SPERM ACTIVATION AND SPAWNING IN ARENICOLA MARINA (L) (ANNELIDA:POLYCHAETA)

Allan Anthony Pacey

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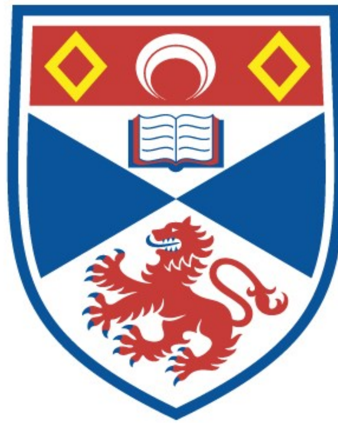

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Sperm Activation and Spawning in *Arenicola marina* (L.) (Annelida: Polychaeta).

by Allan Anthony Pacey

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

Department of Biology & Pre-clinical Medicine

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Abstract

The spermatozoa of *Arenicola marina* are unlike those of most marine invertebrates, in that they become motile in the body cavity prior to spawning. This occurs in response to a Sperm Maturation Factor (SMF) which is released from the prostomium. Prior to activation, spermatozoa are held as morulae with several hundred spermatozoa connected by a common mass of cytoplasm called the cytophore.

Sperm activation by SMF is characterised *in vitro* , in terms of the ultrastructural changes which occur as the sperm become motile, and an active role for the cytophore during sperm activation is suggested. The morphology of these spermatozoa is 'primitive', and ultrastructural observations show that they possess a discoid swelling at the distal end of the flagellum. It is suggested that this may aid in swimming efficiency.

The chemical nature of SMF has been putatively identified as 8,11,14 - eicosatrienoic acid from both *in vitro* and *in vivo* studies. Biochemical investigations demonstrate that sperm activation is linked to an increase in sperm respiration rate, and an elevation of intracellular pH in the order of 0.2 pH units. Levels of ATP in spermatozoa are higher than those reported in other species, and it is considered that quiescence of sperm is not mediated by the deprivation of ATP to the axoneme.

It is reported that sperm activated *in vitro* display a motile life of less than one hour, sperm which has been spawned *in vivo*, however, can have a motile life of up to 48 hours. It is suggested that this extension in sperm motile life may result from capacitation-like events which occur during activation and release *in vivo*.

A hypothesis for the synthesis of 8,11,14 -eicosatrienoic acid, its release from the prostomium, its transportation and mode of action at the level of the spermatozoa is also developed.

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List of Abbreviations

ATP	Adenosine triphosphate
BIS	Brain inhibiting substance
BSA	Bovine serum albumin
C20	20 Carbon
cAMP	Adenosine 3': 5' - cyclic monophosphate (cyclic AMP)
cGMP	Guanosine 3': 5' - cyclic monophosphate (cyclic GMP)
CE	Caudal epididymis
ELH	Egg laying hormone
FMP	Forward motility protein
GLC	Gas-liquid chromatography
GSS	Gonad stimulating substance
GVBD	Germinal vesicle breakdown
HETE	Hydroxyeicosapolyenoic
HPETE	Hydroperoxyeicosapolyenoic
HPLC	High performance liquid chromatography
LT	Leukotrienes
LX	Lipoxins
1-MeAde	1-Methyl Adenine
MH	Maturation hormone
MIS	Maturation inducing substance (Starfish)
MIS	Mitosis inhibiting substance
MS	Mass Spectrometry
MSH	Mitosis stimulating hormone
PA_2	Phospholipase A_2
PG	Prostaglandin
pHi	Intracellular pH

Sat.TFSW	Air saturated TFSW
SMF	Sperm maturation Factor
SMI	Sperm motility initiating factor
TCA	Trichloroacetic acid
TEM	Transmission electron microscopy
SEM	Scanning electron microscopy
sem	Standard error of the mean
TFSW	Triple filtered seawater
TLC	Thin layer chromatography

Chapter 1

Introduction to Sperm Biology and Spawning

1.1 Introduction

Spermatozoa were first described by Leeuwenhoek in 1677, but despite this long history, spermatology has only recently become a popular area for study. It has only been in the last twenty years or so, that detailed investigations on the sperm biology of a considerable number of species has been carried out and in this time the literature on this aspect of reproductive biology has grown enormously. In recent years, the relevance of this reseach to areas such as human infertility has been an added stimulus for the development of spermatology as a discipline.

Polychaetes, on the other hand, have been an area for study for considerably longer. Early investigations began with taxonomic characterisation of these species, but in subsequent years many aspects of their biology have been explored. It is perhaps true to state that no other group of animals show so many different reproductive strategies (Olive & Clark, 1978) and this has made them particularly interesting animals for the study of reproduction.

This thesis investigates aspects of the reproductive biology of the common intertidal polychaete *Arenicola marina* which relate to its sperm biology and the relationship between sperm activation and spawning.

Spermatozoa have an essential role during reproduction in the transfer of the male genetic material to the ovum. In doing so, these cells often have to survive overcrowded conditions, low oxygen tensions, and are required to reach and select and penetrate the eggs' protective barriers (Anderson & Personne, 1975). With such common aims, it might be expected that the sperm of species from different phyla may share many similarities in their biology. It is the aim of this first chapter therefore, to summarise the major aspects of sperm biology, both in terms of their morphology at the ultrastructural level and their biochemistry, with particular reference to how these are related to spawning mechanisms and fertilisation biology.

1.2 Ultrastructure of Spermatozoa

Sperm ultrastructure has been the subject of a number of reviews, which include those of Baccetti, (1970); Baccetti & Afzelius, (1976); Franzén, (1977; 1987). These have identified the existence of a wide variation in the size, shape and form of spermatozoa, but that in spite of this, they are generally composed of similar morphological units. In most spermatozoa, a head, a middle piece and a tail can be distinguished. It has been on the basis of their morphology and their deviation from a simple prototype model (the 'primitive spermatozoon') that sperm have been classified (see Franzén, 1970; Favard &André, 1970; Afzelius, 1972 for detailed classification) and although this is an arbitrary division it is often of use in a comparative study of sperm from different phyla.

i) Primitive spermatozoa have a simple morphology. They generally possess radial symmetry and are composed of the three distinct regions: head, middle-piece, and tail (Franzén, 1956). The head is usually ovoid or rounded and is surmounted by an acrosome, the middle piece is relatively short and contains usually 4 or 5 mitochondria, and the tail consists of a long flagellum (40-65 μm).

ii) Modified spermatozoa differ from the primitive form by some degree of modification to the head, middle piece and tail, to either a greater or lesser extent. In fact, in some cases there may be a complete deviation from the primitive type. In extreme cases, acrosomes or axonemes may be absent, but by far the greatest modifications are to the mid-region and the mitochondria.

These descriptions of primitive and modified sperm types are taken from the review of sperm biology by Baccetti & Afzelius, (1976), who point out that there are a wide variety of modified sperm types and include the ascidian, biflagellate,

aflagellate and apyrene (paraspermatozoon) type of spermatozoa. In almost every case, it is from the primitive condition that modified sperm types have arisen and the relationship of these sperm types in the metazoan phylogenetic tree is shown in figure 1.1 In the following section of this thesis, the ultrastructure of spermatozoa will be described, and where relevant be illustrated with examples of sperm from a variety of these sperm types.

Figure 1.1 (next page). The occurence of sperm types in the metazoan phylogenetic tree (from Franzén, 1987).

1 = primitive type of spermatozoon; 2 = modified spermatozoon; 3 = ascidian type of spermatozoon; 4 = biflagellate spermatozoon; 5 = aflagellate spermatozoon; 6 = apyrene spermatozoon (paraspermatozoon).

Abbreviations: a = acrosome; c = centriole; e = end piece; h = head; m = mitochondrion; mp = middle piece; n = nucleus; t = tail.

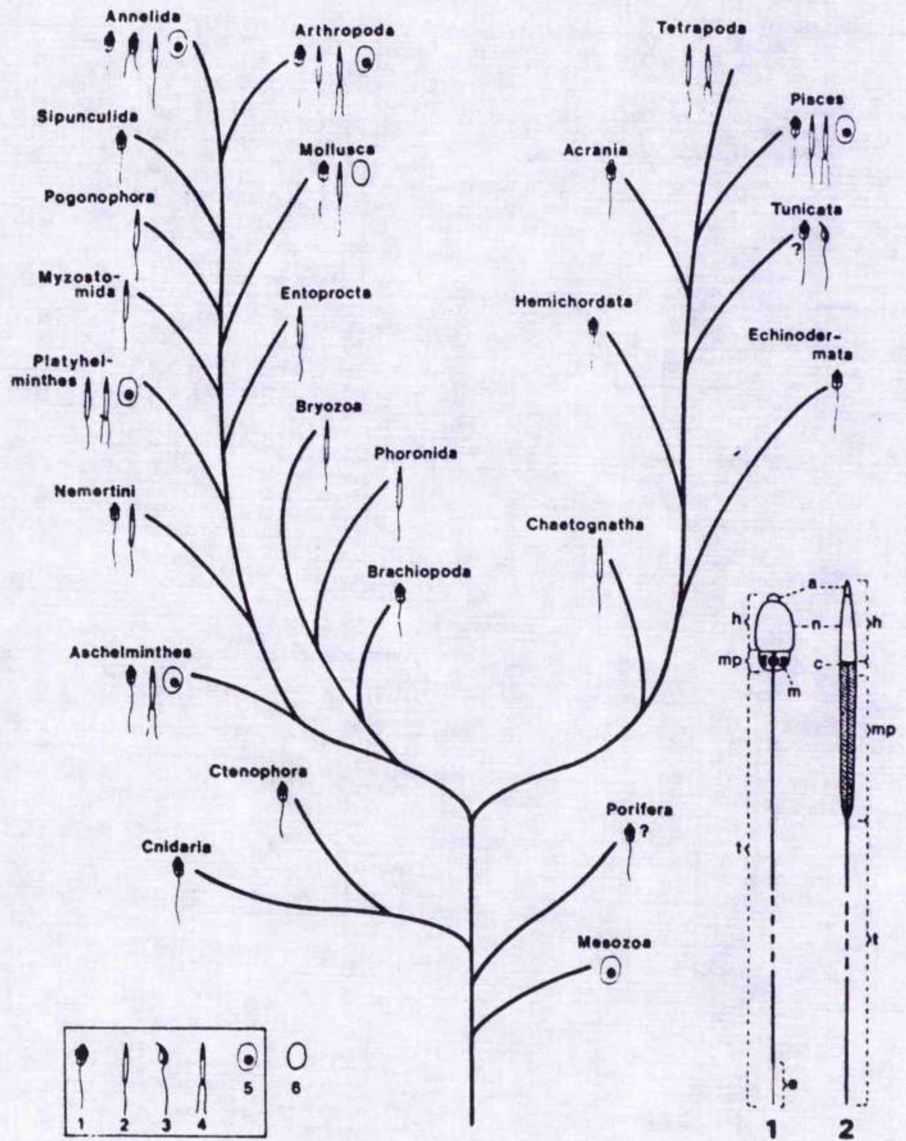


Figure 1.1

1.2.1 The head region

The nucleus occupies the major part of the volume of the sperm head and this is composed of highly condensed nuclear chromatin which is often stabilised by S-S crosslinks (Bedford, 1975). In primitive spermatozoa, the nucleus is generally regular and spherical in shape and although there is a tendency for nuclear elongation as the sperms become modified, many modified sperms retain spherical nuclei (Franzén, 1987). Associated with the nucleus is a sleeve of microtubules called the manchette and these are thought to be important in the final determination of nuclear shape (Myles & Hepler, 1982).

The sperm of most animals is characterised by the presence of an acrosome which is anterior to the nucleus. This is an organelle which is generally considered to facilitate sperm entry into the egg. The structure of the acrosome is highly variable between species (see Baccetti, 1979), but generally consists of two parts, a membrane bound acrosomal vesicle and subacrosomal material. Details of the acrosome reaction will be discussed in section 1.3.4.

1.2.2 The middle piece

The most obvious organelles in the middle piece are the mitochondria. In the primitive condition these number 4 or 5, and occupy approximately 10% of the total cell volume (Baccetti & Afzelius, 1976). In modified spermatozoa the ratio of mitochondrial material to the volume of the rest of the cell is higher than that in primitive spermatozoa (Favard & André, 1970 ; Afzelius, 1972). The mitochondria can vary significantly from that observed in the primitive condition, to those of highly modified sperm. For example, in the hoplonemertean *Emplectonema neesii*, several mitochondria have fused together to form a continuous sleeve of mitochondrial material (Whitfield, 1972). In extreme cases mitochondria may be

totally absent, for example, in the sperm of coccid insects and decapod crustaceans (Afzelius, 1972).

Mitochondria usually surround the centriolar apparatus in the 'neck' region of the spermatozoa. Two centrioles are often present, and in the primitive spermatozoa of protostome animals, the proximal centriole is oriented at 90° to that of the distal. More modified spermatozoa may have different arrangements of the two centrioles, or may possess only a single centriole (Afzelius, 1979). It is the distal centriole which serves as a basal body for the axoneme, although, when two flagella are present, each centriole may serve this role. The proximal centriole is often found in an invagination of the nuclear membrane at the posterior pole (Franzén, 1987). The satellite projections of the basal body function as an anchoring apparatus which enables the distal centriole to resist the torque generated by the movement of the flagellum (Summers, 1970), and it is from these 'paddle like' arms that the nine outer doublets of the flagellar axoneme arise. During spermatogenesis, both centrioles function as organisers of the flagellar protein (Baccetti & Afzelius, 1976), and that the centriole is closely involved with axoneme morphogenesis is also suggested from observations of aflagellate sperm such as the nematodes in which it is reduced or absent (see Baccetti, *et al.*, 1983).

1.2.3 The tail

The forward motion of the spermatozoon is generated by the propulsive forces of the tail (flagellum) as a result of its viscous interaction with the surrounding medium (Taylor, 1951). The cytochemical structure of the flagellum is well understood and is composed of a complex arrangement of microtubules called the axoneme (see Fig. 1.2).

The 9+2 organisation of microtubules is formed of 9 hollow doublet fibrils, equidistant from, and radially surrounding a central pair of single fibrils (see

Gibbons, 1981). Associated with one of the microtubule doublets in each pair are dyenin arms. These usually point in a clockwise direction and the outer arms extend outward to the flagellar membrane and then hook sharply back toward the centre of the axoneme. The inner arms, on the other hand, curve gently inward toward the two central single tubules and have a region of increased density on the terminal end (Allen, 1968). The microtubule to which these arms are permanently attached is termed the A tubule and the other, which has no dyenin arms, is called the B tubule (Gibbons & Grimstone, 1960). The structure of dyenins has recently been reviewed (see Porter & Johnston, 1989; Marchese-Ragona & Johnson, 1990).

Outer dyenin arms of the ciliated protozoan *Tetrahymena* (on which the majority of work has focussed) are composed of two to three heavy polypeptide chains of 400 - 500 kDa, which are termed α, β, & γ, two or more intermediate chains, and four to eight light chains of around 20 kDa. Sea urchin and bovine sperm however, each have two heavy chains (see Marchese-Ragona & Johnson, 1990) *In vitro*, dyenin arms are arranged in the form of a bouquet with the two or three large globular heads connected by a stem to a common globular base (see Johnson, 1985). Each dyenin heavy chain forms a globular head and part of the connecting stem, the globular base is composed of the intermediate chains. *In vivo*, these arms are permanently anchored to the A tubule by the intermediate chains at the base of the bouquet. The globular heads on the other hand, reach out and make contact with the adjacent B tubule in an ATP sensitive process, and results in the generation of motility in the axoneme. The biochemistry of this process will be discussed in section 1.3.2.

The inner dyenin arms are composed of their own set of heavy, intermediate and light chains, and are thought to have a similar structural organisation to those of the outer arms. It appears that they are structurally more complex however, although they have not been as widely studied (see Porter & Johnson, 1989).

In addition to dyenin arms, two further structures are associated with the A tubule of each doublet. Radial spokes, are rigid structures which are attached perpendicularly, radiate toward the central pair of single microtubules, and terminate in an enlarged head (see Haimo & Rosenbaum, 1981). These spokes are thought help to hold the axoneme together and are in addition to a further set of linkages that join the adjacent doublets. These nexin links (Stephens, 1970) connect the A tubule between the inner and outer dyenin arms to the adjacent B tubule. They are highly elastic, and although their normal length is about 30 nm, they can be stretched to 250 nm without breaking (Warner, 1976). The radial spokes occur in groups of either doublets or triplets and along with nexin links have a repeat periodicity of 96 nm along the length of the flagellum (see Gibbons, 1981).

The central pair of single microtubules are themselves connected at their nearest points by two central bridges (Warner, 1976). They are also surrounded by a near complete sheath which arises from each of the central-pair tubules.

The 9+2 organisation of the microtubules, which makes up the axoneme, follows a widespread uniformity, although variations in the patten do exist. Numerous examples have been described, including 9+0, 9+1, 9+3, 9+7 and patterns of 12+0 & 14+0 have been reported (see Baccetti & Afzelius, 1976). Perhaps the most striking modifications to the flagellar axoneme are additions exterior to the 9+2 structure, rather than changes to the 9+2 structure itself. For example, the sperm of many species, including mammals, gastropods, and many insects, possess an additional nine outer tubules giving rise to a 9+9+2 arrangement (see Gibbons, 1981).

Extreme modifications exist where the axoneme is lost completely and this has been reported in several groups such as nematodes (Baccetti *et al.*, 1983) and decapod crustaceans (Pochon - Masson, 1983; Talbot & Chanmanon, 1980).

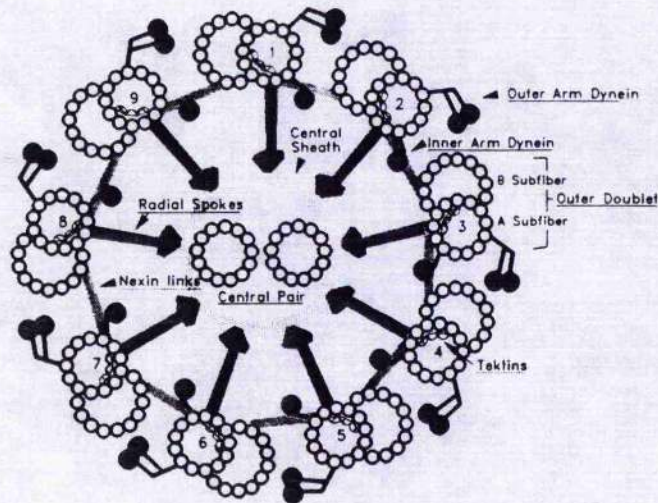


Figure 1.2 Diagrammatic representation of axonemal structures as currently understood (from Tash, 1990).

1.2.4 Sperm ultrastructure in relation to function

The ultrastructure of spermatozoa can generally be related to aspects of the fertilization biology of a given species. The primitive condition for example, is thought to be specifically adapted for swimming in water and is therefore generally retained in those groups which reproduce *via* external fertilization (Franzén, 1987).

Sperm transfer can be achieved through true copulation, pseudocopulation, or by spermatophore transfer, and the increase in mitochondrial volume, which is associated with modified spermatozoa (Favard & André, 1970; Afzelius, 1972), is thought to be an adaptation for the greater energy requirements of locomotion through a more viscous medium (Franzén, 1987). The role of the additional outer coarse fibres within the axoneme of some modified spermatozoa is subject to controversy. It had been suggested that they may function to strengthen the flagellum or may provide additional motor elements during internal fertilization (see Gibbons, 1981). Recently however, in mammals, they have been shown to have a

role in providing additional tensile strength to the sperm tail (Baltz *et al.*, 1990). This is of particular importance in protecting the sperm from the shear stresses encountered during ejaculation. The complete loss of a flagellum has presumably arisen where the sperm no longer has use for a motile flagellum in order to reach eggs. Amoeboid movement occurs in crustacean spermatozoa (Pochon-Masson, 1983) and the acrosome reaction of the lobster generates some forward motility (Talbot & Chanmanon, 1980). The loss of axonemal structures in the polychaete genus *Ophyrotrocha* may be explained by the mode of fertilization and the close association of the male with the egg jelly tubes of the female (Åkesson, 1973). In the nematodes, sperm are inseminated and migrate with an amoeboid like motion into the uterus where the pseudopodia of the sperm interdigitate with the plasma membrane of uterine cells (see Bird, 1971). Here they appear to act as holdfast structures which maintain the position of the spermatozoon in the uterus.

In general, it appears that externally fertilising (primitive) spermatozoa have a larger acrosome and a longer acrosomal filament than more modified spermatozoa (Popham, 1974). Popham suggested that this may be due to the fact that internal fertilisation increases the efficiency of egg fertilsation and therefore less acrosomal material is required to penetrate the protective layers of the ovum. The sperm of teleost fish, the Cnidaria, and a few other species (see Afzelius, 1972; Mattei, 1970) do not possess acrosomes and in the case of teleosts this is presumably because egg entry is gained *via* a micropyle (see Austin, 1965). The presence of a channel through the egg precludes the necessity for a functional acrosome; although the relationship is not clear because the eggs of some species, such as insects, contain micropyles and the sperm retain functional acrosomes (see Longo, 1987).

Phylogenetic aspects are suggested as having a significant effect on sperm morphology., and, in several studies, have been used to question the traditional classification of some groups such as the crustaceans (Wingstrand, 1972; 1978),

and the gastropod molluscs (Thompson, 1973). However, Franzén (1987) points out that it is always important to consider biology of fertilization when using sperm for phylogenetic purposes, and caution must always be exercised.

1.3 Biochemistry of Sperm Function

Studies of the biochemistry of fully differentiated spermatozoa include those on aspects following the completion of spermatogenesis, to the fertilization of an egg. Although the various steps can be outlined individually, it is important to remember that they often do not occur in isolation, but are usually dependent upon preceding steps and may well occur concurrently with them.

It was outlined in section 1.2.4 how sperm of similar fertilization biology have often evolved a common morphological form. In the same way therefore, this might suggest that there may also be common mechanisms in the biochemistry of sperm function, and the following section reviews the present state of knowledge of sperm biochemistry.

1.3.1 The maintenance of sperm quiescence

Sperm are fundamentally excitable cells (Nelson, 1975), but for a considerable proportion of their lives are in a state of dormancy or quiescence and undiluted semen taken directly from the body cavity in nearly all animal groups contains sperm which is immotile. For example, the sperm of sea urchins (see Trimmer & Vacquier, 1987), horseshoe crab *Limulus polyphemus* (Clapper & Brown, 1980), salmonid fishes, and amphibians (see Morisawa, 1987) become motile only when they are spawned. Of these, the sperm of the horseshoe crab and also that of the herring *Clupea harengus* (Yangimanchi, 1957) remain immotile in sea water for a variable time following their release. In some animals, however,

notably the mammals (Morton *et al.*, 1974) and in the lugworm *Arenicola marina*, the subject of this study, (see Howie, 1984) sperm become motile in the body cavity prior to release . In the case of mammals this occurs in the genital tract and in *Arenicola* it occurs in the coelomic cavity.

The biochemistry of quiescent spermatozoa is little understood, although it is probably dependent upon the specific sperm activation mechanism employed by a particular species. For convenience however, the mechanisms which result in the maintenance of sperm quiescence can be divided into two groups.

In some species, sperm dormancy appears to be maintained by the osmotic or ionic conditions of the fluids in the body cavity or testes. Upon release, there is an accompanying change in these conditions and it is at this point that sperm motility is initiated. It is for no group other than sea urchins however, that the intracellular mechanism for the maintenance of dormancy is understood. In sea urchins, a low intracellular pH (maintained by a high CO_2 tension) keeps both respiration and motility suppressed (see Trimmer & Vacquier, 1986). Because the utilization of energy by the flagellum is strictly coupled to its production (Tombes & Shapiro, 1985), then the control over mitochondrial activity is dependent upon whether or not the flagellum is actively using ATP, which in turn depends upon the intracellular pH of the cell.

In other animal groups however, sperm become activated only in response to a specific trigger. The sperm of horseshoe crabs and the herring become motile following interaction with an egg-derived substance. In both mammals, and the lugworm *Arenicola marina* , an internal stimulus is required for sperm activation. The specific details of these activation mechanisms will be discussed in section 1.3.2, but it appears that it is the denial of such a stimulus which is responsible for maintaining sperm dormancy in these animals.

In some species, heavy metals appear to have a role in maintaining the quiescence of sperm, and sperm motility of both starfish and the horseshoe crab is inhibited by the addition of zinc (see Morisawa & Morisawa, 1990).

In mammalian systems, the maintenance of sperm quiescence appears to be more complex. Mammalian sperm acquire the capacity to swim during their transit through the epididymis, but become motile only when they are removed or when they are mixed with seminal fluids at ejaculation. On considering sperm motility in the mammals it is unclear whether the important factor in maintaining quiescence is denial of a trigger stimulus prior to the mixing of sperm in seminal fluid (see section 1.3.2) or whether mechanical immobilisation in the testes is the factor which maintains quiescence. In the bovine spermatozoon, it certainly appears that motility is inhibited by a factor in caudal epididymal fluid. Sperm from the bovine caudal epididymis (CE), when diluted in physiological buffers become motile over a period of about ten minutes, if they are placed back in caudal epididymal fluid however, they quickly become quiescent (Carr & Acott, 1984). The effect is pH dependent , and sperm motility is inhibited at pH 5.5 but not at pH 7.6 (Acott & Carr, 1984). This pH effect occurs not directly on the sperm, but is mediated by an unidentified factor or factors present in the CE fluid and it has been speculated that these interact at the level of the sperm membrane to modulate an ion transport event. Factors from the epididymis of a number of mammals have been implicated in inhibiting sperm motility and include the presence of glycerylphosphorylcholine (Turner *et al.*, 1978), carnitine (Brooks *et al.*, 1974) or proteinaceous factors (Turner & Giles, 1982) in the CE fluid. In the rat, a mucin-like glycoprotein 'immobilin' has been described which imobilizes CE sperm physically (Usselman & Cone, 1983). That immobilin is capable of inhibiting bovine CE sperm motility demonstrates the importance of viscoelasticity, but that the kinetics of this method are unlike that of natural inhibition, suggest that it does not play a major role in this system (Carr &

Acott, 1984). The spermatozoa of the rabbit appears to be an exception to the general rule however, and are motile whilst being stored in the epididymis (Turner & Reich, 1985).

In some species, studies using demembranated spermatozoa have provided interesting suggestions as to the intracellular mechanisms which are involved in the maintenance of dormancy and activation. In many systems it is unclear whether quiescence is maintained by the lack of available ATP, or whether other factors are required before ATP can be utilised. Until very recently, it was thought that because demembranated sperm of many species could be reactivated by the addition of ATP alone (see Brokaw, 1984; see also Morisawa & Morisawa, 1990), it was the lack of available energy that was maintaining sperm quiescence. However, it now seems apparent that membrane debris and soluble cell fractions found following demembranation are capable of synthesising cAMP. In 'naked sperm' preparations in which these have been removed, it is now known that cAMP is required for the initiation of motility (see Morisawa, 1987; Morisawa & Morisawa, 1990; Tash, 1990). The role of cAMP in cellular function will be discussed in greater detail in section 1.3.2.

1.3.2 Initiation of sperm motility

Of the species so far described it is now known that sperm become motile in response to one or more of the following:

a) Dilution

The sperm of sea urchins, salmonid fishes and amphibians become motile upon dilution in the external medium which occurs following release from the body cavity. In the case of sea urchins, a sodium dependent release of protons occurs upon their dilution in sea water and results in an alkalinisation of the cell (see

Trimmer & Vacquier, 1986). This alkalinisation is of the order of 0.4 pH units and motility is stimulated by a change in intracellular pH to that which is within the optimal range for the pH sensitive flagellar dyenin ATPase. The high levels of potassium in salmonid fishes suppress sperm motility in the testes (see Morisawa, 1987) and motility is stimulated due to the lowering of potassium ion concentration upon dilution. It has been shown in trout spermatozoa that this serves to hyperpolarize the cell which in turn stimulates motility (Omoto, pers. comm.). In marine teleosts, it is the osmolarity of the seminal plasma which inhibits sperm motility in the testis, and exposure to the hypertonicity of sea water upon the release of spermatozoa which appears to initiate their motility (Morisawa and Suzuki, 1980). Conversely, in freshwater cyprinid fishes and amphibians, it is the decrease in external osmolarity which is a trigger for the initiation of motility, although, the isosmolarity of the seminal fluid remains as a suppressor of sperm motility in the body cavity.

b) Interaction with egg derived substances

In the horseshoe crab, sperm undergo only a brief flurry of motility when they are released and they remain immotile until they come into contact with a sperm motility initiating factor (SMI) derived from the egg (Clapper & Brown, 1980). The only other example of sperm activation of this kind is observed in the herring (Yangimanchi, 1957). The interaction of sperm with factors that effect sperm motility has been described in over twenty species (see Garbers *et al.*, 1986; Gabers & Kopf, 1980). Unlike the situation in the horseshoe crab or the herring however, these effect the motility pattern of already motile spermatozoa. In sea urchins for example, five classes of egg derived, species specific peptides, that stimulate the respiration and motility of spermatozoa have been isolated and sequenced (see Domino & Garbers, 1990). The two most studied of these peptides (termed

'speract' and 'resact'), act by stimulating a net proton efflux and a transient elevation of cAMP & cGMP concentrations in a receptor-mediated response which lead to modification of sperm behaviour and ultimately fertilisation (see Garbers *et al.*, 1986).

c) Male body fluids

The study of mammalian sperm motility acquisition is hindered by the complex system of cells and fluids in which the process takes place (Mitchell *et al.*, 1976) but it seems clear that sperm leaving the testes are non-functional, immotile and are not capable of fertilisation. Mammalian sperm develop their capacity for forward motility progressively. During transit through the epididymis, binding of a specific component, forward motility protein (FMP) takes place (Brandt *et al.*, 1978). Forward motility protein activity has also been observed in seminal fluid (Acott *et al.*, 1979). It is only when sperm become mixed with fluids from the accessory glands at ejaculation however, that they become motile. This is due either to their release form immobilizing factors present in the epididymis (see section 1.3.1), or due to the mixing of sperm with seminal fluid during ejaculation. Seminal fluid is rich in sodium bicarbonate and this has been demonstrated in the pig, to be a specific stimulator of the sperm adenylate cyclase system; a mechanism now thought to be common to all mammals (Okamura *et al.*, 1987).

In the lugworm *Arenicola marina*, the subject of this investigation, sperm become motile in the body cavity prior to spawning in response to a 'Sperm Maturation Factor' (SMF) released from the prostomium. This will be discussed in greater detail in section 1.5.

From this summary of sperm activation mechanisms, several parameters can be seen to be important at the biochemical level. Intracellular pH (pHi) and cAMP are important in many systems as well as various intracellular and extracellular ionic conditions.

An increase in cAMP levels has been demonstrated to be a stimulator of sperm motility in mammals (Babcock *et al.*, 1983; Babcock & Pfeiffer, 1987), in sea urchins (see Trimmer & Vacquier, 1986; see also Garbers *et al.*, 1986), in the horseshoe crab, (Tubb *et al.*, 1979) and in rainbow trout, *Salmo gairdneri* (Morisawa & Okuno, 1982). A change in pHi is noted during sperm activation of sea urchins (see Trimmer & Vacquier, 1986), and in mammals (Babcock *et al.*, 1983; Babcock & Pfeiffer, 1987). Although an increase in pHi is observed during sperm activation in the horseshoe crab *Limulus*, this is thought to be a side product of the activation sequence rather than a control mechanism (Clapper & Epel, 1982).

Intracellular pH serves to trigger sperm activation by, at least in the case of sea urchins, altering the intracellular pH to that which is optimal for the flagellar dyenin ATPase (see Trimmer & Vacquier, 1986). In rat spermatozoa a sodium dependent control of pHi similar to that of sea urchins has been proposed as a regulator of motility acquisition (Wong *et al.*, 1981). The cellular function of cyclic AMP is known to involve the phosphorylation of protein *via* the activation of a cAMP dependent protein kinase and the role of this in the activation of spermatozoa has recently been reviewed (see Brockaw, 1984; Brokaw, 1987; Morisawa & Morisawa, 1990; Tash, 1990). Work on mammals has identified a heat stable 56 KDa protein in sperm of the dog which is a significant substrate for cAMP phosphorylation. Termed 'axokinin', this protein is able to fully reactivate motility even in the presence of inhibitors to protein kinase (Tash *et al.*, 1986). In the bovine spermatozoa, the same single protein is also thought to be present (Noland *et al.*, 1987). The presence of a protein with axokinin properties has also been

identified in sea urchins, trout and man (see Brokaw, 1987). It has been demonstrated that phosphorylated proteins are localised along the length of the mammalian flagellum and there is evidence that they may play a pivotal role in the second messenger regulatory mechanisms of flagellar movement (see Tash, 1990).

Although each of these variables is singularly important in the stimulation of motility, in some systems factors such as pHi, cAMP levels, various ions, and ATP levels all interact with one another in the regulatory process (see Hoskins & Vijayaraghavan, 1990). In mammalian spermatozoa for example, it is thought that intracellular pH is a permissive event for motility development in that if cAMP levels are elevated without raising pHi no motility is observed until the pHi is raised. Thus, the increase in pHi 'permits' all preceding biochemical events to manifest themselves.

Upon sperm activation, motility results from the transient interaction of the dyenin arm of the A tubule with the adjacent B tubule, which induces a sliding of microtubules which is resisted and coordinated by the other structures of the axoneme to generate the flagellar waveform (see Gibbons, 1981). The dyenin heavy chains, which constitute the globular heads of the dyenin arm bouquet (see section 1.2.4), contain sites of ATP binding and hydrolysis. The binding of ATP induces conformational changes in the dyenin arm. In its absence the dyenin arm is attached to the adjacent subfibre, but in the presence of ATP the arm adopts a relaxed configuration in which it is detached and tilted toward the base of the axoneme. Therefore, the cycle of ATP binding and hydrolysis models suggest that the dyenin arms 'push' the adjacent doublet toward the tip or plus end of the axoneme as they 'walk' toward the minus end (see Porter & Johnston, 1989).

The ATP utilised in this process is thought to be directed from the mitochondria to the flagellum by a phosphorylcreatine (PCr) shuttle (Tombes &

Shapiro, 1985). If ATP was available to the axoneme only by diffusion, then the normal production of flagellar waves would be disrupted at some point along its length due to the preferential hydrolysis of ATP by the more proximal dyenin ATPase. That this occurs in sea urchin spermatozoa in the presence of a specific inhibitor of creatine kinase helps to support the existence of this system in sea urchin spermatozoa. Such a shuttle ensures high concentrations of ADP are present at the mitochondria to permit maximal respiration and that sufficient ATP is present at all points of the axoneme. This is achieved because the diffusing metabolite is not directly accessible to dyenin ATPase molecules along the sperm tail and the energy is available only once transphosphorylation of PCr with ADP has occurred.

1.3.3 Capacitation

Capacitation is a poorly understood aspect of sperm activation that for the most part has been investigated only in mammals. It is generally considered to be an additional maturation step which occurs prior to the acrosome reaction, usually in the female genital tract, and is often associated with the development of a hyperactivated motility (Moore & Bedford, 1983). The cellular events which occur during capacitation are thought to include plasma membrane alterations (such as the rearrangement of intermembranous particles), the removal of sperm surface components, the activation of adenylate cyclase and acrosomal enzymes, and an increase in the permeability of the sperm to calcium (see Clegg, 1983). Calcium appears to be required for an increase in cAMP, and calcium ATPase activity has been demonstrated to be associated with sperm membranes. Changes in the sperm plasma membrane include the masking and / or removal of sperm surface components. Galactosyltransferases on uncapacitated mouse sperm are loaded with

poly-N-acetyllactosamine substrates and these are removed during capacitation (Shur and Hall, 1982). Once exposed the galactosyltransferases may participate in binding to the *zona pellucida*. This process is calcium dependent and is reversible in that the readdition of poly-N-acetyllactosamine glycoconjugates blocks that binding to the *zona pellucida*.

The capacitation reaction in mammalian species has been reviewed recently by Suarez & Pollard, (1990), with particular respect to its relationship with the acrosome reaction . They point out that capacitation was used originally as a general term to describe the processes which prepared sperm for fertilisation and, in this way, also included the acrosome reaction. The two events are now however considered to be quite separate, and indeed, capacitation is now known to be a reversible reaction whereas the acrosome reaction is not. The reversal of capacitation (or 'decapacitation') can usually be acheived by re-exposure of sperm to seminal plasma. This results in the masking of sperm surface components that were exposed during the initial capacitation.

In invertebrates, there is some controversy as to whether the spermatozoa of many species undergo a capacitation reaction. Certainly, in cases where sperm is transferred to females, maturational changes which are similar to those observed during mammalian capacitation have been observed. In the shrimp *Siconia* for example, sperm capacitation has been demonstrated (Clark *et al.*., 1984). However, that sperm which are not transferred to the female reproductive tract undergo a specific capacitation response has not been proven.

1.3.4 The acrosome reaction

The acrosome reaction is an event which assists the penetration of sperm into the egg in order to facilitate fertilisation. Two types of sperm-egg binding have been observed (see Epel & Vacquier, 1978). The first is where binding occurs

between the outer surface of unreacted sperm heads and egg envelopes prior to the acrosome reaction. The second is where binding occurs between the acrosomal process of reacted sperm and egg envelopes after the acrosome reaction has taken place. All too often however, difficulties, which arise during the study of sperm-egg interactions in many species, have all led to an incomplete picture of the site at which the acrosome reaction occurs.

In mammals the acrosome reaction of mice spermatozoa is the best understood. These spermatozoa undergo the acrosome reaction only when an egg binding protein on the sperm head encounters a glycoprotein ZP3 present on the egg's *zona pellucida* (see Wasserman, 1987). In the guinea pig on the other hand, the precise site of the acrosome reaction remains to be determined although sperm have been noted to undergo the acrosome reactions during their ascent of the oviduct (see Yanagimachi & Mahi, 1976). The reaction involved an influx of extracellular calcium which, through a series of steps, results in a rise in intracellular pH and also facilitates membrane fusion and the release of acrosomal contents (see Yanagimanchi, 1987). The acrosome usually contains a variety of hydrolases such as hyaluronidase, proteinases, glycosidases, lipases and phosphatases and as an organelle, has been considered biochemically similar to a lysosome (Wasserman, 1987).

In the invertebrates, acrosome reacted sperm undergo the polymerisation, uncovering or extrusion of an actin filament at their apical region of the head, known as the acrosomal process (see Longo, 1987). Concurrent with its formation is the exocytosis of the acrosomal vesicle and the liberation of its contents in a process similar to that observed in mammals. Perhaps the most spectacular acrosome reaction is that of sperm in the horseshoe crab *Limulus*, where the actin filament is coiled to the posterior of the nucleus and this moves forward through a channel in

the nucleus during the acrosome reaction to form the acrosomal process (Tilney, 1975).

The stimulus for the acrosome reaction in sea urchins is reasonably well understood and is induced by the interaction of two polypeptides located on the surface of sea urchin sperm with a complex gel of fucose sulphate polymers released from the egg jelly layers (Kopf and Garbers, 1980). In starfish, the acrosome reaction is triggered by the co-operation of at least three components from the egg jelly coat: an acrosome reaction inducing substance (ARIS), its co-factor (Co-ARIS) and an oligopeptide (Hoshi *et al.*, 1986). Three major Co-ARIS' have been identified and their chemical nature determined. Both in sea urchins and starfish the acrosome reaction is accompanied by a Na^+ dependent alkalinisation in a mechanism similar to that which induces motility in quiescent sperm. This mechanism is independent of respiratory processes however. The acrosome reaction in numerous species also has a requirement for extracellular calcium (see Epel, 1978) and will not occur in its absence.

1.4 Sperm Release

In species which display more advanced fertilisation biology such as those which reproduce by copulation, the chances of gamete interaction are maximised by direct insemination of spermatozoa into the body cavity of the female. In the case of pseudocopulation, the two sexes undergo close pairing and sperm and eggs are released together. Both these strategies reduce the need for the production of the high number of gametes that are required in species which exhibit free spawning (see Giese & Kanatani, 1987). Such species produce large numbers of gametes and rely upon random interactions to provide successful fertilisation. Usually however, spawning in such species is synchronised with other members of the

population in order to produce the maximum probability of fertilisation (Franzén, 1987).

1.4.1 Environmental influences

Species in which populations undergo synchronous spawning have been reported in a number of different groups and the best documented examples include the crinoid *Comanthus japonicus* (Holland, 1981), the grunion *Leuresthes tenuis*, (Clark, 1925), and in the polychaetes, the pacific palolo worm *Eunice viridis* (Caspers, 1961; Hauenschild *et al.*, 1968) and at some localities, the lugworm *Arenicola marina* (see Howie, 1984). Perhaps the most striking of these examples is that of the pacific palolo worm which has been observed spawning with a fixed and precise relationship to the lunar cycle for the past 100 years (Caspers, 1961).

Such spawning events (and many other reproductive events) are often seen to have strong relationships with environmental phenomena such as lunar or tidal cycles, or daily photoperiodic regimes. These are considered to be better exogenous 'zeitgebers' because they are predictable, invariant and have a fixed phase relationship. Other environmental factors such as temperature, salinity or the abundance of food, are more variable at a given locality from year to year (see Giese & Pearse, 1974). The exact mechanism by which organisms interpret these environmental influences is unknown, although it is thought that a complex hierarchy of different factors may act synergistically to ensure that reproduction takes place at the most suitable time in a given locality. The environmental control of spawning in the polychaete *Eunice viridis* however, has been explained by a model incorporating three endogenous 'gated' rhythms of annual, lunar, and circadian components (Holland, 1981; Olive, 1984). This imposes synchronisation on the population allowing animals to proceed in sexual development only during a relatively broad but restricted time zone called a 'gate'.

By passing through a series of such gates, a greater degree of synchrony is imposed on members of the population as the spawning time approaches (see Olive, 1984 for detailed discussion).

The final stages of spawning in some species may be controlled by the release of pheromones which serve as a signal to other individuals of the same population. Sexual pheromones have been identified in a number of species, including the crustaceans (Bauchau, 1986), in the nematodes and platyhelminths (see Bone, 1986), and in starfish (Miller, 1989). In polychaetes, sexual pheromones have been identified in nereids where they stimulate nuptial behaviour and lead to an increased electrical activity of the brain (Boilly-Marer & Lassalle, 1978). The pheromones are detected by the swollen parapodial cirri that are present in sexually mature animals (Boilly-Marer & Lassalle, 1980) and the integrity of the nervous connections between the brain and the nerve cord are required for the stimulation of the brain but are not required for the stimulation of the nerve cord. In *Platynereis dumerilii* the pheromone has been characterised as an 8-carbon ketone, 5-methyl-3-heptanone. This is highly specific in its nature with the S(+)-enantiomer of this molecule being produced by the males and acts on the females only, whereas the R(-)-enantiomer is produced by the females and acts on the males (Zeek *et al.*, 1988). This substance was also found to be biologically active in the closely related *Nereis succinea* and this species also utilised the same sex specific enantiomers (Zeeck *et al.*, 1990). The swarming activity of these two species overlaps to some extent, but reproductive isolation is maintained by the threshold concentration of response to 5-methyl-3-heptanone in *N.succinea* is about 25 times higher than in *P. dumerilii.*

As well as being important molecules in the timing of reproductive events, pheromones are also of considerable interest to endocrinologists. Although pheromones are not true endocrine substances they are involved in animal

communication and the transmission of information, and therefore they might be closely akin to hormones in their chemical structure and their mode of action (Goldsworthy *et al*, 1981).

1.4.2 Spawning as an endocrine controlled event

Spawning in marine invertebrates has been reviewed recently (see Giese & Kanatani, 1987 see also Schroeder, 1984), and it is at once clear that in only very few species is the endocrine control mechanism understood in any detail.

The endocrine control of spawning in starfish is well documented (see Shirai *et al.*, 1986; Meijer & Guerrier, 1984). It involves a gonad stimulating substance (GSS), released from the radial nerve, acting on the gonad, to produce a maturation inducing substance (MIS). Gonad stimulating substance is a thermostable 22-amino acid neuropeptide of about 2100 Da which is released from neurosecretory granules in the radial nerve (Kanatani *et al.*, 1971). Maturation inducing substance on the other hand was identified as a purine, 1-methyladenine (1-MeAde) (Kanatani *et al.*, 1969) and is released from the interstitial glands of the testes and the follicle cells of the ovary following stimulation by GSS. The action of 1-methyladenine on the oocyte is to reinitiate meiosis, and because oocyte maturation can also be induced by certain fatty acids it is thought that these, or other related acids, may play a role in transducing the signal from 1-methyladenine across the cell membrane (Meijer *et al.*, 1984; Meijer *et al.*, 1986a). It has been demonstrated that exogenous arachidonic acid is converted to hydroxy-metabolites by the oocytes and of these that only (8R)-Hydroxyeicosatetraenoic acid (8-HETE) and not (8S)-HETE are capable of stimulating oocyte maturation (Meijer *et al*, 1986b).

As an endocrine substance, 1-MeAde has a multiplicity of roles in the induction of spawning (see Meijer & Guerrier, 1984 for review). It stimulates not only the maturation of the oocytes, but also their separation from the follicle cells

and the contraction of the ovarian wall (Shirai *et al.*, 1986). The separation of the follicle cells is thought to be attributable to endogenous protease activity, whilst ovarian contraction occurs, probably, by the action of a contraction inducing factor. The action of 1-MeAde on the oocyte is to stimulate the production of an active factor in the oocyte cytoplasm (see Kishimoto, 1986), and it is this 'Maturation Promoting Factor' (MPF) that is responsible for oocyte maturation. As spawning commences, 1-MeAde stimulates reproductive posture in both sexes, and because this can be induced in spent animals, it is assumed that in this aspect 1-MeAde acts on the nervous system directly (Shirai *et al.*, 1986).

Spawning in the mollusc *Aplysia* has also been the subject of detailed investigation (see Giese & Kanatani, 1987) and the mechanism which underlies the release of eggs in the female is reasonably well understood. Egg laying is induced by the action of egg laying hormone (ELH) which is a neurosecretion released from the bag cell cluster of cells associated with the abdominal ganglion (Kupfermann, 1972) and has been identified as a single polypeptide of 36 amino acid residues (Chiu *et al.*., 1979). The hormone is released from stimulated bag cells and the electrical coupling of individual cells is such that the release of ELH is synchronised and is sufficient to stimulate egg laying (Kupfermann & Kandel, 1970). The target cells for ELH are thought to be small muscle cells which surround each follicle in the ovotestis (Coggleshall, 1970; 1972). As well as acting to release oocytes, ELH also modifies the behaviour of the animal and induces such behaviour as puckering of oral musculature, weaving head movements and infrequent locomotion (Arch & Smock, 1977).

In the molluscs *Haliotis rufescens* and *Mytilus californianus,* spawning is thought to be stimulated by the action of a prostaglandin endoperoxide enzyme (Morse *et al.*, 1977; Fitt & Trench, 1981). This observation is however, derived

from the the use of specific inhibitors and stimulators of prostaglandin endoperoxide synthetase and therefore no detail is available on the specific mechanisms involved.

The endocrine control of spawning in other invertebrates has been demonstrated in relatively few species. In the coelenterates, spawning in the medusa *Spirocodon saltatrix* occurs in response to a spawning inducing substance which is produced during darkness (Ikegami *et al.*, 1978). The substance is thought to have a low molecular weight and is produced in the ovaries.

In three species of polychaetes endocrine mechanisms have been identified. In *Nephtys hombergi,* spawning is brought about by the release of a spawning hormone from the supraoesophageal ganglion (Olive, 1976; Olive & Bentley, 1980; Bentley, 1986a). This causes rhythmic contraction of the body wall musculature and results in the ejection of gametes from the coelom to the exterior *via* the pre-pygidial rectal cleft and the anus (Bentley, *et al.*, 1984). Experiments have also suggested that the normal behaviour of spawning animals is modified by spawning hormone and gives way to violent spasmodic contractions of the body during spawning (Bentley, 1984). Preliminary purification of spawning hormone, suggest that it is a trypsin sensitive, heat stable peptide of between 2,000 - 4,000 daltons (Bentley unpublished observations).

In *Pectinaria gouldii* a factor from the suboesophageal ganglia and the cement gland brings about maturation and spawning of gametes in both sexes (Tweedell, 1980). The biological activity of this factor is destroyed by heating, and dialysis suggests that it is a molecule of larger than 12,000 daltons. Tweedell noted that it brought about the maturation of the gametes which includes the release of spermatozoa from sperm packets and germinal vesicle breakdown in oocytes, and he suggested that the entry of oocytes into the nephromixia was possible only once maturation had taken place.

The maturation and spawning events observed in *Pectinaria* are very similar to those of *Arenicola marina.* These will be discussed in greater detail in section 1.5, although it is important to say here that gamete maturation in *Arenicola* occurs in response to a maturation factor released from the prostomium. During maturation, oocytes of *Arenicola marina,* undergo passage from the first prophase stage of meiosis to the first metaphase stage and this is accompanied by germinal vesicle breakdown (GVBD) (Howie 1961b; Meijer 1979a). In males, sperm dissociate from sperm morulae to become free swimming (Howie 1961a; Meijer 1979b). Spawning follows this gamete maturation in that eggs and sperm which have not undergone this final maturation are rejected by the nephromixia which act as gonoducts and are therefore retained within the body cavity. Gametes which have undergone their final maturation however, are accepted automatically and shed (Howie 1961b & c; Howie 1962).

Franke & Pfannenstiel (1984) pointed out that despite the similarity of their function (in that they both bring about spawning in sexually mature animals) the maturation hormones of *Arenicola* and *Pectinaria* both differ considerably from the spawning hormone of *Nephtys* in their mode of action. Only in *Nephtys* does the hormone act as a true spawning hormone by stimulating muscular activities which lead to gamete release whereas, in both *Arenicola* and *Pectinaria,* spawning relies upon maturation of the gametes brought about by the respective endocrine factor.

There are several common characteristics of endocrine substances throughout the examples outlined in this section. The stimulation of muscular contractions which result in gamete release is an important although not exclusive feature of such substances. Often they display a multiplicity of roles in several target tissues and may exert their effects indirectly. In many situations they also act to modify the animals behaviour in some way; the reproductive posture of starfish (see Meijer & Gurrier, 1984), the head waving behaviour of *Aplysia* (see Arch &

Smock, 1977) and the muscular contractions of *Nephtys*(see Bentley, 1984), are all either direct or indirect effects of the spawning hormone.

1.5. Reproductive Biology of the Lugworm *Arenicola marina*

The lugworm *Arenicola marina* which has an annual cycle of reproduction, inhabits soft sediment communities around the coast of Britain and Northern Europe. In many localities, it is present in high numbers and in these situations can be the dominant member of the intertidal community. It has a significant impact on these communities as a major reworker of the sediment and a major food source for birds and fishes (Beukema, 1976; Beukema & deVlas, 1979).

It was perhaps field observations on the spawning of *Arenicola marina* that first drew attention to the reproductive biology of this animal. In many cases, spawning was noted to be an epidemic phenomenon, demonstrated by the appearance of sperm puddles on the sediment during periods of low water (Kyle, 1896; Pirlot, 1933; Newell, 1948; Duncan, 1953; 1960; Howie, 1959). Epidemic spawning could often be correlated with specific tidal or lunar phases and usually occured in the late autumn or early winter. Some populations however, were noted spawning in the late summer and others spawn during both the autumn and spring months, although typically these tended not to be epidemic events and specific information regarding these is limited. In contrast, considerable information exists regarding the biology of autumn breeding populations because it is on these that most attention has been focused (Howie, 1984).

1.5.1 Reproductive biology of males

In autumn breeding animals, spermatogenesis commences in the spring months and an increase in the testis size and the mitotic index has been noted in the period from April to June (Olive 1972a&b). Spermatogonia leave the testis at the eight cell stage (Ashworth, 1904) and undergo spermatogenesis entirely within the coelomic cavity (see Olive, 1983b). Gamete production is not a continuous process, but is synchronised to produce a discrete population of spermatocytes which then undergo spermatogenesis. The control of this is thought to be achieved by a feedback mechanism in which a substance or substances released from the developing spermatocytes which exert an inhibitory effect on further testicular mitoses (Howie & McClenaghan, 1965; Olive, 1972a). Spermatogenesis results in the production of sperm morulae (Howie, 1961a) in which several hundred spermatozoa are cytoplasmically connected to a common mass of cytoplasm, the cytophore (Meijer, 1979b; Olive, 1983b).

Spawning is thought to occur as a direct result of sperm maturation, which involves the dissociation of sperm morulae resulting in free-swimming sperm (Howie, 1961a; Meijer, 1979b; Bentley, 1986b; 1986c). It is only following this process, when sperm have undergone their final maturation, that they are spawned. Unripe gametes are rejected by the ciliated funnels of the nephromixia which act as gonoducts and are therefore retained in the body cavity (Howie, 1961b &c; Howie, 1962). Because spawning is dependent upon sperm maturation in this way the responsible endocrine factor has been termed a 'Maturation Hormone' rather than a 'Spawning Hormone' (Howie, 1984). It has however been suggested that the spawning of both males and females may be a more active process than this hypothesis suggests (Howie 1961a,b&c).

It was noted that the process of gamete maturation and spawning could be stimulated by the lipid extract of whole body homogenates (Howie, 1961a & b).

Howie noted that the active substance in these homogenates could be isolated from the saponifiable lipids (which contain fatty acids) and of this fraction, saturated fatty acids appeared not to induce spawning (Howie, 1961a). It was found that spawning could be stimulated by injecting homogenates of the prostomium into the body cavity of males and that decerebration could inhibit natural spawning (Howie, 1963; Howie, 1966). The lipid nature of the Sperm Maturation Factor (SMF) was confirmed by the fractionation of prostomium homogenates and active factors could be isolated from both the lipid and non-lipid fractions during extraction with ether (Howie, 1963; Meijer, 1979b). Such an observation suggested that there may be more than one active substance present within the prostomium, however Bentley (1985) pointed out that this result could be due to incomplete separation in the solvent system used. The active substance appears to be present in the prostomia of both males and females (Howie, 1966) and Bentley demonstrated the lipid nature of SMF, and that it had very similar TLC characteristics to non-steriod pharmacologically active lipids (Bentley, 1985) such as the unsaturated fatty acid arachidonic acid (Bentley 1986b). The presence of SMF in the brain of *Arenicola* is not constant throughout the year and it is present in the prostomium only during the breeding season. Following spawning, the levels of SMF in prostomia quickly becomes undetectable (Bentley, 1985), and this cyclical activity adds further evidence to the endocrine nature of SMF.

Very little is known about the mode of action of SMF or the mechanism of sperm activation, although sperm maturation and the liberation of free sperm from sperm morulae was noted to be induced by *in vitro* incubation in alkaline sea water (Howie, 1961c). Meijer (1979b) noted that the maturation could be induced *in vitro* following incubation with prostomial extracts at concentrations down to 0.01 prostomium / ml, but also that incubation in calcium free sea water, in ethylenediaminetetracetic acid (EDTA) and in incubations of hyaluronidase (0.5%)

but not trypsin (0.01 - 1%) could induce sperm maturation. It does not appear clear however, whether maturation induced in these instances are comparable or are mechanisms which are utilised during natural sperm activation with prostomial homogenates which contain SMF.

Light microscope observations of sperm maturation induced by incubation with prostomial SMF *in vitro* have been made (Bentley, 1986b), and the maturation process appears to occur in two stages. The first occurs 30 to 35 minutes following commencement of the incubation and is when the sperm flagella become separated, the second, occurs after a further 10 to 15 minutes when the connections between the cytophore and the sperm head are broken and the sperm become free swimming. The whole sperm maturation process *in vitro* and at 14°C takes place over a 50 minute period.

1.5.2 Reproductive biology of females

In comparison to males, more information is available (at least at the biochemical level) on oocyte development and maturation in females. Oogenesis results in the production of mature oocytes which reach a diameter of 180 μm and are held in prophase of their first maturation division (Howie, 1961b; Meijer, 1979a). These are ovulated following the completion of early meiotic stages but prior to the commencement of vitellogenesis (Olive, 1983a). Yolk formation is largely autosynthetic although the existence of a vitellin / vitellogenin system has not been ruled out following the observation that eggs at 100 μm display pinocytotic pits in the plasma membrane (Howie, 1984). During the vitellogenic phase, there is a significant increase in the protein content and the number of protein fractions and the identification of 'female specific' proteins in the coelomic fluid observed by polyacrylamide gel electrophoresis (Howie, 1984). The identification of female

specific proteins, could support the hypothesis of heterosynthetic yolk production in *Arenicola*.

Spawning of mature oocytes is similar to the situation in males in that it is a process which is dependent upon the final maturation of the gametes. The oocytes which are held in prophase following oogenesis, enter the first metaphase stage of meiotic division and this is associated with the breakdown of the germinal vesicle and a morphological change in shape of the oocyte (Howie, 1961b; Meijer, 1979a). The morphological changes which occur at maturation then allows them to be accepted by the ciliated funnels of the nephromixia and they pass through the gonoducts and are spawned (Howie, 1961b &c; Howie, 1962).

The nature of the maturation substance in females, which serves to promote oocyte maturation is unknown. Lipid fractions of whole worm homogenates fail to promote oocyte maturation and spawning as they do in males, which suggests that it has a different chemical nature (Howie, 1961a & b).

The mode of action of the maturation hormone in females has been investigated and Meijer and Durchon (1977) found that oocytes could be induced to undergo Germinal Vesicle Breakdown (GVBD) between 0.5 and 3 hours after the addition of prostomium extracts *in vitro*. As well as the associated morphological changes (Meijer, 1979a), GVBD is also accompanied by the 'cortical reaction' (Rashan & Howie, 1982). Biochemical observations have shown that calcium is involved in maturational events, because pharmacological agents which act on membrane calcium permeability and which modify intracellular free calcium levels are known to stimulate oocyte maturation (Meijer, 1980). The involvement of sulphydryl groups is also implicated by the fact that sulphydryl reducing agents can also induce oocyte maturation.

1.5.3 Reproductive endocrinology of *Arenicola*

The known endocrinological steps which serve in both gametogenesis and spawning are summarised in figure 1.3 . No attempt has been made to identify the chemical nature of hormones regulating the formation of spermatocytes and all attempts to identify the site of production of the maturation hormones of either sex has so far been unsuccessful.

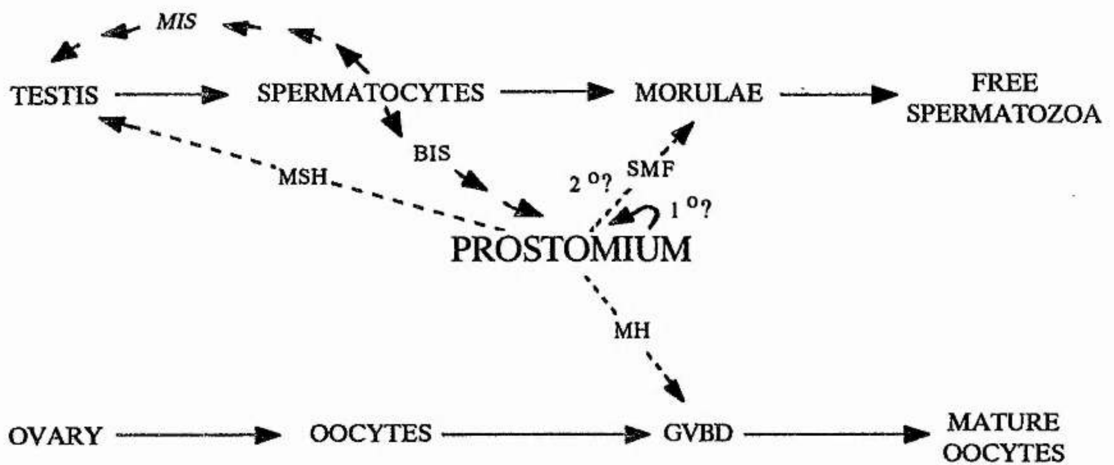


Figure 1.3 Schematic diagram of the endocrine control of reproduction in *Arenicola marina* as currently understood (after Bentley, 1986b). This includes the alternative hypothesis of mitosis inhibition in the testis and a primary or secondary action of sperm maturation factor. Abbreviations: *MIS* = Mitosis inhibiting substance (*sensu* Howie & McClenaghan, 1965; Olive 1972a,b); MSH = Mitosis stimulating hormone (Howie & McClenaghan, 1965); BIS = Brain inhibiting substance (Howie & McClenaghan, 1965) ; MH = Maturation hormone (Howie, 1961; Meijer, 1979); SMF = Sperm maturation factor (Howie 1961; Meijer 1979); GVBD = Germinal vesicle breakdown.

Ultrastructural observations of the histology of the prostomuim found relatively few cerebral neurones (Howie, 1966), although cells having the morphological characteristics of peptidergic neurones were noted chiefly in the neuroectodermal connectives (Howie, 1977). Ablation experiments have identified the site of secretion of SMF as being in the posterior lobes of the brain (Howie, 1966) and possibly in a region of nervous tissue associated with the nuchal nerves (Howie, 1984). Howie points out however that such observations do not correlate with the identified sites of neurosecretion in the prostomium of *Arenicola*.

1.5.4 Spawning of gametes

The release of maturation hormone from the prostomium of both sexes occurs immediately prior to spawning and in that case requires an element of synchronisation between individuals to ensure an epidemic spawning event. An adequate environmental trigger has long been sought which would account for this. For example, it was suggested that the autumn spawning at St. Andrews may be triggered by a sudden drop in environmental temperature, because spawning at this locality is mostly coincident with frosty mornings (Howie, 1959). Pirlot (1933), Newell (1948) and Duncan (1953) also suggested that there was some relationship between the onset of spawning and a particular phase of the tidal cycle following a similar correlation. However, although a more remote external timing device, for example a 'zeitgeber', may serve to synchronise breeding when it is epidemic, no immediate environmental stimulus appears to be necessary as worms maintained in the laboratory spawn at the same time as corresponding animals on the shore (Howie, 1963; Farke & Berghuis, 1979; see also Howie, 1984).

1.5.5 Other considerations

In order to clarify terminology which will be used in this thesis it is important here to make the distinction between the terms sperm maturation and sperm activation. As has been pointed out in section 1.5, spawning in *Arenicola marina* occurs as a result of the dissociation of sperm morulae into free swimming spermatozoa. Sperm activation, is used to describe the acquisition of motility in individual spermatozoa and is clearly only one aspect of sperm maturation.

1.6 Aims of the Thesis

A well structured thesis should have clear objectives and this section, therefore, aims to identify those which were used as a framework in the organisation of this research programme. Sperm activation and spawning in *Arenicola marina* is of considerable interest and forms the basis of this study for the following reasons.

i) Until very recently, the wealth of literature surrounding sperm activation in sea urchins appointed this system as a model for the description of animal sperm activation in general. It had been suggested that because flagellated spermatozoa utilise similar axonemes as motile organelles that perhaps some of the intracellular events during motility initiation may be similar (Clapper & Epel, 1982). As work progressed however, it has become apparent that this was not always the case and that in some species sperm activation was unlike that observed in sea urchins. Mature spermatozoa in *Arenicola* are unlike those of most other species in that they become motile in the body cavity prior to spawning. In this way their spermatozoa are more like those of mammals where sperm motility is initiated in the genital tract, during ejaculation, when the sperm become mixed with seminal fluid and secretions from accessory glands.

ii) Sperm activation is an endocrine mediated process and this raises many questions such as how the sperm receive, interpret and respond to the endocrine signal and the chemical nature of the factor involved (SMF). Also, because no annelid hormone has been fully identified and characterised, the identification of SMF would represent a significant advance in the field of invertebrate endocrinology.

iii) Sperm activation and spawning in *Arenicola* are closely linked (see section 1.5). Spawning in some *Arenicola* populations is epidemic and therefore an understanding of sperm activation can only lead to a greater understanding of the role of sperm, SMF, and environmental influences in the regulation of this phenomenon.

The objectives of this research are therefore two fold. To characterise the sperm activation response, and to identify the nature and the mode of action of the endocrine principle involved (SMF).

Sperm activation can be characterised in a number of ways. On a morphological basis this can be at the level of the light or electron microscope or both. Biochemical analysis on the other hand can include any number of cellular parameters. This thesis begins with a detailed ultrastructural investigation of the morphological changes which occur when spermatozoa become motile and uses both scanning and transmission electron microscopic techniques (Chapter 3).

The identification of SMF involves the use of a number of purification techniques and assay procedures that will be detailed in Chapter 2 and in Chapter 4. The response of spermatozoa to SMF and to other factors will be quantified by the measurement of oxygen consumption (Chapter 5). The intracellular pH and cellular ATP levels of spermatozoa will also be determined because these parameters have

often been linked with sperm motility initiation (Chapter 6). All measurements in these experiments will be on the sperm activation response and the mode of action of SMF *in vitro*. Chapter 7 however, investigates the role of SMF on spermatozoa and on spawning *in vivo*.

Chapter 2

Materials & Methods

2.1 Introduction

A wide number of experimental techniques were employed during the course of the following investigation and this chapter describes them in detail. This removes the need for an extensive Materials and Methods section at the beginning of each experimental chapter, and also prevents the repetition of methods which are common between chapters. Any modifications to the materials and protocols outlined here will be referred to in the chapter to which they apply.

2.2 Collection and Maintenance of Animals

Specimens of *Arenicola marina* were obtained at various sites around the British Isles, by digging in the sand during low water of spring tides at the following times of year: Between the months of July and October, mature animals were obtained from St. Andrews Bay or from the Eden Estuary, St. Andrews, Fife, and between October and December specimens were obtained from Budle Bay, Northumberland. During the spring months, February to May, small numbers of animals were collected from Fairlie Sands, Ayrshire and from Kirkcolm, Wigtownshire.

Animals were maintained in the laboratory, individually, in filtered seawater, under constant illumination, at 5°C, until required for use. On alternate days, the seawater was replaced and the plastic holding containers were washed to remove mucus or bacterial deposits. Animals in the laboratory survived well and could be used for experimentation often for several months after collection. This reduced the need for excessive field collection, and it was often possible to bridge the gap between the breeding seasons of various field populations allowing a more or less continuous experimental programme to be carried out. There was a tendency however, as the time of natural spawning of field populations approached, for

animals kept in the laboratory to begin spawning spontaneously at the same time as members of the same population in the field. This phenomenon has been described by Howie (1984).

2.3 Removal of Sperm Samples

Sperm samples were removed from the coelomic cavity of mature male *Arenicola* using a 1ml disposable syringe, fitted with a size 25 g hypodermic needle. The body wall was usually penetrated at the thicker area between the end of the trunk region and the beginning of the tail. Between 1 and 9 ml of sperm suspension could be extracted from a healthy animal, depending on its size, and consequently sperm samples, from the same individual, for replicate experiments could be readily obtained. Animals which suffered internal damage, evidenced by the presence of blood in the coelomic fluid, were discarded.

Coelomic sperm samples for use in experiments were collected immediately prior to use, although these remained stable for several hours following removal from the animal. Eventually however, the morula structure breaks down and some spermatozoa are observed swimming, whilst other morulae appear to undergo lysis.

2.4 *In vitro* Bioassay for SMF Activity

Bioassays for SMF activity were carried out according to Bentley (1985). From its initial design the bioassay has since been modified to use the 96 well, flat bottom, multi-well plates (BIBBY) which reduces the volume in each test well to approximately 200 µl. Coelomic sperm samples (10 µl) are incubated with 200 µl TFSW (Triple Filtered Seawater) or an assay sample which has been dissolved or resuspended in TFSW. After the incubation period (50 minutes at 14°C), each well

is observed individually and a positive sperm activation response indicated by either an 'oily droplet' appearance of aggregated mature sperm, or the presence of free motile sperm when examined with a light microscope. A negative sperm activation response is indicated by the absence of motile spermatozoa when examined with a light microscope, the sperm morulae remaining undissociated. All assays are scored blind.

By introducing 400 µl of sample to be assayed for SMF activity into the first well and transfering a 200 µl aliquot and mixing with 200 µl of TFSW contained in the second well (and so on), a dilution series of sample consisting of double dilutions can be created along the wells of the plate. In this way the assay requires only an initial 400 µl of sample in order to determine a minimum active concentration. Multiple assays of samples for SMF activity are performed using the sperm from the same animal because the response of any one male is constant between parallel series but there may be a marked difference in the extent to which sperm of different males respond (Bentley, 1985).

In order to provide adequate controls, each test plate, was always accompanied by a dilution series of quinacrine (double dilutions from 10^{-4} M) as a positive control and a corresponding number of wells which contain TFSW as a negative control. Incubation with TFSW acts as a negative control because sperm diluted in this way do not usually undergo activation. Quinacrine, on the other hand, acts as a positive control and fully differentiated spermatozoa usually become activated in response to quinacrine concentrations greater than 10^{-5} M. Assays in which the sperm do not respond as expected in the control wells are disregarded.

The response of spermatozoa to quinacrine will be investigated during this thesis (see Chapter 5 and Chapter 6), although it is necessary to point out here that its ability to stimulate sperm maturation was derived from observations which were

made during experiments in which it was used as a phospholipase A_2 inhibitor (see section 4.4) but was found to be a stimulator of sperm activation.

2.5 Preparation of Prostomial Homogenate

Prostomia were ablated from mature specimens of *Arenicola marina* using iridectomy scissors. Animals were immobilised by grasping firmly with fine forceps on either side of the nuchal groove (Fig. 2.1), this forced out the prostomia which could then be easily removed. If a clean cut was made, without the removal of too much surrounding tissue, this process appeared to damage the animals very little and the animals could survive indefinitely.

Excised prostomia were stored on ice, and homogenized at 0°C in TFSW by sonication in a MSE Soniprep 150 ultrasonic disintegrator. The resulting homogenate was then transferred to Eppendorf tubes and centrifuged for 60 seconds at 6500 g in an MSE microcentrifuge. The resulting supernatant was collected for use in bioassay or in other experiments. Prostomial homogenates were prepared immediately prior to use, and stored on ice until required.

The dose of prostomial homogenates is expressed as the number of prostomia that would be present in a 1ml volume of an equivalent dose. Therefore, a total of five prostomia homogenised and made up to a final volume of 10 ml is equivalent to 0.5 prostomia per ml of homogenate (or 0.5 $Pr/Eq.ml^{-1}$). Dose was calculated in the same way for the lipid extracts of prostomial homogenates (prepared as in section 2.7) assuming that there was 100% partition into, and recovery of lipids from the organic phase.

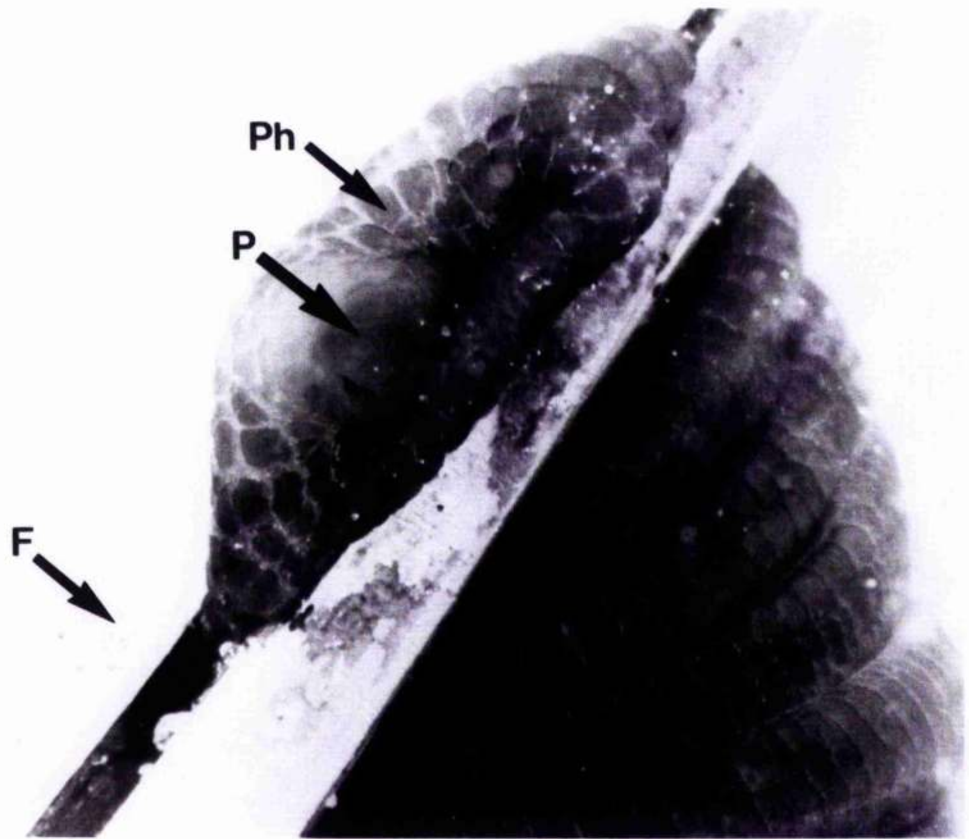


Figure 2.1. A specimen of *Arenicola marina*, (ventral surface uppermost), immediately prior to the removal of the prostomium. The photograph shows the position of the prostomium, and demonstrates how the animal is immobilised in order to facilitate the decerebration procedure. (F = forceps ; P = prostomium (containing cerebral ganglion); Ph = position of pharynx).

2.6 Fixation Methods for Electron Microscopy

Aliquots of sperm morulae suspension were fixed for both scanning and transmission electron microscopy by the addition of an excess of 3% glutaraldehyde (v/v) in 0.1M phosphate buffer (pH 7.2), with 0.26 M NaCl for 90 minutes at 20°C. This was followed by a rinse in 0.1M phosphate buffer (pH 7.2) with 0.25 M sucrose for 10 minutes, before post fixation for 30 minutes with 1% $Os0_4$ (v/v) in 0.1M phosphate buffer (pH 7.2) and 0.13 M NaCl at 2°C.

Samples for scanning electron microscopy were dehydrated through an alcohol series (see Glauert, 1975). Double sided adhesive tape was found to be more effective than glue in holding the dried cells in place, this was placed on the top of a brass stub and surrounded by a collar of PVC tape making a small boat (see Fig. 2.2). A small volume of dehydrated cells in absolute alcohol was added to the boat before critical point drying, and it was found that sperm samples adhered to the adhesive tape when critical point drying was complete. Critical point drying was carried out in a Tousimis Samdri 780CPD critical point drier and the stubs were sputter coated in a Emscope SC 500 sputter coater to a depth of 8 - 10 nm.

Samples for transmission electron microscopy underwent an identical fixation process, but were dehydrated through an acetone series before being placed in an araldite : acetone mixture (4:1 v/v) overnight, and then embedded in araldite. This was allowed to polymerise at 60°C for three days. Sections were cut on an A. F. Huxley pattern ultramicrotome and mounted on G300 mesh copper grids (EM Scope). Prior to observation, the sections were double stained according to the methods of Lewis & Knight (1977); for 10 -15 minutes with saturated uranyl acetate in 70% ethanol and then, following washing, with Reynolds' lead citrate (Reynolds, 1963) for 10 - 15 minutes.

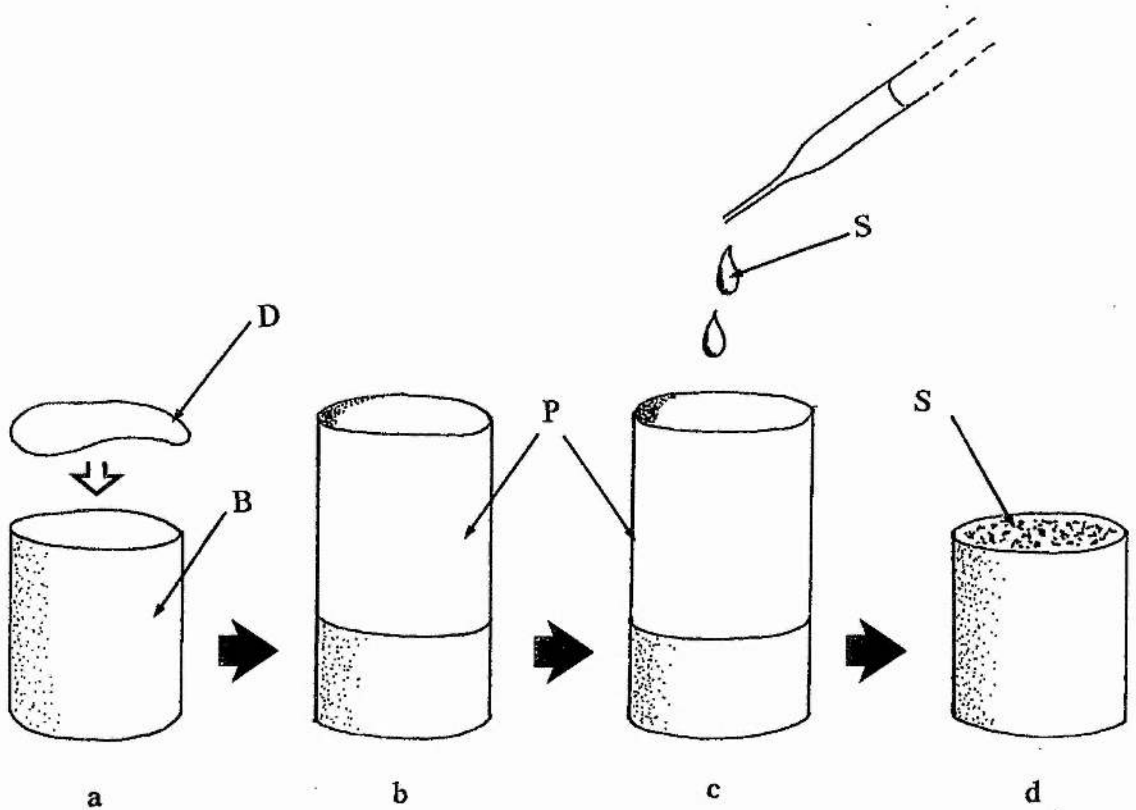


Figure 2.2. Diagram of the procedure used to retain the fixed sperm samples on the brass stubs during critical point drying. (a) A disc of double sided adhesive tape is applied to the top of a brass stub. (b) A collar of PVC tape is then made around the top of the stub. (c) An aliquot of sample following dehydration in absolute alcohol is then added dropwise into the 'boat' formed by the PVC tape. (d) Following critical point drying the PVC tape can be removed and sperm are retained on the stub by the adhesive tape. The stub is then sputter coated prior to observation. (B = brass stub; P = PVC collar ; D = double sided adhesive tape; S = sample)

2.7 Preparation of Lipid Extracts

The lipid fractions of prostomial homogenates, enzyme incubations, or localised areas of TLC plates were partitioned from non-lipids using a modified Folch method (Christie, 1982). A chloroform / methanol mixture (2:1 v/v) was added to the sample in equal volumes and the organic fraction was removed. This step was repeated, and the organic fractions pooled. The organic layer was then washed, by the addition of 0.88% potassium chloride (25% by volume), the mixture shaken, and then allowed to settle. The upper (aqueous) layer was removed, and the washing step repeated by the addition of one fourth the volume of the lower layer of methanol - saline solution (1:1). The lower organic layer was dried by the addition of anhydrous sodium sulphate, and the solvent evaporated on a rotary evaporator to yield the purified lipids (Büchi RE111 Rotavapor). These were then either resuspended in TFSW for bioassay or in solvents (usually methanol) if required for thin layer chromatography (TLC) or gas-liquid chromatography (GLC) analysis. Sonication was often necessary to resuspend any lipids which had adhered to the glass.

This basic method was followed for all lipid extractions. For GLC analysis it was found necessary to modify this procedure somewhat and this will be discussed in Chapter 4.

2.8 Thin Layer Chromatography

Thin layer chromatography (TLC) was performed using 20 x 20 x 0.25cm pre-coated silica gel 60 F_{254} (Merck) TLC plates. These were prepared for TLC by first soaking the plate in the solvent system to be used for its full length, in order to clean the plate of any contaminants. After allowing the solvent to evaporate, the plate was activated in an oven at 120°C for 30 minutes. For argentation TLC the

plates were prepared in the same way, and the plates then impregnated with silver nitrate using the method described by Christie (1982). Samples for TLC were prepared as described in section 2.7 and were applied to the plates using disposable micropipettes (Blaubrand).

The plates were run vertically for 12 cm up the plate and then were removed from the chamber and the solvent allowed to evaporate. Fatty acids were visualised by spraying the plate with 10% Phosphomolybdic acid in ethanol (v/v). Because phosphomolybdic acid reacts irreversably with lipids, in experiments where it was necessary to identify the region of biological activity, a second plate was run in parallel which was not visualised. The regions on this plate corresponding to the visualised lipid spots on the first, were then scraped off. Lipids were prepared for bioassay as described in section 2.7.

2.9 Enzyme Incubations

Details of the incubations using enzymes of the cyclooxygenase and lipoxygenase pathways are outlined in Chapter 4. Cyclooxygenase enzymes were obtained from fresh bovine lung (provided by St. Andrews abattoir), Soyabean lipoxygenase was obtained from Sigma Chemicals C°.

2.10 Oxygen Consumption Experiments

These were performed *in vitro* using a system that had been used by Havenhand and Todd (1988), to measure the respiration rate of nudibranch molluscs, but with some minor modifications (described in section 2.10.1).

2.10.1 Apparatus

Measurements of the oxygen consumption during the sperm activation were made polarographically, by monitoring the depletion of dissolved oxygen of sperm morulae suspensions contained in a sealed chamber, using a Clark type oxygen electrode. The observations were made using a Radiometer PHM 71 MkII amplifier fitted with a pO_2 module, and a Radiometer E5046 oxygen electrode.

All determinations were made in a variable volume semi-through flow chamber which had a final volume of approximately 7 ml. Measurements were made at 14-15°C, and the temperature was maintained by immersing the chamber in a constant temperature water bath (see Fig. 2.3). At the beginning and end of a days experimental observations the electrode was calibrated using a combination of 'zero solution' (0.01M disodium tetraborate in a solid watchglass, with < 0.5 mg of crystalline sodium sulphite added) and a fully air saturated solution. A fully saturated solution of TFSW was pumped continously through the chamber at a flow rate sufficient that dissolved oxygen replacement was greater than the depletion caused by the electrode itself. During experimental observations fully air saturated TFSW (sat. TFSW) was also pumped into the chamber during the sealing process and whilst the chamber was being manoeuvred into position within the water bath. This helped to maintain a slight positive pressure within the chamber sufficient to prevent air bubbles entering through the capillary outlets. Once in position the pump was switched off which effectively isolated the chamber. Havenhand and Todd (1988) demonstrated previously that no oxygen diffusion occurs through the

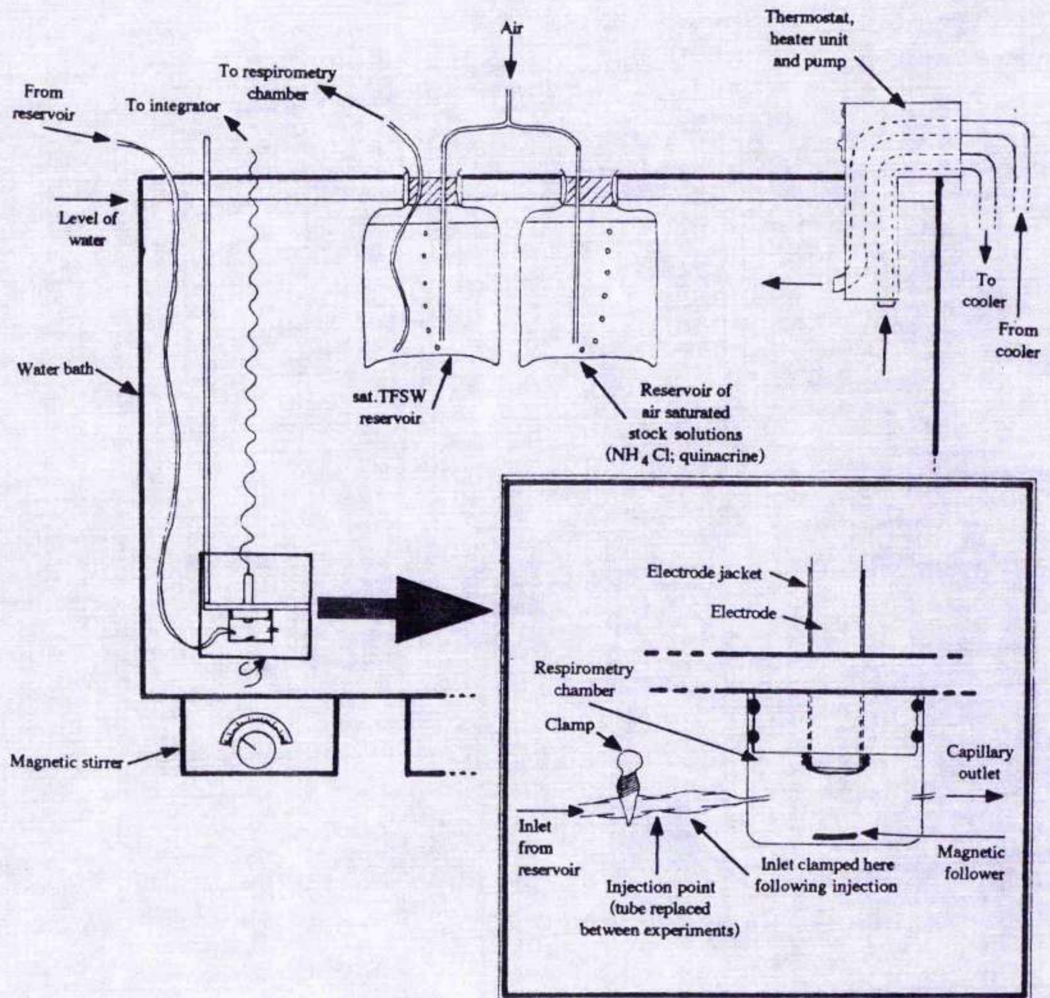


Fig. 2.3 Diagram of respirometry apparatus. The water bath contains stock solutions and respirometry chamber at constant temperature (14°C), and the pump allows fully air saturated TFSW from the reservoir to be pumped continuously through the chamber for calibration purposes. The respirometry chamber is held in position over a magnetic stirrer throughout oxygen consumption determinations. Inset shows details of respirometry chamber and position of clamps which enable direct chamber injections without removal of electrode.

capillaries at this time. Throughout all determinations the incubation was stirred by a small glass mounted magnetic follower within the chamber, and a magnetic stirrer beneath the waterbath. This helped to maintain a uniform oxygen tension within the experimental chamber throughout the duration of the measurements.

The capillary inlets and outlets allowed substances to be introduced into the chamber and the oxygen consumption to be recorded continuously both before and after their introduction. If the chamber was opened, the resealing process would mean that no readings would be possible for approximately ten minutes whilst the electrode underwent a settling period. By slowly injecting samples into the chamber however, the electrode remains largly undisturbed, and needs only two or three minutes in which to settle. A continuous series of measurements can therefore be made by this method both before and after the introduction of a sample to the incubation.

2.10.2 Experimental procedure

Dry sperm (undiluted coelomic sperm) was diluted 1:100 (v/v) with sat. TFSW to give a final volume of approximately 10 ml and a final sperm density of 1.0 - $9.0x10^7$sperm.ml^{-1}. The sperm suspension was sealed in the chamber, and the excess was vented through the capillary outlets fitted to the chamber. Once sealed, the electrode was allowed to settle for fifteen minutes, after which the basal oxygen consumption was monitored for a further fifteen minutes. A sample was then introduced into the chamber, by injection through the replaceable section of wall in the capillary inlet tube as described previously (see Fig. 2.3), the oxygen consumption of sperm morulae suspensions was then monitored for a variable length of time depending on the experiment. All samples were either dissolved or resuspended in sat. TFSW and were injected into the chamber in a total volume of 0.7 ml and as the final chamber concentration was 7 ml, it was therefore necessary

for them to be 10 fold more concentrated than the final chamber concentration required.

An experiment using sperm suspensions were followed with a control incubation using a similar aliquot from the same experimental animal, but with the oxygen consumption monitored following injection of 0.7 ml of sat. TFSW. In this instance, the sperm would not be expected to undergo activation and therefore any experiments in which the control incubation showed evidence of spontaneous activation during incubation with sat. TFSW, were disregarded. As a further control, each test substance was injected into the chamber in the absence of spermatozoa and the oxygen consumption monitored for an equivalent length of time.

Between each experiment or control, 'blank' (chamber only) oxygen consumption rates were obtained. These determined any oxygen consumption due to bacteria or other small organisms within the TFSW, and also determined the oxygen consumption of the electrode itself. These adjacent 'blank' values were subtracted from the experimental determinations, in order to obtain a value for the sperm oxygen consumption alone.

After each experiment or 'blank', the volume of the respirometer chamber was measured to the nearest 100 μl, and in the case of experimental or control incubations the sperm density was determined using a Neubauer haemocytometer.

The injection of test substances into the chamber displaced an equal volume of the existing chamber contents, and this was vented through the capillary outlets. From control incubations where sat. TFSW was injected into the chamber it appears that injection has very little effect on the oxygen consumption rate and because the injection would have displaced a proportion of the incubating sperm suspension through the capillary outlet this is perhaps surprising. However, the injection facilitated the temporary removal of the chamber from its position over the magnetic

stirrer, and the sperm are seen to sediment quickly to the bottom of the chamber. It is therefore likely that very few are vented to the exterior at this time. The injection procedure did however upset temporarily the stability of the electrode, which produce a slight 'blip' on the time trace, and it was for this reason that no oxygen consumption rate was determined at this time point.

Output recordings were obtained from the apparatus using a Vitatron 2001 series flat-bed pen recorder. These were then processed and then the oxygen content of the water calculated at five minute intervals according to the methods of Hitchman (1978).

2.11 Measurement of Intracellular pH (pHi)

Intracellular pH (pHi) determinations were made by using the fluorescent probe 9-amino acridine and following a method used by Christen *et al*, (1982) for the measurement of intracellular pH in sea urchin spermatozoa. Fluorescent measurements were made using a Kontron SMF-Spectrofluorimeter with excitation at 400 nm and emission at 452 nm. Sperm suspensions (c.10^8sperm ml^{-1}) were loaded with fluorophore by incubation in 9-amino acridine at a concentration of 1 $\mu g.l^{-1}$ dissolved in TFSW.

For fluorescence measurements sperm suspensions were transferred to quartz cuvettes (3.5 ml volume with lid). Due to the tendency of the spermatozoa to settle out within the cuvette the suspensions were mixed every five minutes during measurements. Mixing was achieved by inverting the cuvette two or three times. Membrane permeabilisations were performed by the addition of 40 µl of Triton X100 to give a final concentration of 0.04% (v/v). For the calculation of pHi, fluorescence measurements were made both before and after membrane

permeabilisations and calculations were made according to Christen *et al.*, (1982) using the formula:

$$pHi = pHe - \log\left(\frac{Q}{1-Q} \times \frac{1-Q'}{Q'} \right)$$

Where Q is the fraction of fluorescence quenched in intact sperm, and Q' is the fraction quenched when the sperm membrane is permeabilised. pHe is the pH of the medium within which the sperm are incubated.

2.12 Measurement of Cellular ATP Concentration

Levels of cellular ATP were determined employing the firefly luciferin-luciferase technique using an Adenosine 5' - triphosphate bioluminescent assay kit (Sigma Chemical Co FL-AA). The assay procedure was carried out in accordance with Sigma technical bulletin No BAAB-1 as follows:

An aliquot (100 µl) of assay mix was placed in a reaction vial and left at room temperature for 3 minutes. During this period, any exogenous ATP is hydrolysed and therefore will decrease the background luminescence and hence the error of the assay. The assay was commenced by addition of 100 µl of sample (or standard) and following mixing, measuring the bioluminescence produced. Despite thorough cleaning of vials prior to commencing the experiment and the procedure outlined above, any exogenous ATP remaining can be determined by assay of a blank which contains no cellular material or added ATP. Such blank determinations are identical to the assay procedure except 100 µl of freshly distilled deionised water is added in place of the sample or standard. The values determined during blank readings are subtracted from those obtained for the sample or standard in order to give an accurate measurement of their ATP content.

Absolute values of ATP concentration are calculated with reference to a standard curve following an appropriate dilution of the sample, if required. The standard curve was freshly prepared from a $1x10^{-3}$M solution of Adenosine-5'-triphosphate disodium salt, grade 1 (Sigma Chemicals Co) and the mean bioluminescence values were determined from triplicate readings at each concentration using the same stock of reagents that are used for bioluminescence measurements of sperm samples.

The reaction vessels used for all bioluminescence measurements were polypropylene 'pony vials' (Canberra Packard), normally used for liquid scintillation counting and the relative light intensity of the reaction mixture was measured using a Packard Tri-Carb Liquid Scintillation Analyzer Model 2000, set to protocol 9 and counting for one minute. The use of liquid scintillation counters to determine the light emission of luciferin-luciferase bioluminescence is well established (see for example Idahl *et al.*, 1986) and relies on the property of light produced in this assay system being stable for several hours.

All reaction vials, pipette tips and other glassware which had any contact with samples or reagents were cleaned of exogenous ATP and bacterial contamination by soaking overnight in 1N HCl followed by a thorough rinse in freshly distilled deionised water and drying in an oven. All stock solutions were prepared in freshly deionized water and at all times samples were handled using surgical gloves to prevent ATP or bacterial contamination from the skin.

Adenosine triphosphate was extracted from spermatozoa using trichloroacetic acid (TCA) in the ratio of 1:1(v/v). For the extraction of ATP from human spermatozoa, Lyons *et al.*, (1986) used a TCA concentration of 6% (w/v) which gave a maximal ATP extraction. Extractions were performed for 10 minutes at room temperature and were followed by centrifugation at 3000 rpm for 15 minutes. Lyons *et al.*, (1986) point out that the supernatant should be diluted to give a final

TCA concentration of less than 0.1%, because at higher concentrations TCA can inhibit the firefly luciferase reaction. For the purposes of this study the optimum TCA concentration required for maximal ATP extraction, and hence the dilution required in this system, will be investigated in Chapter 7.

2.13 Spawning Experiments

The spawning response of animals was estimated by counting the number of gametes spawned into a standard volume of 180 ml TFSW in a twenty-four hour period.

2.13.1 The male spawning response

Males release a considerable number of gametes which therefore facilitates the use of a Neubauer haemocytometer to measure sperm numbers. The number of sperm per ml was estimated from three subsamples, each of 100 µl, taken from the standard volume into which the animals spawned after adequate mixing. Because of the high densities of sperm, these subsamples were usually diluted between 1:1000 to 1:5000 (v/v) to give a total sperm number of between 200 and 500 sperm counted in a total of ten 1mm squares. It is in this range that the estimate of cell density using a haemocytometer is most accurate.

Due to the highly motile nature of the sperm cells, estimates of sperm numbers were carried out after cell motility had ceased (usually within 48 hours of spawning).

2.13.2 The female spawning response

Oocytes could be counted on a microscope slide using a x10 objective of a compound microscope. Using this method a total number of oocytes in three

replicate sub-samples, each of 1 ml were estimated. This provided a mean number of oocytes per ml of standard volume (180 ml), and the spawning response of females could therefore be expressed as the total number of oocytes spawned per animal.

Chapter 3

Sperm Activation:
Ultrastructural characteristics

3.1 Introduction

Spermatogenesis and sperm ultrastructure in the Polychaeta have been reviewed a number of times and, in recent years, have included those of Olive (1983b), Sawada (1984), Franzén (1987), and Franzén and Rice (1988). Such reviews have highlighted the striking variation in sperm morphology which exists in the class and has demonstrated that the sperm of related species often display widely different morphologies. Even in the same family, some genera have retained primitive spermatozoa whilst others have evolved modified forms (Sawada, 1984). Where modifications in sperm ultrastructure do occur however, these are generally associated with a modified mode of reproduction, and Franzén (1956) identified five out of twenty-two polychaete families with aberrant spermatozoa which, in all cases, were associated with a modified mode of reproduction.

Recent ultrastructural investigations in species which have evolved modified sperm types include those on *Polydora* spp., *Streblospio benedictii* (Rice, 1981), the *Capitellid* spp. (Eckelbarger & Grassle, 1987) and the interstitial polychaete *Hesionides arenaria* (Westheide, 1984). These show the characteristic modifications expected of spermatozoa of this type (see section 1.2 for review). Extreme modifications have been observed in some polychaetes including that of *Ophryotrocha puerelis* (Berruti *et al.*, 1978) which is aflagellate. The majority of polychaetes, however, have sperm of the primitive type and this is thought to reflect the common mode of reproduction in the group of a whole, that of external fertilisation (Sawada, 1984). Primitive spermatozoa have been described recently occurring in species such as *Chaetopterus pergamentaceus* (Anderson & Eckberg, 1983), *Cirriformia tentaculata* (Sawada, 1984), *Eulalia* spp. (Rouse, 1988), *Eurythoe complanata* (Rouse & Jamieson, 1987), *Phragmatopoma lapidosa* (Eckelbarger, 1984), *P. californica* (Kopp, 1985) and *Cistenides okudai* (Sawada, 1984).

The spermatozoa of the Arenicolidae have a classically primitive structure and spermatogenesis within this family is particularly interesting because it results in the formation of a disc of mature spermatozoa called a morula (see Newell, 1948; Olive, 1983; Sawada, 1984). Within a morula, individual spermatozoa are cytoplasmically connected to a central mass of cytoplasm called the cytophore and it is in this syncitial condition that the spermatozoa remain until the structure dissociates immediately prior to spawning (see section 1.5). The function of the cytophore remains unknown although the formation of a cytophore has been described in a number of polychaete species including the terebellids (Eckelbarger, 1975; Smith, 1989) and in the Opheliidae (Ochi *et al.*, 1977).

The ultrastructure of sperm morula and spermatozoa in the sibling species *Arenicola brasiliensis* was investigated by Sawada (1975). Meijer (1979b) published micrographs of sperm morula in *Arenicola marina* but said very little of the ultrastructural detail of the morula or of mature spermatozoa. This study however, investigates the ultrastructure of mature spermatozoa in *Arenicola marina* and observes the ultrastructural changes which occur during the dissociation of morulae to free active spermatozoa. This is of particular interest as the spawning mechanism of *Arenicola* relies upon the dissociation of sperm morulae immediately prior to spawning, and that free swimming spermatozoa are automatically spawned from the body cavity (see section 1.5). Bentley (1986a) made some preliminary light microscopic investigations of sperm activation in *A. marina* and began ultrastructural observations using electron microscopy (Bentley, 1986c). This study is an extension of these preliminary observations and incorporates both scanning electron microscopy (Bentley & Pacey, 1989) and transmission electron microscopy.

3.2 Materials and Methods

Sperm samples for both transmission and scanning electron microscopy were incubated with prostomial SMF, and sequential fixations were carried out at five minute intervals from commencement of the incubation throughout the activation period. During each experiment, thirteen activation and thirteen control incubations were carried out in parallel, and sperm was incubated with either 500 µl of prostomial homogenate at a final concentration of 0.4 Pr/Eq.ml^{-1}, or 500 µl TFSW respectively. At time zero, 50 µl of 'dry sperm' (undiluted coelomic sperm), was added to the incubates and at five minute intervals from zero to sixty minutes, one experimental and one control incubation were fixed by the addition of fixative (see section 2.6). These samples were then processed for electron microscopy, the samples being halved prior to the dehydration stage and each half undergoing preparation for either TEM (transmission electron microscopy) or SEM (scanning electron microscopy). Five experiments were carried out which gave a total of 130 fixed sperm samples which were then examined using either a JEOL JSM - 35CF scanning electron microscope or a Phillips EM301 transmission electron microscope.

In addition coelomic samples were taken from male *Arenicola* which were not yet sexually mature and contained early stages of spermatogenesis. These were fixed for both TEM and SEM in the same way.

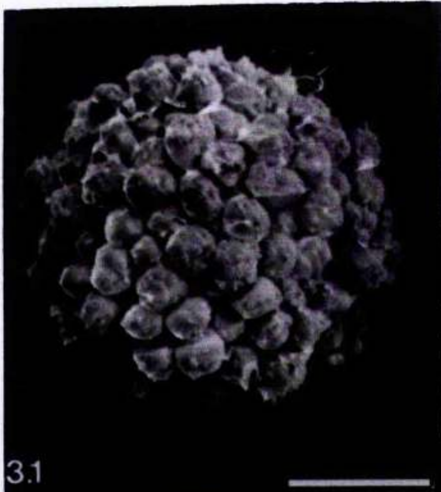
3.3 Results

Figures 3.1 to 3.24 show the results of the TEM and SEM investigation of spermatogenesis and sperm activation in *Arenicola marina.*

3.3.1 Development to the sperm morula

The male germ cells of *Arenicola* are released from the testes at the eight cell stage and, within the coelomic fluid, these undergo spermatogenesis (Olive, 1983b). The earliest stages seen by scanning electron microscopy consist of a spherical ball of cells which at their largest are 25 µm in diameter (Fig. 3.1). It is unclear from scanning electron microscopy whether such cells are spermatogonia or whether they have completed meiosis and are spermatids. Transverse sections of the larger clusters however, show some electron dense vesicles and what appears to be a single centriole, with a pre-acrosomal vesicle forming nearby (Fig.3.2), indicating that cell division in this cluster is complete. Figure 3.2 also shows that cytoplasmic continuity between individual spermatids is maintained and these cytoplasmic bridges are seen in the centre of the spermatocyte cluster forming the cytophore. At this stage the nucleus of the spermatid is elongate and nuclear condensation is yet to take place.

As differentiation of the spermatids continues, the spermatid cluster flattens and becomes disc-like. Spermatids acquire a longitudinal axis and a polarity with the rudiments of flagellum becoming visible at their distal end (Fig. 3.3). Figure 3.4 shows a transverse section through a spermatid disc at this stage of development. Within these spermatids, nuclear condensation is partially complete, the pre-acrosomal vesicle being visible near to the centrioles. The cytophore at this time is still quite large and granular although now flattened, and the spermatid cluster, has become more disc-like. Sawada (1975) suggests that the flattening of the cytophore to a disc of cytoplasm provides a greater surface area for the attachment of spermatozoa than a ball like mass. As development proceeds the flagellum increases in length and in some cases pairs of flagella become associated at their distal ends, this is illustrated in figure 3.5.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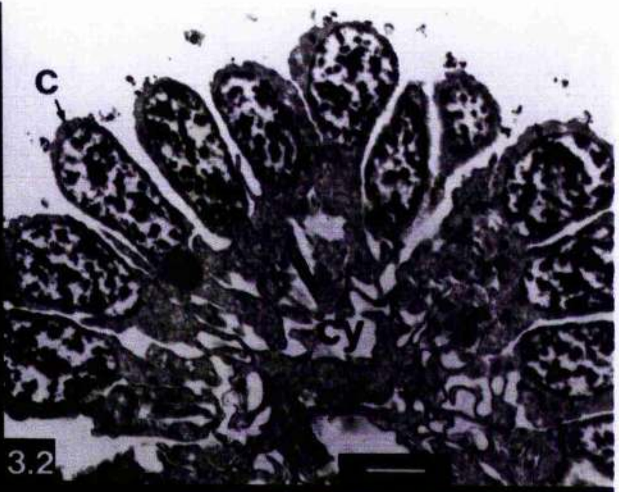
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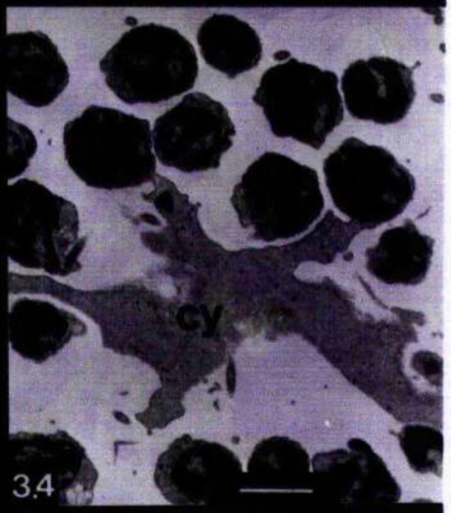
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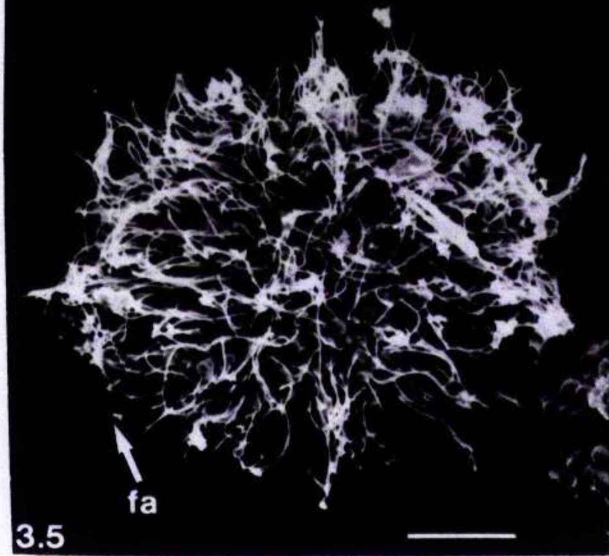
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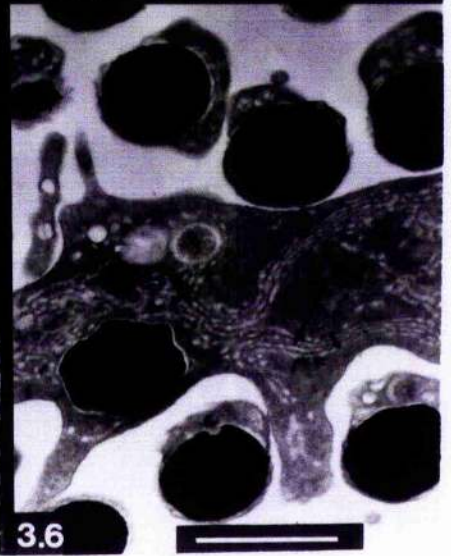
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The cytophore appears to play a key role in the development of the spermatids, as in later stages it is seen to contain many active cell organelles. Figure 3.6 shows spermatids which have completed nuclear condensation and in which the acrosomal vesicle, although not yet fully differentiated, has migrated to the anterior end of the spermatozoa. At this stage, endoplasmic reticulum is widely distributed throughout the cytoplasm of the cytophore and suggests that active synthesis is occuring within the cell. Electron dense spherical vesicles are also visible within the cytophore although it is unclear whether these are lysosomes or storage organelles.

As the development into mature spermatozoa proceeds, the cytophore shrinks in size, until it contains very little in the way of cytoplasmic inclusions apart from membrane bound vesicles which are possible lysosomes. Figure 3.7 is of a mature sperm morula and it is within this structure the spermatozoa remain, until they are activated and become free swimming either just prior to spawning or when incubated with SMF.

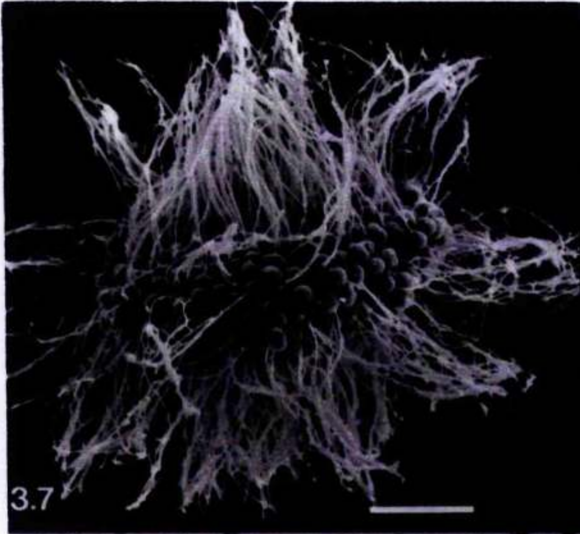
3.3.2 Ultrastructure of the mature spermatozoa and the sperm morula

Figure 3.7 shows the structure of a fully differentiated sperm morula. The individual sperm heads are closely associated and lie at the centre of the disc. The flagella from individual spermatozoa radiate outwards and often lie closely associated along a considerable proportion of their length. These form the characteristic bunched or bundle like appearance of flagella observed in morulae at this stage. Whilst this may be as a result of the fixation process, it seems unlikely because the flagella of morulae in later stages of incubation with SMF, and the flagella of free spermatozoa, do not aggregate in such a manner. By observing sperm morulae that have become fractured during preparation it is possible to see

some of the internal detail and figure 3.8 shows part of one half of such a morula. The cytophore is visible as a laminar structure with individual spermatozoa oriented with their acrosomes towards it. Cytoplasmic connection is made between the cytophore and spermatozoa, although the actual point of contact with the spermatozoa is not visible.

Figure 3.9 is a transverse section through a mature sperm morula showing the orientation of the mature spermatozoa with their acrosomes toward the cytophore. The cytophore is seen to contain little in the way of cytoplasm or cytoplasmic inclusions, but does make connection with sperm heads. The details of these connections are seen more clearly in figure 3.10 (TEM), with a stalk of cytoplasm from the cytophore making connection with the spermatozoa on the lateral surface of the sperm head, just behind the acrosome. This figure also shows detail of the head and mid-region of a mature spermatozoa in *Arenicola marina*.

The spermatozoa of *Arenicola* conform to a typical primitive ultrastructure. The head region of mature spermatozoa consist mainly of nuclear material surmounted by a cup-like acrosome (Fig. 3.10) (see Sawada, (1984) for acrosome types). At the apex of the acrosome is a small acrosomal button or apical vesicle. This is anterior to the acrosomal vesicle which lies in the space between the acrosome and the nucleus, and in *Arenicola* contains material which is moderately granular. The nucleus both in transverse and longitudinal section appears ovoid in shape and posteriorly there are four to six mitochondria with prominent cristae. Figure 3.11 shows a transverse section through the mid region of a spermatozoon in which there are five. Located within the mid region of two spermatozoa are two centrioles oriented at 90° to each other (Figs. 3.12 & 3.13). The distal centriole lies along the central vertical axis of the spermatozoon and has nine paddle-like arms. From these arise the nine outer doublets of the flagellar axoneme, the two central singlets arising distal to the centriolar apparatus (Fig. 3.14).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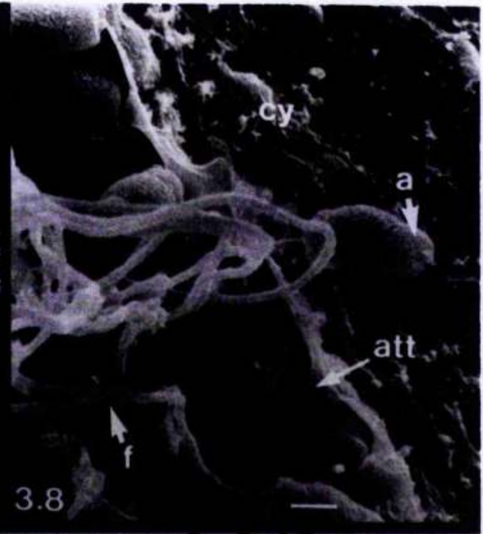
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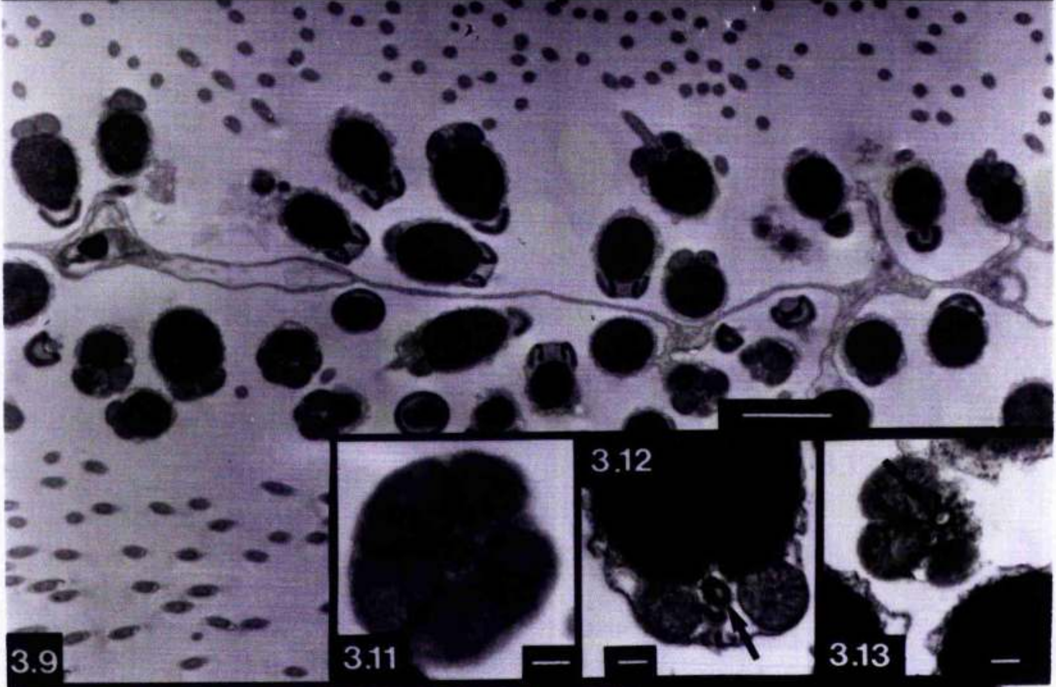
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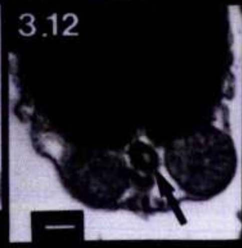
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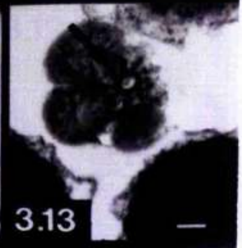
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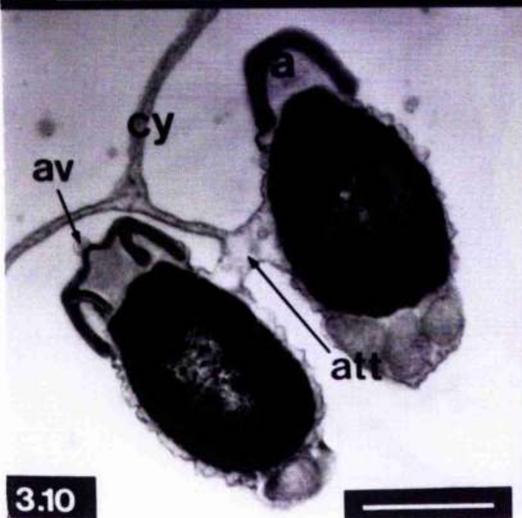
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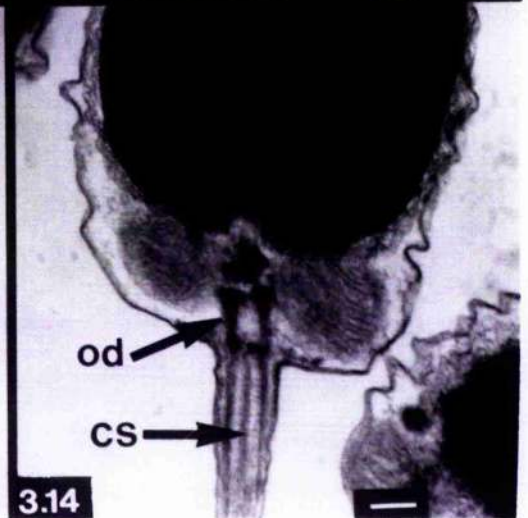
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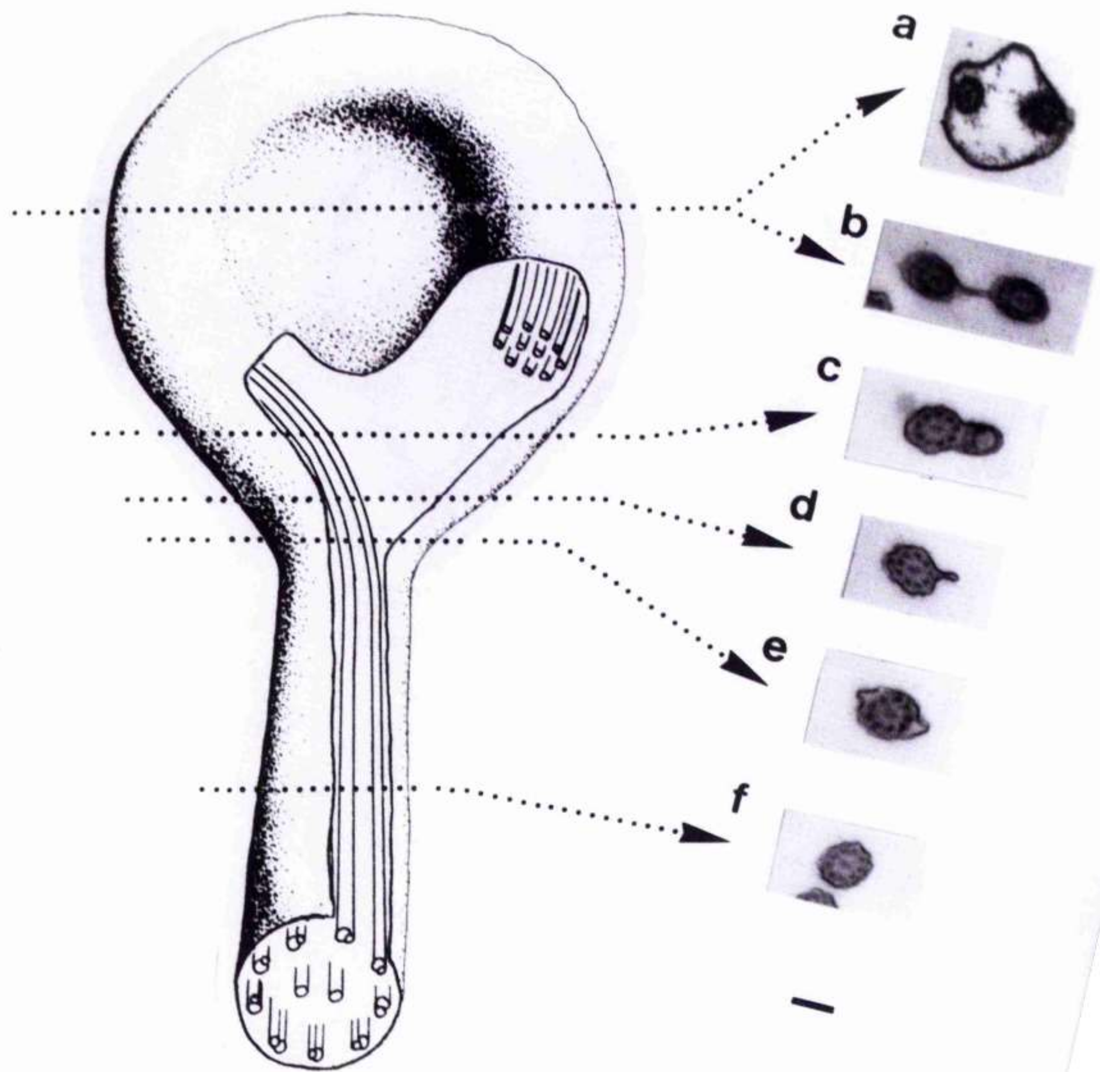
The sperm flagella of *Arenicola* are relatively unmodified along their length and consist of the conventional arrangement of 9 + 2 microtubules tightly enclosed within a membrane sheath. Occasionally however, modifications of the flagellum are observed. These usually occur with regard to the membrane surrounding the flagellar axoneme which may become flared, expanded, or occasionally, forms are observed where two axonemes are surrounded by a single membrane. The various transverse sections observed suggest that the axoneme is coiling within the flagellar part of the cell membrane at its distal end (Fig. 3.15).

3.3.3 Activation of spermatozoa *in vitro*

When coelomic sperm samples of *Arenicola* are incubated with SMF *in vitro*, free spermatozoa are liberated from the sperm morulae after approximately 50 minutes at 14°C. Sperm morulae present within sperm samples, which are fixed at the commencement of incubation, show no ultrastructural changes and have an appearance unchanged from that of the unactivated morula (Fig. 3.16). Only after twenty minutes of incubation with prostomial extracts do any changes become apparent (Fig. 3.17). This corresponds to the time when flagellar beating commences in some of the spermatozoa (observed by light microscopical examination *in vitro*). The general morula structure is still maintained, although there is a suggestion that the structure is beginning to loosen (arrow). The bundles of flagella have also become disorganized, as beating has commenced, and there is less tendency for flagella to be as closely associated as they were in the unactivated morula. However, the fact that the flagella are not beating individually suggests that they may still be associated in pairs at this time.

Transverse sections of sperm morula show that significant changes have taken place with respect to the state of the cytophore. At this time the cytophore becomes swollen and significantly increases in size (Fig. 3.18 cf. Fig. 3.9). The



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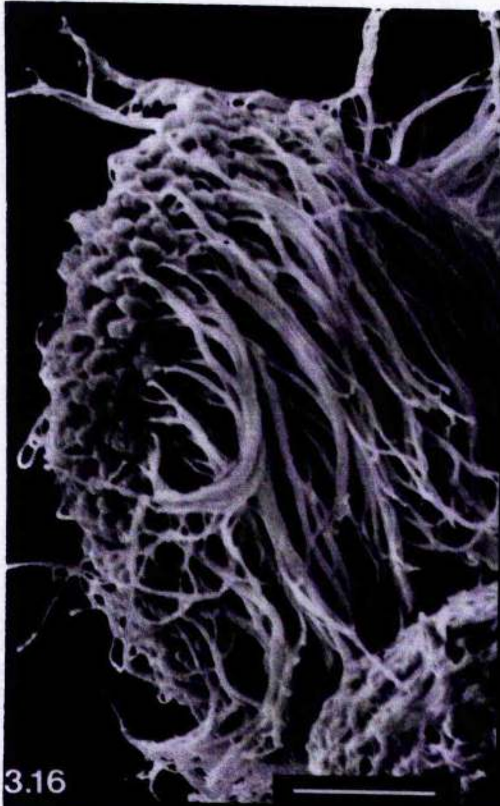
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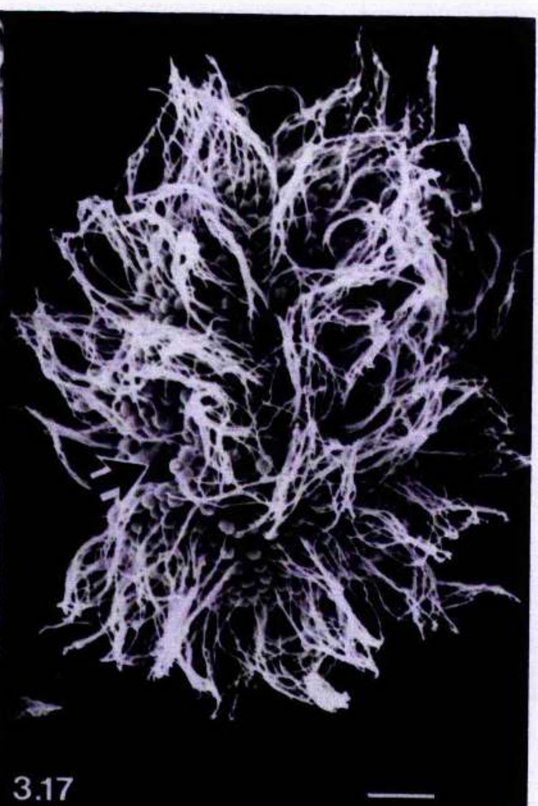
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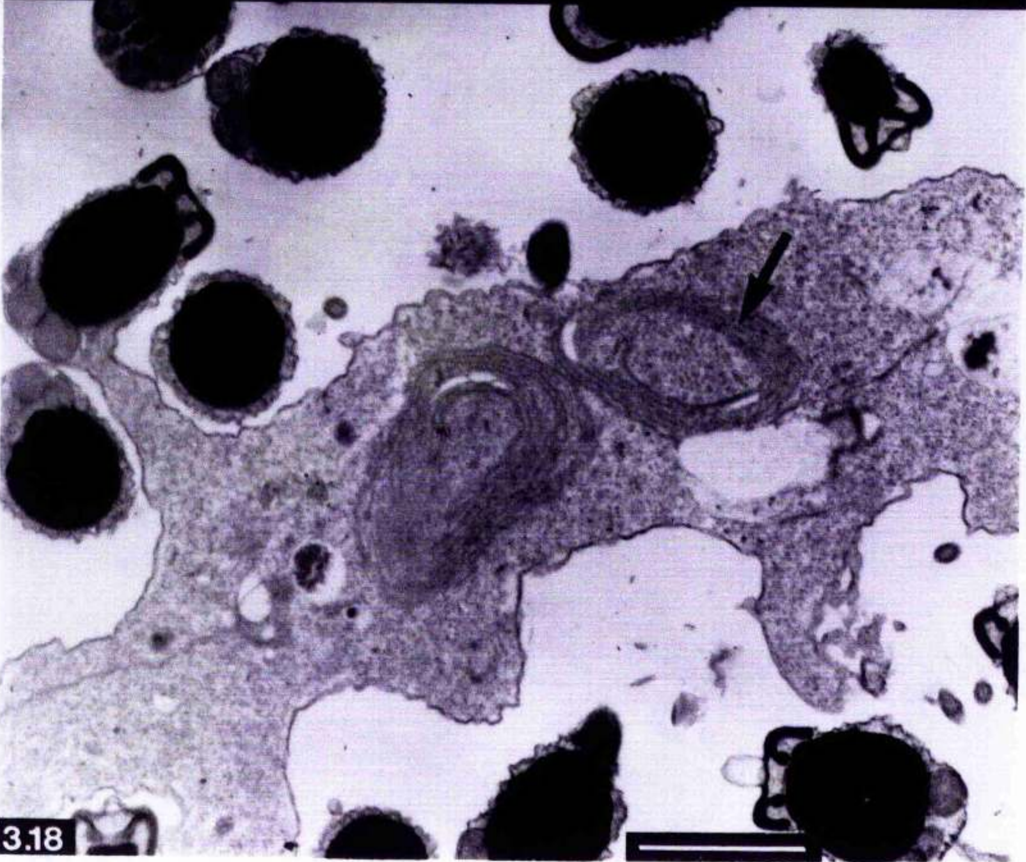
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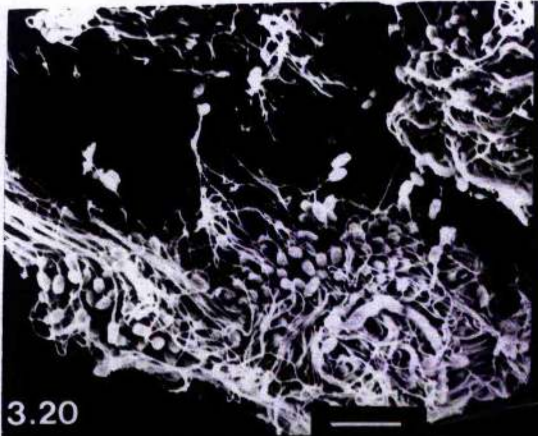
stalks of cytoplasm which connect the cytophore to the spermatozoa and the spermatozoa to each other are similarly swollen. Both the cytophore and the connecting stalks are filled with a granular electron dense material, and whorls of membrane within the cytoplasm are visible (arrowed).

After 30 minutes incubation, there is a noticeable increase in the degree of disorganization of the flagella, evidenced by both transverse and longitudinal sections of flagellum visible within the same micrograph, and fewer sperm heads are associated with the cytophore (Fig. 3.19). The cytophore at this point is very swollen, contains some clear vacuoles, and appears to be undergoing degeneration, possibly through the action of enzymes released from lysosome activity. After forty minutes there is a marked increase in the degree of disorganisation within the morula (Fig. 3.20). Observations of live spermatozoa at this point, reveal that spermatozoa in most of the morulae are becoming activated, individual flagella are beating, and many of the sperm heads have broken contact with the cytophore. This is the 'swimming morula' stage, although Bentley (1986b) indicates that the distal binding of sperm flagella is first broken, to produce a swimming morula, and only when the symplasmic connections between sperm heads are broken are free sperm liberated. This study has indicated however, that the connection of sperm with the cytophore breaks prior to the breaking of distal flagellar binding. Figure 3.20 shows that although the degree of disorganisation has significantly increased, and sperm heads are seen to have broken contact with the cytophore, free sperm have not yet been liberated, but, how the association of spermatozoa is maintained is unknown. Perhaps the spermatozoa still remain bound at the tips of their flagella as was observed in earlier stages of morula development.

Ultimately spermatozoa become totally independent and free swimming, and these are visible in live sperm samples or samples fixed after 50 minutes incubation with SMF (Fig. 3.21); remnants of the cytophore are also visible (Fig. 3.22). The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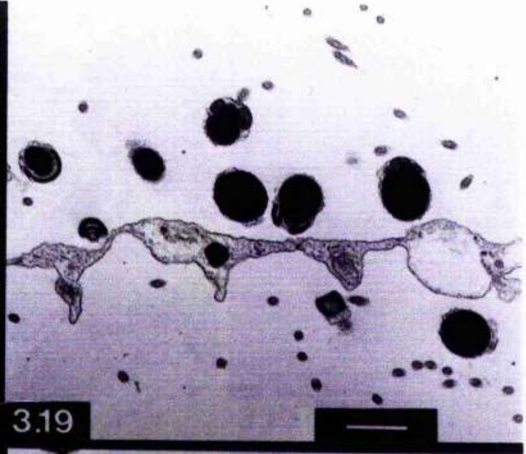
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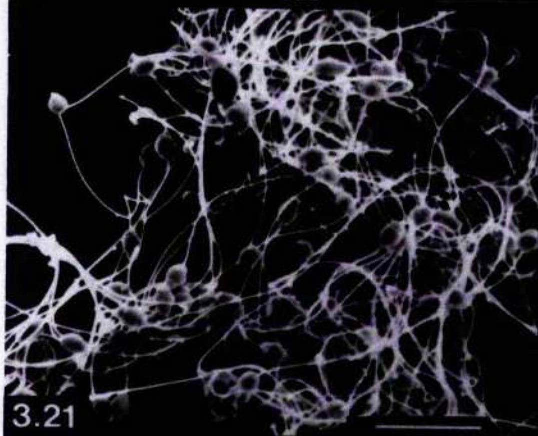
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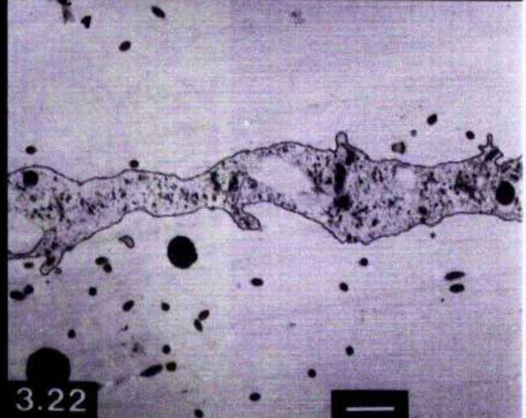
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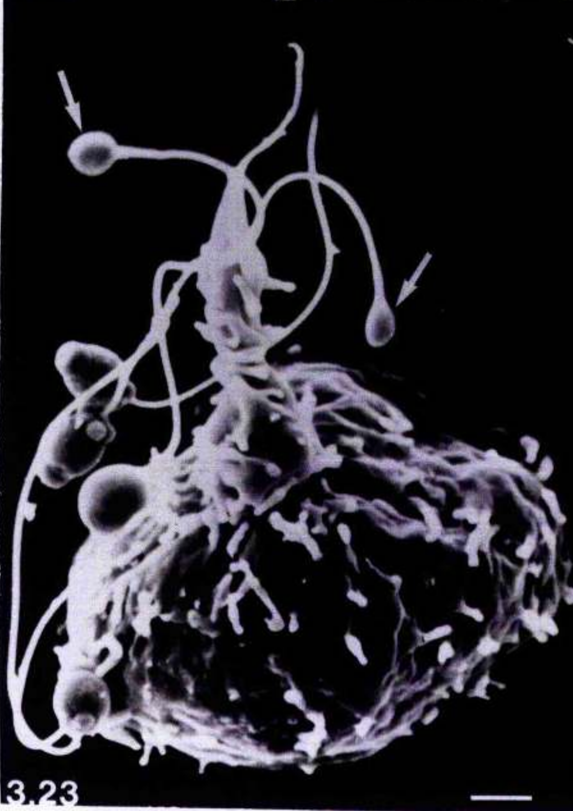
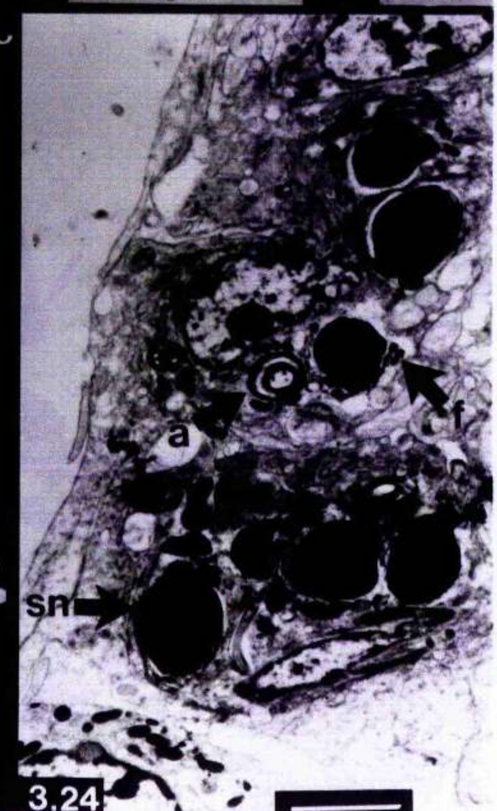

3.23


a
f
sn
3.24

presence of free spermatozoa at 50 minutes is the basis of the *in vitro* bioassay, indicating the sperm activation has taken place (see section 2.4). Over the activation period the ultrastructure of mature spermatozoa does not change, with the ultrastructure of free swimming spermatozoa being identical to that of morula bound spermatozoa.

Following incubation of coelomic sperm samples with TFSW there are no free spermatozoa present at this time, and SEM observations of such control incubations, show that all sperm morulae remain undissociated and have undergone no visible ultrastructural changes.

During experimental incubations there may be some morulae which remain undissociated after 50 minutes incubation with SMF and do not liberate free spermatozoa. In the experimental fixations described here, spermatocyte clusters, plates of spermatids and other developmental stages of sperm morula are also present within experimental fixations and these are noted to undergo no ultrastructural changes.

3.3.4 Other ultrastructural observations

During TEM and SEM observations of the fixed *in vitro* incubations, cell types other than spermatozoa are visible. Present within the coelomic cavities of Polychaeta are coelomocytes which function within the immune response system, and in general microbial defence reactions (see Dales & Dixon, 1981; Dhainaut & Porchet-Henneré, 1989 for review). It has been suggested that coelomocytes may 'clean' the coelomic cavity of unspawned spermatozoa (Bentley & Pacey, 1989). During *in vitro* activation free spermatozoa are observed associated with coelomocytes and figure 3.23 shows a coelomocyte which is c. 16 μm in diameter, with a large pseudopodial process becoming entangled with the flagella of some free

sperm. This figure also clearly shows discoflagella, the bulbous swellings at the distal ends of some sperm flagella (see section 3.3.2).

Figure 3.24 is a transmission electron micrograph showing an aggregate of up to eight coelomocytes within which the nucleus or acrosomes of seven mature spermatozoa are visible. It is unclear whether these spermatozoa have been phagocytosed by a single, or several cells or have been encapsulated by them and are currently lying within the intercellular space. All these observations were made at the latter end of the incubation 40 - 50 minutes after the point at which spermatozoa had become free swimming.

3.4 Discussion

Gametogenesis in the Polychaeta most commonly occurs within the coelomic fluid, with the male germ cells being liberated from the testes at an early stage of spermatogenesis (Sawada, 1984). It is often relatively easy, therefore, to follow the ultrastructural changes that accompany spermatogenesis.

Spermatogenesis in *Arenicola marina*, results in the development of sperm morula in the same manner as described in *Arenicola brasiliensis* (Sawada, 1975), with the exception, that sperm differentiation in *A. marina* results in the formation of a rounded sperm head rather than an elongate form. Olive (1983b) points out that the long and narrow sperm head observed in *A. brasiliensis* is unusual for spermatozoa of the primitive type and the significance of the difference unknown. In the case of *A. brasiliensis* however, it leads to a more tightly packed arrangement of sperm heads around the cytophore than is possible with *A. marina*. A similar dimorphism of sperm types is observed between the spermatozoa of the closely related species of nereids, *Platynereis massiliensis* & *P. dumerilli* (Lücht & Pfannensteil, 1988), but the authors could suggest no reasons as to why such

differences should occur. Despite the differences between the shape of the sperm head, both *A. marina* and *A. brasiliensis* have an inverted cup-like acrosome which has a vesicle like structure on its apex. Sawada (1984) classified polychaete sperm acrosomes as being either of the conical, beret-like, cup-like or pouch-like types. The functional significance of these different types is unclear, but Franzén & Rice (1988) suggested that they are probably related to species specific fertilization mechanisms. If the similarity between acrosome types suggests that their fertilization biology is very similar, then it is perhaps surprising to find such a difference with regard to the shape of the sperm head in these two species.

The formation of a cytophore is a common feature in the Polychaeta and is thought to be characteristic of the annelids, but its role seems unclear (Sawada 1984). In the earthworms, *Eisenia foetida* and *Bimastus pana*, Sareen and Verma (1983) describe the cytophore containing phospholipids, RNA, carbohydrates and proteins, but ascribe no function or role to these substances. Sawada (1984) suggested that the cytophore may serve as a 'sink' for residual cytoplasm for the dividing spermatogonia, and may serve to synchronise their development. This study shows that by the presence of endoplasmic reticulum and other organelles within the cytophore of developing spermatogonia, but not in the mature morula,that the cytophore may have a nutritive or metabolic role in spermatogonial development. Cytoplasmic continuity between developing germ cells is not a feature exclusive to the Annelida. Intracellular bridges between developing spermatogonia have been observed in a number of animals, for example in the millipedes (Reger & Cooper, 1968) and also in many mammals (Holstein & Roosen-Runge, 1981). Weber & Russell (1987) suggested that intracellular bridges between developing spermatogonia in the rat, facilitated physiological communication between cells. Such communication would ensure synchronous germ cell development, possibly by the spread of regulatory substances throughout the clone (Huckins, 1978). It is

conceivable that the cytophore of *Arenicola* could have a comparable role. The cytophore is clearly involved in the activation of spermatozoa since ultrastructural changes are closely allied with the acquisition of motility and the release of spermatozoa. The significance of the cytophore swelling, the role of the granular material, and the whorls of cytoplasm remains unclear, although the possibility that enzymes are being synthesised which are involved in the breaking of the cytoplasmic connections between spermatozoa, and substances which stimulate sperm activation, should not be overlooked. Since sperm activation is mediated by SMF, the reception and translation of endocrine signals may well be modulated by the cytophore.

The observations made that the axoneme is coiling within the flagellum membrane at its distal end is very much like the transverse sections of a discocilium (see Laverack, 1988) . These are modified cilia which are described as being associated with the sensory epithelia of many marine invertebrates (Haszprunar, 1985) and although there are several variations ('club footed', 'spatulate' or 'paddle' type), these cilia generally bear a discoid, biconcave or round swelling at their tip. Within sensory cilia such swellings are thought to increase the membrane surface area and consequently the opportunity for interaction with chemical substances (Davis & Matera, 1982). Perhaps, in spermatozoa, such an increase in surface area aids its movement through the water. Discocilia have been observed located within the sensory cilia of many species including larval sponges, polychaetes, cephalopods, nudibranchs, crabs, insects, frogs and mice (see Davis & Matera, 1982). Sawada (1975) made no mention of the ultrastructure of the sperm flagellum in *A.brasiliensis* but concentrated more on the ultrastructure of the sperm head, and the arrangement of spermatozoa around the cytophore. It would be interesting to see if this species contained such structures. Examples of sperm flagellum displaying expanded membranes have not been found within the literature,

although Lücht & Pfannensteil (1988) published micrographs of the spermatozoa of the protandrous polychaete *Platynereis massiliensis* showing lateral folds of the flagellar membrane, but this occurs in the proximal region of the flagellum and not at its distal end.

Coiling of the flagellar axoneme has been observed in the flagellum of mammalian spermatozoa (see Drevius, 1975 for review), but was exclusively an artefact due to osmotic stress within hypotonic media. It seems unlikely that the structures in *Arenicola* sperm flagella are caused by osmotic effects since the osmolarity of the fixatives were closely kept to that of sea water and of coelomic fluid.

Bentley and Pacey (1989) suggested that the appearance of two axonemes within a single membrane was due to the binding of axonemes from separate spermatozoa, since pairing of flagella is observed during sperm morula development. The pairing of spermatozoa has been observed in many species but is usually observed with the sperm heads of highly modified spermatozoa and pairing usually occurs within sperm storage organs such as testes, seminal vesicles or female spermathecae (see Bawa, 1975 for review). It now seems unlikely that the pairing of flagellum in *Arenicola* results in the binding of axonemes and consequently how the association is maintained remains unclear.

Coelomocytes play a vital role in the removal of unwanted cellular & non-cellular material from the coelomic cavities of polychaetes (see Dhainaut & Porchet-Henneré, 1989 for review). For example, it has been shown that coelomocytes play a central role in the clearance of bacteria from the coelomic fluid in *Arenicola marina* (Fitzgerald & Ratcliffe, 1989) and coelomocytes have been seen to proliferate following tissue damage and degeneration (Clark & Clark, 1962). Resorption of gametes within the Polychaeta, whether due to reproductive failure or partial spawning, is a common occurrence (Olive *et al.*, 1981a&b), and it has been

Fig 3.7: Structure of a mature sperm morula. The sperm remain bound together in this way until just prior to spawning or *in vitro* activation with SMF. Scale bar = 10 μm.

Fig 3.8: Fully developed spermatozoa lying on one side of the cytophore (a = acrosome, att = attachment of cytophore to sperm head, cy = cytophore, f = flagellum). Scale bar = 1 μm.

Fig 3.9: Orientation of mature spermatozoa toward the cytophore in mature sperm morula. Scale bar = 2 μm.

Fig 3.10: Ultrastructure of acrosome and attachment of mature spermatozoa to the cytophore (a = acrosome, av = acrosomal vesicle, cy = cytophore, att = cytoplasmic attachment of spermatozoa to cytophore). Scale bar 1 μm.

Fig 3.11: Mid-region of mature spermatozoon (m = mitochondria, arrow shows position of distal centriole). Scale bar = 0.2 μm.

Fig 3.12: Longitudinal section of mature spermatozoon showing distal centriole (arrowed). Scale bar = 0.2 μm.

Fig 3.13: Mid-region of mature spermatozoon showing distal centriole (arrowed). Scale bar = 0.2 μm.

Fig 3.14: Mid-region of mature spermatozoon showing position of centriolar apparatus and the outer doublet (od) and central singlet (cs) microtubules of the flagellar axoneme.

Scale bar = 0.2 μm.

Fig 3.15: Diagrammatic representation of the distal end of mature sperm flagella showing the coiling of the axoneme within an expanded portion of the flagellar membrane. The diagram shows the membrane tightly adhering to the microtubules, although sections a shows that an alternate form, where the membrane is expanded is often observed. The other transverse. sections (c -f), correspond to interpretation to the diagram. Scale bar = 0.2μm

Fig 3.16: Mature morula fixed immediately following the beginning of incubation with SMF. Scale bar = 10 μm.

Fig 3.7: Structure of a mature sperm morula. The sperm remain bound together in this way until just prior to spawning or *in vitro* activation with SMF. Scale bar = 10 µm.

Fig 3.8: Fully developed spermatozoa lying on one side of the cytophore (a = acrosome, att = attachment of cytophore to sperm head, cy = cytophore, f = flagellum). Scale bar = 1 µm.

Fig 3.9: Orientation of mature spermatozoa toward the cytophore in mature sperm morula. Scale bar = 2 µm.

Fig 3.10: Ultrastructure of acrosome and attachment of mature spermatozoa to the cytophore (a = acrosome, av = acrosomal vesicle, cy = cytophore, att = cytoplasmic attachment of spermatozoa to cytophore). Scale bar 1 µm.

Fig 3.11: Mid region of mature spermatozoon (m = mitochondria, arrow shows position of distal centriole). Scale bar = 0.2 µm.

Fig 3.12: Longitudinal section of mature spermatozoon showing distal centriole (arrowed). Scale bar = 0.2 µm.

Fig 3.13: Mid-region of mature spermatozoon showing distal centriole (arrowed). Scale bar = 0.2 µm.

Fig 3.14: Mid-region of mature spermatozoon showing position of centriolar apparatus and the outer doublet (od) and central singlet (cs) microtubules of the flagellar axoneme.

Scale bar = 0.2 µm.

Fig 3.15: Diagrammatic representation of the distal end of mature sperm flagella showing the coiling of the axoneme within an expanded portion of the flagellar membrane. The diagram shows the membrane tightly adhering to the microtubules, although sections a shows that an alternate form, where the membrane is expanded is often observed. The other transverse. sections (c -f), correspond to interpretation to the diagram. Scale bar = 0.2µm

Fig 3.16: Mature morula fixed immediately following the beginning of incubation with SMF. Scale bar = 10 µm.

Fig 3.17: Morula fixed at 20 minutes incubation with SMF *in vitro*. As beating commences the flagella become disorganized along their length but are still bound together at their distal ends. Sperm heads are beginning to detach from the cytophore (arrowed). Scale bar = 10μm.

Fig 3.18: Section of a sperm morula at 20 minutes incubation with SMF *in vitro*. The cytophore appears swollen and contains whorls of cytoplasm (arrowed), also the spatial distance between sperm heads has increased noticeably . Scale bar = 2 μm.

Fig 3.19: Section through a sperm morula following 30 minutes incubation with SMF. Relatively few sperm heads are associated with the cytophore and the cytophore appears to be undergoing lysis. Scale bar = 2 μm.

Fig 3.20: Free spermatozoa being liberated from a sperm morula after 40 minutes incubation with SMF *in vitro*. Scale bar = 10 μm.

Fig 3.21: Free spermatozoa following breakdown of the morula. Scale bar = 10 μm.

Fig 3.22: Remains of the cytophore are visible after 50 minutes incubation with SMF *in vitro* . Scale bar = 2 μm.

Fig 3.23: Fifty minutes after commencement of incubation with SMF, free spermatozoa are observed in association with coelomocytes. The fine structure of the sperm head and the swelling at the distal end of the flagella are visible (arrowed). Scale bar 2 μm.

Fig 3.24: Section through an aggregation of coelomocytes which contain several spermatozoa, sperm nuclei, acrosomes and flagella are visible (sn = sperm nucleus; a = acrosome; f = flagella). Scale bar 2 μm.

Chapter 4

Sperm Activation:

The Role of 20-Carbon Fatty Acids

4.1 Introduction

Lipids have been implicated in the activation of spermatozoa in *Arenicola* for some time. Howie (1961 a&b) noted that the lipid component of whole body homogenates could induce spawning and Bentley (1985; 1986b) later demonstrated that the chromatographic characteristics of SMF were similar to those of arachidonic acid. From these observations (see section 1.5.1 for detailed review), this chapter investigates the role of fatty acids in the activation of spermatozoa and looks specifically at the action of eicosapolyenoic (polyunsaturated 20 - carbon) fatty acids using a variety of biological and chemical techniques. Some of the work outlined in this chapter has been published recently (Bentley *et al.*, 1990).

Lipids can be classified as the fatty acids and their derivatives (Christie, 1987) and therefore a review of fatty acid structure and chemistry is usually included in general reviews of lipids as a whole. In the last twenty years these have included Christie (1982); Gurr & James (1980); Harwood & Russell (1984); Gunstone (1986); Mead *et al.*, (1986); Christie (1987). Such reviews generally include a section on nomenclature, and several schemes are seen to exist by which the structure of the fatty acid can be identified. To prevent confusion however, only one scheme will be used throughout this thesis and this will be outlined below.

Fatty acids generally contain an even number of carbon atoms in a straight chain with a carboxyl group at one extremity. Along the carbon chain double bonds are often present and these are usually in a *cis* configuration and are methylene interrupted (ie —C=CH—CH_2—CH=C—). The common fatty acids vary in chain length from 2 to 36 carbon atoms, and contain between 1 and 6 double bonds. These straight chain fatty acids are named according to a systematic nomenclature which identifies both the number of carbon atoms that make up the chain and the number of double bonds within it. The position of these bonds are indicated numerically with reference to the carbon atom from which they begin when counting

from the carboxyl group. Eicosanoic acid (arachidic) is therefore a 20 - carbon skeleton on which there are no double bonds, whereas 5,8,11,14 - eicosatetraenoic acid (arachidonic) is a twenty carbon skeleton which contains four methylene interrupted double bonds at carbons 5,8,11 & 14. Often, these acids are referred to using trivial names (see Robinson, 1982) and where these are relevant they will be referred to in parentheses. A drawback of this system however, is that it is not possible to derive structural information from the trivial name alone

4.2 Materials and Methods

4.2.1 Thin layer chromatography of SMF

The free fatty acids, eicosanoic, 11-eicosenoic, 11, 14 -eicosadienoic, 8,11,14 -eicosatrienoic, 11,14,17- eicosatrienoic, 5,8,11,14 -eicosatetraenioc, and 5,8,11,14,17 -eicosapentaenoic acids, were obtained from Sigma Chemical Co. Stock solutions (1×10^{-2}M) were prepared in HPLC grade methanol (BDH), and were stored under helium at -20°C until required for use. An aliquot of total prostomial lipid, and an aliquot of each of the 20-carbon (C20) fatty acid standards were applied to two cleaned and activated TLC plates using a 100 µl disposable micropipette. The solvent system used was the upper phase of; ethyl acetate : 2,2,4 trimethylpentane : acetic acid : water (45:25:10:50 v/v) (Salmon & Flower, 1982). The region of biological activity in prostomial lipids was then compared with the elution of the free fatty acid standards when the plate was visualised (see section 2.8).

Aliquots of prostomial lipids and free fatty acid standards were then applied to silver impregnated plates for argentation TLC. The same solvent system was used and again, the region of biological activity within the prostomial lipid sample was compared with the elution characteristics of the free fatty acid standards.

4.2.2 Assay of 20-carbon fatty acids

The sperm activating properties of C20 fatty acids were investigated *in vitro.* Aliquots of the stock solution used for TLC were diluted 100 fold in TFSW, to give a free acid concentration of $1x10^{-4}$ M and a solvent concentration of 1% (v/v) or less. This was taken as the starting concentration for the dilution series in the bioassay, and double dilutions of this were used to determine the biological activity of each acid *in vitro.* A total of nine replicate experiments were performed.

4.2.3 Effect of cyclooxygenase and lipoxygenase pathway inhibitors

The ability of prostomial homogenates to stimulate sperm activation in the presence of inhibitors of the cyclooxygenase and lipoxygenase pathway was investigated. Stock solutions of the cyclooxygenase inhibitors aspirin, indomethacin, and tolazoline at 10 mM, and the lipoxygenase inhibitors butylated hydroxytoluene and quecertin at 1 mM & 200 μM were prepared in TFSW.

Prostomial homogenates were prepared as described in section 2.5, except homogenisation of the prostomia was carried out in an aliquot of stock solution of a selected inhibitor. Individual prostomia were homogenised in this way in solutions of each inhibitor and were then assayed for SMF activity. A second series of experiments were performed where the prostomia were homogenised in the usual way, following which an aliquot of inhibitor was added to give a comparable concentration to that in the first experiment. Four prostomia were homogenised in 1ml TFSW and 100 μl aliquots were added to 300 μl of stock solution. In this way

the distinction can be made between the metabolism of fatty acid substrate by the prostomial homogenate and metabolism by the spermatozoa themselves.

In order to check for any non-specific SMF activity of the various inhibitors used in these experiments, dose responses on each were performed using the bioassay described in section 2.4.

4.2.4 Incubation of biologically active fatty acid with cyclooxygenase and lipoxygenase enzymes

Cyclooxygenase enzymes and soyabean lipoxygenase were obtained (see section 2.9) and incubated with biologically active fatty acid as described below.

a) Cyclooxygenase enzymes

Fresh bovine lung was incubated with arachidonic acid to synthesise a variety of products of the cyclooygenase pathway using a method outlined by Powell (1982). One gram of bovine lung was homogensied with 5 ml 0.05 M Tris HCl buffer, pH 7.4, on ice. One ml of this homogenate was incubated with arachidonic acid at a final concentration of $1x10^{-2}$ M, at 37°C for 5 minutes. The reaction was terminated by adding 5 ml ethanol and then 16 ml H_2O was added and the mixture centrifuged at 400 g for 10 minutes. The supernatant was then removed and an aliquot assayed for biological activity. A further sample was prepared for thin layer chromatography.

b) Soyabean lipoxygenase

Soyabean lipoxygenase (Sigma Chemical Co) was incubated with arachidonic acid to synthesise 15-L-Hydroperoxy 5,8,11,14 - eicosatrienoic acid (15 - HPETE). A total of 250,000 units were incubated with arachidonic acid at a final concentration of 5 x 10^{-3} M at 25°C for 15 minutes. After the incubation, the

reaction was terminated by heating, the extract was centrifuged, and the supernatant was assayed for biological activity. Parallel incubations containing denatured lipoxygenase and in the absence of lipoxygenase were also carried out.

Soyabean lipoxygenase was also incubated with 8,11,14 - eicosatrienoic acid. A total of 30,000 units were added to 3.0 ml 0.2 M borate buffer at a pH of 9.0 (0.2 M boric acid with pH adjusted with NaOH) which contained 3.0×10^{-4} M 8,11,14 - eicosatrienoic acid. The incubation was carried out at 25°C for three hours following which, the reaction was terminated by brief heating. Parallel incubations containing denatured lipoxygenase and in the absence of lipoxygenase were also carried out. Following the incubation, 2 M citric acid was added to give pH 4 - 5 and the lipids were extracted by the addition of two volumes (6 ml) chloroform. The organic layer from two extractions were pooled and the lipids were prepared for TLC following the method outlined in section 2.8.

c) Incubation of prostomial SMF with soyabean lipoxygenase

Aliquots of prostomium homogenate, each containing the equivalent of 0.36 prostomium were incubated with 250,000 units of lipoxygenase at 20°C for 60 minutes. After incubation, the extract was assayed for biological activity. Parallel incubations containing denatured lipoxygenase and in the absence of lipoxygenase were also carried out.

4.2.5 Incubation with bovine serum albumin (BSA)

The biological activity of prostomial homogenate, 8,11,14- eicosatrienoic acid and quinacrine were determined *in vitro*, in the presence of dissolved bovine serum albumin (BSA). Each dose response series was performed in BSA concentrations of 0, 0.1, 1 and 10 $mg.ml^{-1}$ respectively and the assay identified the minimum dose required to stimulate sperm activation in each of the BSA

concentrations used. Three experiments were performed and each experiment was replicated three times on sperm samples from a single animal.

4.2.6 Extraction of SMF on octadecylsilyl silica (ODS) cartridges

Sperm maturation factor, present in prostomial homogenate, was separated using Sep-Pak® C_{18} Cartridges (Waters Associates). Freshly prepared prostomial homogenates or free fatty acid standards were applied to a pre-wet cartridge . Pre-wetting was achieved by using 2 ml of methanol followed by 5 ml of H_2O before application of the sample or standard. These were applied to 10% aqueous ethanol with the pH adjusted to 4.0 using a 1 M stock solution of citric acid. Fractions were partitioned using solvent mixtures described in the protocol of Powell (1982): 20 ml 10% aqueous ethanol, 20 ml H_2O, 10 ml petroleum ether, 10 ml petroleum ether : chloroform (65:35 v/v) and 10 ml methyl formate. The fractions obtained using each solvent were collected and prepared for bioassay by removing the solvent and resuspending the lipids in TFSW as described in section 2.7. The Sep-Pak® was regenerated for subsequent use by flushing with 10 ml of 80% aqueous ethanol.

4.2.7 Gas-liquid chromatography of prostomial lipids

a) Sample collection

Gas-liquid chromatographic (GLC) analysis was carried out on two samples of prostomial lipids: one taken from animals collected on spring tides between March 7th to 12th 1989, and the second from animals collected between September 29th to October 2nd 1989. On each occasion two hundred and fifty animals were collected giving a total of 0.9653 g and 0.8348 g wet wt. of prostomia respectively. On each occasion, the coelomic cavities of a number of the animals collected were inspected for the presence of gametes. The prostomia were removed from the animals

obtained in each day's collection and were stored frozen in TFSW at -20°C under helium until required. Ten prostomia were retained for bioassay of SMF activity, the remaining prostomia were prepared for GLC.

b) Preparation of prostomial lipids

Prostomial lipids were extracted and prepared for gas chromatography using a modification of the method described in section 2.7. The prostomia were homogenised on ice in 5 ml TFSW and the lipids were extracted by the addition of chloroform : methanol (2:1 v/v). Cold methanol (8 ml) was added, prior to the addition of 16 ml chloroform. Between each stage the mixture was centrifuged in an MSE MISTRAL 3000 centrifuge at 6000 g for 10 minutes at 4°C. The organic layer was removed and washed as described in section 2.7. No repeat extractions or washes were performed on these samples for GLC. The solvent was removed in a rotary evaporator (Büchi RE 111 Rotavapor), 4 ml petroleum ether was added, and the sample sonicated to resuspend the lipids.

c) Methylation of prostomial lipids

The purified prostomial lipids were methylated using a method modified from Christie (1982). The sample was dissolved in 1 ml of dichloromethane, and refluxed for 2 hours with 2% methanolic sulphuric acid. After cooling, 4 ml saturated NaCl was added and the fatty acid methyl esters extracted with 2 ml petroleum ether (40°-60°C). Gas chromatographic analysis was performed using a Hewlett Packard 5890A gas chromatograph fitted with a flame ionisation detector (FID). Samples were separated on a capillary non-polar column (fused silica, 25m x 0.25 mm i.d., 0.12df, CP-sil 5CB) following on-column injection. A linear thermal gradient program from 90°-300°C at 20°C min^{-1} was used with helium as the carrier gas (25 $cm.s^{-1}$).

Using this protocol, the sample was partitioned, and the components were compared with the retention times of authenticated standards. As an internal standard, 8,11,14 - eicosatrienoic acid methyl ester was added to an aliquot of the sample. This helped in the identification of the retention time of the acid, and the isolation of a peak in the sample which corresponded to that of 8,11,14 - eicosatrienoic acid.

4.3 Results

The results of the experiments outlined in this chapter are shown in figures 4.1 to 4.5 and in tables 4.1 to 4.3

4.3.1 Thin layer chromatographic analysis of prostomial lipids

The elution characteristics of SMF present in prostomial homogenate in the solvent system described is identical to that of the C20 fatty acid standards (Rf 0.78 - 0.82), see figure 4.1a. Typically, the biologically active metabolites of C20 fatty acids, such as the prostaglandins, hydroxy-acids and thromboxanes are generally less polar and so in this solvent system would have Rf values lower than that of the parent fatty acid (e.g. 0.18 - 0.78) (see Salmon & Flower, 1982). In this TLC system it is not possible to separate the various fatty acids since they are identical in their polarity and hence in their TLC characteristics.

Argentation TLC permits the separation of such closely related molecules on the basis of their degree of saturation and using this technique, the active substance within prostomial lipids is shown to have identical elution characteristics to the two eicosatrienoic fatty acid standards (Rf 0.75 - 0.78)(see Fig. 4.1b). Argentation TLC separates C20 fatty acids, because the silver ions interact with the double bonds present on the carbon skeleton of the fatty acid molecule and therefore impedes its

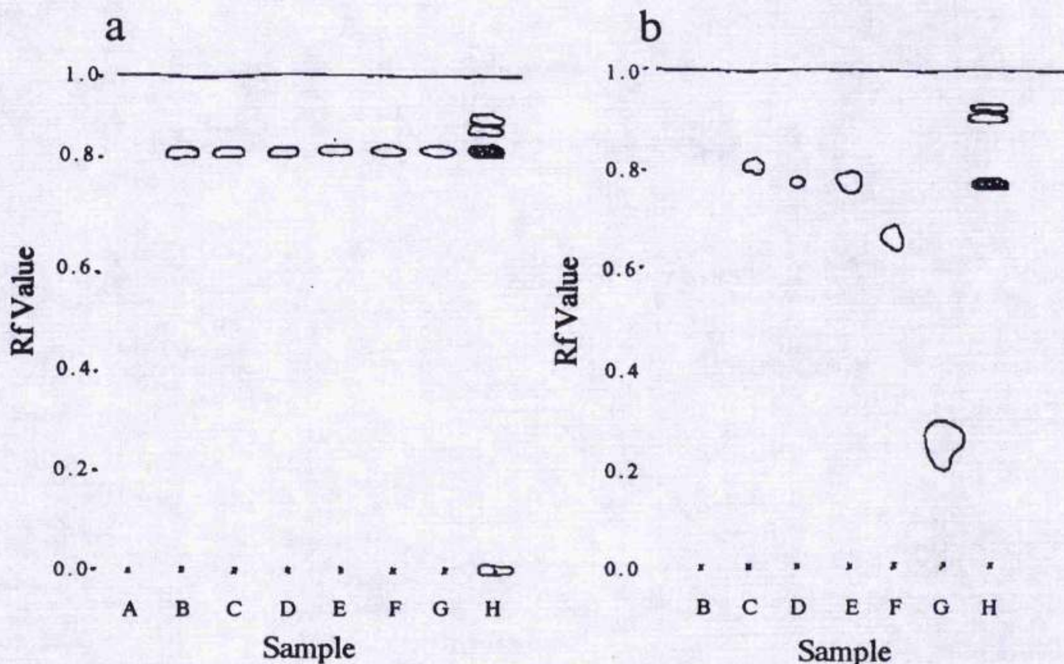


Figure 4.1a (left). Thin layer chromatography of 20-carbon fatty acids (spots A to G) and prostomial lipids (spot H). The active region of SMF in prostomial lipids (shaded) has an Rf value which is identical to that of the fatty acids (Rf 0.78 - 0.82), which in this TLC system all have identical chromatographic characteristics.

Figure 4.1b (right). Argentation TLC of 20-carbon fatty acids (spots B to F) and prostomial lipids (spot H). In this solvent system the silver ions interact with the double bonds on the carbon skeleton of the fatty acid and those with the greatest degree of saturation interact least and therefore migrate further up the plate. The active region in prostomial lipids (shaded) has an Rf value identical to that of the eicosatrienoic acid standards (Rf 0.75 - 0.78) The two eicosatrienoic acid standards cannot be separated however because they have the same number of double bonds.

Key: (A) eicosanoic, (B) 11, eicosaenoic acid, (C) 11,14 - eicosadienoic acid, (D) 8,11,14 - eicosatrienoic acid, (E) 11,14,17 - eicosatrienoic acid, (F) 5,8,11,14 - eicosatetraenoic acid, (G) 5,8,11,14,17 - eicosapentaenoic acid, (H) prostomial lipids. The solid line in both figures indicates the extent of the solvent front.

progress up the plate (see Christie, 1982). It is not possible however to separate the two isomers 8,11,14 - eicosatrienoic acid and 11,14,17 - eicosatrienoic acid, because they are identical in terms of their degree of unsaturation (both molecules contain 3 double bonds).

4.3.2 Bioassay of 20-carbon fatty acids

Table 4.1. Sperm activation by 20-carbon fatty acids.

	Fatty acid	Activity	Minumum effective concentration for sperm activation. (Mean ± s.e.m; n=9)
A	eicosanoic	—	
B	11 - eicosenoic	—	
C	11,14 - eicosadienoic	—	
D	8,11,14 - eicosatrienoic	+	$4.47 \pm 1.46 \times 10^{-5}$M
E	11,14,17 - eicosatrienoic	—	
F	5,8,11,14 - eicosatetraenoic	+	$2.28 \pm 1.78 \times 10^{-4}$M
G	5,8,11,14,17 - eicosapentaenoic	—	
H	TFSW (negative control)	—	
I	Quinacrine (positive control)	+	$7.33 \pm 1.68 \times 10^{-5}$M

The ability of the various C20 fatty acids to stimulate sperm activation *in vitro* is shown in Table 4.1. Of these, only two displayed biological activity: 5,8,11,14 -eicosatetraenoic (arachidonic acid) and 8,11,14 -eicosatrienoic acid. The mean minimum concentration required for a response was $2.28 \pm 1.78 \times 10^{-4}$ M and

$4.47 \pm 1.46 \times 10^{-5}$ M respectively, showing that 8,11,14-eicosatrienoic acid was about five times more biologically active in this system than 5,8,11,14 -eicosatetraenoic.

4.3.3 Studies of cyclooxygenase and lipoxygenase pathways

Inhibitors of either the cyclooxygenase and lipoxygenase pathways had no effect on the SMF activity of prostomial extracts, when added prior to, or following the homogenisation of entire prostomia. This result suggests that there is no conversion of the fatty acid to biologically active metabolites *via* the prostomium.

Dose response experiments indicated that two of the lipoxygenase pathway inhibitors, namely quercetin and aspirin, had non-specific effects on spermatozoa. At concentrations of 5 and 10 mM, aspirin caused sperm lysis and although the reasons for this are not clear, it is probable that this effect is not related to the lipoxygenase inhibitory property of the aspirin. Quercetin on the other hand, stimulated sperm activation at concentrations down to 1×10^{-5} M, although this appeared on a visual basis to be unlike natural sperm activation and therefore is thought to be unrelated to its properties as a lipoxygenase inhibitor (see section 8.7.1).

The incubation of fatty acid and/or prostomial homogenates (which contain SMF) with cyclooxygenase or lipoxygenase enzymes confirms the results obtained using the inhibitors of these enzymes. The incubations were carried out to examine whether there was a reduction, an enhancement, or the same level of sperm activation following the conversion of fatty acid substrates to metabolites. Table 4.2 shows that incubation with bovine lung (which contains cycloxygenase enzymes) and with soyabean lipoxygenase brought about a diminution in the incubated fatty acids ability to activate spermatozoa. This suggests that the fatty acid has been largely converted to the metabolites of these enzymes which are themselves unable to activate spermatozoa.

Table 4.2. The effects of cyclooxygenase and lipoxygenase enzymes on sperm activation by 5,8,11,14 - eicosatetraenoic acid

Incubation	Activity	Minimum effective concentration for sperm activation.
a) Cyclooxygenase enzymes:		
Arachidonic acid with bovine lung	—	
Arachidonic acid only	+	$1.25x10^{-5}$ M
Bovine lung only	—	
b) Soyabean lipoxygenase:		
Arachidonic acid with lipoxygenase	+	$2.5x10^{-3}$ M
Arachidonic acid only	+	$4.0x10^{-5}$ M
Soyabean lipoxygenase only	—	

Thin layer chromatography confirms the formation of metabolites, and incubation with bovine lung results in the formation of a series of products with Rf values of 0.62, 0.57, 0.52, 0.46, 0.44, 0.21, 0.19 and 0.14 respectively (Fig. 4.2a), whereas incubation with soyabean lipoxygenase results in the formation of 15-HPETE (see Hamberg & Samuelsson, 1967) with an Rf value of 0.62 - 0.60 (Fig. 4.2b). Thin layer chromatography also demonstrates that prostomial lipid extracts show no spots which correspond to either cyclooxygenase or lipoxygenase. metabolites which may have formed as a result of the action of endogenous enzymes.

The incubation of prostomial homogenate with soyabean lipoxygenase results in a total loss of SMF activity. The conversion of fatty acid in the prostomial homogenate to non-active 15-HPETE, suggests that it is the fatty acid component of prostomial homogenate which stimulates sperm activation *in vitro*.

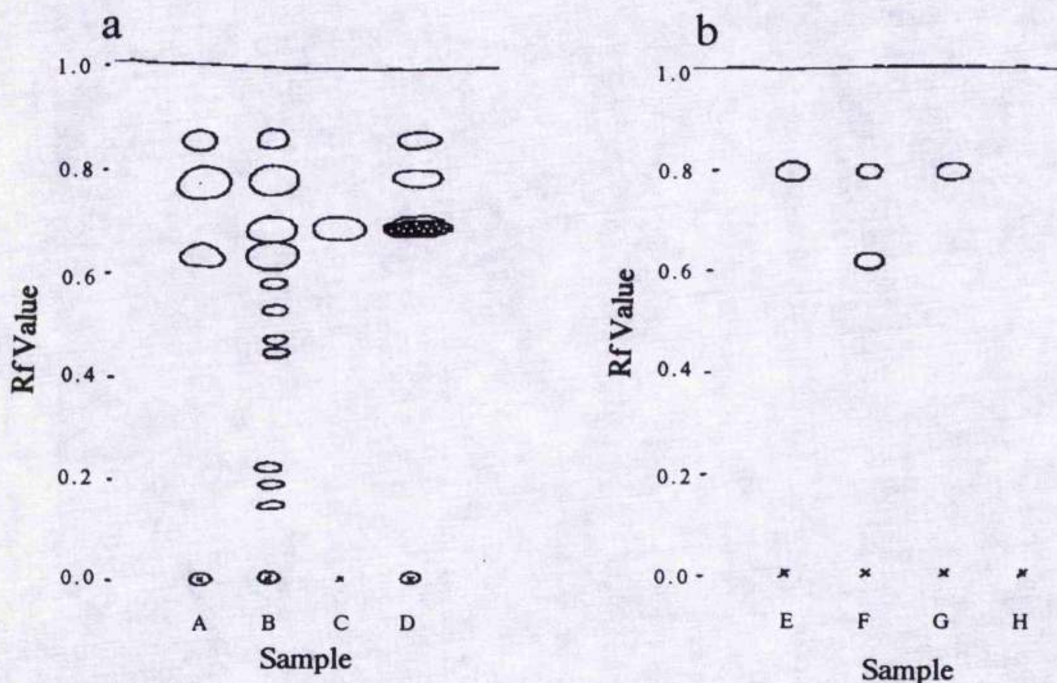


Figure 4.2a (left). Thin layer chromatography following incubation of fatty acid with cyclooxygenase enzymes. Samples are: (A) Bovine lung homogenate (containing cyclooxygenase), (B) 5,8,11,14 - eicosatetraenoic acid incubated with bovine lung, (C) 5,8,11,14 - eicosatetraenoic acid, and (D) prostomial lipids. The region of SMF activity in prostomial homogenates is indicated by shading and corresponds to an Rf value identical to that of the fatty acid standard.

Figure 4.2b (right). Thin layer chromatography following incubation of fatty acid with soyabean lipoxygenase. Samples are: (E) 8,11,14 - eicosatrienoic acid incubated with denatured soyabean lipoxygenase, (F) 8,11,14 - eicosatrienoic acid incubated with soyabean lipoxygenase, (G) 8,11,14 - eicosatrienoic acid, and (H) soyabean lipoxygenase.

In both cases, the figures indicate that prostomial lipids show no spots which correspond to either cyclooxygenase or lipoxygenase products that have arisen through the action of endogenous enzymes. The solid line in both figures indicates the extent of the solvent front.

4.3.4 Purification of SMF using ODS silica cartridges

After application of either SMF or 8,11,14-eicosatrienoic acid to pre-wet Sep Pak® cartridges, and following the protocol of Powell (1982), both are recovered from the same solvent fractions (see Table 4.3).

These cartridges are a simple form of low pressure reverse-phase liquid chromatography and the solutes are retained on the basis of hydrophobic interaction with the stationary phase (Powell, 1982). The fatty acids and their metabolites are separated from sugars, peptides or other polar lipids by elution with aqueous media containing relatively small amounts of miscible organic solvents (fractions 1-2). By removal of the water and elution with increasingly polar organic solvents, fatty acids and monohydroxy fatty acids can be separated from prostaglandins and other moderately polar substances (fractions 3-5). Petroleum ether : chloroform (65:35) and methyl formate, elutes fatty acids and monohydroxy fatty acids, and prostaglandins and thromboxanes respectively.

Table 4.3. Purification of prostomial SMF on ODS silica (Sep-Pak®) cartridges.

Fraction	Eluent from cartridge	Activity of prostomium extract	Activity of 8,11,14 - eicosatrienoic acid
1	20ml 30% ethanol	—	—
2	20ml H_2O (distilled)	—	—
3	10ml petroleum ether : chloroform (65 : 35 v/v)	+	+
4	10ml methyl formate	+	+
5	10ml 80% ethanol	—	—

Both the SMF activity of prostomial homogenate and 8,11,14 -eicosatrienoic acid are eluted from the cartridge by both petroleum ether : chloroform (65:35) and by methyl formate and are therefore of a similar chemical nature. That they are associated with two solvents and are recovered from the fraction expected to contain prostaglandins as well as that containing fatty acids is probably due to overspill and incomplete removal by petroleum ether : chloroform rather than the presence of both biologically active fatty acid and prostaglandins in prostomial homogenate.

4.3.5 Bovine serum albumin (BSA) incubations

During incubation with BSA, the biological activity of both prostomial SMF and of 8,11,14 - eicosatrienoic acid are inhibited in a dose dependent manner (see Fig. 4.3 a&b). This is in contrast to sperm activation by incubation with quinacrine (see Figure 4.3c) which at all concentrations is unaffected by the presence of BSA.

Fatty acids are known to interact strongly with serum albumin (Goodman, 1958; Nugteren, 1975) and therefore suggests that the active substance in prostomial SMF and 8,11,14 - eicosatrienoic acid are chemically very similar. Quinacrine on the other hand clearly has different chemical characteristics.

Figure 4.3. (next page) Minimum concentrations of prostomial homogenate (graph a), 8,11,14 - eicosatrienoic acid (graph b) and quinacrine (graph c) required to bring about sperm activation in the presence of bovine serum albumin. Both the fatty acid and prostomium homogenate concentration is decreased in a dose dependent manner whereas the dose of quinacrine required to stimulate sperm activation is independant of BSA concentration. Data shown are mean ± s.e.m.

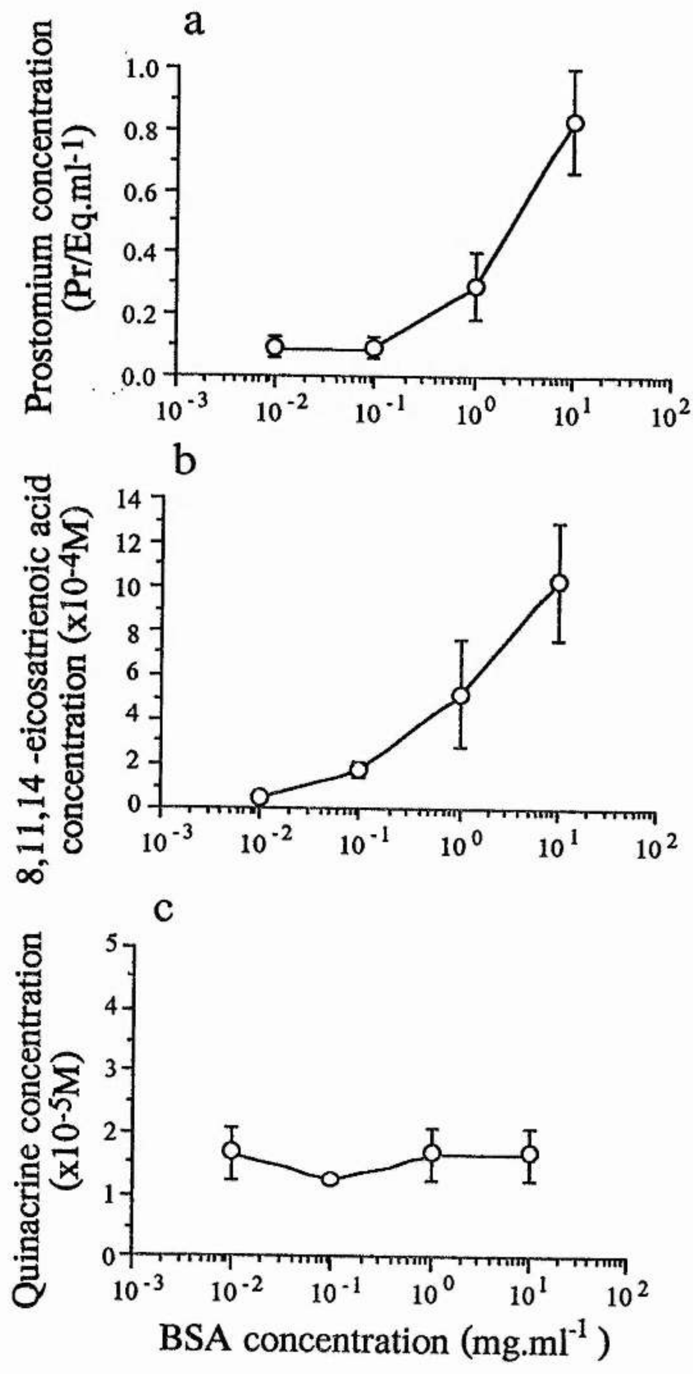


Figure 4.3

4.3.6 Gas-liquid chromatographic analysis of prostomial lipids

The prostomia taken from animals in March show no SMF activity, with the mean minimum dose required for a response being greater than 2.0 Pr/Eq. ml^{-1}. Examination of the coelomic cavities of these animals demonstrated that they are in the latent phase of their reproductive cycle because they contain no gametes. This is in contrast to the prostomia taken from animals during September - October 1989, the coelomic cavities of which indicted that they were close to spawning, (the coelomic cavities contained either fully differentiated sperm morula or oocytes of 180 μm). The prostomia from these animals displayed SMF activity at concentrations as low as 0.05 Pr/Eq.ml^{-1}.

Figure 4.4 shows the traces obtained from the GLC analysis of the two samples, and figure 4.5 shows the interpretation of these two GLC traces. Figure 4.5 compares the analysis of the sample (trace a) with that of authenticated standards (trace b) and with that of a sample which has been 'spiked' with a small quantity of 8,11,14 - eicosatrienoic acid as an internal standard (trace c). The retention times of

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Figure 4.5. (Before page 87) Diagrammatic representation of GLC traces showing the identification of fatty acid peaks in prostomial lipid samples (trace a) by comparison with methyl esters of authentic standards (trace b) and the addition of 8,11,14 - eicosatrienoic acid as an internal standard to prostomial lipids (trace c). The retention times of 5,8,11,14 - eicosatrienoic, 8,11,14 - eicosatrienoic and 11,14,17 - eicosatrienoic acids are indicated by the dotted lines 1,2 & 3 respectively. This demonstrates that a small area peak corresponding to the retention time of 8,11,14 - eicosatrienoic acid is present in the biologically active lipid sample (Sept - Oct 1989), but is not visible in the biologically inactive lipid sample (March 1989)(see traces a of both samples). Using this method, absolute retention times of peaks can be visually compared in relation to each other. This is of particular use when the small area peaks were not identified by the integrator and therefore absolute retention times were unavailable.

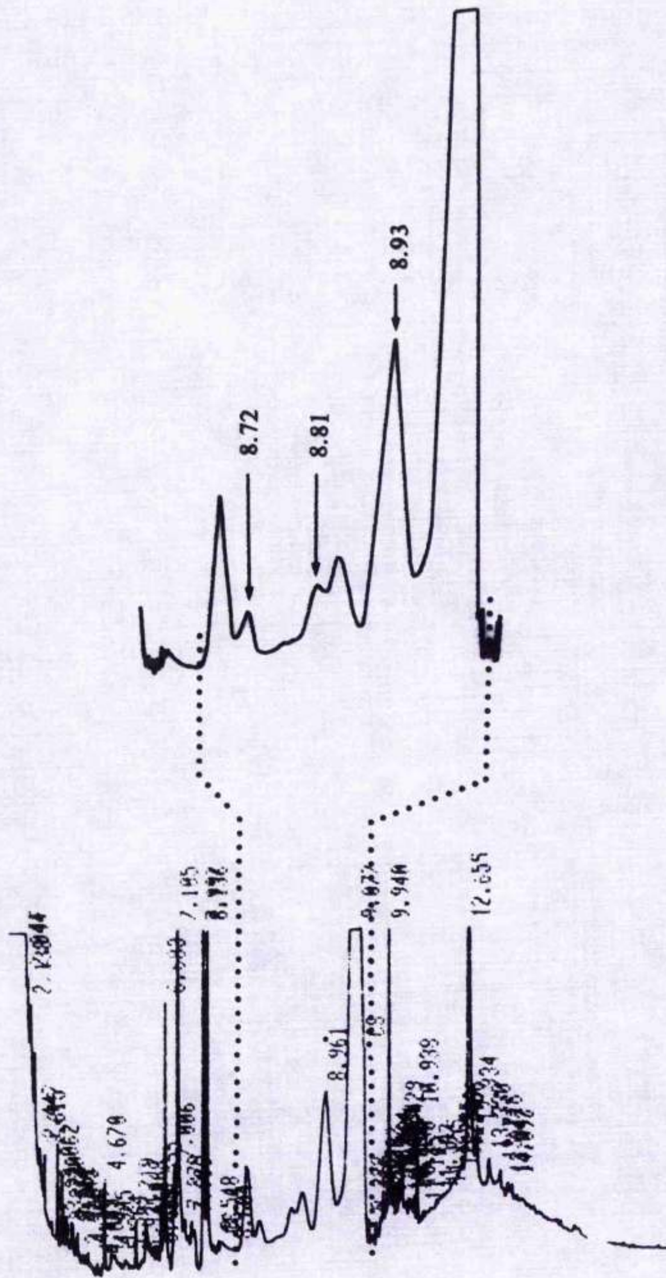

Figure 4.4a Gas chromatograph of fatty acid methy esters of biologically active prostomial lipids (Collected September - October 1989). In the sample, peaks çan be identified which have identical retention times to 5,8,11,14 - eicosatetraenoic acid (8.72 min), 8,11,14 - eicosatrienoic acid (8.81min), and 11,14,17 - eicosatrienoic acid (8.93min).

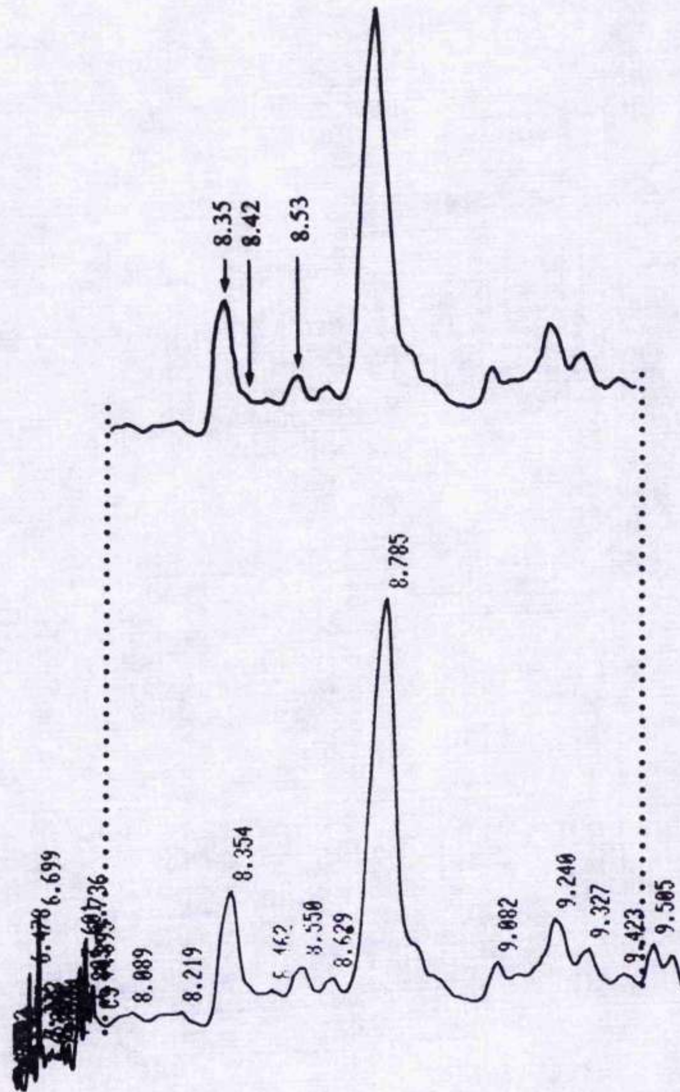

Figure 4.4b. Gas chromatograph of fatty acid methyl esters of biologically inactive prostomial lipids (collected March 1989). In the sample, peaks can be identified which have identical retention times to 5,8,11,14 - eicosatrienoic acid (8.35min), and 11,14,17 - eicosatrienoic acid (8.53min), but not 8,11,14 - eicosatrienoic acid (8.42).

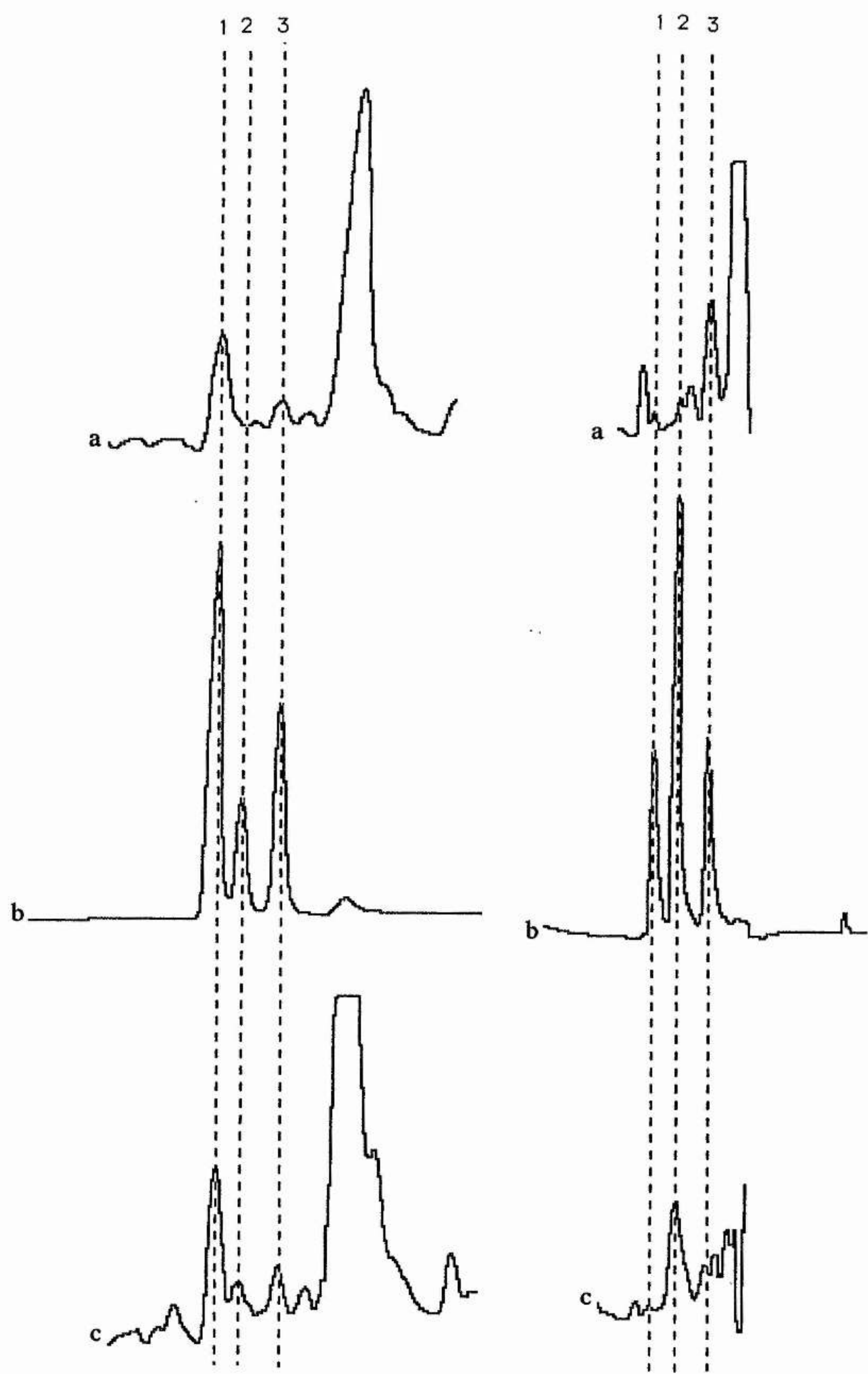

Figure 4.5

the components, when compared, identifies a small peak present within the October 1989 sample which has an identical retention time to 8,11,14 - eicosatrienoic acid. Such a peak cannot be identified in the March 1989 sample. Correlating these results with the data for the biological activity of the GLC samples suggests that in biologically active samples, there is a component which has the same GLC characteristics as 8,11,14 - eicosatrienoic acid, but this is absent in biologically inactive samples.

Peak identification by this method was considered to be the best way for trace analysis, because the quantities of biological material available were very small and automatic peak detection by the gas chromatograph was at the limits of its resolution. The integrator did not always identify some of the smaller area and size peaks, consequently precise numerical data as to the retention times of the peaks, and their percentage composition within the sample were not available.

4.4 Discussion

Eicosapolyenoic (20-carbon) fatty acids are a group of very important molecules which over the past twenty years have become the focus of increasing attention. Three of this family of acids are of particular interest because they are the precursors of a group of highly biologically active molecules, the eicosanoids (Corey *et al.*, 1980). There are four major groups of eicosanoids: the prostaglandins (PGs), the hydroperoxy and hydroxyeicosapolyenoic acids (HPETES or HETES), the leukotrienes (LTs) and the lipoxins (LXs). These molecules have been found to play an important role in many vertebrate (see Horrobin, 1978; Hansson, *et al.*, 1983; Samuelsson, 1983; Serhan, *et al.*, 1985; Das, 1987), and invertebrate systems (see Bundy, 1985; Srivastava & Mustafa, 1987; Stanley-Samuelson, 1987).

The fatty acids (a) 8,11,14 - eicosatrienoic acid (dihomo-γ-linolenic), (b) 5,8,11,14 - eicosatetraenoic acid (arachidonic acid) and (c) 5,8,11,14,17 - eicosapentaenoic acid are metabolised into eicosanoids *via* two major pathways. The biochemistry of both the cyclooxygenase and the lipoxygenase pathways is well understood and recent reviews include Gibson (1977); Samuelsson *et al.*, (1978); Hammarstrom (1983) and Mitchell (1990). A third pathway of arachidonic acid metabolism has recently been described and this has been termed the epoxygenase pathway (Capdevia *et al.*, 1981; Oliw *et al.*, 1982), but specific biochemical details of this pathway are little understood.

The synthesis of prostaglandins involves a number of steps in which oxygen is introduced at carbons 9,11 and 14, and a double bond is formed between carbons 8 and 12 to give a five membered ring. This involves the loss of two double bonds and the classification of prostaglandins is based, in part, upon the number of double bonds which remain in the aliphatic chain following their synthesis. Series one prostaglandins (which contain one double bond) are therefore produced from

8,11,14 - eicosatrienoic acid, and series two and three prostaglandins from 5,8,11,14 - eicosatetraenoic acid, and 5,8,11,14,17- eicosapentaenoic acid respectively. It is interesting that the two C20 fatty acids that are capable of activating sperm in *Arenicola* are those which give rise to series one and series two prostaglandins. Eicosapentaenoic acid, which gives rise to series three prostaglandins, and is considered to be the most widespread of these acids in the marine environment (Stanley-Samuelson, 1987), displays no biological activity. The reason for this is not clear, but it may be due to the particular configuration of double bonds within 8,11,14 - eicosatrienoic and 5,8,11,14 - eicosatetraenoic acids which is important in either their metabolism or reception.

Thin layer chromatography suggests that the biological activity in prostomial homogenates is associated with the parent C20 fatty acid and not with an eicosanoid metabolite. Argentation TLC indicates that the biological activity is with the eicosatrienoic acids in particular and bioassay of these acids indicate that of these only 8,11,14 - eicosatrienoic acid is biologically active. The use of inhibitors to cyclooxygenase and lipoxygenase enzymes and the use of the enzymes themselves confirms that the biological activity is with the fatty acid component of prostomium homogenate, and suggests that there is no extracellular metabolism of the fatty acid by the spermatozoa. The use of enzyme inhibitors of these pathways assumes however that polychaete enzymes which metabolise fatty acids, are the same as those observed in vertebrates. There is evidence that the precise nature of these enzymes may vary between phyla. For example, in the pond snail *Lymnea stagnalis*, the inhibitors indomethacin and aspirin fail to inhibit the synthesis of prostaglandins, which suggests that the prostaglandin synthetase (cyclooxygenase) differs from that of mammals and other invertebrates (Clare *et al.*, 1986).

Eicosanoids are often considered as being 'local hormones' and for the most part are short lived and usually exert their effects at their site of synthesis (Mead *et*

al., 1986). In the invertebrates they have been documented in several Phyla (see Bundy, 1985; Srivastava & Mustafa, 1987; Stanley-Samuelson, 1987) and in a number of instances are noted as being important in reproductive processes.

Prostaglandins are known to cause spawning of the abalone *Haliotis rufescens* and the mussel *Mytilus edulis* (Morse *et al.*, 1977; see also section 1.4.2), and a tri-hydroxy metabolite of arachidonic acid has been identified as the hatching factor in the barnacle *Semibalanus (Balanus) balanoides* (Clare *et al.*, 1982; 1985; Holland *et al.*, 1985).

Arachidonic acid has been implicated in starfish oocyte maturation and this has been reviewed in section 1.4.2. In the starfish, a purine 1-Methyl Adenine (1-MeAde) is released from the follicle cells of the ovary and acts on ooyctes to bring about their maturation. It was found however that arachidonic acid could mimic the action of 1-MeAde *in vitro* (Meijer *et al.*, 1986a) and that the action of this could be attributed to the metabolism of arachidonic acid to 8(R)-HETE by the oocyte (Meijer *et al.*, 1986b). That the action of arachidonic acid, but not 1-MeAde was inhibited by BSA, suggested that their mode of action was by different mechanisms and that the action of arachidonic acid was at the level of the plasma membrane. In this study, the action of both prostomial SMF and 8,11,14 - eicosatrienoic acid are inhibited by BSA in a dose dependent manner and suggests a similarity in their response.

Serum albumin is known to strongly bind fatty acids (Goodman 1958; Nugteren 1975) and therefore is of further support to the inference that 8,11,14 - eicosatrienoic acid and prostomial SMF are chemically identical. That the action of quinacrine in this system is not inhibited by BSA suggests that it is chemically quite different and that its mode of action, at least extracellularly, is not mediated by a fatty acid. Quinacrine was initially employed in its role as a phospholipase A_2 inhibitor (Hofman *et al.*, 1982) to investigate the possibility that fatty acids were perhaps

liberated from membrane phospholipids of the prostomium during homogenisation (Bentley - unpublished observations). It was found however to have a non specific effect on sperm activation at concentrations down to 10^{-5} M. The action of quinacrine in this system will be discussed further in section 5.4 (see also sections 6.6 and 7.4.1), although it is relevant to say here that it is thought to have no role in natural activation of spermatozoa.

During oocyte maturation in echinoderms, arachidonic acid is metabolized to 8-HETE (Meijer et al., 1986b). Whether 8,11,14 - eicosatrienoic acid is similarly metabolized by *Arenicola* spermatozoa must remain a possibility, although there is no evidence in this chapter to indicate that it is. It may appear unusual that it is a fatty acid rather than a metabolite which stimulates activation in spermatozoa. The action of fatty acids on cellular metabolic control mechanisms has, however, been observed in other systems. Arachidonic acid and other fatty acids have been noted to trigger oxidative metabolism in human neutrophils and inhibitor studies have revealed that this response is not mediated by a metabolite (see McPhail & Snyderman, 1984 for review). In other situations fatty acids are noted to have a direct effect upon cell processes and this includes the opening of potassium channels in ischemic cells (Donghee & Clapham, 1989) and in smooth muscle cells (Ordway *et al.*, (1989).

The results presented in this chapter suggest that the fatty acid 8,11,14 - eicosatrieonic acid has an important role in the activation of spermatozoa in *Arenicola marina*. To unequivocally demonstrate that this and prostomial SMF are identical however can only come from GLC-MS structural analysis of a purified sample. In order to achieve this, one of the many problems to overcome is to obtain sufficient starting material before purification can proceed. The capillary GLC techniques that are outlined in this chapter represent a significant advance over conventional GLC

methods in that it can detect much smaller concentrations. It may therefore be by this method that a full structural analysis is obtained.

During the remaining chapters of this thesis, the mode of action of SMF is compared to that of 8,11,14 - eicosatrienoic acid in an attempt to determine if they are comparable in terms of the elevation in respiration rate observed during sperm activation (Chapter 5) and their ability to induce spawning *in vivo* (Chapter 7).

Chapter 5

Sperm Activation:

Oxygen Consumption

5.1 Introduction

Chapter 4 identified a number of chemical factors that are capable of activating the spermatozoa of *Arenicola marina*, *in vitro*. It demonstrated that 8,11,14 -eicosatrienoic acid has identical chemical characteristics to SMF present in prostomial homogenates, and it indicated that other substances, such as quinacrine, are also capable of stimulating sperm activation. However, bioassay and dose response experiments, upon which such observations are based, supply very little quantitative data on anything other than the minimum concentration of these substances required to stimulate sperm activation.

Techniques which quantify sperm activation are of particular use because they allow the comparison of sperm activation under different conditions. An estimate of sperm motility during sperm activation for example, would provide data on the time that motility was acquired, and the level to which motility was stimulated following incubation with prostomial SMF and 8,11,14 - eicosatrienoic acid. Such data can be obtained using photographic (Makler, 1980), video (Katz & Overstreet, 1981), or laser (Steiner *et al.*, (1978); Pusch *et al.*, (1986)) techniques, but these are both specialised and expensive. An alternative, however, is to monitor the oxygen consumption that accompanies the increase in respiration rate as the sperm become motile. Because the ATP generated by respiration is used for motility, and the ADP produced by motility is required for respiration, then it seems likely that both motility and respiration may have the potential for being linked (Christen *et al.*, 1982). Therefore, it is possible that observations of respiration rate of spermatozoa bear a direct relationship to sperm motility, and consequently give an indication of the state of activation of the sperm themselves.

This chapter, therefore, describes the oxygen consumption of sperm as they are activated by prostomial SMF, 8,11,14 - eicosatrienoic acid and quinacrine in an attempt to investigate any differences in the response to these substances.

5.2 Materials and Methods

Details of the technique used to measure the oxygen consumption rate in the following experiments is outlined in section 2.10.

5.2.1 Oxygen consumption during sperm activation

The oxygen consumption of spermatozoa during incubation with prostomial SMF, 8,11,14 -eicosatrienoic acid and quinacrine at a final concentration of 1 Pr/Eq.ml^{-1}, $5x10^{-5}$M, $1x10^{-4}$ M respectively was investigated. The doses used were chosen from previous *in vitro* dose response experiments, and were typical mid-range doses sufficient to stimulate sperm activation. For compariative purposes, the response of spermatozoa during incubation with $1x10^{-2}$ M NH_4Cl was also investigated. Incubation with NH_4^+ has been carried out in a number of systems such as sea urchin spermatozoa (see Christen *et al.*, 1982), to elevate the pHi, and this parameter has been demonstrated to be important during sperm activation in a number of species (see section 1.3.2).

All observations were made for 80-100 minutes following the beginning of incubation. Stock solutions of quinacrine and NH_4Cl were made in TFSW and aerated to saturation. Prostomial homogenate was freshly prepared as described in section 2.5 and the required concentration of 8,11,14 -eicosatrienoic acid was obtained by the dilution of a frozen stock solution. Both prostomial homogenate and 8,11,14 - eicosatrienoic acid were brought to volume using sat.TFSW (fully air saturated triple filtered sea water).

Each test incubation was followed by a control incubation, and a chamber only incubation, where the test substance was injected in the absence of spermatozoa. Six replicate experiments were performed for each test substance and along with the controls gave a total of 72 experimental procedures.

In an attempt to relate oxygen consumption with the gross morphological changes which occur during sperm activation (see Chapter 3), light microscopic examinations of a parallel incubation were carried out throughout the observation period of each experiment.

5.2.2 *In vitro* response of spermatozoa to prostomial lipids

The effect of non-lipid components of prostomial homogenate and their effect on the sperm activation response was investigated. A crude prostomial homogenate, containing the prostomia from 14 sexually mature *Arenicola,* was divided into two equal aliquots, each of 700 µl and containing 7 prostomial equivalents. The prostomial lipids were extracted from one aliquot, (as described in section 2.7) and these were resuspended in 700 µl sat.TFSW. The oxygen consumption of replicate sperm suspensions, were determined in response to this 'prostomial lipid extract', and also to the unpurified aliquot of prostomial homogenate. During the lipid extraction procedure, it is assumed there is full recovery of prostomial lipids. Consequently, the doses of the two aliquots should be identical in terms of the total lipid content and have a final concentration of 1 $Pr/Eq.ml^{-1}$ following injection into the respirometry chamber.

An incubation with 8,11,14 -eicosatrienoic acid at a final chamber concentration of $5x10^{-5}$ M was also carried out. Control incubations were performed using the aqueous fraction which remained after the lipids had been removed from the crude prostomial homogenate. This extract was dried in a rotary evaporator at 30°C, and resuspended in 700 µl sat.TFSW. It contained some solid protein residues which were not collected or injected, as they may have blocked the capillary inlets and outlets of the chamber.

5.2.3 Dose response of spermatozoa to activating substances

The oxygen consumption of spermatozoa to dose responses of SMF (1.25 to 0.025 Pr/Eq. ml^{-1}); 8,11,14 -eicosatrienoic acid ($4.8x10^{-4}$ to $1.6x10^{-5}$ M); quinacrine ($1x10^{-4}$ to $1.05x10^{-6}$ M); and NH_4Cl ($5.7x10^{-2}$ to $4.5x10^{-3}$ M) were measured. The doses used, where possible, were over a range from higher than the dose required to stimulate sperm activation, to a dose lower than the threshold concentration which can elicit activation. This range was determined prior to the incubations in the *in vitro* assay, using aliquots of spermatozoa from a single animal.

Replicate sperm samples from a single animal were tested over each dilution series, but, because each substance required observations over usually 8 or 9 dilutions (equivalent to 4 days experimental time), a different animal was used for each series. A total of 31 experimental observations were carried out over the four substances tested.

In this experiment, the control incubations were doses which, in the *in vitro* bioassay, were lower than the threshold concentration required for activation. If such a dose showed evidence of sperm activation during incubation, lower doses were tested until the sperm remained unactivated throughout.

5.3 Results

The results of these experiments are shown in figures 5.1 to 5.6 and also in tables 5.1 to 5.3.

5.3.1 Oxygen consumption during sperm activation

The oxygen consumption during incubation with prostomial SMF (Fig. 5.1a), 8,11,14 -eicosatrienoic acid (Fig. 5.2a), quinacrine (Fig. 5.3a) and NH_4Cl

(Fig. 5.4a) is not uniform, but usually rises to reach a maximum value before returning to near basal levels by the end of the incubation period. In this respect, the oxygen consumption rates of sperm activated by prostomial SMF and 8,11,14 - eicosatrienoic acid are similar in a number of ways. First, in both cases the oxygen consumption at its maximum point is increased by a factor of 3.7 above the basal rate observed prior to the commencement of the incubation (Table 5.1). Secondly, the period of maximum oxygen consumption corresponds exactly to the time period, in parallel incubations, where spermatozoa are being liberated from the morulae and the sperm are becoming free swimming (see section 3.3.3). There are differences with respect to the time at which these events occur however, and maximum oxygen consumption during incubation with 8,11,14 eicosatrienoic acid occurs after 25 minutes (Fig. 5.2a), whereas, during incubation with prostomial SMF, it is observed some 60 minutes later, after 85 minutes incubation (Fig. 5.1a). During incubation with prostomial SMF there is also a period of some 50 minutes after the beginning of the incubation, when the oxygen consumption remains at basal levels. Such latency is not observed during incubation with 8,11,14 -eicosatrienoic acid and the reasons for this are discussed further in section 5.3.2.

During incubation with quinacrine and NH_4Cl, the sperm oxygen consumption is markedly different, and in both cases rises suddenly and rapidly, immediately following the commencement of incubation. Maximum values are reached after 10 and 15 minutes for quinacrine and NH_4Cl respectively (Fig. 5.3a & 5.4a), but unlike the incubation with prostomial SMF and 8,11,14 -eicosatrienoic acid, this does not correspond to the time at which sperm are becoming free-swimming (usually occuring after 20 to 30 minutes incubation). These incubations also stimulate a much greater rise in oxygen consumption, and mean increases of 9.9 fold and 5.5 fold above basal levels respectively are recorded (Table 5.1). In the case of quinacrine this corresponds well to the hyperactivation and more vigorous

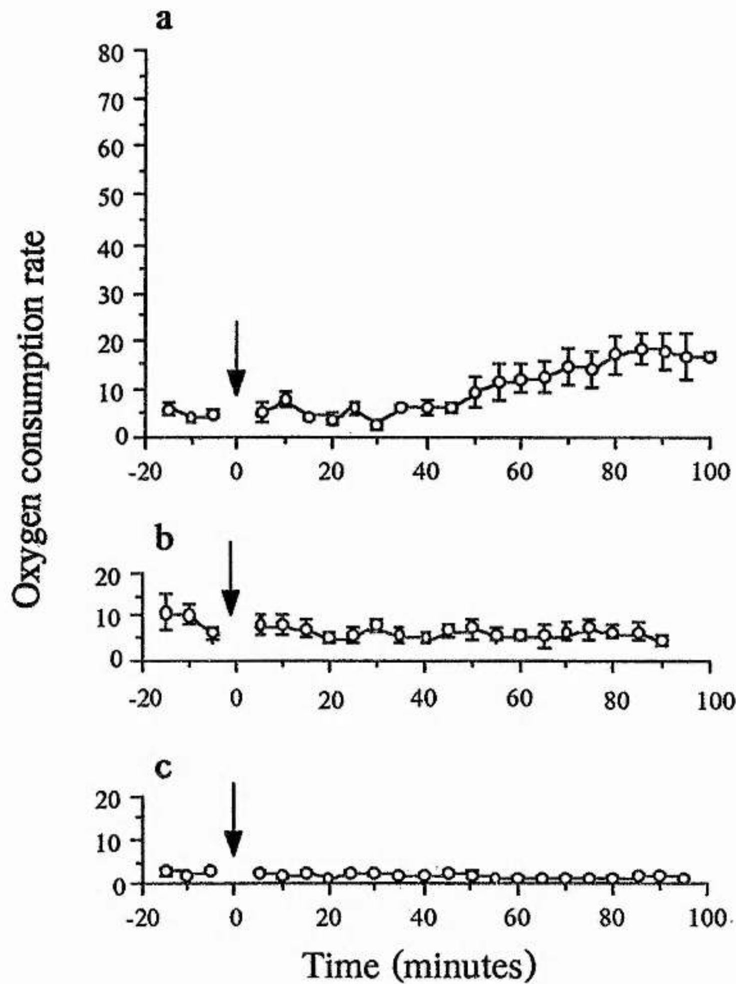

Figure 5.1. Oxygen consumption of spermatozoa during, (a) incubation with prostomial SMF, (final concentration 1 Pr/eq.ml^{-1}), and (b), control incubation of replicate sperm samples with TFSW. Graph (c) shows oxygen consumption of prostomial homogenate injected into the chamber in the absence of spermatozoa, (final concentration 1 Pr/Eq. ml^{-1}) showing no spontaneous oxygen consumption by the homogenate. In each case, the arrow denotes the time of injection and the data shown are the means of six replicate experiments ± s.e.m. Oxygen consumption is expressed as μl $O_2.10^8$sperm^{-1}.h^{-1} except for (c) which is expressed as μl O_2.h^{-1} in the absence of spermatozoa.

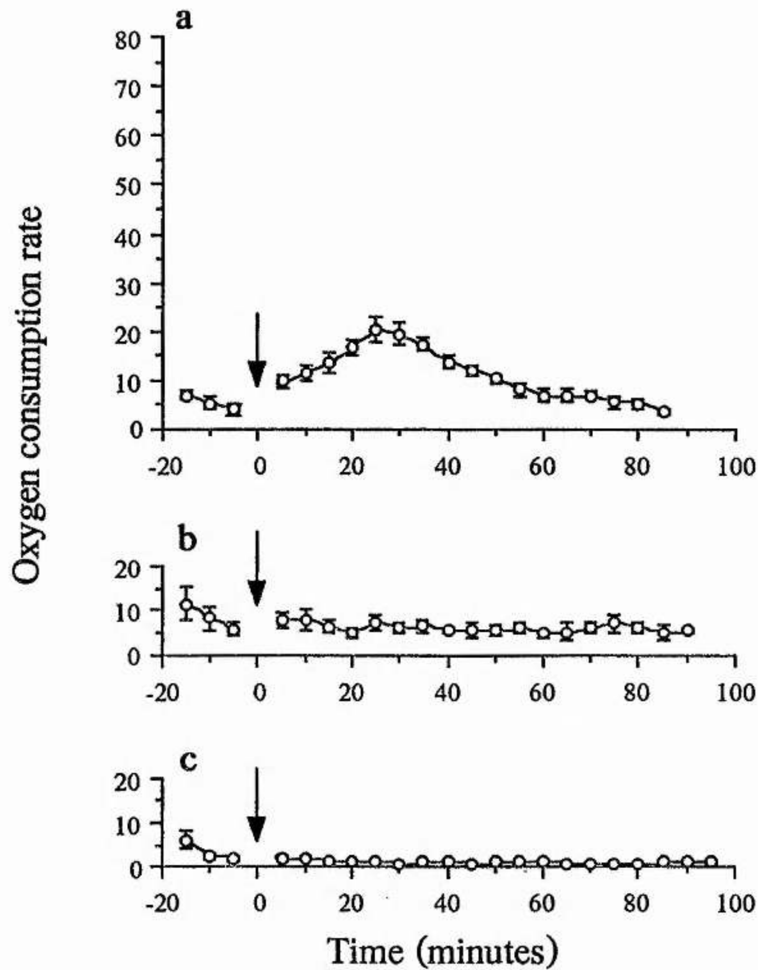

Figure 5.2 Oxygen consumption of spermatozoa during, (a) incubation with 8,11,14 - eicosatrienoic acid (final concentration $5x10^{-5}$ M), and (b), control incubation of replicate sperm samples with TFSW. Graph (c) shows no oxygen consumption resulting from autoxidation when injected into the chamber in the absence of spermatozoa (final concentration $5x10^{-5}$M). In each case, the arrow denotes the time of injection and the data shown are the means of six replicate experiments ± s.e.m. Oxygen consumption is expressed as µl $O_2.10^8 sperm^{-1}.h^{-1}$ except for (c) which is expressed as µl $O_2.h^{-1}$ in the absence of spermatozoa..

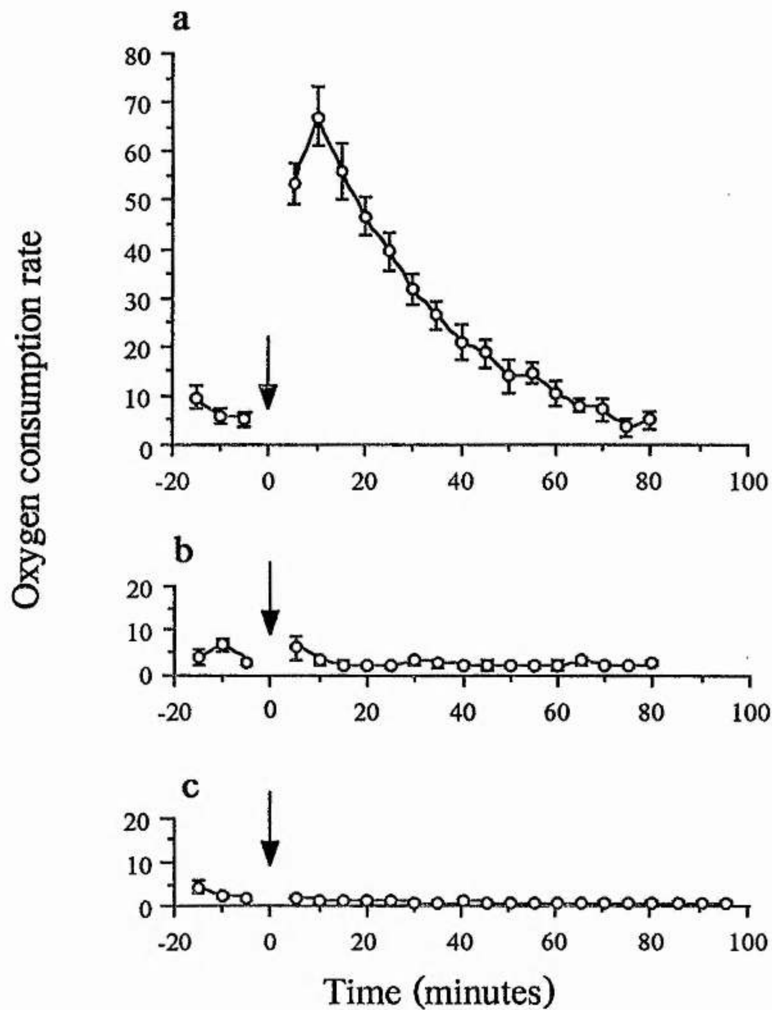

Figure 5.3 Oxygen consumption of spermatozoa during, (a) incubation with quinacrine, (final concentration 10^{-4} M), and (b), control incubation of replicate sperm samples with TFSW. Graph (c) shows oxygen consumption of quinacrine injected into the chamber in the absence of spermatozoa (final concentration 10^{-4} M), and shows no spontaneous oxygen consumption under these conditions. The arrow denotes the time of injection, and oxygen consumption is expressed as µl $O_2.10^8 sperm^{-1} .h^{-1}$ except for (c) which is expressed as µl $O_2.h^{-1}$ in the absence of spermatozoa. Data shown are the means of six replicate experiments ± s.e.m.

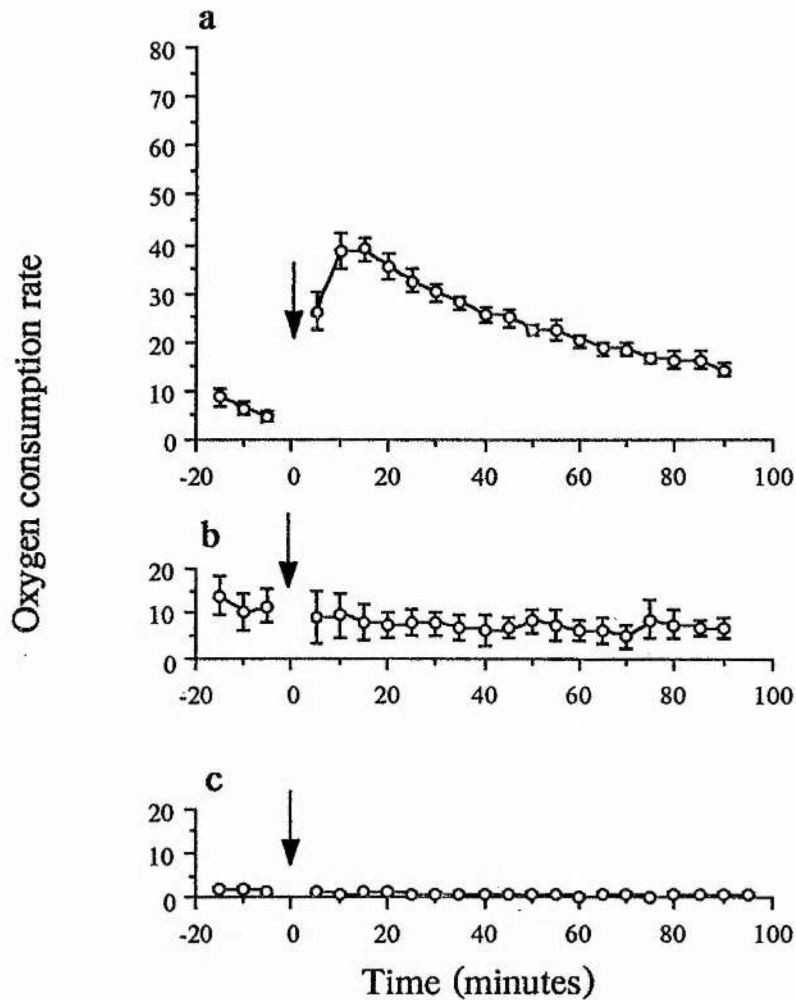

Figure 5.4. Oxygen consumption of spermatozoa during, (a) incubation with NH_4Cl, final concentration 10^{-2} M and (b), control incubation of replicate sperm samples with TFSW. Graph (c) shows oxygen consumption of NH_4Cl injected into the chamber in the absence of spermatozoa (final concentration 10^{-2}M), showing no spontaneous oxygen consumption by NH_4Cl under these conditions. In each case, the arrow denotes the time of injection and the data shown are the means of six replicate experiments ± s.e.m. Oxygen consumption is expressed as $\mu l\ O_2.10^8 sperm^{-1}.h^{-1}$ except for (c) which is expressed as $\mu l\ O_2.h^{-1}$ in the absence of spermatozoa.

motility pattern of spermatozoa that is observed when compared with other incubations.

Table 5.1: Summary of basal and maximum oxygen consumption rates, and the relative increase in oxygen consumption following incubation of spermatozoa with the substances listed. The oxygen consumption rate is expressed as $\mu l\ O_2 . 10^8\ sperm^{-1}.h^{-1}$ (mean ± s.e.m.). The basal rates of unactivated spermatozoa were calculated as the mean of the oxygen consumption rates determined prior to the beginning of incubation. The relative increase in oxygen consumption is the ratio of the maximum value observed to that of the corresponding basal rate. The time to maximum oxygen consumption is the mean time observed over six replicate experiments, and the ranges quoted, in parentheses, are the variations observed between the individual replicate experiments.

	OXYGEN CONSUMPTION			Mean time to
Incubation	Basal Rate (inactive sperm)	Max. Rate (active sperm)	Increase (Max / Basal)	max. rate (ranges)
Prostomial SMF	4.934 ± 0.601	18.285 ± 3.199	3.7	85 (55 - 95)
8,11,14 - eicosa-trienoic acid	5.567 ± 0.739	20.367 ± 2.536	3.7	25 (25 - 35)
Quinacrine	6.767 ± 1.410	67.100 ± 6.118	9.9	10 (5 -10)
NH_4Cl	6.639 ± 1.225	38.959 ± 2.244	5.9	15 (10 - 15)

Table 5.1 also shows that there is little variability between the time of maximum oxygen consumption observed during replicate experiments, with the exception of incubation with prostomial SMF. In these incubations the oxygen consumption maximum varies up to 40 minutes. Unlike incubations with 8,11,14 - eicosatrienoic acid, quinacrine and NH_4Cl however, a precise dose and purity of prostomial homogenate cannot be assured. The SMF content of the homogenate will vary according to, for example, the size of the prostomium, the sexual maturity

of the donor animal (Bentley, 1985), and the efficiency of the homogenisation. These parameters will vary between homogenates used and may account for the differences observed. This will be investigated further in section 5.3.2.

Figures 5.1b - 5.4b show that during control incubations there is no change in oxygen consumption above basal rate for the 90 minute observation period. Neither was there any change in oxygen consumption during blank (chamber only) incubations of any substance (Figs. 5.1c - 5.4c). This demonstrates that the increase in oxygen consumption observed in these experiments, is solely as a result of the activation of the spermatozoa and not the result of either spontaneous activation of the spermatozoa *in vitro*, autoxidation by fatty acids or unpurified homogenates, or any other chemical reactions or respiratory stimulation of contaminating substances, micro-organisms or bacteria.

In each case, the observation of parallel incubations indicated that sperm motility *in vitro* usually ceased within 30 to 40 minutes following activation. Therefore, with the exception of incubation with prostomial SMF, sperm were generally quiescent by the end of the incubation period.

5.3.2 The effect of non-lipid contaminants

This experiment investigates the effect of soluble proteins and other non-lipid contaminants on the sperm activation response in this experimental system. Figure 5.5a shows the response of spermatozoa during incubation with prostomial SMF. Figure 5.5b shows the response of a replicate sperm morula suspension, incubated with prostomial lipids extracted from a replicate aliquot of prostomial homogenate. The doses in terms of prostomial lipid content should be identical, the responses obtained however, were not.

The removal of non-lipid contaminants from a prostomial homogenate accelerates the sperm activation response. The peak of oxygen consumption is

advanced by 105 minutes by the removal of non-lipids , but is still 25 minutes later than that observed with 8,11,14 -eicosatrienoic acid (Fig. 5.5c).

The prostomial homogenate used in the incubation, stimulated an increase in oxygen consumption which was much later than that observed in the previous experiment (section 5.3.1). This may simply be due to differences in the composition of the homogenate between the two experiments, possibly in terms of dose and purity as already outlined.

Using the absolute maximum value of oxygen consumption as an index of the response time is perhaps not the most accurate comparison that can be made between the two treatments. The oxygen consumption rises rapidly during incubation with prostomial lipids (Fig. 5.5b), but rises more gradually during incubation with unpurified homogenate (cf. Fig. 5.5a). Table 5.2 therefore quotes values for the time taken for the oxygen consumption to increase by a factor of two above the basal rate. This reduces the latency of the response time between unpurified and purified prostomial homogenate to 55 minutes.

The response time of sperm morulae incubated with total prostomial lipids alone is not identical to that of 8,11,14 -eicosatrienoic acid, which is perhaps surprising if prostomial SMF and 8,11,14 -eicosatrienoic acid are the same substance. Whether the time of absolute maximum oxygen consumption, or the time taken for the rate to increase two fold is taken as a comparative figure, prostomial lipids have a 25 or 20 minutes later response time than 8,11,14 - eicosatrienoic acid, respectively. The presence of non-biologically active lipids within the purified homegenate may slow the reception of the signal or the metabolism of the fatty acid by the spermatozoa, possibly by acting competetively.

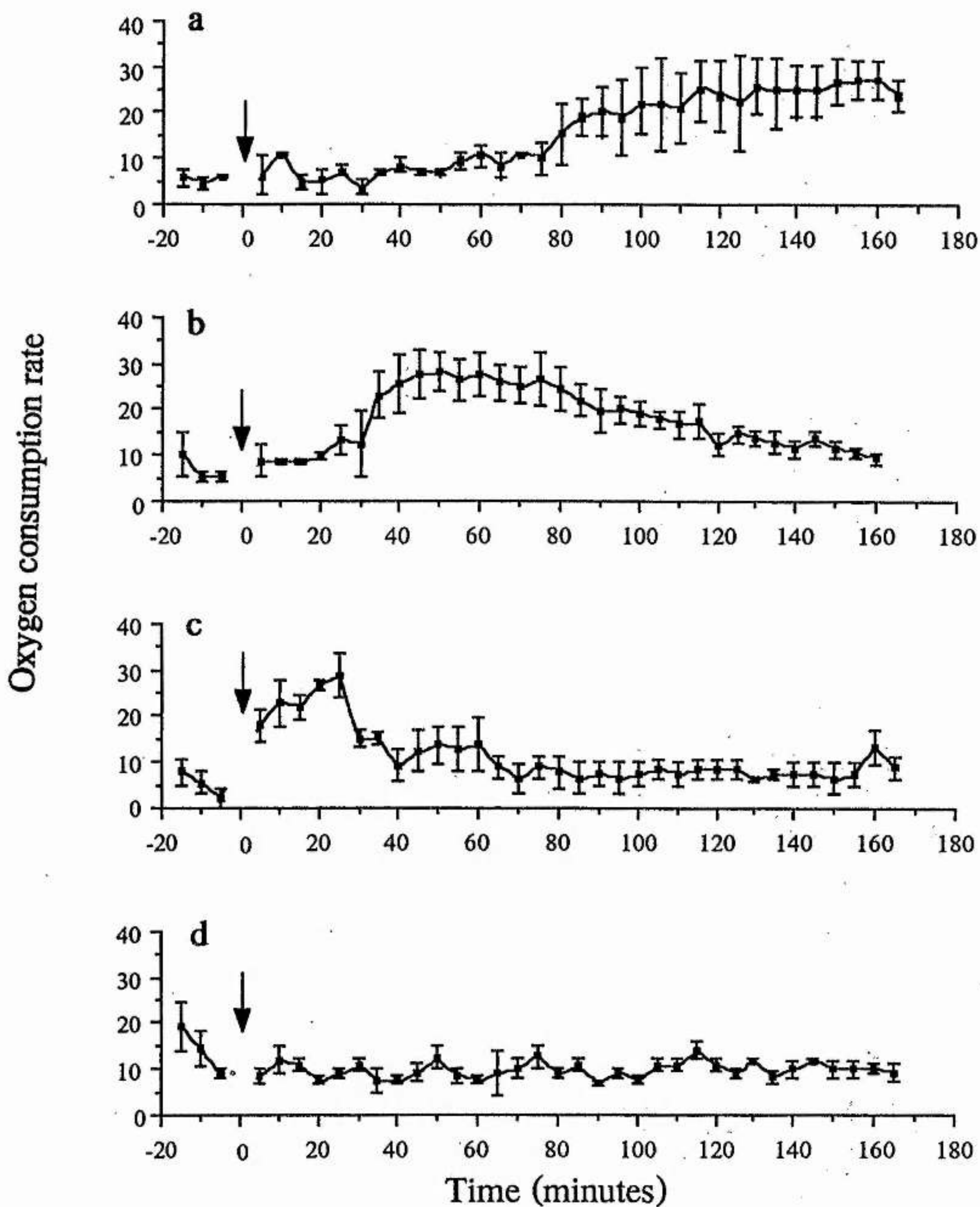

Figure 5.5 Oxygen consumption rates of spermatozoa during incubation with (a) prostomial homogenate (which contains SMF) and (b) the lipid component of prostomial homogenate, both at a final concentration of 1 Pr/Eq.ml^{-1}. Graph (c) shows incubation with 8,11,14 - eicosatrienoic acid (final concentration $5x10^{-5}$ M) and (d) shows a control incubation using the aqueous fraction of the lipid extract. In each case, the arrow denotes the time of injection and the data shown are the means of three replicate experiments ± s.e.m.

Table 5.2. Summary of basal and maximum oxygen consumption rate and the relative increase in oxygen consumption in during incubation of spermatozoa with prostomial SMF (crude homogenate), prostomial lipids and 8,11,14 - eicosatrienoic acid. Oxygen consumption is expressed as $\mu l\ O_2 .10^8$ $sperm^{-1}.h^{-1}$ (mean ± s.e.m.) and all times are expressed in minutes. The basal rate of oxygen consumption is calculated as in Table 1.

Incubation	OXYGEN CONSUMPTION Basal Rate (inactive sperm)	Max. Rate (active sperm)	Increase (Max / Basal)	Time to doubling	Mean time to Max. Rate (Ranges)
Prostomial SMF	5.753 ± 0.434	27.556 ± 4.230	4.8	80	155 (120 - 160)
Prostomial lipids	5.426 ± 1.671	28.072 ± 4.270	5.2	25	50 (40 - 70)
8,11,14 - eicosatrienoic acid	5.890 ± 1.707	28.903 ± 4.847	4.9	5	25 (15 - 25)

5.3.3 Dose response experiments

Variations in dose of activating substance has very little effect on the sperm activating characteristics, each substance has its own particular response time which variations in dose do not radically effect. The responses between quinacrine and NH_4Cl are very similar, in that they have a rapid response time over a wide range of activating doses (Fig. 5.6c & 5.6d). The upper limiting dose above which sperm activation would not occur, was in either case not found. Prostomial lipids and 8,11,14 -eicosatrienoic acid both have a narrow window of activating doses (Fig. 5.6a & 5.6b) with both upper and lower doses, above and below which sperm activation does not occur.

Such a fixed response time over a wide range of activating doses confirms that the sperm activation response is an all or nothing event. It was possible to

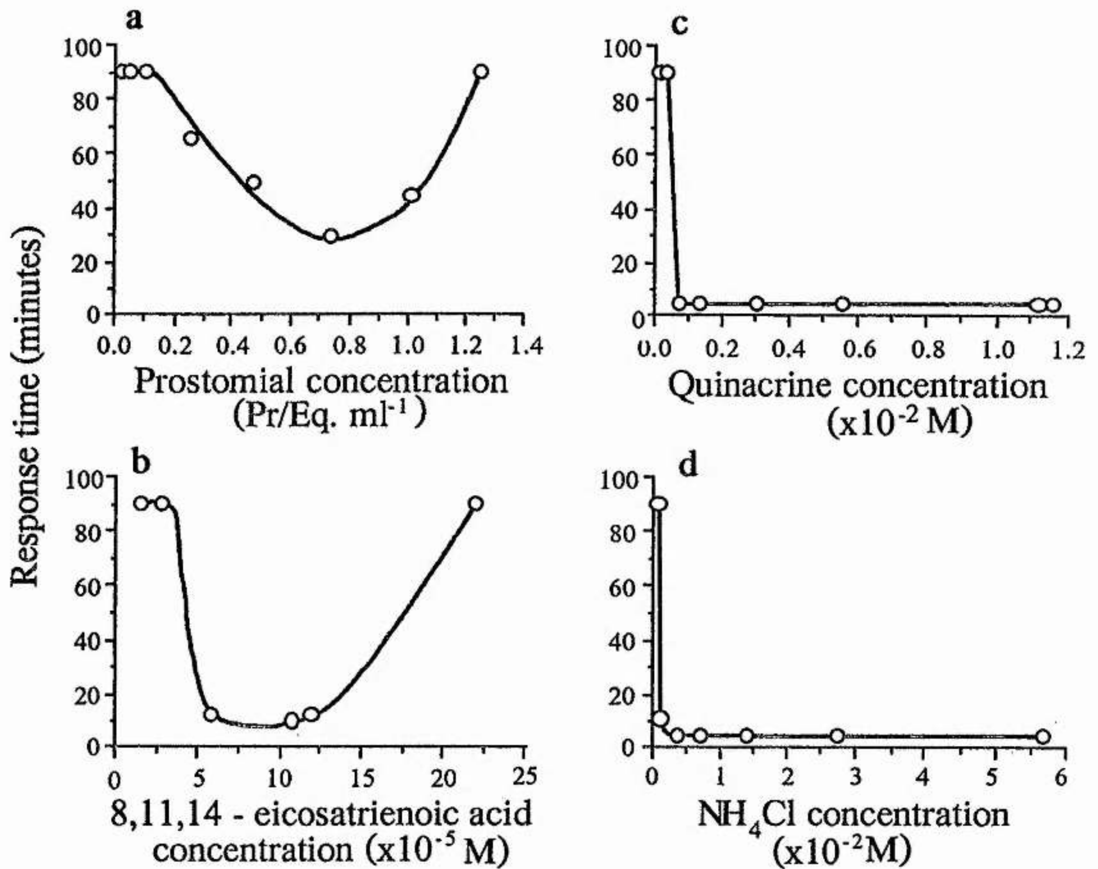

Figure 5.6. Response time of spermatozoa incubated with dilution series of (a) prostomial SMF; (b) 8,11,14 -eicosatrienoic acid; (c) quinacrine; and (d) NH_4Cl. The response time is defined as the period of time taken from the beginning of the incubation to the oxygen consumption increasing twofold above basal levels. The basal levels were calculated as previously described in Table 5.1. Each dilution series is based on replicate sperm aliquots from a single experimental animal.

perform only a single dose response on sperm morulae from one animal for each dose response series however, which means that comparisons between series may not be strictly valid. However, taking the response of each test substance in isolation demonstrates the fundamental 'all or nothing' properties of sperm activation.

5.4 Discussion

This experimental system allows the continous monitoring of sperm oxygen consumption during activation. Monitoring the oxygen consumption in this way identifies two classes of response. First, there is the response observed during incubations with prostomial SMF, and secondly the response obtained during incubation with quinacrine and with NH_4Cl. The response of sperm morulae to prostomial SMF and 8,11,14 -eicosatrienoic acid are very similar, although they differ with respect to the time scales over which sperm activation occurs. The effect of non-lipid contaminants on this has been demonstrated and it appears that the presence of non-lipids in prostomium homogenates serves to slow the response of sperm to SMF. Fatty acids have been shown to interact strongly with soluble proteins (Goodman, 1958, see section 4.3.5) and therefore their removal may make 8,11,14 -eicosatrienoic acid more readily available to the sperm. During transport *in vivo* it is unlikely that fatty acids would be found in their free form, but are usually bound to albumin or other proteins in a fatty acid-protein complex (Mead *et al.*, 1986). These function to solubilise the fatty acids for transport and act to maintain the actual concentration of fatty acid below that producing a haemolytic effect. Fatty acids then transfer from binding sites on the albumin, to sites on the cell membrane (Goodman, 1958). If internalised, the fatty acids are transported through the cytoplasm *via* binding to small molecular weight proteins, the fatty acid

binding proteins (Vahouny *et al.*, 1987). In this system, how the acid causes sperm activation, and whether it is internalised, metabolised, or interacts with an external receptor, remains to be investigated (see also section 4.4).

The response obtained during activation with quinacrine is very different in several respects from that obtained during incubation with the natural activator, prostomial SMF; the kinetics of activation are clearly not the same. Ammonium ions (NH_4Cl) and other weak bases increase the pHi of spermatozoa (Christen *et al*, 1982), and such a rise has been seen to stimulate respiration and activation of spermatozoa in a number of species (see section 1.3.2). The similarity of the activation by quinacrine, and with NH_4Cl suggests that quinacrine may be stimulating sperm activation by the same mechanism. The rise in oxygen consumption however is much greater than that observed after incubation with NH_4Cl, which may suggest a multiple stimulation for this substance.

In other studies monitoring the increase in respiration rate has been used to look at the biochemical and ionic requirements for sperm activation, and in many species there is a correlation between respiration rate and motility (Bohensack & Halangh, 1986; Halangh & Bohensack, 1986; Cardullo & Cone, 1986; Christen *et al.*, 1982). In some species however, this is not the case, rat spermatozoa show no lowering of their respiration rate when they are immobilised mechanically (Cardullo & Cone, 1986), and the sperm of *Xenopus laevis* shows no relationship between motility acquisition and oxygen consumption (Bernardini, 1988). Sperm activation in *Arenicola* however does appear to show a correlation with motility when activated by prostomial SMF and by 8,11,14 - eicosatrienoic acid, but not during activation by quinacrine or NH_4Cl.

In this study oxygen consumption has been used as a tool to look at the differences in response of spermatozoa to activating substances and, throughout this chapter, the oxygen utilisation by spermatozoa during activation has been termed

'oxygen consumption'. Presumably a proportion of this is a result of respiratory processes, but specific metabolic studies are needed to establish that fact. The oxygen consumption rates obtained in this study are comparable to the respiration rates of ejaculated rat, rabbit and ram spermatozoa in the study of Foley and Williams (1967), but much higher than that seen for motile sea urchin spermatozoa (Cardullo & Cone, 1986) (see Table 3). However, in many studies the way that the results are expressed makes direct comparisons difficult.

Other processes may contribute to the total oxygen consumption observed in this study, such as the biosynthesis of eicosanoids from fatty acids for example. The formation of eicosanoids following both the cyclooxygenase and lipoxygenase pathways are oxygen consuming reactions which result in the oxidation of fatty acids (see Gibson, 1977; Samuelsson *et al.*, 1978; Hammarstrom, 1983; and Mitchell, 1990) and that these may be involved in sperm activation by SMF and 8,11,14 -eicosatrienoic acid has already been discussed (see section 4.4). In the sea urchin egg, Perry and Epel (1985) described a calcium activated polyunsaturated fatty acid oxidase (probably lipoxygenase) which converts free fatty acids to hydroxy-fatty acids. In this system, the addition of arachidonic acid to egg homogenates resulted in a two to fourfold increase in oxygen consumption which could not by inhibited by cyanide. Similar enzymic conversion of 8,11,14 -eicosatrienoic acid by the spermatozoa in this system cannot be ruled out as a possible mode of action or mechanism through which the fatty acid exerts its effect, and by which oxygen is, perhaps, only in part being utilised.

Table 5.3: A comparison of sperm respiration and oxygen consumption rates in some animal species.

Species	Respiration rate	Notes	References
Sea urchin	8 µl O_2 10^8 $sperm^{-1}.h^{-1}$	Sperm alone	Cardullo & Cone (1986)
	0.9 ng atom O_2 .mg $sperm^{-1}.min^{-1}$	Sperm alone (pH 7.5)	Respaske & Garbers (1983)
	4.1 ng atom O_2 .mg $sperm^{-1}.min^{-1}$	Sperm plus 11mM NH_4Cl	
	17.5 µmole $O_2.min^{-1}$	Sperm alone	Christen *et al* ., (1983)
	0.5 µmole $O_2.min^{-1}$	Sperm alone	
	5.0 nmoles O_2 mg $sperm^{-1}.min^{-1}$	Sperm plus speract	Suzuki *et al* .,(1988)
Lugworm *(Arenicola marina)*	18.285 ± 3.199 to 27.556 ± 4.230 µl $O_2 10^8$ $sperm^{-1}.h^{-1}$	Sperm plus Prostomial SMF	This study
	4.934 ± 0.601 to 6.639 ± 1.225 µl $O_2 10^8$ $sperm^{-1}.h^{-1}$	Sperm alone (unactivated)	
Rat	20 µl $O_2 10^8$ $sperm^{-1}.h^{-1}$	Sperm in ringer	Cardullo & Cone (1986)
Rabbit	33 µl $O_2 10^8$ $sperm^{-1}.h^{-1}$	Sperm in ringer	Foley & Williams (1967)
Ram	30 µl $O_2 10^8$ $sperm^{-1}.h^{-1}$	Sperm in ringer	
Boar	19 µl $O_2 10^8$ $sperm^{-1}.h^{-1}$	Sperm in ringer	
	2.9 ± 0.6 µl O_2 $.10^8$ $sperm^{-1}.h^{-1}$	Sperm in ringer	Stone *et al* .,(1973)
	4.1 ± 0.6 µl O_2 $.10^8$ $sperm^{-1}.h^{-1}$	Sperm plus oviductal fluid	
Bull	2 - 4 nmol $O_2.min^{-1}$.µl $cells^{-1}$	Sperm alone	Bohensack & Halangk (1986)

Chapter 6

Sperm Activation:

Observations on pHi and ATP Levels

6.1 Introduction

The review of the biochemistry of sperm motility acquisition in section 1.3.2 identified several important biochemical events, which are important in its control and regulation. An elevation of intracellular pH (pHi) for example, is known to be associated with the onset of sperm motility in many species, but most notably in the spermatozoa of sea urchins (see Trimmer & Vacuier, 1986), and various mammals (Babcock *et al.*, 1983; Babcock & Pfeiffer, 1987). Levels of cellular adenosine triphosphate (ATP) on the other hand have been positively correlated with sperm motility and hence are critical in determining the fertilising ability of sperm (Bilgeri *et al.*, 1987). ATP levels are also thought to be important in the activation of sperm motility although it is not always clear whether effectors of motility act first on the flagellum, with metabolism responding to signals generated by the change in motility (such as a decrease in ATP concentration), or whether they change the rate of energy production with motility responding secondarily to the supply of ATP (Ford & Rees, 1990).

A wide variety of methods have been employed to investigate both of these parameters. Intracellular pH measurements have been made utilising several fluorescent probes and radiochemical methods and estimates of ATP have utilised both bioluminescent and chromatographic techniques. This chapter utilises the techniques of the fluorescent probe 9-amino acridine for the measurement of intracellular pH and the luciferin-luciferase bioluminescent assay for the measurement of cellular ATP, during sperm activation *in vitro*.

6.2 Materials and Methods: (pHi Determination)

Intracellular pH measurements were made using the fluorescent probe 9-amino acridine and specific details of the technique are given in section 2.11.

6.2.1 Fluorescent properties of incubation media

All solutions used in pHi determinations were scanned for any fluorescence activity (which may overlap with that of 9-amino acridine) using an excitation wavelength of 400 nm and scanning the emission from 300 to 600 nm. Similarly 9-amino acridine was assayed for effects on sperm activation, as a control to identify any non-specific sperm activating properties, before pHi determinations were carried out using this method. Double dilutions of 9-amino acridine from 10^{-3} M were incubated using the *in vitro* bioassay described in section 2.4.

6.2.2 Quenching of intracellular fluorescence

Determination of pHi by this method relies upon the property of 9-amino acridine to enter the cell, in its uncharged form, according to the trans-membrane pH gradient. The uptake of the fluorophore into the cell can be measured directly by monitoring the quenching of fluorescence as 9-amino acridine enters the cell. To test whether any 9-amino acridine fluorescence remains following the uptake of fluorophore into the spermatozoa, the fluorescence was measured both before and after the removal of spermatozoa from the incubation medium.

Dry sperm samples were loaded in 9-amino acridine for 30 minutes prior to fluorescence measurements and sperm were removed from spectrofluorimeter cuvettes to Eppendorf tubes and centrifuged at 6000 g for one minute to remove the sperm cells. Following centrifugation the supernatant was returned to the cuvettes for fluorescence measurements. A total of three determinations, using the sperm from three males, were carried out.

6.2.3 Uptake and binding characteristics

The movement of 9-amino acridine across the cell membrane occurs by passive diffusion (Christen *et al.*, 1982) at rates which are dependent upon the ionic constitution and pH of the medium in which the fluorophore is contained. The calculation of pHi requires the measurement of free cytosolic 9-amino acridine and therefore an accurate estimate of pHi can only be made once an equilibrium of fluorophore inside the cell has been reached.

Uptake and binding characteristics of 9-amino acridine in this system were therefore investigated by the sequential measurement of total intracellular concentration of the fluorophore (as observed through fluorescence quenching) and the proportion of this which is bound to intracellular binding sites (as observed following membrane permeabilisation). Three incubations, each of 30 ml volume, were carried out with sperm incubated with prostomium homogenate (1Pr/Eq ml^{-1}), NH_4Cl (10^{-2} M) both prepared in a stock solution of 9-amino acridine, and a control incubation of sperm, incubated with an equivalent volume of stock solution of fluorophore. Aliquots (3 ml) were removed from each incubation medium at five minute intervals and the fluorescence measured both before and after membrane permeabilisations. The fraction of fluorescence which is not recovered following membrane permeabilisation is that which is bound to intracellular binding sites within the cell. The sperm suspension was not pre-loaded with fluorophore and therefore time zero and the beginning of the incubation also signals the beginning of loading.

6.2.4 Parameters of intracellular binding

To determine the binding parameters of 9-amino acridine to intracellular binding sites, permeablised sperm aliquots (100 μl of c.10^8cells ml^{-1}) were added to increasing concentrations of fluorophore between $2.5x10^{-6}$ and $2.5x10^{-5}$M. The

decrease in fluorescence due to the binding of 9-amino acridine with intracellular binding sites was noted and the concentration of unbound fluorophore remaining calculated with reference to a standard curve of known 9-amino acridine concentration. In that way the concentration of bound fluorophore could be determined.

6.2.5 Determination of pHi during sperm activation

Three aliquots of 9-amino acridine stock solution (10 ml), containing dry sperm (c.10^8 cells ml^{-1}), were left at 14°C for 30 minutes to facilitate loading of the fluorophore into the cells. Incubations were then commenced by the addition of 1ml of either prostomium homogenate or NH_4Cl, prepared in stock solution of 9-amino acridine and added to give final concentrations of 1Pr/Eq.ml^{-1} or 10^{-2} M. Incubations with quinacrine were not carried out because it was found that quinacrine had similar fluorescent properties to that of 9-amino acridine (see section 6.4.1).

A control incubation consisted of the addition of an equivalent volume of fluorophore, and all incubations were left at 14°C for the duration of the experiment. Aliquots (3 ml) were removed at 30, 50 and 70 minutes and the relative fluorescence of intact, membrane permeablised sperm were measured and the values used to calculate the intracellular pH of the sperm at this time. A total of five incubations using sperm aliquots from five separate males were carried out.

6.3 Materials and Methods: (Measurement of ATP)

ATP concentration was determined using the luciferin-luciferase bioluminescent assay system which is described in detail in section 2.12.

6.3.1 Stability of luciferin-luciferase bioluminescence

In order to determine the stability of bioluminescence produced in this assay system, five standard solutions of ATP concentration (1.0×10^{-6} - 1.0×10^{-10} M in ten fold steps) were subject to an assay and the bioluminescence produced measured repeatedly over the following 3.5 hours. Measurements were made at 0, 30, 65, 85, 100 and 225 minutes following the commencement of the assay.

6.3.2 Extraction of ATP

The optimum trichloroacetic (TCA) concentration required to extract ATP from live spermatozoa was determined following the methods of Lyons *et al.*, (1986). Extractions using replicate aliquots of dry sperm were carried out using TCA over a concentration range (2 - 13% (w/v)). An equal volume of TCA was added to 100 µl aliquots of sperm suspension (10^8 sperm ml^{-1}) and extractions were performed at room temperature for ten minutes. Prior to ATP assay, the sperm were removed by centrifugation at 6000 g for ten minutes and the supernatant was diluted 50 fold to reduce the TCA concentration to less than 0.1% (v/v). A total of three such extractions were performed using the sperm from three sexually mature *Arenicola*.

6.3.3 ATP levels during sperm activation

Using the optimum TCA concentration for the extraction of cellular ATP in this system (see section 6.3.2), ATP levels in spermatozoa were determined during sperm activation by incubation with prostomial SMF. Dry sperm were diluted to

give a concentration of 10^8 sperm ml^{-1} and an incubation volume of 16 ml. Standard aliquots of 100 μl were removed at ten minute intervals and ATP was extracted and assayed. Prior to the beginning of the incubation, three aliquots were assayed and following the addition of prostomial SMF, ATP levels were determined for 100 minutes.

Prostomial SMF was added in a total volume of 1ml to give a final concentration of 1 Pr/Eq.ml^{-1} and the concentration of spermatozoa both before and after its addition were determined in order to allow for the correction of the ATP concentration for a standard number of cells. The ATP levels of sperm in a control incubation running in parallel were also determined. In the control incubation, 1ml of TFSW was added at time zero.

6.4 Results: (pHi)

6.4.1 Bioassay of 9-amino acridine

Bioassay of 9-amino acridine demonstrates that it causes activation of spermatozoa above concentrations of $4.58 \pm 1.667 \times 10^{-5}$ M (8.88 mg/litre). This does not preclude its use in these experiments however, because this concentration is much higher than the tracer quantities used in pHi determinations (1 μg/litre or 4.33×10^{-9} M).

Of the solutions which could be used to activate spermatozoa during pHi determinations, only quinacrine was found to exhibit fluorescent properties. At the excitation wavelength of 400 nm, the emission maximum of quinacrine is c.495 nm. At the emission maximum of 9-amino acridine used for pHi determination (c. 450 nm) the fluorescence of 1×10^{-5}M quinacrine solution was 50 % of this value whereas that of a 1×10^{-4}M was equivalent to 80%. Such residual fluorescence is sufficient to preclude the use of 9-amino acridine for pHi measurement of quinacrine

activated spermatozoa at this wavelength (Fig. 6.1). At the second emission maximum of 9-amino acridine, c. 430 nm, there is only a small amount of fluorescence (< 5%), emitted by quinacrine solutions of 10^{-4} or 10^{-5}M. The use of this wavelength for pHi measurement therefore remains a possibility, however, additional problems may exist and these will be discussed in section 6.6.1.

The fluorescence produced by a solution was quantified in terms of relative fluorescence, based upon an arbitrary pre-set value. In these experiments a 90% relative fluorescence is equal to the maximum fluorescence of a 9-amino acridine solution (1 μg / litre) at an excitation wavelength of 400 nm, and an emission wavelength of 452 nm.

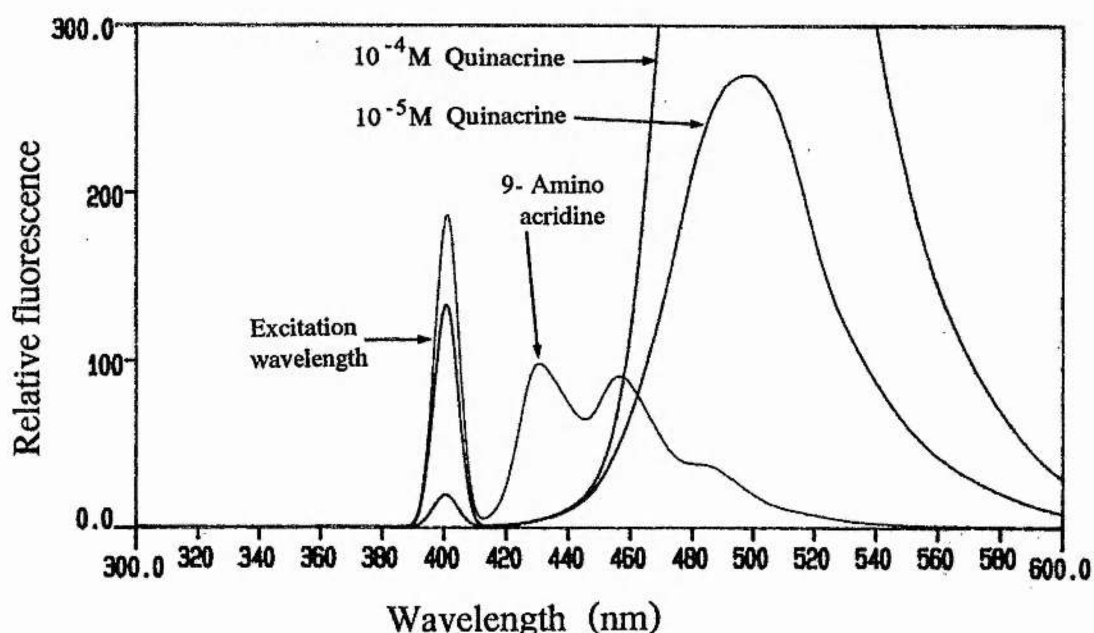

Figure 6.1: Emission scan of 9-amino acridine (1 μg/l) and quinacrine (10^{-4} & 10^{-5} M) with excitation fixed at 400 nm and emission measured between 300 and 600 nm. Maximum 9-amino acridine fluorescence is observed at c. 430 and 450 nm and the value observed at 450 nm was used to calibrate the instrument to 90% relative fluorescence. Maximum quinacrine fluorescence is observed at a single wavelength of c. 495 nm.

6.4.2 9-amino acridine as a probe to measure pHi

a) Quenching of intracellular fluorescence

As 9-amino acridine is loaded into spermatozoa there is a decrease in the fluorescence of the incubation medium as the extracellular concentration of fluorophore declines. The removal of fluorophore loaded spermatozoa from a solution of 9-amino acridine has little effect on the relative fluorescence of the supernatant, and therefore demonstrates that the uptake of fluorophore into cells quenches its fluorescence (see Table 6.1).

Table 6.1: Fluorescence measurements of 9- amino acridine loaded sperm suspension prior to and following sperm removal, demonstrating that uptake of fluorophore intracellularly completely quenches its fluorescence. Loading of 9-amino acridine was carried out by sperm incubation in fluorophore (1 μg/l) for thirty minutes before fluorescence measurements began.

Fluorescence prior to sperm removal	Fluorescence following sperm removal	Percentage change in fluorescence
69.26	69.34	0.12
70.46	71.20	1.04
76.38	82.58	7.51

b) Intracellular binding sites

Figure 6.2 shows the binding characteristics of permeable sperm to 9-amino acridine over a range of concentrations. There is a linear increase in the amount of 9-amino acridine bound up to a concentration of 1.5×10^{-5}M, which suggests that there is an excess of intracellular binding sites below this value. Therefore over the range of cytosolic 9-amino acridine concentrations at which pHi determinations are

made, a suitable equilibrium between the fluorophore which is bound and that which is cytosolic should exist.

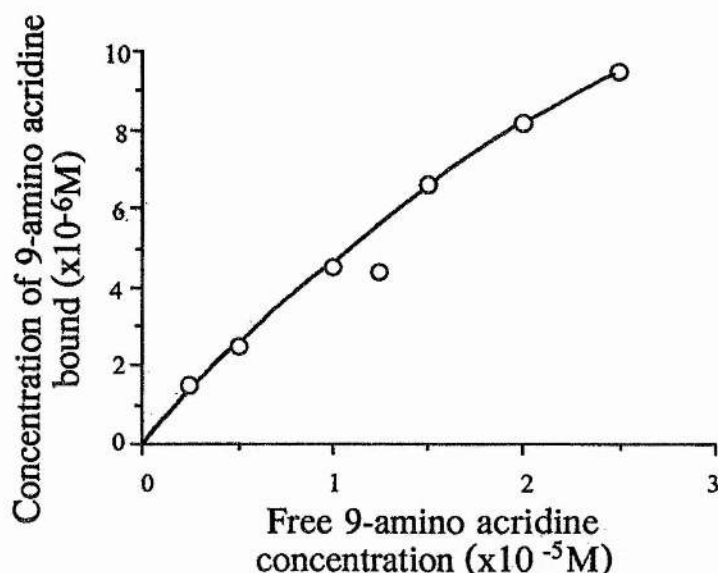

Figure 6.2: Intracellular binding of 9-amino acridine in *Arenicola* sperm showing a linear increase in binding up to a concentration of 1.5×10^{-5} M. The concentration of bound 9-amino acridine was determined by measuring the residual fluorescence, after fluorescence quenching following membrane permeabilisation with Triton X-100 (v/v) and calculating the remaining free 9-amino acridine concentration with reference to a standard curve.

c) Uptake and binding characteristics of 9-amino acridine into sperm

Figure 6.3 shows the uptake curves of 9-amino acridine into spermatozoa and the calculated values for pHi for each incubation based on these data. Figures 6.3a - c indicate the proportion which is bound to intracellular binding sites, during incubation with TFSW and with prostomial SMF and NH_4Cl. Binding of 9-amino acridine to intracellular binding sites (predominantly to cellular DNA (see Christen *et*

al, 1982)) remains constant throughout the incubation. As the incubation proceeds however, the amount of free intracellular 9-amino acridine increases until an equilibrium is reached. Incubation with TFSW control and prostomial SMF in these experiments, show that equilibrium is achieved 30 - 40 minutes after commencement of the incubation with 9-amino acridine (Fig. 6.3a & Fig. 6.3c) and, in the case of NH_4Cl, the equilibrium was established relatively quickly, after about 20 minutes (Fig. 6.3e).

d) Calculation of pHi

Figure 6.3 (b,d,f) shows the pHi values calculated at each time point from the uptake, and binding data (Figure 6.3a,c,e) obtained during the incubation. This demonstrates that only after complete loading of the fluorophore are pHi values stable and therefore accurate. In the case of incubation with TFSW (Fig. 6.3b) a value of pH 7.5 was recorded 40 minutes after the commencement of the incubation, and pH 7.6 after a similar incubation with prostomial SMF (Fig. 6.3d). Intracellular pH values determined prior to this for each incubation are higher which is a function of the low intracellular concentration of fluorophore before establishing equilibrium. In the case of incubation with NH_4Cl, equilibrium was established

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Figure 6.3 (next page) Uptake, binding and pHi of sperm suspensions incubated with TFSW (a & b); prostomial SMF (1 Pr. Eq/ml^{-1}) (c & d); and NH_4Cl (10^{-2}M)(e & f). Left hand figures (a,c,e) show quenching of 9-amino acridine solution during the loading of fluorophore into the sperm (open circles) indicating the total 9-amino acridine uptake. The proportion of intracellular 9-amino acridine which is not recovered, following membrane permeabilisation, indicates that which is attached to intracellular binding sites (closed circles). Right hand figures (b,d,f) show the corresponding values for pHi, at each time point, calculated from the intracellular concentration of fluorophore concentration, determined in the left hand figure.

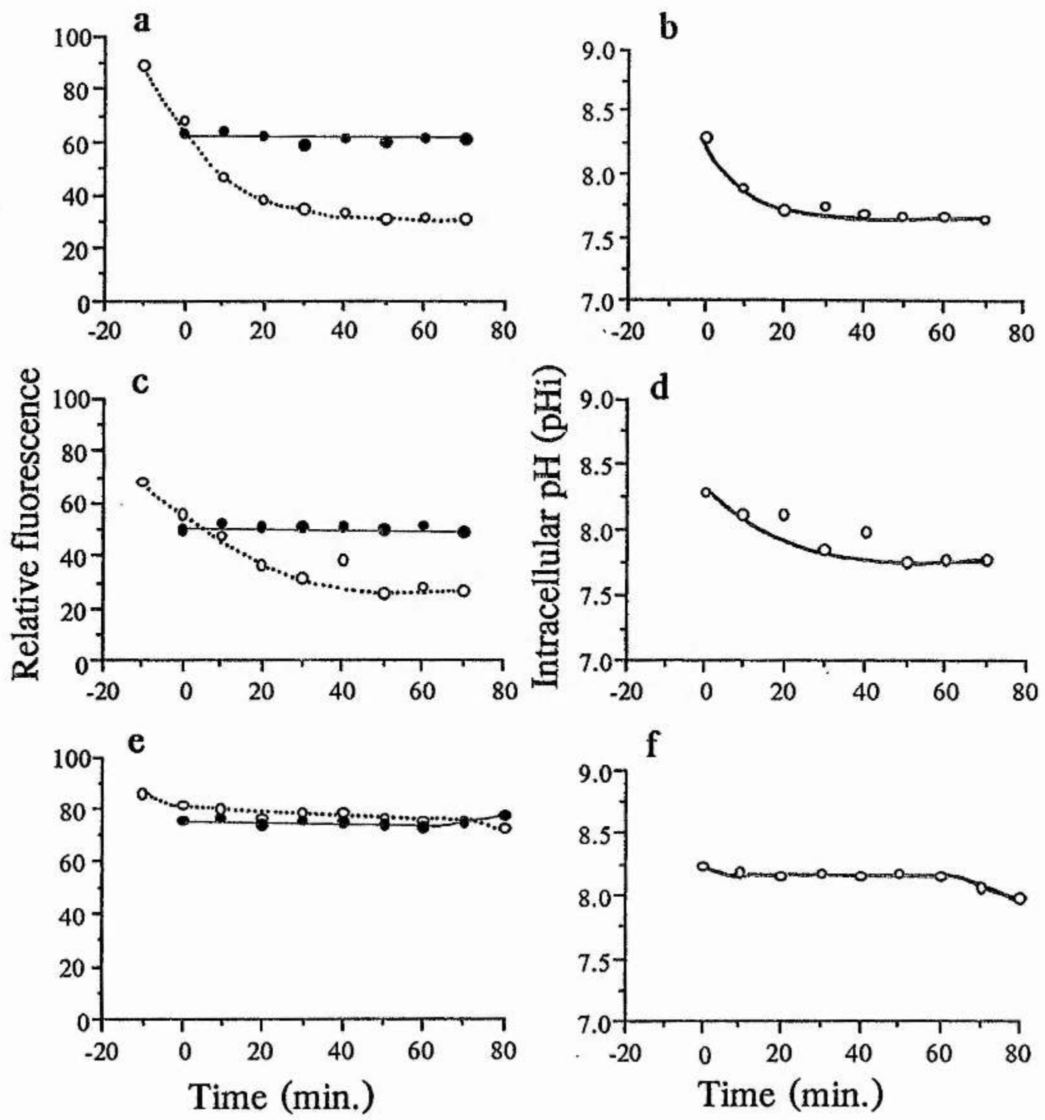

Figure 6.3

after 20 minutes (Fig. 6.3f) and this is probably due to the low intracellular concentration of fluorophore (apparent by little fluorescence quenching) by virtue of the high pHi values observed. During sperm activation with NH_4Cl, a pHi value of 8.1 was observed after 20 minutes and this value remained constant until 60 minutes after commencement of the incubation after which the intracellular 9-amino acridine concentration increased (therefore increasing quenching) and the pHi began to fall toward 8.0 pH units. This may reflect pH changes within the cell which occur at this time, the biological reasons for this are not clear, but it suggests that care should be exercised when calculating pHi by this method.

6.4.3 pHi values during sperm activation

Values for pHi during incubations with prostomial SMF, NH_4Cl and during a control incubation of TFSW, measured from incubations in which spermatozoa were fully loaded with fluorophore, prior to the beginning of the incubation, are shown in figure 6.4. At all times, there is a significant increase in pHi above both the control incubation and the incubation with prostomial SMF following incubation with NH_4Cl ($p= 0.0122$, after 30 minutes and 0.0119 after 50 and 70 minutes incubation respectively). Although a slight elevation in pHi is observed above controls following incubation with prostomial SMF, at all times it is not significant ($p = 0.0937$, 0.0601, and 0.0937 after 30,50 and 70 minutes incubation respectively). This trend is observed consistently and therefore suggest that a larger sample size may indicate a significant difference between treatments. During incubation with prostomial SMF there is generally an elevation in pHi in the order of 0.1 - 0.2 pH units but this is considerably lower than values observed with NH_4Cl, which suggest that their mechanisms of action are different.

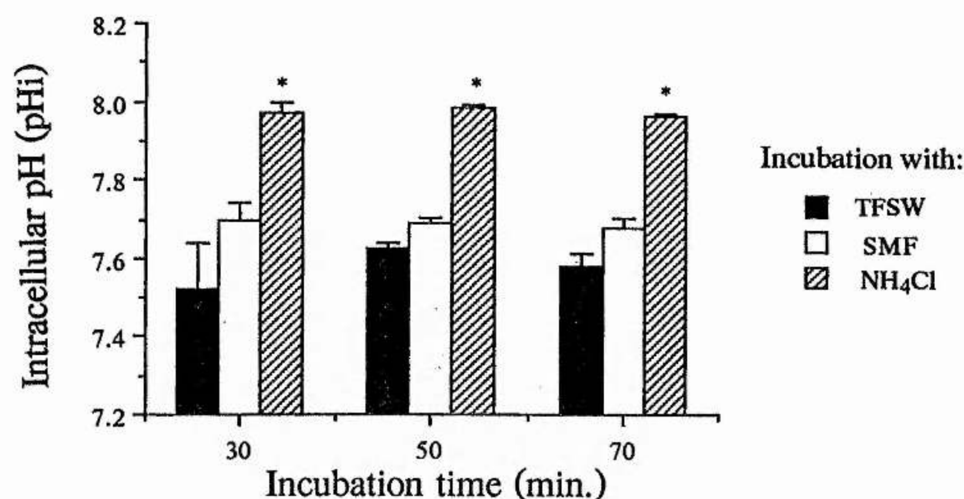

Figure 6.4 Intracellular pH of *Arenicola* sperm following incubation with TFSW, SMF (1 Pr/Eq.ml^{-1}), NH_4Cl (10^{-2} M). Spermatozoa were loaded with 9-amino acridine prior to the commencement of the incubation and pHi was determined at 30,50 and 70 minutes by removing replicate aliquots from the incubation medium. Data shown are the means ± s.e.m for five experiments using the sperm from five *Arenicola*. Asterisk indicates $P<0.05$, compared with pHi of control incubation, with TFSW (Mann - Whitney U test).

6.5 Results: (ATP)

6.5.1 Evaluation of bioluminescence and extraction procedure

The stability of ATP bioluminescence in the luciferin-luciferase assay system used in these experiments is shown in figure 6.5. Although there was a gradual decline in light intensity over the 3.5 hours following the mixing of the reagents, there is only a small change in bioluminescence over the few minutes taken to load and process a sample. Therefore, it appears likely, that few errors would be incurred in utilizing this method as a result of a rapid decrease in light intensity.

The optimum TCA concentration for the extraction of ATP from spermatozoa was found to be 9% (w/v) (Fig. 6.6) and, therefore, this concentration was used in subsequent ATP extractions.

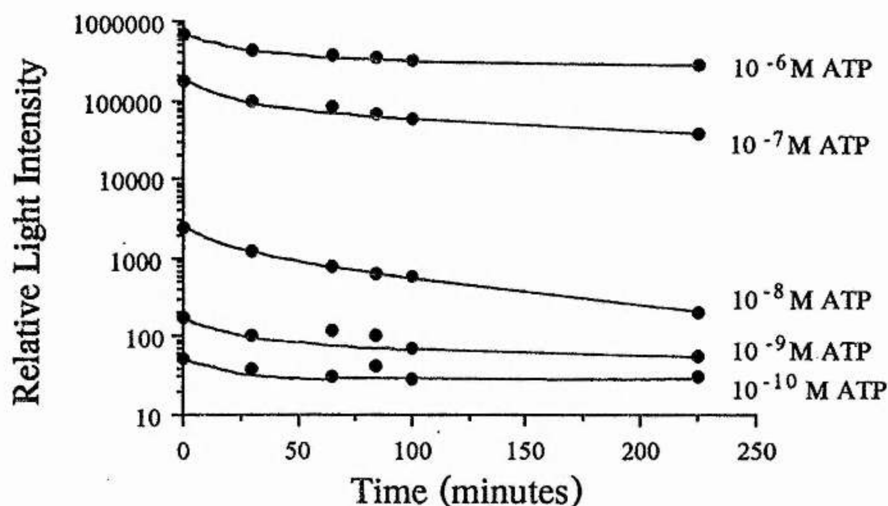

Figure 6.5 Stability of luciferin - luciferase bioluminescence over 225 minutes following commencement of ATP assay. Relative light intensity was measured using a liquid scintillation counter over one minute.

Figure 6.6 (next page) Effect of varying TCA concentration (0 to 15 % (w/v)) on the extraction of ATP from live sperm. Extractions were performed over a ten minute period at room temperature by mixing an equal volume of TCA with the sample, followed by centrifugation and dilution of the supernatant, sufficient to reduce the TCA concentration to less than 0.1% prior to assay. Data shown are from a single experiment and are typical of replicates (not illustrated), indicating an optimum TCA concentration of 9% for extraction of ATP in this system.

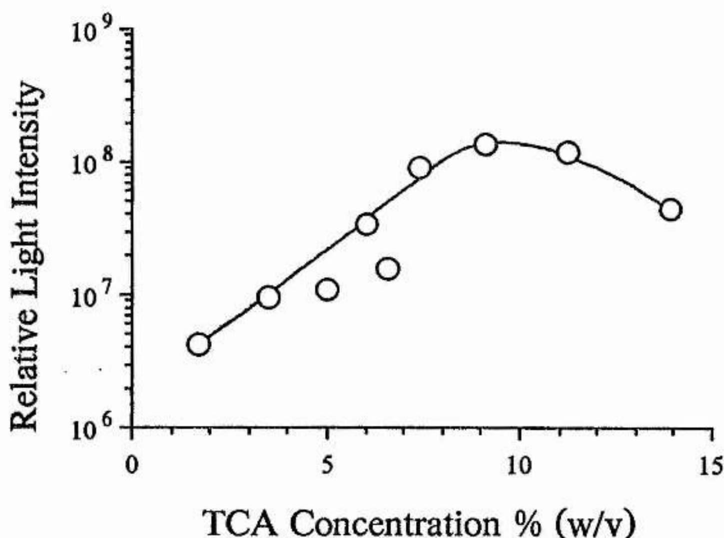

Figure 6.6

6.5.2 ATP concentration during sperm activation

Incubation of spermatozoa with prostomial SMF appears to result in few points at which ATP levels differ from those of sperm which are incubated with TFSW (Fig. 6.7). Only at 35, 40 and 100 minutes following the commencement of the incubation are there any differences between the standard errors of the two incubations. These may well result from errors or inaccuracies of the ATP assay rather than being a biological event. Over the whole incubation period the cellular ATP levels ranged from 9.831×10^{-6} - 2.100×10^{-5} $M.10^8$ $sperm^{-1}$, taking the lowest and highest mean values observed in both incubations.

Figure 6.7 (next page) ATP concentration of spermatozoa during activation by incubation with prostomial SMF (1 $Pr.eq.ml^{-1}$). There is no significant difference between ATP content of activated spermatozoa (closed circles) and quiescent spermatozoa (open circles). ATP content varies between 9.831×10^{-6} to 2.100×10^{-5} M taking the lowest and highest recorded mean values for both activated and control incubations. Data shown are means of ATP content per 10^8 spermatozoa ± s.e.m for four replicate experiments using the sperm from four sexually mature *Arenicola*.

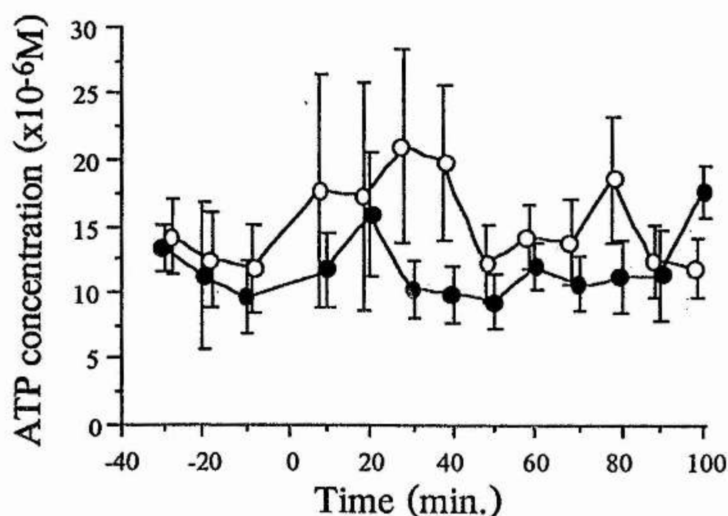

Figure 6.7

6.6 Discussion

6.6.1 The role of pHi

In their evaluation of the use of 9-amino acridine as a fluorescent probe for the determination of pHi, Christen *et al*, (1982) concluded that it was a useful technique for cells with large nucleocytoplasmic ratios such as sea urchin sperm. The sperm of *Arenicola marina* are morphologically very similar to those of sea urchins, and the experiments outlined in this chapter also indicate that they have very similar 9-amino acridine uptake, binding and fluorescence quenching characteristics. This would, therefore, suggest that 9-amino acridine is of potential use as a means of determining intracellular pH of *Arenicola* sperm.

Christen *et al.*, (1982) indicated that the slow uptake of 9-amino acridine into spermatozoa of sea urchins, precluded its use for kinetic analysis, and the uptake and binding characteristics outlined in this chapter for the sperm of *Arenicola*, indicate that this is also the case. Once spermatozoa are fully loaded with the fluorophore, however, pHi values obtained are relatively constant. Values of pHi

for non-motile sperm are 0.3 pH units lower in *Arenicola* when compared with sea urchins, although such differences may be species specific and themselves not significant. Upon activation, following dilution in seawater however, sea urchin spermatozoa undergo an increase in pHi which is in the order of 0.4 - 0.5 pH units (Christen *et al.*, 1982; Lee *et al*, 1983). This is similar to the elevation of pHi brought about artificially in this study by incubation with 10^{-2} M NH_4Cl. This suggests that respiration and motility of *Arenicola* sperm can be stimulated by an increase in pHi similar to that in sea urchins, and it is striking therefore, that during incubation with the natural inducer, prostomial SMF, that there is only a slight (0.2 pH units), but not significant, elevation of pHi. This suggests that natural activation of spermatozoa occurs by a different mechanism, and that such a difference exists, indicates that in terms of pHi changes, the mode of action of SMF and NH_4Cl induced activation are not the same. This supports observations made during respirometry studies described in Chapter 5.

Measurements have yet to be made regarding the pHi changes which occur during activation induced by 8,11,14 -eicosatrienoic acid or quinacrine. Following respirometry studies outlined in Chapter 5, it was suggested that because the oxygen consumption of sperm, in response to activation with quinacrine and NH_4Cl were similar, they may both be acting in the same way. Ammonium ions are known to increase the pHi of many cell types *in vitro* and likewise have been used in spermatozoa for this purpose (eg. Christen *et al*, 1982). It is therefore suggested that quinacrine may function by stimulating a rise in the intracellular pH of spermatozoa, and further support for this hypothesis comes from a discussion of the chemical properties of quinacrine.

Quinacrine [†] (d,1-9-(4-Diethylamino-1-methylbutylamino)-7-methoxy-3-chloroacridine), is a planar heterocyclic compound of 400 daltons molecular weight (see Wolfe, 1975) which was originally developed as an antimalarial drug (Mauss & Mietzsch, 1936). Quinacrine is a 9-alkylamino acridine, is in the same family of compounds as 9-amino acridine and has itself been been utilized in measuring the pHi of membrane vesicles in plant systems (Kasamo, 1986), by virtue of its fluorescent properties. It follows therefore that such closely related molecules may have similar properties. The results described in section 6.4.1 indicated that, above concentrations of 4.58×10^{-5} M, 9-amino acridine is capable of stimulating sperm activation in *Arenicola marina*. Christen *et al.*, (1982) have pointed out that since amines enter the cell in their unprotonated form, they can themselves increase the pHi at sufficiently high concentrations because they become protonated intracellularly and overcome the buffering capacity of the cell. It seems likely that this is the mode of action by which both 9-amino acridine, and therefore quinacrine, exert their effects. Definitive proof, of course, can only be obtained by measuring the pHi of quinacrine activated spermatozoa. This procedure may however prove complex and it may be impractical to determine the pHi in this situation using a closely related molecule as the probe. They overlap in their fluorescent properties, and this could therefore lead to difficulties in accurate fluorescent measurements. However, antagonisms between the two molecules may result in difficulties in reaching uptake equlilbrium as it has been demonstrated previously that a heirarchy of acridine uptake exists in some cell systems (Silver, 1967). Further experiments are clearly required to establish and overcome these problems. In this case it may be possible to measure pHi using radiochemical means, or by using the recently

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† Quinacrine is also known as atebrin, atabrine, mepacrine or acrichin (see Wolfe, 1975)

developed probes, such as carboxyfluorosin (Thomas *et al*, 1979). Both of these techniques however, will require a full evaluation in this system.

Metabolic regulation in a wide variety of cell types has been shown to be controlled *via* pHi (see Busa & Nucetti, 1984). In spermatozoa, pHi has been shown to be involved specifically in both the control of the acrosome reaction and the control of sperm motility. The acrosome reaction of sea urchins, as in many invertebrates and some vertebrates, involves an increase in pHi (Schackmann *et al.*, 1981) which can stimulate a polymerisation of actin (Tilney *et al.*, 1978). In the marine shrimp *Sicyonia ingentis* however, the acrosome reaction is accompanied by a decrease of intracellular pH (Griffin *et al.*, 1987). Sperm motility in many systems including sea urchins (see Trimmer & Vaquier, 1986) and mammals (Babcock *et al.*, 1983; Babcock & Pfeiffer, 1987), is triggered by an elevation of pHi. In the case of sea urchins this acts directly on the pH sensitive flagellar dyenin ATPase (Christen *et al.*, 1982), whereas in higher animals such as mammals, it interacts with other factors such as cAMP, in order to stimulate motility (see Hoskins & Vijayaraghavan, 1990). That artificially elevating pHi can stimulate sperm activation in *Arenicola*, but that sperm activation by SMF does not result in such a rise in pHi is interesting. It appears therefore that any role of intracellular pH in the sperm activation mechanism of *Arenicola* is more complex than the situation in sea urchins, and it may be the case that it is interacting with other cellular parameters in the control of sperm motility.

6.6.2 Levels of ATP during sperm activation

During sperm motility, intracellular ATP is the immediate source of energy for the spermatozoa (Summers, 1974; Summers & Gibbons, 1971) and high levels of ATP have been positively correlated with the motility and viability of the sperm (Comharie *et al.*,1983; Orlando *et al.*, 1982). However, many authors have pointed

out that far more factors influence the motile life and viability than this one parameter (Bilgeri *et al.*, 1987).

Values of intracellular ATP levels of sperm in other studies have been published and include 76 nmol.10^8sperm^{-1} for bovine sperm (Foulkes & McDonald, 1979), for humans 3.30×10^{-2} nmol.10^6sperm^{-1} (Bilgeri *et al.*, 1987), and Ford & Rees (1990) publish a range of values (10 - 60 nmol.ATP.10^8sperm^{-1}). The concentration of ATP in *Arenicola* sperm by comparison is greater than all these values, although the significance of this is not clear.

In this study during sperm activation by SMF *in vitro*, ATP levels do not alter from those of the control, both prior to and during the incubation. The existence of a system which maintains high levels of ATP has been described in the spermatozoa of sea urchins (Tombes & Shapiro, 1985), but despite such a mechanism, however, a decline in the ATP concentration of motile spermatozoa of sea urchins has been observed (Christen *et al.*, 1983), although no comparable figures are available. In human sperm, Bilgeri *et al*, (1987) concluded a positive correlation between a percentage drop in viability, motility and ATP levels. ATP levels were seen to fall by a maximum of 0.24 nM per million sperm over a period of 90 minutes (Bilgeri *et al.*, 1987), however, a change of this magnitude in our system would be overlooked by the variability which exists in the assay at this time.

During sperm activation in *Arenicola* described in this chapter, ATP levels between the control and experimental incubation do not radically alter, and this suggests that (a) sperm quiescence prior to incubation and (b) the cessation in motility following *in vitro* activation, are not events which are mediated by depleted cellular ATP levels.

6.6.3 Further considerations

In terms of both cellular ATP levels and pHi, this chapter makes significant observations which suggest that these parameters warrant further evaluation in the sperm activation system in *Arenicola*. When considering the relationship between these two parameters, particularly in comparison to other systems, it is important to consider the observations made in the next chapter (Chapter 7), that *Arenicola* sperm are characterised by two motility patterns. The first accompanies sperm morulae breakdown and is determined by the fact that sperm motility *in vitro* ceases after 30 - 40 minutes (see Chapter 5). The second is that which is observed following spawning and continues for up to 48 hours (see Chapter 7). Certainly, on the basis of their motile lives, the motility pattens of sperm under these conditions appear to be very different and it may be that their ATP utilization and pHi environments may differ also.

Chapter 7

Spawning:

The Role of 8,11,14-eicosatrienoic Acid

7.1 Introduction

The biological investigations described in Chapters 4 to 6 have implicated 8,11,14 -eicosatrienoic acid as the sperm maturation factor of *Arenicola marina*. In the light of its molecular structure, and the structure of other endocrine factors which stimulate spawning in other invertebrates (see section 1.4.2), 8,11,14 -eicosatrienoic acid is unusual. In order, therefore, to establish a role for 8,11,14 -eicosatrienoic acid in the natural spawning process, *in vivo* investigations are also required. This chapter investigates the experimental induction of spawning by the fatty acid *in vitro*.

It has been noted that SMF is present in the brains of sexually mature male and female *Arenicola* (Howie, 1966), although the lipid substance that can stimulate spawning in males does not induce spawning in females (Howie, 1961 a & b). In light of these observations, therefore, this chapter investigates the effect of the fatty acid 8,11,14 -eicosatrienoic acid on the spawning of both male and female animals. This chapter also reports observations of the spawning of *Arenicola marina* in the field, and aspects of experimentally induced spawning are discussed in relation to naturally spawning animals.

7.2 Materials and Methods

7.2.1 Spawning of *Arenicola marina* at St. Andrews, East Sands

During periods of low water, observations were carried out in order to determine the spawning dates of *Arenicola marina* at the East Sands, St. Andrews, between the four years of 1986 to 1989. During the breeding season for these four years, the beach was regularly sampled and animals were collected for use in laboratory experiments. Inspection of the coelomic contents, for the presence of gametes, indicated whether spawning in these animals had occurred or had yet to take place. Consequently, the spawning date could be estimated by the appearance

of spent animals on occasions when spawning was not observed directly. Natural spawning in these animals is visible by the presence of sperm puddles on the beach during periods of low water.

Tidal data were obtained from the Admiralty Tide tables Vol 1 (published by the Hydrographer of the Navy), for the corresponding years and using Leith as the nearest standard port to St. Andrews.

7.2.2. Experimentally-induced spawning of *Arenicola marina*.

Experiments were carried out in order to determine the spawning response of sexually mature specimens of *Arenicola marina* following injection of either prostomial homogenate, 8,11,14 -eicosatrienoic acid, 11,14,17 -eicosatrienoic acid and quinacrine.

The first consisted of four treatment groups of eight sexually mature male and eight sexually mature females. Individuals were injected with 200 µl of either (a) crude prostomial homogenate, (b) 8,11,14 -eicosatrienoic acid or (c) quinacrine, to give a final concentration of 0.1 prostomia, 13 µg or 16 $\mu g.g^{-1}$ body weight respectively. Animals in the control group were injected with 200 µl TFSW. In the second experiment, the quinacrine treatment group was replaced by 11,14,17-eicosatrienoic acid, and 200 µl of the fatty acid was injected to give a final concentration of 13 $\mu g.g^{-1}$ body weight. The doses used were calculated on the basis of known *in vitro* dose response experiments of these substances.

During each experiment animals were maintained individually in plastic baths containing 180 mls TFSW at 10°C and after 24 hours the sperm and egg densities in the seawater bathing the specimens were estimated as described in section 2.13.

For analysis, the data from the two experiments were combined giving a total of sixteen replicates for injection with prostomial homogenate and 8,11,14 -

eicosatrienoic acid, and eight replicates for quinacrine and 11,14,17- eicosatrienoic acid treatment groups, for each sex, respectively.

7.2.3 The effect of dose on the spawning response

Further experiments were carried out in order to determine the effect of dose on the spawning response of sexually mature *Arenicola*. For each dose response analysis, a total of 48 animals in 8 treatment groups were injected with either prostomial SMF, 8,11,14- or 11,14,17- eicosatrienoic acid.

Injections were carried out to give a final concentration of 0.25, 0.125, 0.0625, 0.0125, and then tenfold dilutions down to a concentration of 1.25×10^{-6} prostomia.g^{-1} body weight for prostomial SMF, or 13, 2.6, 1.3, 0.26, and then tenfold dilutions down to a concentration of 2.6×10^{-5} µg.of the respective fatty acid.g^{-1} body weight.

The experimental protocol was unchanged to that described in section 7.2.2, however, each treatment group consisted of six individuals. This reduced the total number of animals required for the experiment, and was possible because little inter-individual variability in the spawning response is observed (see section 7.3.2).

7.3 Results

The results obtained from observations on the spawning of *Arenicola marina* on St. Andrews East Sands are seen in figures 7.1 to 7.3, and the results from the experimentally induced spawning of sexually mature animals seen in figures 7.4 to 7.8.

7.3.1. Observations on spawning of *Arenicola* at St. Andrews

During each year that observations were made, spawning was observed directly by the appearance of sperm puddles on the surface of the beach sediment. Spawning of animals within the East sands, St. Andrews, was highly synchronised, with the peak of the observed spawning occurring during low water over a one or two day period (Fig 7.1). This was identified as the peak of spawning, although for several days before and after this date one or two individuals released spermatozoa.

Sperm puddles were usually found in either the head or the tail hole depressions which are characteristic of *Arenicola* burrows (Fig 7.2). Within these depressions, the free spermatozoa often has an 'oily droplet' appearance which is characteristic of activated spermatozoa at high densities and has been observed during sperm activation using the *in vitro* bioassay (Bentley, 1985).

Whether the spermatozoa appears in the head or tail hole depression of the *Arenicola* burrow presumably depends upon where the animal is lying within the burrow during spawning, since there appears to be no discernible patten. The release of spermatozoa to the sediment surface is not a continuous process but occurs in bursts, often separated by several minutes of quiescence.

At this location, sperm puddles are generally observed after the time of low water when the tide and water level are rising. In this way sperm release is not only synchronised to the same few days of the year, but also to the same phase of the

Figure 7.1. Sperm pools of many male *Arenicola marina* observed spawning simultaneously. Sperm pools are indicated by an arrow and 13 are clearly observed within the photograph. (Location: St.Andrews, East Sands. Date: 25/10/88)

Figure 7.2. Sperm pools created by two male *Arenicola marina* spawning during a period of low water, (location and date as Fig. 7.1).

tidal cycle. Observations were only made during the period of low water which occured during daylight, and so consequently it is not possible to say whether *Arenicola* undergo spawning during all periods of low water throughout the spawning period. Sperm puddles were only observed on the beach sediment, and unlike spawning in some other polychaete species, spawned eggs were never seen.

Figure 7.3 correlates the peak of spawning with the cycle of spring and neap tides at this locality. During all the years in which observations were made spawning is observed occurring during the periods of spring tides and is usually those tides toward the end of October or beginning of November.

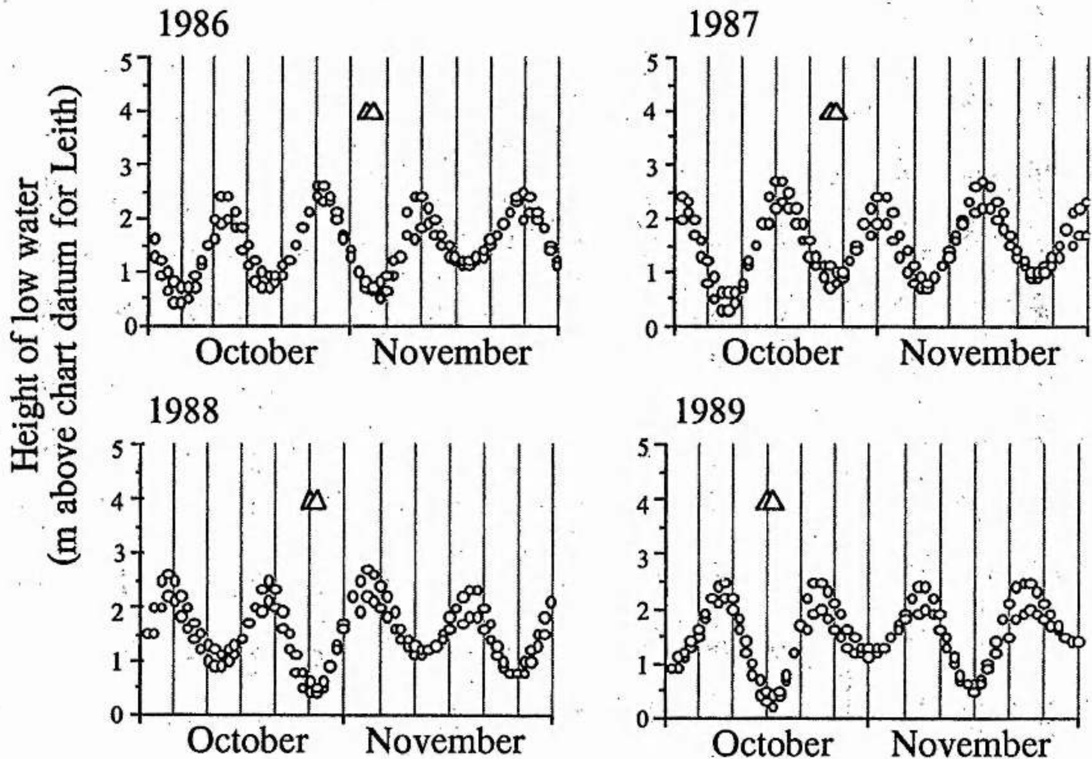


Figure 7.3. Correlation between the observed spawning of *Arenicola marina* on St. Andrews, East Sands and tidal cycle for the months of October and November (1986 to 1989 inclusive). The dates of spawning are indicated by (Δ) symbol. Open circles denote the daily height of low water (m above chart datum), using Leith as the nearest standard port to St. Andrews. Vertical lines divide every five day period from October 1st.

7.3.2. Experimentally induced spawning

Spawning in sexually mature male *Arenicola* was induced by a coelomic injection of either prostomial homogenate or 8,11,14 -eicosatrienoic acid (Fig 7.4). The spawning response observed was identical in every respect, and in the first experiment both treatment groups released a similar number of spermatozoa (2.53 ± 0.58 x10^8 and 1.51 ± 0.22 x10^8 sperm.ml^{-1}) following injection with prostomial homogenate and 8,11,14 -eicosatrienoic acid respectively.

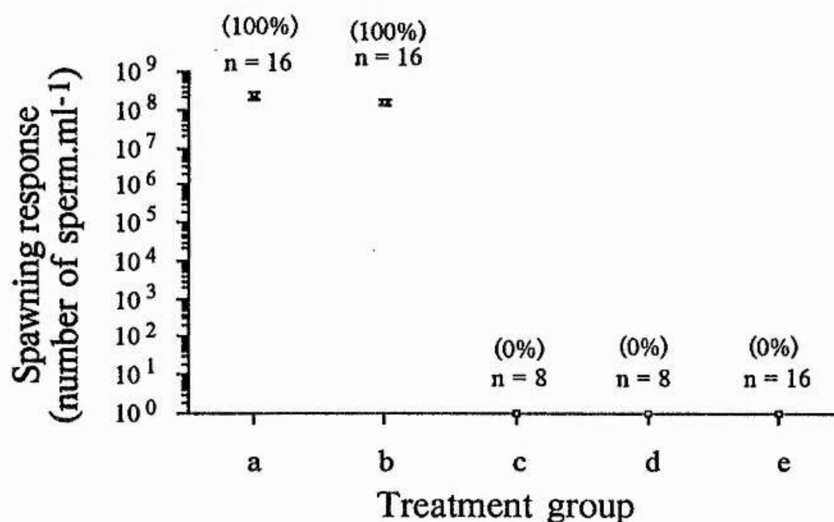


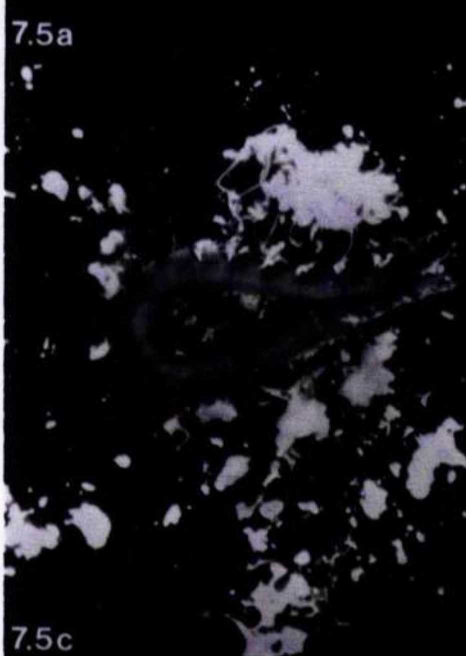
Figure 7.4 Spawning response of sexually mature male *Arenicola* to the injection of (a) prostomial homogenate; (b) 8,11,14- eicosatrienoic acid; (c) 11,14,17- eicosatrienoic acid; (d) Quinacrine or (e) TFSW. Spawning response is indicated as the number of sperm/ml released into 180 mls of bathing medium. Figures in brackets indicate the percentage of animals in the treatment group which underwent a spawning response. Data shown are the mean spawning responses ± s.e.m, n = number of individuals in treatment group.

Spawning in males, was accompanied by intermittent muscular contractions of the body wall, which causing ejaculation through six pairs of modified nephridia in the trunk region, which act as functional gonopores. Powerful ejaculations usually resulted in sperm release from all nephridia in synchrony, and ejaculations were often separated by periods of quiescence, sometimes lasting several minutes, during which there were no ejaculations (see Fig 7.5 a-f). In both treatments, ejaculations began and spawning commenced c. 50-60 minutes after injection and ejaculatory bursts often continued for more than one hour or until the animal was spent. Microscopic examination of the ejaculate shows large numbers of free swimming sperm which remained motile for up to 48 hours after spawning. No spawning was observed following injection of either quinacrine, the fatty acid isomer 11,14,17 - eicosatrienoic acid or TFSW and no rhythmic muscular contractions were observed.

Figure 7.5 (Next page). Spawning of male *Arenicola marina*, following injection of 8,11,14 - eicosatrienoic acid. The sequence of photographs (a-f) illustrate the progressive release of sperm from the coelom *via* the nephridia. (a) shows the animal following an initial burst of spawning activity 50 minutes following injection with the fatty acid. Periods of relative quiescence are followed by bursts of ejaculatory activity (b,c) during which sperm suspensions are clearly visible as they are released to the exterior. (c) shows a powerful ejaculation and the simultaneous release of sperm through all six nephridia along one side of the animal. Spawning takes place over a period of more than an hour after commencement (d), at which point, the worm begins to be obscured by the cloud of spermatozoa in the surrounding sea water (e,f)

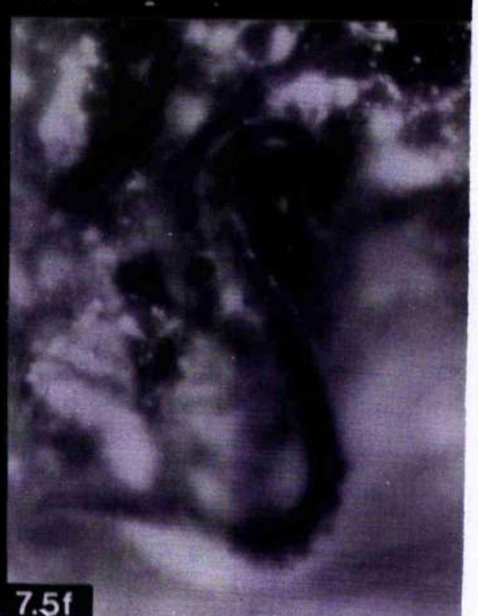
7.5a

7.5b


7.5c

7.5d

7.5e


7.5f

Spawning in females differs in several respects from that observed in males. Only females injected with prostomial homogenate underwent spawning (Fig. 7.6) and like males, all females injected, responded, and the mean spawning response was $1.17 \pm 0.259 \times 10^5$ oocytes (mean ± s.e.m). However, unlike males, the timescale over which spawning occured was significantly different, with females not commencing spawning until at least five hours, or longer, following injection, and all individuals had ceased spawning after 19 hours. Spawning in females was not accompanied by any noticeable muscular contractions. Microscopic examination of spawned oocytes indicated that they had undergone germinal vesicle breakdown (GVBD) and consequently maturation of the oocytes had occured. Following injection of either of the eicosatrienoic fatty acid isomers, quinacrine or TFSW no spawning was observed. However, one of the sixteen females injected with 8,11,14 -eicosatrienoic acid did release a small number of oocytes. These however, only numbered c 2,500 and therefore could not be considered as a true spawning response.

Figure 7.6 (Next page). Spawning response of sexually mature female *Arenicola* to the injection of (a) prostomial homogenate; (b) 8,11,14- eicosatrienoic acid; (c) 11,14,17- eicosatrienoic acid; (d) quinacrine or (e) TFSW as in Figure 4. Spawning response is indicated as the total number of spawned oocytes (mean ± s.e.m) Figures in brackets indicate the percentage of animals in the treatment group which underwent a spawning response. n = number of individuals in treatment group.

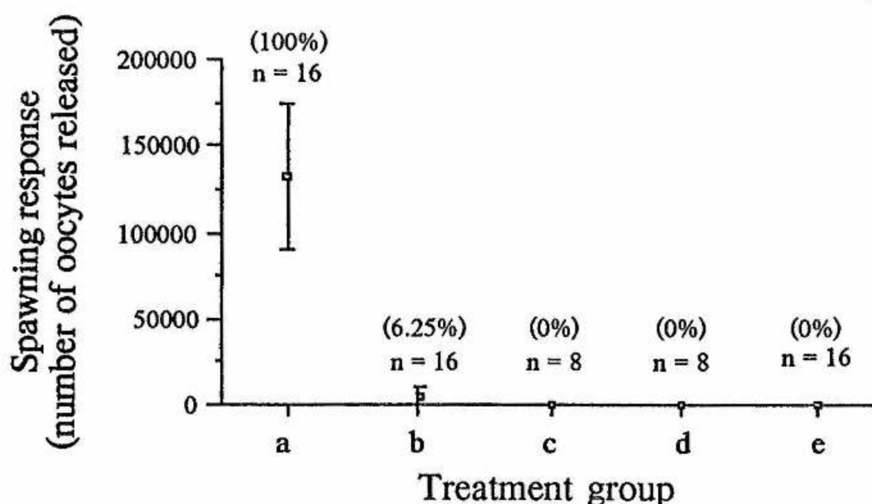


Figure 7.6

7.3.3 The effect of dose on spawning

Figure 7.7 shows that in these experiments, spawning in male *Arenicola* was stimulated above concentrations of 1.25×10^{-2} µg.prostomia. g^{-1} body weight of prostomial extract and 1.3 µg.g^{-1} body weight of 8,11,14- eicosatrienoic acid. At these concentrations however, only 2 out of 6 animals spawned in response to the fatty acid (33.33% response) and only 1 out of 6 animals spawned in response to prostomial SMF (16.67% response). No spawning was observed at any dose of 11,14,17 -eicosatrienoic acid and in this respect confirms the observations made in 7.3.2, that this eicosatrienoic isomer is unable to stimulate spawning. Above the

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Figure 7.7 (Next page). Spawning response of male *Arenicola* in response to doses of (a) prostomial SMF, (b) 8,11,14-, and (c) 11,14,17 -eicosatrienoic acid. Doses is expressed in N° of prostomia.g^{-1} body weight for prostomial SMF and µg.g^{-1}body weight for the two fatty acids. The spawning response is expressed as number of sperm.ml^{-1} released. Data shown are spawning responses of all six individuals per treatment group and figures in parentheses show the number of individuals which did not release spermatozoa at a given concentration.

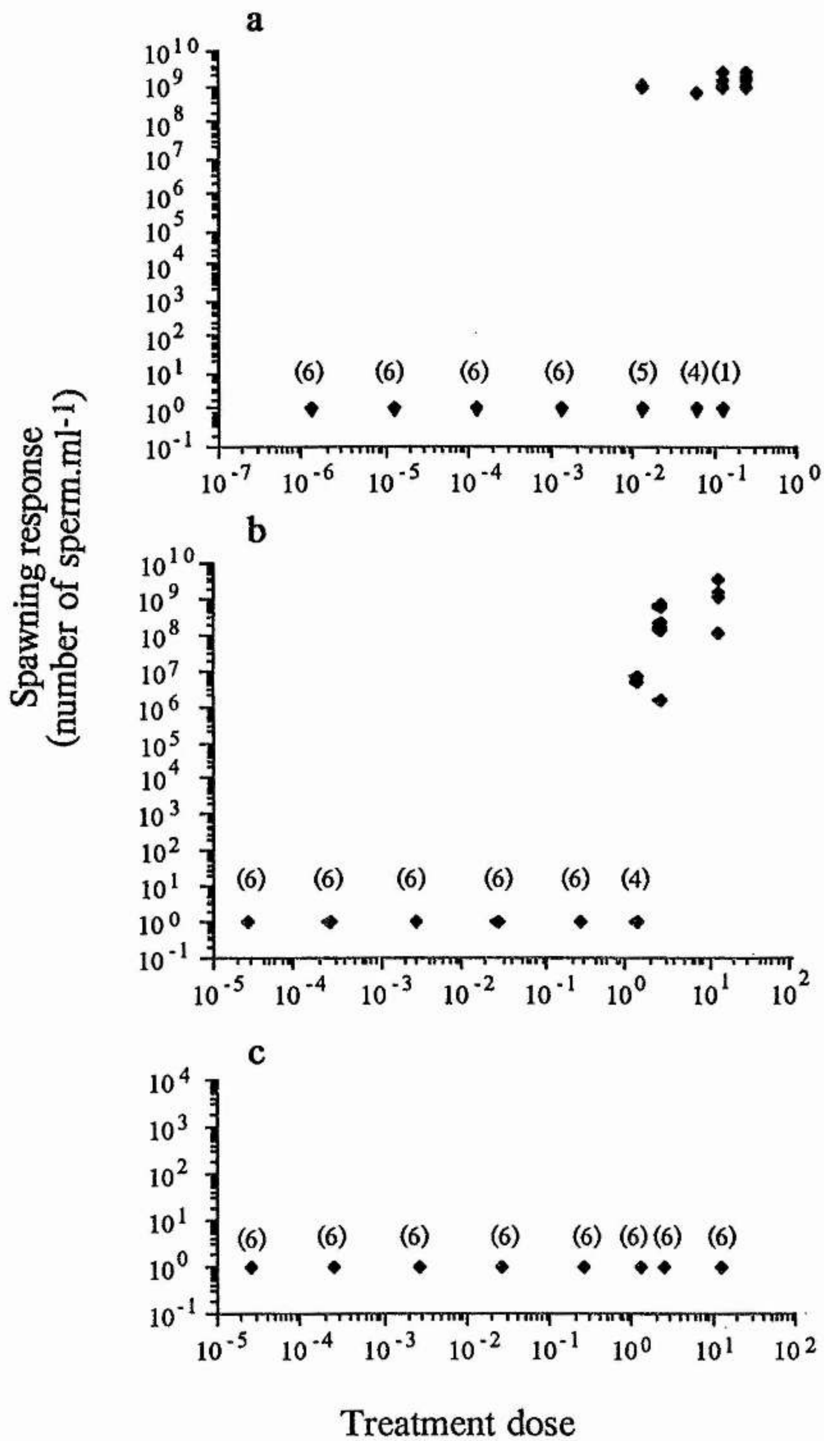

Figure 7.7

threshold concentrations of prostomial SMF, figure 7.7a clearly shows that spawning is an 'all or nothing' event, and a similar number of sperm are released over a range of concentrations. Slightly more variation is observed, however, at above threshold concentrations of 8,11,14 -eicosatrienoic acid (Fig 7.7b), both in terms of the number of animals responding and the number of sperm released, although this response can be still regarded as 'all or nothing'.

The response observed with female animals over comparable doses confirms the results obtained in section 7.3.2. Spawning is not stimulated by either of the fatty acid isomers, but only occurs in response to prostomial homogenate (see Fig 7.8). Spawning was stimulated above a concentration of 6.25×10^{-2} μg.prostomia.g^{-1} body weight, although, at this concentration, only 1 animal out of 6 underwent spawning (16.67% response), and at the maximum concentration tested (0.25 μg.prostomia.g^{-1}body weight) only an 83.33% response was obtained. In comparison to the spawning response observed in males, these data suggest that there is a greater variation in the number of gametes released.

During the dose response experiment to 8,11,14 -eicosatrienoic acid (Fig 7.8b) one female released c. 25000 oocytes, but these had not undergone GVBD, and were therefore not considered to be the result of a true spawning response, but probably the result of leakage from the body cavity.

Figure 7.8 (Next page). Spawning response of female *Arenicola* in response to doses of (a) prostomial SMF, (b) 8,11,14 -eicosatrienoic acid and (c) 11,14,17 -eicosatrienoic acid. Treatment doses are expressed in number of prostomia.g^{-1} body weight for prostomial SMF and $\mu g.g^{-1}$body weight for the two fatty acids. The spawning response is expressed as the total number of eggs released per animal. Data shown are spawning response of all six individuals per treatment group and figures in parentheses show the number of individuals which did not release eggs at a given concentration.

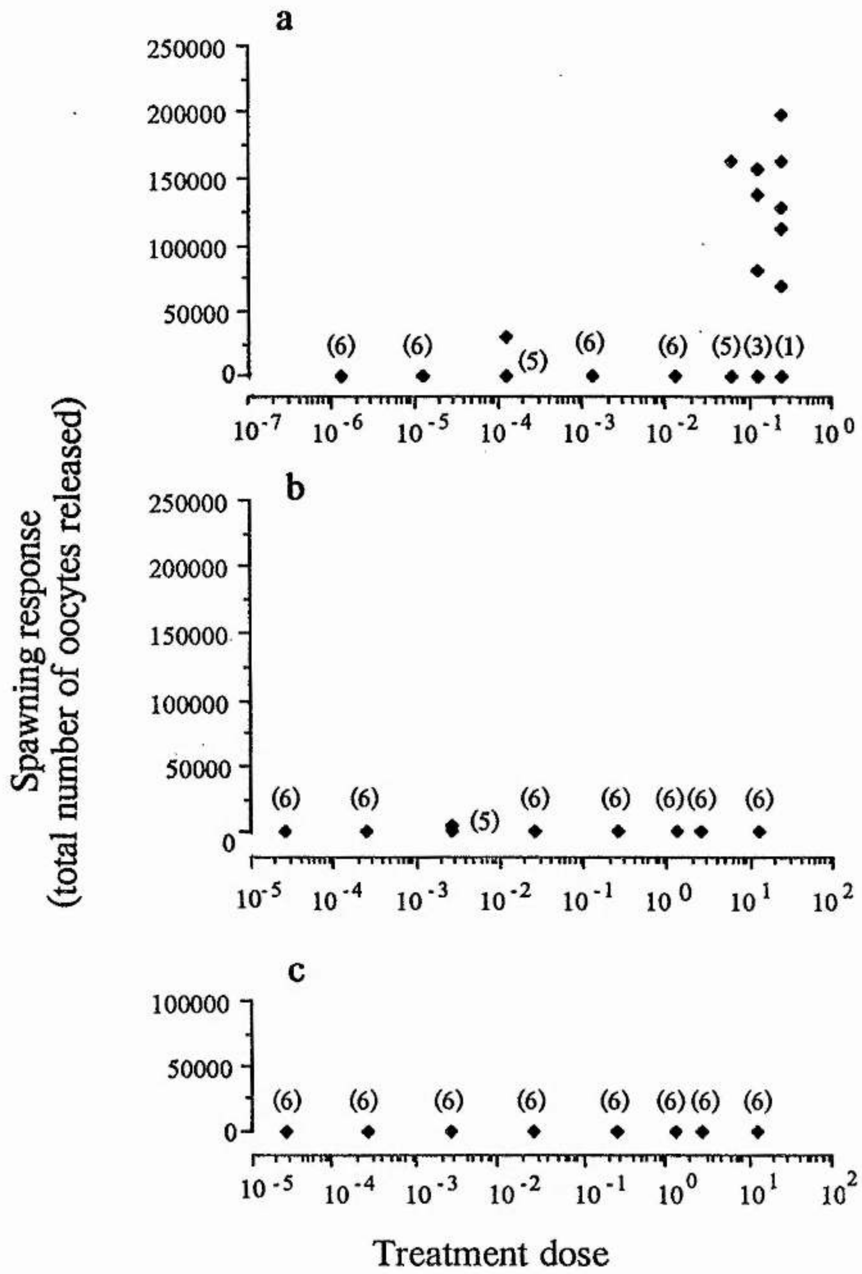

Figure 7.8

7.4 Discussion

The results described in this chapter support the observations made by Howie (1959; 1961a; 1961b; 1961c) that were outlined in section 1.5, both with regard to the experimental induction of spawning, and observations on the spawning of populations of *Arenicola marina* in the field.

7.4.1 Induction of spawning

Sexually mature specimens of *Arenicola marina* have been induced previously to spawn following the injection of macerated extracts or 'saponifiable lipid' extracts of whole body homogenates (Howie, 1961b). The saponifiable lipid fraction includes the fatty acids, and therefore, that the fatty acid 8,11,14 - eicosatrienoic acid can induce spawning, supports these observations.

Following the injection of active tissue extracts, Howie (1961b) noted that worms underwent violent contractions of the body wall which led him to suggest that a muscle stimulant must be present in such tissue extracts. The utilisation of muscular contractions during spawning has been observed in other polychaete species. For example, during spawning in *Nephtys caeca* and *Nephtys hombergi*, violent muscular contractions result in the emission of gametes from the anus (Bentley *et al.*, 1984). To discharge gametes in this way is not common within poychaetes, and to date, has been described only in these species. More commonly, polychaetes not forming epitokes, undergo gamete discharge through a coelomoduct system with most ducts leading into the nephridial canal rather than directly to the exterior (Schroeder and Hermans, 1975).

The number of gametes observed being released in these experiments is large, and is comparable with studies in other broadcast spawners. For example, *Asterias rubens* and *Strongylocentrotus purpuratus* have been shown to liberate $2x10^8$ and $8x10^{10}$ sperm respectively during a breeding season (Giese & Kanatani,

1987). In *Arenicola* there is little variation, both between individuals and between treatment groups, in the number of spermatozoa released, although, over lower doses of 8,11,14 -eicosatrienoic acid which are capable of stimulating spawning, the number of sperm released begins to decline. Above threshold doses however, spawning generally appears to be an 'all or nothing' response and the variability of gamete release that has been observed in other polychaete species (Olive *et al.*, 1981a ; Olive *et al.*, 1981b) does not occur.

Chapter 4 of this study presented evidence which suggested that the chemical characteristics of prostomial SMF and 8,11,14- eicosatrienoic acid were identical. Data presented in Chapter 5 however, suggested that the responses of spermatozoa *in vitro,* to incubation with pure 8,11,14 -eicosatrienoic acid and prostomial homogenate, were slightly different. Principally, there was a difference in the time taken for sperm to become independently motile and the corresponding oxygen consumption rate to rise above basal levels. During incubation with 8,11,14-eicosatrienoic acid, this time was significantly shorter than during incubation with prostomial SMF. During spawning experiments however, no such time differences are observed and this suggests that the repsonse may be modulated *in vivo*. That this might occur due to carrier proteins has already been suggested (see section 5.4). In fact, in every respect, the spawning response of male *Arenicola* to either 8,11,14 -eicosatrienoic acid or SMF is identical and this adds further support to the evidence derived from chromatographic, enzymic and respirometric experiments, that SMF is 8,11,14 -eicosatrienoic acid. That the fatty acid isomer 11,14,17 -eicosatrienoic acid is unable to induce spawning (and is unable to induce sperm activation *in vitro* - see section 4.3.2), is probably a reflection of its stereochemistry. Whilst 8,11,14 -eicosatrienoic acid is the parent molecule of a wide variety of biologically active substances, due to the differences in the position of the conjugated double bonds, the 11,14,17- isomer is not (see section 4.4). Quinacrine is unable to induce

spawning, but it has been demonstrated clearly that its mode of action differs from that of the natural activator (see sections 4.3.5; 5.3.1 & 5.3.3).

Howie (1961c) described that free spermatozoa, when injected into the coelomic cavities of male *Arenicola*, were quickly released to the exterior, presumably as a result of the spermatozoa being accepted automatically by the ciliated funnels of the nephromixia. A similar series of events have been suggested for the spawning mechanism of *Pectinaria gouldii* (Tweedell, 1980), and an endocrine factor activates the admission of fully developed gametes into the nephromixia where they then undergo their final maturation. In the terebellid *Lanice conchilega* however, there is no evidence for selective uptake of spermatozoa and both ripe and unripe gametes are observed in the nephromixia of post spawning animals (Smith, 1989). In *Arenicola*, only free swimming spermatozoa or morulae in which the sperm are swimming (Howie 1961c) and oocytes which have undergone germinal vesicle breakdown, are observed being spawned (Howie, 1961b), so clearly some selection of gametes is occuring. The observations of muscular contractions and periods of definite ejaculation described in this chapter, suggest that an active spawning mechanism is involved.

Either during passage through the nephromixia or during sperm activation within the coelomic fluid of *Arenicola*, the spermatozoa undergo changes in which their motile life and viability are prolonged over and above that observed during *in vitro* activation (section 5.3.1). Presumably these enhance the success of interaction with oocytes and subsequent fertilisation. Additional cytochemical changes often occur following sperm motility acquisition in many animals and these prepare the spermatozoa for the acrosome reaction and fertilisation (reviewed in section 1.3.3). Such events, known as capacitation, usually occur in the female genital tract, but in invertebrates, which undergo 'external fertilisation' and no sperm transfer to females, it has been suggested that capacitation and the acquisition of

motility may occur concurrently (Longo, 1987). The precise moment of sperm motility acquisition is subject to significant intraspecies variation and is reviewed in section 1.3.2. Sperm motility acquisition in *Arenicola* occurs in the coelomic cavity, but is not a simple process, and the dissociation of sperm morulae and the activation of spermatozoa is clearly quite separate from the changes which result in the increase in sperm viability.

That 8,11,14- eicosatrienoic acid is capable of stimulating spawning in males, but not in females, supports the observations made by Howie (1961a & b). He described that the lipid fraction of whole worm homogenates, which can induce spawning in males, cannot do so in females. Spawning in females can be induced by a maturation factor present within prostomial homogenates but this clearly is not 8,11,14 -eicosatrienoic acid. Meijer (1979a; 1979b; 1980) investigated an oocyte maturation factor in the prostomia of sexually mature females. He concentrated however on describing its biochemical and morphological effects on oocytes, rather than on its chemical identity and these are reviewed in section 1.5.2. There are, therefore, clear sex specific differences between the endocrine signalling mechanisms which surrounds gamete maturation and spawning in this animal.

7.4.2 Spawning of field populations

Spawning was only observed in males and spawned oocytes were never observed in the field. Previous studies have demonstrated this to be the case and it has been suggested that females retain their eggs in the burrows after spawning (Howie, 1961c). Within males, observations on the spawning at St. Andrews, East Sands, indicate that there is a high degree of 'within population spawning synchrony' at this location. Both Howie (1959) and Duncan (1960) suggested that spawning in *Arenicola* at St. Andrews was correlated with a sudden drop in environmental temperature. Howie (1984) has since pointed out however, that

worms maintained in the laboratory at many localities, often spawn at the same time as the corresponding population on the shore. Many environmental variables can therefore be eliminated as being the final synchronising trigger in this species.

Observations that the population at St. Andrews undergoes such a regular spawning on spring tides suggests a tidal or lunar component existing in the spawning control mechanism. It appears however, that St. Andrews may be unusual in this respect, since Duncan (1960) noted that all other populations she observed, with this exception, spawned on neap tides. The isolation of any single environmental variable which effects spawning in *Arenicola* may be difficult, and different populations may utilise a hierarchy of different factors, each acting to ensure that spawning occurs at the most suitable time at any locality. This has been reported previously with some polychaetes on North Sea and Atlantic coasts showing tidal periodicity, and those on Mediterranean coasts showing lunar periodicity (Geise and Kanatani, 1987).

Section 1.4.1 reviews the role of environmental influences on spawning, but it is important to point out that environmental factors, which are involved in endocrine signalling, may be more complex or subtle than the observation of a single clearly recognisable or measurable parameter. The speculation on the nature of such a trigger by the relation of conclusions drawn from the coincidence of specific events can be misleading (Olive, 1984). Such observations do not permit the causal factors responsible for the timing of such events to be determined, particularly since spawning is the terminal event of a long process of gametogenesis which occupies most of the year .

Following the submission of this thesis, the author has become aware of a paper by Bailey - Brock which describes the spawning and larval development of *Arenicola Brasiliensis*(Nonato). In this species, fertilized eggs are retained in cocoons which are extruded from burrows but remain attached by a stalk. Bailey - Brock notes that there is a correlation between lunar phase and spawning, with more cocoons appearing during neap tides than during springs.
(Ref: Bailey - Brock, J.H., (1984) Spawning and development of *Arenicola brasiliensis* (Nonato) in Hawaii (Polychaeta; Arenicolidae). In: *Proceedings of the First International Polychaete Conference.* (Ed) P.A. Hutchins. The Linnean Society of New South Wales. pp. 439 - 449.)

Chapter 8

General Discussion

8.1 Introduction

The work outlined in this thesis was undertaken with two major objectives in mind; to characterise the sperm activation response of *Arenicola marina* and to identify the chemical nature and the mode of action of the endocrine principle involved. As was outlined in section 1.6, sperm activation in *Arenicola* is of particular interest because, first, as a process it is inextricably linked to spawning and, second, it is an endocrine mediated response which has more characteristics in common with sperm activation in higher animals (eg. mammals) than do other model invertebrate systems such as sea urchins. It is the aim therefore, of this discussion, to draw together the results described in the preceding chapters into some kind of order, to determine their significance in terms of the reproductive biology of *Arenicola* and the sperm biology of other species.

8.2 Ultrastructure of Sperm and Sperm Activation

The sperm morphology of *Arenicola* is of a primitive type. It is slightly unusual however in that the distal part of the axoneme is coiled which gives rise to a discoid swelling at the posterior of the sperm flagellum. The function of this is not clear and it appears not to have been reported in sperm from any other species of either primitive or modified sperm types. It is possible that this may assist in the propulsion of the spermatozoon in that it allows the interaction of a greater surface area of the flagellum with the surrounding medium.

The sperm of *Arenicola* are also unusual from animal groups outside the Annelida in the respect that they are held together in a morula, both throughout their development and during their phase of quiescence in the period prior to spawning. Within a morula, spermatozoa are cytoplasmically connected by a common mass of cytoplasm called a cytophore, but the function of this remains unclear. The

ultrastructural description of sperm activation presented in Chapter 3 however, suggests that the cytophore appears to be actively involved in spermatogenesis, during which it is observed to contain organelles such as endoplasmic reticulum which are usually associated with biosynthetic activity. It is also demonstrated that the cytophore may play an active role in the dissociation of sperm morulae.

Sperm activation in *Arenicola* is therefore more complex than in other species in that it includes ultrastructural changes as well as the switching on of the motile apparatus of the cell. The morphological changes which are observed during this investigation are those which are observed *in vitro* during incubation with prostomial SMF. It seems doubtful that these observations differ greatly from those that would be observed during sperm activation *in vivo* despite the fact that differences in the duration of swimming activity of such sperm have been shown to exist. Such differences may occur as a result of changes which probably occur at the molecular level and these will be discussed in section 8.7.3.

8.3 Sperm Maturation Factor - A 20-Carbon Fatty Acid?

Central to the work outlined in this thesis is the inference that the chemical nature of SMF is that of a straight chain polyunsaturated fatty acid 8,11,14 - eicosatrienoic acid. All chromatographic, enzymatic, biochemical, and respirometric evidence from *in vitro* studies support this conclusion, and that 8,11,14 -eicosatrienoic acid stimulates spawning *in vivo* is of particular significance. This conclusion is in agreement with the previous observations on the chemical nature of SMF carried out by Howie (1961a) (see section 1.5.1) who noted that the active substance of whole worm homogenates was found in the fatty acid fraction, and of this, saturated fatty acids appeared to be biologically inactive.

The mechanism by which sperm of *Arenicola* are activated by 8,11,14 - eicosatrienoic acid, is at present not understood, but it is thought that it may act in one of two ways. First, either as a metabolic substrate which stimulates sperm metabolism directly, or secondly by either a receptor mediated response or by being metabolised intracellularly and acting as a second messenger itself. These will be outlined in the following sections.

8.3.1 Fatty acids as metabolic substrates

Fatty acids have been known to have a role in sperm function for a number of years. They have been demonstrated to be rapidly incorporated into the sperm phospholipids, neutral lipids and diglycerides of a number of species including the bovine sperm (Payne & Masters, 1968) and sperm of the ram (Mills & Scott, 1969).

One function of fatty acids in spermatozoa is as an energy source. Ford and Rees (1990) point out that sperm are capable of metabolising a wide range of extracellular substrates including pyruvate, lactate, acetate, amino acids and fatty acids. The demands on these substances however, may vary during the life of the spermatozoon, and in mammalian sperm, phospholipids and fatty acids are important at various stages. Studies in rats for example, have shown that fatty acid is the main endogenous substrate (Mann & Lutwak-Mann, 1981) and sperm may also depend on exogenous phospholipids during epididymal transit (Volmayr, 1976). At ejaculation, the sperm are mixed with seminal plasma which contains a variety of substances and semen fructose has been shown to provide about 60% of the sperm energy requirements (Peterson & Freund, 1971). That fatty acid oxidation occurs however has also been suggested (Abdel Aziz *et al.*, 1983). Such metabolic studies have been carried out in few other species outside the mammals however, although it has been demonstrated that ATP production in sea urchin sperm occurs primarily as a result of fatty acid oxidation (Mita & Yasumasu, 1983).

It is possible that in the the stimulation of sperm activation in *Arenicola*, the mode of action of 8,11,14 -eicosatrienoic acid is *via* the supply of available substrate for mitochondrial respiration and that this alone is the trigger for sperm activation. Certainly sperm activation by incubation with the fatty acid induces a rise in respiration rate which is probably (although not conclusively) as a result of oxidative phosphorylation. Two lines of evidence, however, suggest that this is unlikely. First, fatty acid oxidation occurs by the conversion of the acid into acyl-CoA derivatives, and subsequent degradation by either the β, ω, or α pathways (see Mead *et al.*, 1986) is not stereospecific. Secondly, Mead *et al.*, (1986) point out that the limiting factor in fatty acid metabolism appears to be the availability of the fatty acid itself. That sperm activation by 8,11,14 -eicosatrienoic acid is stereospecific (the 11,14,17 -eicosatrienoic isomer cannot stimulate sperm activation) and that it is an 'all or nothing' response would therefore rule out this mechanism as a control step for sperm activation in *Arenicola*.

8.3.2 The role of eicosanoids

The chemical nature of the active molecule in question (8,11,14 -eicosatrienoic acid), strongly implicates eicosanoids as having a role in sperm activation. Eicosanoids, and principally those formed from 5,8,11,14 -eicosatetraenoic (arachidonic) acid, have been shown to have a variety of signalling roles in cell systems (Needleman *et al.*, 1986). Those such as prostaglandins are particularly interesting because they differ from other second messengers in that they can leave the cell in which they were generated, and act as primary messengers on neighbouring cells (Hedqvist, 1977). Prostaglandins have been suggested as being important in sperm motility because they are present in high concentrations in the

seminal fluid of many animals including humans[†] (Iwamoto *et al.*, 1990). Their effects on sperm motility and sperm function at physiological concentrations is however unclear.

Eicosanoids have been implicated as being involved in the reproductive biology of several invertebrate species, for example, during oocyte maturation of starfish (Meijer & Guerrier, 1984), in the spawning of two species of molluscs (Morse *et al.*, 1977) (see also section 1.4.2) and in the hatching of barnacles (Holland *et al.*, 1985). Pathways of prostaglandin synthesis have also been identified in the tissues of many invertebrate species including *Arenicola* (Srivastava & Mustafa, 1984).

The mode of action of 8,11,14 -eicosatrienoic acid in sperm activation of *Arenicola* may therefore be *via* an eicosanoid metabolite of the fatty acid. This thesis however, includes no evidence to support such a hypothesis which could only be achieved by a detailed biochemical analysis of the sperm metabolism of labelled 8,11,14 -eicosatrienoic acid.

8.3.3 The role of other tissues

Not all endocrine responses occur by single step mechanisms and it therefore remains a possibility that the action of 8,11,14 -eicosatrienoic acid *in vivo* occurs *via* another endocrine tissue. It has been suggested, that any type of endocrine control in the lower invertebrates however, occurs by neurohormones which act directly on peripheral targets (Highnam & Hill, 1977). In the polychaetes, this was

† The name prostaglandins is in fact derived from the observation of Von Euler (1934; 1935a&b) of an acidic component of the lipid extracts of the seminal fluid of sheep. This component had the biological properties of lowering the blood pressure and stimulating the contraction of smooth muscle. He coined the term "prostaglandin" for this active substance in the belief that it was synthesised in the prostate gland.

certainly considered to be the case, as in both the Arenicolidae and the Nereidae germ cells develop in isolation without intimate associations with other cell types.

During sperm activation in *Arenicola* the endocrine mechanism is not considered to conform to this type because 8,11,14 -eicosatrienoic acid cannot be regarded as a neurosecretory substance (see section 8.5). The action of 8,11,14 -eicosatrienoic acid or SMF is however, probably that of a primary signal because it is capable of stimulating sperm activation directly *in vitro*. However, endocrine substances in other invertebrate groups are known to act on a multiplicity of target tissues (see section 1.4.2 for some examples) and therefore, that 8,11,14 -eicosatrienoic acid may function on several target tissues *in vivo* is discussed in section 8.4.

8.4 Sperm Maturation Factor - A Spawning Hormone?

The data presented in Chapter 7 of this thesis, suggests that spawning, as induced by prostomial SMF or by 8,11,14 -eicosatrienoic acid, may be a more active process than previously considered. Periods of definite and vigorous ejaculatory behaviour, during which the sperm are ejected through the nephromixia, are separated by several minutes of quiescence before sperm release continues. During these ejaculations, muscular contractions are observed, and therefore, as well as acting directly to stimulate sperm activation, sperm maturation factor (or 8,11,14 -eicosatrienoic acid) may act at other levels, either directly or indirectly (*via* a feedback mechanism from the sperm themselves for example), to stimulate this behaviour. This may be at the level of the body wall musculature, the nephromixia or at other tissues.

It was outlined in section 1.5, that spawning in *Arenicola marina* is an event which was thought to be entirely dependent on gamete maturation. Because SMF

induces spawning in this way, the maturation hormones of male and female *Arenicola* are thought to differ greatly from the spawning hormones of other polychaete species (Franke & Pfannensteil, 1984). It is suggested however, that the action of SMF at least, is not entirely passive, although this has been suggested previously (Howie 1961a,b&c).

The interaction at several different target sites is a characteristic of endocrine substances which induce spawning in many species (see section 1.4.2). The maturation inducing substance of starfish (1- MeAde) acts on the gamete, the gonad and the nervous system of both males and females to bring about spawning. In this respect, it is interesting that the sperm maturation factor of *Arenicola* is present in the brains of both sexes (Howie, 1966), although the lipid substance (SMF) which can induce spawning in males (Howie 1961a &b) or 8,11,14 -eicosatrienoic acid, cannot do so in females (Chapter 7). It has been suggested that because SMF is present in the brains of both sexes that it may function in a yet unidentified role in the spawning response of females (Howie, 1961b). However, the action of 8,11,14 - eicosatrienoic acid as a trigger for spawning in *Arenicola*, is certainly sex specific.

8.5 The Site of SMF Secretion or Production

From previous work, it is evident that the site of SMF synthesis in the prostomium of *Arenicola* does not appear to correspond to any of the identified areas of neurosecretory activity (see section 1.5.3). From the results outlined in this thesis, concerning the nature of SMF, it seems unlikely that a fatty acid such as 8,11,14 -eicosatrienoic acid would be released from, or stored within, neurosecretory structures. Fatty acids are usually produced enzymatically in cells by the action of phospholipase A_2 (PA_2) on membrane phospholipids (see Hostetler,

1985). At the time of spawning, PA_2 is presumably liberated from lysosomes and acts on membrane phospholipids to produce SMF.

The cyclical nature of SMF titres in prostomia may therefore be derived through seasonal variability in the lipid composition of the prostomium, or the cyclical production of PA_2. In animals prior to spawning, that SMF activity is observed in prostomial homogenates, is possibly due to the action of stored PA_2 which is liberated during homogenisation. Evidence to support this has been derived, in part, from observation that prostomia, homogenised in a solvent such as methanol (which disrupts enzyme function), can result in a total loss of SMF activity (Bentley, unpublished observations).

Assay of PA_2 activity (see Gatt *et al.*, 1981) could be utilized to investigate the level of this enzyme in relation to the SMF activity of prostomia and therefore help to establish the role of PA_2 in SMF production. Similarly, ultrastructural localization of PA_2 activity can be carried out using antibodies, raised to this enzyme, and it would be interesting to observe if these areas correspond to the areas of SMF production, which have been identified through previous ablation experiments (see Howie, 1984).

8.6 A Fatty Acid as an Endocrine Substance

The concept that a fatty acid has a function as an endocrine factor is unusual, because in terms of its chemical nature it does not fit in to the conventional categories of hormonal substances. Hormones were once assumed to be, exclusively, either peptides or steroids (for example see Hoffmann & Porchet, 1984), but in recent years however, a whole variety of molecules have been implicated as endocrine factors, and many have been demonstrated to be involved in reproductive processes.

It was outlined in section 1.4.2, how eicosanoids have a role in the spawning of molluscs and starfish. A tri-hydroxy metabolite of 5,8,11,14 - eicosatetraenoic acid has been identified as the barnacle hatching factor (Holland *et al.*, 1985) and the purine 1-MeAde as the maturation inducing substance of starfish. These factors are certainly outwith the peptide / steroid classes. With a close relationship to the eicosanoids, that a fatty acid is an endocrine substance is therefore not as strange as it might first appear.

The cyclical nature of SMF production, demonstrated by Bentley (1985), and the fact that SMF is produced by the prostomium and acts on a distant target organ (the gametes), supports the fact that it is an endocrine substance. SMF may be produced in the prostomium by the action of phospholipase A_2 on membrane phospholipids (see section 8.5), and may be released either into the blood or directly into the coelomic cavity. That the endocrine response is not dependent on transport *via* the blood system, is demonstrated by the spawning experiments (Chapter 7), where spawning is stimulated by the direct injection of either SMF or 8,11,14 - eicosatrienoic acid into the coelomic cavity.

In species where free fatty acids are transported around the body for metabolic purposes, they are usually found bound to albumin in an albumin-fatty acid complex (see Mead *et al.*, 1986). The effect of serum albumin on 8,11,14 - eicosatrienoic acid and SMF has been demonstrated in a number of experiments *in vitro* (see sections 4.3.5 & 5.3.2), and can modulate the response of sperm to these substances considerably. The role of serum albumin in the spawning response of *Arenicola* is however not understood.

It was once thought that the endocrine systems of invertebrates were much simpler and were completely different from those of vertebrates, but the current knowledge of invertebrate reproductive endocrinology alone, demonstrates that this is not always the case. The evolution of endocrine systems appears to have been far

more conservative and the presence of many vertebrate hormones, both within and outwith reproductive endocrinology, have been identified within the invertebrates (see deLoof & deClerk, 1984). Basic principles of endocrinology are far more universal and therefore invertebrate systems may have more to contribute to endocrinology as a whole, than was once considered possible.

8.7 Biochemistry of Sperm Activation

The experiments described in Chapter 6, although only preliminary, make several significant observations regarding sperm activation biochemistry in *Arenicola*. The relationship between these observations and their relevance to sperm biology in other animals is discussed in the following sections.

8.7.1 The role of pHi

The importance of intracellular pH in the sperm activation of a number of species has been described (see section 1.3). Although the precise role of pHi in many systems remains unclear, in some species such as the sea urchins it is of fundamental significance in the control of sperm activation.

The measurements undertaken in Chapter 6 indicate, that following sperm activation in *Arenicola* by SMF, there is a slight elevation of intracellular pH. That substances such as NH_4Cl, which are known to elevate the intracellular pH, can also stimulate sperm activation, could suggest that the mode of action of SMF occurs *via* this mechanism. However, experiments have also demonstrated that the characteristics of sperm activation by SMF and by NH_4Cl are clearly different, in terms of the accompanying motility patterns, respiration rates (Chapter 5) and also in terms of the pHi values themselves (Chapter 6). This would therefore seem to suggest that whilst the sperm of *Arenicola* are capable of activation in response to an

increase in pHi alone, it does not appear to be the mechanism by which natural activation (as stimulated by SMF) occurs.

The sperm of *Arenicola* are therefore clearly quite different from those of the sea urchin (see Trimmer & Vacquier, 1986) and appear to display a converse relationship between pHi and motility than that observed in the horseshoe crab *Limulus polyphemus* (Clapper & Epel, 1982). In this species, the elevation of sperm motility is accompanied by an increase in intracellular pH, but artificial elevation with NH_4Cl does not bring about sperm activation. It may be that in this respect sperm activation in *Arenicola* may be more like mammalian species, where pHi interacts with other parameters such as Ca^{2+}, and cyclic AMP in order to bring about sperm activation (see Hoskins & Vijayaraghavan, 1990).

An elevation in the intracellular pH is probably the mechanism by which quinacrine exerts its sperm activating properties (see section 6.6) and it remains a possibility that it is the mechanism by which quercertin also activates spermatozoa (see section 4.3.3). That sperm respond to such a trigger is interesting, and no biological explanation can be offered on the basis of the data presented in this thesis. Clearly the data presented only refers to sperm activation *in vitro*, and that pHi has a role in the period of extended sperm motility following activation *in vivo*, remains to be determined (see 8.7.3).

8.7.2 The role of ATP

Adenosine triphosphate has been implicated in having vital importance in the maintenance of sperm quiescence in the sea urchin (see section 1.3.1). ATP also has potential in determining the motile life and fertilizing ability of sperm by virtue of being the immediate source of energy to drive the motile apparatus of the cell. Chapter 6 however, outlines that in *Arenicola* the ATP concentration of sperm, remains more or less at constant levels, prior to and during sperm activation by SMF

in vitro. This suggests, that both the maintenance of sperm quiescence and the cessation of sperm motility following activation, are not events which are mediated by low levels of ATP.

8.7.3 A capacitation-like response

Capacitation is an important step in the preparation of sperm for the acrosome reaction and for fertilisation in many species. That it occurs at all in sperm of invertebrates has, however, been the source of much speculation, although it has been pointed out that it may occur concurrently with other aspects of sperm activation and therefore may not be apparent as a discrete event (Longo, 1987). The molecular changes which occur in sperm during capacitation have been reviewed in section 1.3.3.

One aspect of capacitation is the effect that it has on the motility pattern of the sperm and capacitated sperm usually develop a 'hyperactivated' motility pattern. The fertilizing ability of the sperm is also affected and generally sperm which have not undergone capacitation are incapable of fertilising oocytes. These events are similar to those observed in *Arenicola marina*, in that sperm activated *in vitro* rapidly become immotile whereas sperm which has been spawned, by comparison has a considerably extended motile life (Chapter 7). It has also been noted that fertilisation of mature oocytes is rarely possible with sperm activated *in vitro*, whereas sperm which has been spawned readily fertilises oocytes (Bentley - unpublished observations). That these events are comparable to those of mammalian capacitation are particularly interesting and warrant further investigation.

8.8 *Arenicola* : A Model System for Sperm Activation?

It was pointed out in section 1.6 how sperm activation in *Arenicola* has more characteristics in common with sperm activation in higher animals than it does with other invertebrate systems such as sea urchins. The data presented in this thesis further suggests that this may be the case. Measurements of intracellular pH during sperm activation indicate that there is not a simple relationship between pHi and the acquisition of motility. The observation that sperm, following spawning, have undergone a capacitation - like response also suggests a more complex activation mechanism occurs *in viva*

The fact that *Arenicola*, a widely available species which is easy to maintain and has gametes which are easily manipulated, is of particular advantage for spermatologists. Although the animal has an annual cycle of reproduction, by harnessing the different populations at different sites around the British Isles, sexually mature animals can be obtained for a large proportion of the year. Clearly, more work is required in order to fully evaluate the sperm activation mechanisms in *Arenicola marina*, but on the basis of these observations it appears to be a particularly interesting species in which to do so. Research into the sperm biology of *Arenicola* can only be fruitful, and has possibilities in leading to significant advances in our knowledge of its reproductive biology, to that of the polychaetes, and to the knowledge of sperm biology in general.

8.9 Future Research

On the basis of the discussions in this chapter, several areas of further work can be suggested.

(i) Identifying the mechanism and mode of action of this novel sperm activating substance is of particular interest, and these can be identified in a number of ways. Using ^{14}C labelled fatty acid, any metabolism by the spermatozoa can be identified by the partition of the label in compounds identified and separated by chromatographic techniques. Fundamental to the understanding of the mode of action of 8,11,14 -eicosatrienoic acid is discovering its site of action, and this could be achieved in part, by using demembranated studies. The removal of the sperm membrane, but keeping the axoneme and associated cellular apparatus intact (see Gibbons, 1981), allows the activation conditions of the axoneme to be experimentally defined. It is possible therefore, to quickly establish whether the fatty acid is being internalised and is acting directly on the axoneme or mitochondria, or, whether it functions at the level of the cell membrane *via* either a receptor mediated response, or by metabolism into a further metabolite.

(ii) With a greater understanding of the chemical nature of SMF, identifying its site of secretion, storage or production becomes a real possibility. Using antibodies, raised to phospholipase A_2, should help in the localisation of this enzyme, at the level of the electron microscope. Assays for phospholipase activity would help to determine any seasonality in the titre of this enzyme, which may be responsible for seasonal variations in the SMF content of prostomia.

(iii) The purification and full structural characterisation of SMF still remains to be completed, although preliminary purifications have made practical contributions towards achieving this goal. The capillary GLC techniques which have been described (Chapter 4) are probably most suited to this kind of analysis

and the data obtained in this thesis on the chemical characteristics of SMF, should make final purification procedures easier.

(iv) The effect of pHi in this system should be fully investigated along with the role of ATP and cAMP which all interact together in the regulatory process of sperm motility in many animals (Hoskins & Vijayaraghaven, 1990).

(v) The capacitation-like events which are observed in sperm following spawning are of particular interest. The sperm activation system in *Arenicola* may therefore represent a system in which capacitation can be investigated in an invertebrate system. And finally,

(vi) Qualitative data suggests that the spawning mechanism of *Arenicola* may be a more active event than was previously considered. Interesting questions are therefore raised as to the events which underlie this spawning behaviour, and the role that 8,11,14 -eicosatrienoic acid plays in the stimulation or mediation of these events.

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Publications Arising from this Thesis

Bentley, M.G., & A.A. Pacey., (1989) A scanning electron microscopical study of sperm development and activation in *Arenicola marina* (Annelida: Polychaeta). *Invertebr. Reprod. Dev.* **15**: 211-219.

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