported previously (Meijer & Durchon, 1977). The oocytes of *A. marina* investigated during this study could not, however, be stimulated to undergo maturation *in vitro* in the presence of prostomial homogenate. This observation either means that there are inter-population physiological differences or it could mean that Meijer & Durchon (1977) and Meijer (1979a; 1979b;1980) were working on the new species of *Arenicola, A. defodiens* (Cadman & Nelson-Smith, 1993).

A. defodiens oocytes incubated in a prostomial extract of the same species underwent GVBD *in vitro* ; this provides evidence in support of the second hypothesis. Oocytes from both *A. marina* and *A. defodiens* were stimulated to undergo GVBD artificially, using calcium agonists *in vitro*. This implies that the underlying cellular mechanism for meiosis reinitiation is similar in both species. The oocytes of *A. defodiens* will undergo maturation *in vitro* when incubated with prostomial homogenate of either *A. defodiens* or *A. marina*. The oocyte maturation factor is therefore present in the prostomia of *A. marina* but does not act on the *A. marina* oocyte. Meiosis reinitiation is more staightforward in *A. defodiens* because a substance that is present in the prostomium is able to act directly on the oocyte and induce maturation *in vitro*. Injection of prostomial extract of female *A.*

defodiens into gravid female *A. marina* induced spawning. It is therefore suggested that a cofactor or secondary substance produced *in vivo* acts on, or with, the oocyte maturation factor in *A. marina* to alter it slightly and thereby rendering it active. The experiments with *Arenicola* from Wimereux in France (Chapter 5) showed them to respond in the same way as *A. defodiens* from Wales, which is further evidence that Meijer and Durchon (1977) and Meijer (1979a; 1979b; 1980) were actually working on *A. defodiens* and not *A. marina.*

Experiments in the laboratory have shown that male prostomial homogenate did not induce spawning when injected into gravid females, whereas injection of female prostomial homogenate into gravid males induced sperm activation and spawning (Chapter 6). This agrees with observations made by Howie (1963) and implies that the female prostomium contains a sperm activating factor. The results of the *in vitro* experiments suggest that there is a sperm activating factor in the aqueous and organic phase. It is likely that the organic phase contains 8,11,14-eicosatrienoic acid or a similar fatty acid. Evidence is provided for this by the fact that incubation of the organic phase with BSA inhibits activity. The positive bioassay results observed for the aqueous phase are either due to a putative female hormone which affects the males in some way, or an incomplete separation of the crude extract. If the separation was incomplete only a very small amount of the organic phase, and hence sperm activating substance, would be present in it. The concentration of the aqueous phase required for sperm activation was in fact lower than that of the organic phase. Incubation of the aqueous phase with pronase inhibited sperm activation, suggesting an active protein, and the results of the *in vivo* spawning experiments with the females tentatively suggest the presence of a spawning inducing substance in the fraction eluted with 30% acetonitrile.

The key to successful isolation and purification of endocrine substances is a reliable biological assay on which to test putative factors. The *in vivo* spawning assay using female *Arenicola marina* proved to be unreliable in that not all of the individuals responded in the same way. This is a problem that was also encountered by Howie (1963) and is different from the *in vivo* spawning assay using male *A. marina* which has proved much more useful (Howie, 1961a, c, 1963; Pacey & Bentley, 1992). An *in vitro* assay is ideal because it provides the opportunity of using the gametes of one individual for multiple assay. The *in vitro* sperm activation assay proved invaluable in the identification of 8, 11, 14- eicosatrienoic acid, the male sperm maturation factor (Bentley, 1985; Bentley *et al.*, 1990; Pacey & Bentley, 1992). It is very unfortunate that the oocytes of *A. marina* cannot be stimulated to undergo maturation *in vitro*. The fact that the oocytes of *A. defodiens* do undergo maturation *in vitro*, and in the presence of prostomial homogenate of both *A. marina* and *A. defodiens*, is an important discovery for the continuation of the work that has been started in Chapter 6.

7.1 Areas for Future Research

1) A genetic survey of a few beaches that are close together and where the tidal effects are well known would provide useful information regarding the extent of gene flow by larval transport. Genetic fingerprinting would provide a more detailed study of the amount of genetic variation that occurs within and between populations from different localities.

2) There is great potential for advancing the work on establishing the chemical nature of the oocyte maturation hormone that is present in female *Arenicola marina* and *A. defodiens*. *A. defodiens* oocytes could be used as an *in vitro* assay to extend the experiments carried out in Chapter 6.

3) The cofactor or secondary product which is essential for reinitiation of meiosis to take place in *Arenicola marina* oocytes requires further investigation. It may be associated with the coelomic fluid and could potentially be isolated and purified using HPLC or possibly gas chromatography.

4) Information regarding the site and mechanism of action of the oocyte maturation factor could be established using fluorometric techniques. Intracellular changes in calcium ion concentration in the oocyte, which may occur at the onset of meiosis reinitiation, may be measured in this way.

5) Work on the reproductive cycle and distribution of *Arenicola defodiens* is required because at present there is very little information available on this species. It would also be interesting to look at other species of *Arenicola* with regard to the control of spawning in comparison with *A. marina.*

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