FERTILIZATION ECOLOGY OF BROADCAST SPAWNING MARINE INVERTEBRATES

Mark Elliott Williams

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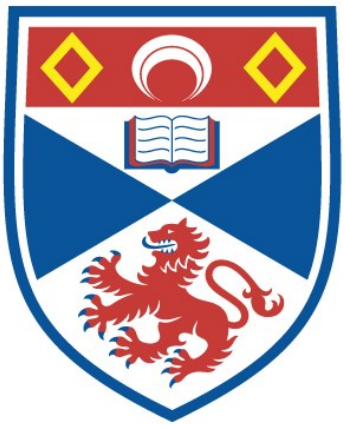

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Abstract

In situ measurements of the fertilization success of broadcast spawning marine invertebrates have been conducted mainly on shallow subtidal echinoderm and coral species. In this study, field fertilization success was measured for two infaunal, intertidal polychaetes *Arenicola marina* and *Nereis virens*. Both species are epidemic spawners, with populations of *A. marina* spawning over a few days in autumn while *N. virens* spawns in early spring. The fertilization success of female *Arenicola marina* is highly variable, ranging from 0 to 100%, and is determined by male spawning density. It is hypothesised that fertilization success over the entire spawning period is the result of an accumulation of fertilizations each day in the spawning period. Fertilization success was measured indirectly in *Nereis virens* by transplanting oocytes into the field. At least two spawning periods occurred for the population studied here. Fertilization success was uniformly high during the first period, and in the second fertilization success was highest among those eggs located high in the water column as opposed to those at substratum level. The fertilization strategies of these polychaetes are discussed in the light of these results.

Comparative laboratory experiments were performed on factors that affect fertilization success in *Arenicola marina*, *Nereis virens*, *Asterias rubens* and *Echinus esculentus*, including sperm:egg ratio, sperm-egg contact time and sperm concentration. The extent to which each of these factors affects fertilization success varies with species, and this is explained by gamete attributes and mathematical models. Gamete longevity significantly affects fertilization success, and eggs and sperm of *Arenicola marina* are extraordinarily long lived compared to those of the other species studied here and elsewhere. Eggs are viable for 5 days after spawning, while dilute sperm remains capable of fertilizing eggs for more than 48 hours. The laboratory data are discussed in terms of the fertilization strategies of each of the species.

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Chapter 1

The Biology and Ecology of Reproduction in Marine Invertebrates

1.1 Introduction

The reproductive biology of marine invertebrates is a well established and productive area of research. A rich literature exists from across the globe detailing the variety of reproductive modes demonstrated by marine invertebrates. From the early work in the last century that provided qualitative descriptions of reproductive behaviours, cycles and gross reproductive morphology, the field has blossomed into a number of advanced disciplines. These include (amongst others) studies into the molecular basis of fertilization, endocrinology and the control of reproduction, cellular physiology, developmental biology and population genetics and gene flow. In many cases (e.g. fertilization biology) it is marine invertebrates (and in particular the Echinodermata) that have provided the model for study in the animal kingdom.

The study of reproductive ecology, in particular supply side (larval) ecology, has advanced considerably in recent years. The importance of larval ecology to general community ecology is now well recognised among marine benthic ecologists. Consideration of this aspect of biology is an integral part of the study of the life cycle of a given invertebrate species. It is curious therefore to note the general paucity, at least until the last 10 years, of quantitative information in the literature concerning life cycle stages prior to larval release, namely spawning and fertilization.

With a few notable exceptions (e.g. Belding 1912, Lillie 1915) who attempted to quantify this vital life cycle stage, the impact of fertilization success upon the concomitant reproductive success of marine invertebrates has been largely ignored. Instead, the older (pre-1980s) literature on this subject is mostly comprised of assumptions about the nature of the spawning behaviour and likely outcome (fertilization success) of the event in the life cycle. Indeed, the eminent biologist Gunnar Thorson, whose work is still highly relevant to larval biologists today, believed it likely that through the behaviour of many marine invertebrates (synchronous spawning, aggregative behaviour) almost all eggs of marine benthic invertebrates are fertilized (Thorson 1946). This view was widely accepted until recently, mainly because of the absence of evidence to the contrary.

In 1985, however, J.T. Pennington published the first work which attempted to quantify the fertilization success of marine invertebrates (in this case, the echinoid *Strongylocentrotus purpuratus*) under field conditions. With the advent of new field and laboratory techniques, and an improved understanding of the spawning behaviour of marine invertebrates, a new awareness of the importance of this stage has arisen through a recent proliferation of work on the subject. Coupled with mathematical models, these practical studies have given the first firm evidence that the fertilization of oocytes from free-spawning invertebrates is far from certain. Rather it is an unpredictable event, which may be prone to a range of stochastic phenomena. In this way, it may be equally important to the recruitment success of a species as more established factors such as larval survival, and settlement success.

Through the course of this chapter, I shall attempt to show how fertilization ecology has brought a new aspect to the study of marine invertebrate reproduction. I similarly shall discuss the factors which affect fertilization success, a theme central to the aims of this thesis. These will be placed in context with the overall reproductive strategies of marine invertebrates, with particular emphasis given to the animals under study here - the polychaetes *Arenicola marina* and *Nereis virens*, and the echinoderms *Echinus esculentus* and *Asterias rubens*.

1.2 Reproductive Strategies of Marine Invertebrates

Reproduction can be regarded as the ultimate "goal" of an organism. Central to the reproductive success is the reproductive strategy of a species. A huge variety of strategies exist throughout the various marine invertebrate phyla. Indeed, in his 1991 review of the reproductive strategies of the Polychaeta alone, W.H. Wilson recorded a total of 18 distinct strategies and combinations of strategies. The following account is not intended to be exhaustive, but will compare and contrast strategies that apply to the sexually reproducing gonochoristic, annual iteroparous and semelparous broadcast spawning invertebrates studied in this thesis.

1.2.1 Sexual and Asexual Reproductive Strategies

Asexual and sexual reproduction are obviously the most distinct strategies for propagation in invertebrates. However in this course of this thesis I shall be concerned solely with sexually reproducing species, and shall therefore make only a brief mention on this subject. There is still a great deal of controversy regarding the appearance of sexual reproduction, and Ghiselin (1987) points out that theoretically, sex should not exist. An asexually reproducing organism will always out-reproduce a sexually reproducing one.

Historically, the persistence of sexual reproduction in nature has been explained by invoking group selection. Much of the early literature on the subject is extensively reviewed by Ghiselin (1974) and Bell (1982). Williams (1966) provided the first real criticisms of the group selection theory, and instead proposed the "penny-stock" model. This model predicts that in an unpredictable environment, organisms should diversify (reproduce sexually) to increase the probability of producing a phenotype with greater fitness. Bell (1982) took this further with the suggestion that saturation of niches required the production of more diverse phenotypes. In an unsaturated (under-exploited) environment, an asexual strategy is favoured, whilst a saturated environment favours sexual strategies.

Some evidence exists in nature to support this view, and this is reviewed by Ghiselin (1987). For example, the main body of the sea can be taken to be a stable, saturated environment, and by comparison the estuaries or rivers (or other semi-isolated bodies) which flow into the sea less stable and thus under-saturated. Ghiselin (1987) reports that there is an increased incidence of asexual reproduction amongst freshwater and terrestrial organisms than in their marine counterparts (see also Jaenike and Selander 1979). He cites the particular examples that asexual reproduction is rare among marine turbellarians, and parthenogenesis in tardigrades and prosobranchs is restricted to the freshwater forms. The evidence available to date would thus seem to bear out the hypothesis of Bell (1982).

Another factor which may determine the sexual strategy of the organism is its mode of life. Schroeder and Hermans (1975) suggest that asexual reproduction is

generally more common in sessile, or colonial filter feeding forms. This would enable those sessile forms that have settled in a suitable habitat to exploit the available resource fully. Amongst marine polychaetes, the process of asexual reproduction is most often reported in sessile forms, although many sessile worms (e.g. *Spirorbis* spp.) show no form of asexual reproduction.

Asexual propagation is demonstrated by marine invertebrates in a number of ways. As none of the animals under study here reproduce in this way, this subject will not be considered, although for some polychaetes this is an important method of reproduction. The various processes are reviewed for marine invertebrates in general by Ghiselin (1987), and is reviewed for polychaetes by Schroeder and Hermans (1975), and by Chia and Walker (1991) and Pearce and Cameron (1991) for asteroid and echinoid echinoderms respectively.

Sexual Reproduction

During the 1950s, 1960s and 1970s the study of life history strategies became increasingly theoretical, giving rise to a number of complex mathematical models. Many of the models are surrounded by controversy, and are extensively reviewed by Stearns (1976). A full account of the models is unnecessary, and reference will only be made where it is pertinent to do so. A number of different strategies for sexual reproduction exist in marine invertebrates, and some of these which apply to the animals under investigation here are discussed below briefly.

Hermaphroditism is not considered here, because both echinoderms and polychaetes are fundamentally gonochoristic, although there are exceptions. The reader is referred, therefore, to reviews for accounts of hemaphroditism in these invertebrate groups (Schroeder and Hermans, 1975 Ghiselin, 1987 Chia and Walker, 1991, Pearse and Cameron, 1991).

1.2.2 Iteroparous and Semelparous Reproductive Strategies

One of the key characteristics of the life history strategy of an organism is the number of reproductive episodes that occur before senescence. A strategy that involves a single event, followed by death is termed semelparous, whilst a strategy that includes multiple breeding episodes is termed iteroparous (see Cole 1954, Gadgil and Bossert 1970, Stearns 1976 for terminology). These strategies are described widely in marine invertebrates, and particular classes or groups may show examples of both strategies. A number of authors have applied mathematical models to the study of semelparous and iteroparous life history strategies in an attempt to understand their nature and occurrence (Cole 1954, Charnov and Schaffer 1973, Stearns 1976, Young 1981). The complex models published to date are beyond the scope of this review, but the implications of these models have greatly advanced our understanding of the strategies observed in nature.

From life history theory, one would expect in the case of semelparity that there would be a clear benefit to reproducing only once before death, with "fitness" measurable in terms of numbers of individuals produced. It is often suggested that semelparous organisms have a much greater investment in reproduction at any one time that an iteroparous species. Recently, Brenchley *et al.*. (1996) reported on the reproductive allocation (RA) in two fucoid species, one semelparous, one iteroparous. The iteroparous *Fucus serratus* has a RA of 38.6% in its first year, rising to 50% in its second. In contrast, the semelparous *Himanthalia elongata* has 98% of its body mass given over to reproduction.

Often, this increased reproductive allocation will result in a trade off of sorts between somatic and reproductive investment. Normal somatic maintenance is neglected in favour of increased parental investment. This is adequately summed up by Fischer *et al.*. (1996) with reference to the semelparous polychaete *Nereis virens* which demonstrates "catabolism of somatic tissue and anabolism for the benefit of gametocytes". No such trade off would exist for an iteroparous species, and normal somatic maintenance takes place throughout the reproductive season.

Usually it is a lack of somatic maintenance that ultimately leads to senescence of the adult. This has been demonstrated in a number of species from cephalopods to the nereid polychaete *Nereis virens* studied here. The lack of somatic maintenance has been most accurately measured in freshwater planarians by Romero *et al.*. (1991). They report that the rate of mitosis of somatic tissues of mature semelparous planarians during the breeding season is significantly lower than a sympatric iteroparous planarian species, indicating a decreased somatic maintenance of the semelparous species.

Perhaps the best understood examples of semelparous strategies among marine invertebrates are the nereid polychaetes. These show a number of morphological changes during gamete development (epitoky). The gut becomes non functional, and there will typically be degeneration of many somatic tissues as most energies are directed to reproduction (see Schroeder and Hermans 1975, Olive and Clark 1978, Olive 1984). This will be further discussed during the consideration of the reproductive biology of *Nereis virens* in section 1.7.

Within the Polychaeta, the terms iteroparous and semelparous have been, in the past, replaced by monotelic, polytelic and semi-continuous (Clark and Olive 1973, Clark 1979, Olive and Clark 1978). These differ slightly from iteroparous and semelparous in that they describe the nature of the reproductive event in more detail. Monotelic describes a single reproductive event before death. Polytelic describes a strategy where the organism breeds several times, but each breeding cycle is a discrete, often annual, event (i.e. discrete, annual, iteroparous). Semi-continuously reproducing polychaetes breed as and when gamete maturation occurs, often throughout much of the year. Both the latter examples could be considered to be iteroparous.

1.2.3 Other strategies

The species studied here are either annual iteroparous (*Arenicola marina*, *Asterias rubens* and *Echinus esculentus*), or annual semelparous (*Nereis virens*) strategists with reproduction occurring in a discrete spawning period measurable in

days or a few weeks. Gamete development is synchronised throughout the population over an extended period of time, and gamete release occurs over a short spawning season (days or weeks) at the same time each year. However, reproduction in other species may be continuous or semi-continuous within a population, with cohorts of gametes developing and being released throughout an extended breeding season.

Copulatory or pseudocopulatory behaviour, unlike the broadcast spawning animals under investigation here is a feature of many polychaete and echinoderm species. Interstitial polychaetes in particular show a high incidence of copulatory behaviour (Schroeder and Hermans 1975). Pseudocopulation in relation to polychaetes can be defined as the forming of a male-female pair and the release of gametes in close association, and is a feature of several members of the Nereidae. The nereid polychaete *Platynereis dumerilii* begins a nuptial dance prior to spawning which is instigated by pheromones (Hauenschild 1966). The male and female swim around each other in very close spirals, and eggs and sperm are spawned into the water in close association. Such behaviour is also seen in *Nereis succinea* (Hardege *et al.*. 1990). Pseudocopulation in its truest sense has been rarely described for echinoderms. Slattery and Bosch (1993) report male-female pair formation in the brooding Antarctic asteroid *Neosmilaster georgianus*, which they term as pseudocopulation. Although many other species form large spawning assemblages in which there may be intimate contact between individuals, this cannot be termed as pseudocopulation because there is no evidence to suggest male-female pair formation. In the starfish *Leptasterias hexactis*, males and females form pairs or larger aggregations, and spawn in synchrony to maximise the chances of successful fertilization (Hamel and Mercier 1995). However, contact between this normally solitary species is not limited to simple male-female pairs, but occurs between all individuals. Some pairing behaviour is shown in the deep sea hermaphroditic holothurian genus *Paroriza*, aiding reproduction in these continuously reproducing species (Tyler *et al.* 1992). Similar aggregative behaviour is also shown in the seasonally reproducing bathyal echinoid *Stylocidaris lineata* (Young *et al.* 1992).

1.3 Environmental Control of Reproduction

There are many examples of marine invertebrates whose reproduction is under the influence of external (exogenous) factors. These can be physical factors such as photoperiod, temperature and lunar phase, or biotic factors, for example phytoplankton blooms or control through conspecifics (pheromones). In a review of annual rhythms in marine invertebrates, Halberg *et al.*. (1969) reported that many marine invertebrates exhibit circannual rhythms which may be influenced by the environment. In some cases, these environmental cues have been shown to entrain endogenous endocrine controlled rhythms, and are termed "zeitgebers". Once entrained in the individual, control of reproduction becomes endogenous, under the direct control of the various endocrine mechanisms (see Bentley and Pacey 1992 for a review of the control of reproduction in polychaetes), and this leads to the synchronisation of reproduction in a population.

Temperature in particular has long been implicated in the control of reproduction of many temperate marine invertebrates, where there are wide fluctuations in the annual sea temperature, as for example in the North Sea (range 4 - 15°C. Indeed, Orton (1920) cites temperature as the most important factor controlling reproduction. Townsend (1940) reports the stimulation of Summer spawning sea urchins (*Arbacia punctulata*) to produce ripe gametes during the Winter by keeping them at an elevated temperature. This artificial ripening is similarly reported in molluscs (Loosanoff and Davis 1950, 1952).

It is perhaps not surprising that temperature should play such a part in the regulation of reproduction, since, along with photoperiod, it regulates the annual growth of phytoplankton and hence food availability along the food chain in the marine environment. However, Olive (1995) states that it is unlikely to be the single controlling factor in any marine invertebrate. He cites other factors which may override the direct effects of environmental temperature. These include nutrient availability and photoperiod. This was demonstrated in the polychaete *Nereis diversicolor* which shows identical oocyte development rate across a range of temperatures, indicating that the reproductive rhythm was entrained at an early stage

of development and then regulated endogenously, independent of temperature (Olive and Garwood 1983).

Temperature undoubtedly influences the time of year that many invertebrate populations spawn. Minchin (1992) correlated the spawning of a range of marine invertebrates with environmental temperature. He argued that rising temperature induces more frequent spawning. North sea Nereidae in particular appear to require temperatures above 6-8°C to begin spawning (Goerke 1984), and it is well known that many bivalve species can be induced to spawn in the laboratory by raising the water temperature.

Photoperiod is one of the most important factors governing the reproductive cycles of invertebrates, and has been demonstrated most comprehensively in polychaetes (Fong and Pearse 1992, Olive 1995). In particular, the reproductive cycle of the polychaete *Nereis virens* has been shown to be under the control of photoperiod, and this is discussed in section 1.7. It is also believed to be influential in the annual reproductive cycle of *Asterias rubens* (Chia and Walker 1991). Photoperiodic control of reproduction and spawning in marine invertebrates is discussed by Giese and Kanatani (1987), where table VII (page 290) lists a range of species from sponges to tunicates whose reproductive cycle and spawning correlates with photoperiod. Of all invertebrate groups, induction of spawning by exposure to light seems to be most prevalent in ascidians (see Lambert and Brandt 1967) and hydroids (Baker 1936, Ballard 1942, Yoshida 1952, 1954).

The reproductive cycle and spawning times of many marine invertebrates can be correlated with lunar periodicity (e.g. Korringa 1947). Indeed, the exact hour of spawning of some species can be predicted by the lunar cycle. Such cycles are discussed by Giese and Kanatani (1987), and appear in invertebrates ranging from corals to littorinid molluscs. Lunar periodicity is particularly well documented in tropical species where annual changes in temperature and photoperiod are less pronounced. Indeed, the spawning time of many species of corals and other tropical invertebrates correlate so well that they can be predicted annually (Harrison *et al.*. 1984, Babcock *et al.*.1986, Alino and Coll 1989, Babcock *et al.*. 1992). Dan and Kubota (1960) working on the tropical crinoid *Comanthus japonica* found that its

spawning time is directly dependent upon the lunar phase. Similarly, Illiffe and Pearse (1982) describe the lunar periodicity of reproduction in a tropical sea urchin. They report that the annual cycle of reproduction of a population is indistinct, and variable between years, probably influenced by a range of factors. However, when it occurs, gamete development and spawning time correlate well with lunar periodicity. The literature regarding lunar periodicity in echinoderms is reviewed by Pearse (1975).

One of the most famous examples of lunar controlled spawning event is that of the Pacific palolo worm, *Eunice viridis* in Western Samoa. Populations of these polychaetes exist around the coasts of many of the islands in this region of the Pacific. Epitokes of the worm swarm on specific nights during the lunar cycle, and different populations spawn on different days in the cycle. The spawning time is predictable to specific dates during October for each population (Caspers 1961, 1984). This event is extremely important to islanders who regard the worm as a delicacy.

Seasonal reproduction also exists in the deep sea, an environment not subject to fluctuations in temperature or photoperiod. This has been demonstrated in a number of species, and is reviewed by Tyler (1988). In the species studied to date, the precise cue that initiates the cycle of reproduction is not well understood. An obvious choice in the search for a reproductive cue is the annual pulse of phytodetrital material falling to the sea floor, but the reproductive cycles observed to date are often out of phase with the pulse of material (Tyler 1988, Tyler *et al.*. 1992, Young *et al.*. 1992).

1.4 Fertilization in Broadcast Spawners

1.4.1 Fertilization Strategies

The term reproductive strategy is commonplace, and is used to describe the mode of reproduction of a species, be it iteroparous or semelparous reproductive strategies, or planktotrophic or lecithotrophic larval development. There are also a

number of different strategies employed to achieve fertilization in animals which broadcast their gametes into the environment. I shall therefore introduce the term "fertilization strategy" to describe the means by which a species bring eggs and sperm into contact. Often, an individual strategy is unique to a particular species, and is the result of the evolution of particular behaviours, gamete attributes and the physiology of the organism.

Fertilization strategy describes all the steps between spawning and larval development including spawning cues, spawning behaviour, gamete release, gamete physiology and fertilization kinetics. In recent years, consideration of these aspects of the reproduction of marine invertebrates has led to the establishment of fertilization ecology as a discipline in its own right, alongside larval ecology (e.g. Levitan 1995). It is this sphere of research with which the remainder of this chapter, and indeed the rest of this thesis is principally concerned.

1.4.2 Spawning Behaviour

In order to ensure successful fertilization, a number of strategies must be employed by free spawning invertebrates to increase the probability of sperm-egg interaction. In particular, it is essential that a population synchronises gametogenesis and spawning. The synchronisation of spawning is essential in free spawning invertebrates so that sperm and eggs are released and interact before they are swept away or become senescent. Synchrony of the reproductive cycle is evident in a range of marine invertebrates, and is usually controlled (initially) by an environmental cue (zeitgeber). Such cues were discussed in section 1.3. Once entrained in the individual, the reproduction cycle is often modulated externally and controlled endogenously through endocrine systems.

As stated earlier, the spawning cues for many invertebrates are also derived from the environment. However, other mechanisms have evolved to synchronise spawning behaviour, specifically the induction of spawning by conspecifics. This is a well established feature of reproduction in marine algae (Clifton 1997), and mate attraction is well studied in Crustacea, particularly lobsters (Dunham 1978, Atema

1995). Synchronous spawning in marine invertebrates induced by pheromones, or the presence of gametes in the water column is common, although investigations into the chemical nature of the pheromones are in their infancy. In nereid polychaetes, the same volatile organic compound is implicated as the spawning pheromone of *Platynereis dumerilii* and *Nereis succinea* (Zeeck *et al.*. 1988, 1990, Hardege *et al.*. 1990). Sexual pheromones are also believed to exist in echinoderms, and Beach *et al.*. (1975) and Miller (1989) report the induction of spawning in several asteroid species. As well as inducing spawning, pheromones can control the synchrony of gametogenesis in holothurians (Hamel and Mercier 1996) and opisthobranchs (Painter 1992). In addition, it is reported that chemicals derived from phytoplankton induce spawning in some mollusc and echinoderm species (Himmelman 1975, Starr *et al.*. 1992).

Aggregation of individuals of a free-living population prior to spawning is another strategy which is employed to improve the chances of fertilization success. It has been reported in a number of species (Thorson 1946), and is most frequently recorded in echinoderms (e.g. Pennington 1985, Levitan 1988 Tyler *et al.* 1992, Young *et al.* 1992), although it is unclear whether such behaviour is related to reproduction. Perhaps this aggregative behaviour is taken to extremes by swarming polychaetes, where spawning individuals or epitokes leave their burrows and swarm freely in the water column, interact and release gametes (Schroeder and Hermans 1975).

1.4.3 Sperm : Egg Interaction

Chemical attraction and subsequent taxis of a sperm towards an egg is a phenomenon that had been suspected and implied in animal phyla for many years before it was unequivocally demonstrated (Lillie 1912, 1919). It has, however, been known for over a century in plants, and much is known of the nature of the chemical attractants, particularly in the marine brown algae (Maier and Müller 1986). Chemotaxis in marine invertebrates was first shown to occur in a medusoid cnidarian by Dan (1950), and extensive work by Miller during the 1960s and 1970s

demonstrated chemotaxis in a number of other species of Cnidaria (Miller 1970, 1978). It has since been demonstrated in a number of phyla including molluscs (Miller 1977, Miller *et al.*. 1994), arthropods (*Limulus* sp., Clapper and Brown 1980), echinoderms (Miller 1985) and tunicates (Miller 1975). Whether such chemotaxis exists in annelids is presently inconclusive.

Chemical nature of attractants

A number of chemicals have been implicated in the attraction of sperm in marine invertebrates. The characteristics of sperm attractants were reviewed by Miller (1984), before the actual identification of the chemicals involved. He noted that the basic characteristics of attractants from various phyla were similar, in that they were small (in the range of 2 - 12 kDa), highly potent at low concentrations and generally species specific.

The first sperm attracting substance to be identified was resact, a small peptide isolated from the jelly layer of the sea urchin *Arbacia punctulata* eggs (Ward *et al.*. 1985). This peptide fails to attract sperm from other species of echinoderm. Latterly, this peptide has become known as SAP-IIA (SAP = sperm-activating peptide) (Yoshino *et al.* 1990), the structure of which has been found to vary between different species of echinoderm (Punnett *et al.*. 1992).

The classes of sperm attracting chemical appear to differ between phyla. While peptide attractants are apparent in echinoderms (Ward *et al.*. 1985, Punnett *et al.*. 1992), and siphonophore cnidarians (Carre and Sardet 1981), lipids ((-)-epi-thunbergol) are implicated for corals (Coll *et al.*. 1994) and the molecule involved in the chemoattraction of sperm from the tunicate *Ciona* sp is neither a peptide or a lipid (Yoshida *et al.*. 1993). The chemotactic pheromones of algae show even more variety, and are comprehensively reviewed by Maier and Müller (1986).

Effects of attractants upon sperm swimming

Chemicals derived from the egg can have three types of effect upon the swimming of the sperm. First they can initiate swimming of the sperm (e.g. *Limulus* sp., Clapper and Brown 1980), second they can lead to an increase in sperm swimming velocity and finally, they can cause the sperm to swim in a directed manner towards the egg (see Miller 1984 for review of sperm swimming behaviour). The outcome of these behaviours, is simply to increase the probability of sperm - egg interaction. It is believed that the principal effect of a sperm attractant which is released from the spawned egg is to create a greater surface area of the egg, thus making "larger target" for passing sperm (see Cosson 1990). However, the distance over which the attractants are effective will be limited by the constraints of sperm swimming capacity, and hydrodynamic effects.

1.4.4 Biochemistry of Fertilization

Sperm-egg fusion is the end result of a complex process of chemical interactions. Most of the studies of fertilization in marine invertebrates have centred around the Echinodermata, and very little literature exists for the Annelida.

Sperm-egg binding

The sperm head of many groups of organisms contains an acrosomal vesicle at its tip which has been known to biologists for over a century (see Tilney 1985 for review). It was not until the mid-1950s, however, that its role in fertilization became apparent. Studies by Dan (1954) and Colwin and Colwin (1956) revealed structural changes within the vesicle, leading to the formation of a pointed acrosomal process upon sperm-egg contact. Investigation of cross-fertilization with sympatric echinoid species have revealed that the acrosome reaction is not species-specific (Summers and Hylander 1975) and the block to cross fertilization occurs at a later stage (Gao *et al.*. 1986, Metz *et al.*. 1994). In starfish, at least three organic

components of the egg jelly are responsible for inducing the acrosome reaction. These have been identified to be a sulphated glycoprotein named ARIS (acrosome reaction inducing substance), a group of sulphated steroid saponins named Co-ARIS, and a sperm activating oligopeptide (SAP) (Hoshi *et al.*. 1990a,b, Okinaga *et al.*. 1992, Ushiyama *et al.*. 1993).

The acrosomal vesicle contains a protein which coats the surface of the extruded process. It was first isolated from sea urchin spermatozoa, and upon addition to egg suspensions was seen to cause agglutination (Vacquier and Moy 1977). The protein was therefore called "bindin". Subsequent studies have revealed that bindin is a 51 kDa precursor protein which cleaves during the acrosome reaction to form "mature" 24 kDa bindin (Gao *et al.*. 1986). More recently both the cDNA responsible for the bindin (Glabe and Clark 1991) and the bindin itself (Lopez *et al.*. 1993) have been sequenced. These and other studies have emphasised the species-specific nature of bindin, reinforcing observations by Vacquier and Moy (1977) and Glabe and Vacquier (1977) that agglutination of eggs by purified bindin is species-specific.

Egg receptors to sperm

Upon release of the bindin from the acrosomal vesicle, it interacts with the proteins of the plasma membrane, binding the sperm to the egg to facilitate fusion (Vacquier 1981). It has been suggested that there are a specific number of binding sites on the surface of the egg (Dale 1985), in agreement with Vacquier and Payne's (1973) estimate that an average of 1744 sperm are able to bind to an egg of the echinoid *Strongylocentrotus purpuratus*. It has since been found that specific sperm receptor proteins exist on the surface microvilli of the egg plasma membrane (Folz and Lennarz 1990). Subsequent molecular studies have revealed this protein to be a complex, high molecular weight glycoprotein consisting of four identical sub-units (Folz *et al.*. 1993, Ohlendieck *et al.*. 1994, Ohlendieck and Lennarz 1995). Ohlendieck *et al.*. (1993, 1994) took the yield of purified receptor to calculate that the egg plasma membrane of *Strongylocentrotus purpuratus* contains approximately

10^6 receptor molecule complexes. They estimate that from Vacquier and Payne's (1973) value of 1744 sperm per egg that each sperm has around 180 receptor molecules available for binding.

Oocyte activation

Once bound, the fertilizing sperm induces an immediate de-polarisation of the egg membrane (Dale *et al.*. 1978). Initially, this prevents attachment of other sperm to the egg (polyspermy). In sea urchins this leads to a rapid rise in calcium within the egg, commencing at the site of sperm-egg fusion (Whitaker and Steinhardt 1985). The calcium wave is the trigger for a number of different systems within the oocyte to re-activate meiotic division, leading to cell-cycle progression. Different systems appear to operate between deuterostomes and protostomes. In deuterostomes (e.g. sea urchins) internal calcium stores appear to be the source of the calcium wave (Jaffe 1983, Whitaker and Swann 1993), whilst in protostomes the source is believed to be external (Jaffe 1983).

Gamete fusion

Following membrane fusion and male pronuclear development, the time for which varies between phyla (Longo 1981), the male and female pronuclei then begin their association in one of two ways. The *Ascaris* type of pronuclear fusion occurs in oocytes which are fertilized during meiotic arrest, and involves the intermixing of condensed parental chromosomes on the metaphase plate of the mitotic spindle (Wilson 1925). The *Sea urchin* type of fusion occurs in eggs which have completed meiotic maturation, where male and female pronuclei fuse to form a zygotic nucleus (Longo 1973, reviewed in Longo 1987).

Polyspermy

Polyspermy (the entry of more than one sperm nucleus into the oocyte) can be a common occurrence. It may have no detrimental effects, and lead to the fusion of only one male pronucleus with the female, or it can be pathologic and lead to abnormal development of the embryo. The latter type is often the case with marine invertebrates (Longo 1987). As mentioned above, polyspermy is initially inhibited by the depolarisation of the oocyte membrane, but this is a transient depolarisation and upon re-polarisation the egg is prone to further sperm binding. A number of mechanisms are thus employed to avoid this, and these are discussed extensively by Longo (1987). They include rapid exocytosis of cortical granules leading to the formation of a physical barrier to further sperm entry. This is characteristic of sea urchins and higher deuterostomes. They also include specialised functioning of the cytoplasm to prevent further sperm movement within the egg.

1.5 Fertilization Kinetics

The level of fertilization success attained by free-spawning marine invertebrates is a topic which is beginning to receive a good deal of attention. Previously, the importance of this step in the life-cycle of marine invertebrates was overlooked. Lillie (1915) suggested that fertilization success might be a limiting factor in the maintenance of populations of free-spawning sea urchins. Mortenson (1938) further suggested that the reason for the low recruitment levels of highly fecund species such as asteroids was because of the lack of successful sperm-egg interactions following spawning. However, Thorson (1946) countered this by postulating that through the highly synchronous nature of the spawning behaviour of many marine invertebrates, the majority of spawned eggs are fertilized. He stated that the principal factor limiting the recruitment in marine invertebrates was mortality of the larval stage. Although there were no empirical data upon which to base this assumption, it became widely accepted in later literature.

1.5.1 Theoretical models

Vogel *et al.*. (1982) formulated a series of mathematical models to describe the probability of sperm-egg encounters and hence the likely fertilization success of echinoid eggs. The models assumed a static situation, and were tested *in-vitro* through artificial fertilizations in beakers. The models confirmed early work by Lillie (1915), and Rothschild and Swann (1951) who showed that sperm dilution leads to a reduction in the fertilization success of eggs *in vitro*. The mathematical models of Vogel *et al.*. (1982) were utilised by Levitan *et al.*. (1991) to further show the relationship between sperm availability and fertilization success.

Denny and Shibata (1989) improved upon Vogel *et al.*.'s (1982) work, by formulating models to predict the level of fertilization success likely in nature. These models took account of the turbulent nature of the marine environment to give a more accurate estimate of the fertilization success of natural populations. Few improvements have since been made to the original models of Vogel *et al.*. (1982) and Denny and Shibata (1989), and most work has concentrated instead in determining the factors that affect fertilization success.

1.5.2 Laboratory studies of fertilization success

Many factors in the marine environment are outwith the control of the marine ecologist. This is particularly true of the study of fertilization success, requiring not only a complete understanding of the local conditions of water flow, but a suitable population to study and a means of measurement which does not unduly affect the results of the study. Consequently, a great deal of work into the factors that affect fertilization success in free spawning invertebrates has been conducted in the laboratory.

Three main factors have been implicated in the restriction of fertilization success in marine invertebrates. These are sperm concentration, sperm : egg ratio and gamete longevity. Sperm limitation has been shown to be the main cause of reduced female fertilization success, and is reviewed by Levitan and Petersen

(1995). In the laboratory, the effect of sperm dilution upon the concomitant fertilization of eggs *in vitro* has been demonstrated in echinoderms (Lillie 1915, Rothschild and Swann 1951, Pennington 1985, Levitan *et al.*. 1991, Benzie and Dixon 1994), bivalve molluscs (Clotteau and Dubé 1993, Andre and Lindegarth 1995), tunicates (Bolton and Havenhand 1996), hydroids (Yund 1990) and the polychaete *Arenicola marina* (Williams *et al.*. 1997).

More recently, the importance of sperm : egg ratio has been investigated for the asteroid *Acanthaster planci* (Benzie and Dixon 1994) and the echinoid *Strongylocentrotus franciscanus* (Levitan *et al.*. 1991). Levitan (1995) points out that there are differences in the sperm : egg ratio required to achieve the same level of fertilization in the two species, and that *A. planci* requires an order of magnitude less sperm to achieve the fertilization success of *S. franciscanus*. It would thus appear that there are interspecific differences in the fertilization kinetics of marine invertebrates. Reasons for these differences will be discussed in Chapter 6.

Gamete longevity has long been considered an important limiting factor in the fertilization success of marine invertebrates. To date, gamete longevity has been investigated in a number of free-spawning invertebrates, including hydroids (Yund 1990), molluscs (André and Lindegarth 1995), echinoderms (Pennington 1985, Levitan *et al.*. 1991, Benzie and Dixon 1994), arthropods (Brown and Knouse 1973) and ascidians (Havenhand 1991). These results have indicated that, in general, sperm are viable for a far shorter period of time than eggs. Often, egg longevity is measured in a few hours whilst sperm longevity can, in some cases, be measured in minutes. One exception to this was reported recently for a didemnid ascidian, where dilute sperm successfully produced one larva 48 hours after release (Bishop 1998).

Lillie (1915) demonstrated that sperm longevity was determined to some extent by its concentration in the water column. This was reviewed by Chia and Bickell (1983) who termed the phenomenon the "respiratory dilution effect". Increasing the dilution of a sperm suspension leads to a decrease in the half life of the sperm, and the actual time of the half life varies between species (reviewed in

Levitan 1993). It thus appears that there are both spatial and temporal constraints upon the fertilization of free-spawned eggs.

Most of the aforementioned studies were conducted under static conditions in beakers or petri dishes. Some workers, however, have attempted to model the fertilization success of marine invertebrates under turbulent flow conditions to simulate the natural situation. Denny and Shibata (1989) investigated the behaviour of gametes in variable flow conditions. This enabled them to produce improved mathematical models of the dispersal of sperm downstream from spawning males, and hence fertilization success. They showed that fertilization success was highly sensitive to turbulent flow and thus predicted that in the wave swept environment, fertilization success was likely to be very low. More recently, however, Denny *et al.*. (1992) demonstrated that in some cases fertilization success can actually be enhanced in the field through turbulent mixing in surge channels. These topographical features of the shore seem to act as containment vessels, lowering the rate of sperm dilution, thereby increasing the probability of sperm-egg interaction.

1.5.3 Fertilization success in the field

Since the first report of fertilization success under field conditions by Pennington (1985), the literature has grown rapidly. Table 1.1 shows some of the studies that have been conducted to date and also the levels of fertilization success recorded. Generally, the animals studied so far are shallow sub-tidal, epifaunal free spawning species, and most studies have been conducted upon echinoderms. This is presumably because of the ease of manipulation, high fecundity and ready availability of these animals. As can be seen from table 1.1, other species examined for fertilization success in the field include several cnidarian species.

Taxon	Fertilization Success	Reference
Cnidaria		
Pseudoplexaura porosa	0% - 98%	Coma and Lasker 1997
Plexaura kuna	<20%	Lasker *et al.* 1996
Briareum asbestinum	0.01% - 6.3%	Brazeau and Lasker 1992
Scleractinia		
Montipora digitata	32% - 75%	Oliver and Babcock 1992
Echinodermata		
Asteroidea		
Acanthaster planci	23.1% - 82.6%	Babcock and Mundy 1992
Holothuroidea		
Actinopyga lecanora	72%	Babcock *et al.* 1992
Bohadschia argus	1% - 90%	Babcock *et al.* 1992
Holothuria coluber	9% - 83%	Babcock *et al.* 1992
Cucumaria miniata	92%	Sewell and Levitan 1992

Table 1.1. Fertilization success recorded from the field during natural spawning events for a variety of species (adapted from Levitan 1995)

Studies of field fertilization success can be divided into two main categories. In some, fertilization success can be measured during a natural spawning event, in others it is measured in a an artificial, controlled experiment. Many of the studies conducted to date with echinoid and asteroid echinoderms are of the latter type. Most are similar to the work of Pennington (1985), in which the experimental animals are placed at pre-determined positions on the sea floor and induced to spawn by the injection of potassium chloride (sea urchins) or 1-methyladenine (starfish). This method has enabled workers such as Levitan *et al.*. (1992), Benzie *et al.*. (1994) and Babcock *et al.*. (1994) to demonstrate some of the factors that affect fertilization success. These include population density and proximity to spawning

individuals. In addition, because it is possible to select the site of the experiment, the influence of varying flow velocity on gamete dispersion can be examined.

The study of fertilization success during a natural spawning event presents a much more difficult task. It is necessary to select for study a species that has an entirely predictable spawning pattern, that is present in sufficient numbers to sample effectively, and whose habitat is accessible to the field ecologist.

To date, fertilization success in a naturally spawning population has been observed in a number of free spawning species, and the values obtained during such studies are given in table 1.1. Babcock *et al.*. (1992) conducted a comprehensive survey of the spawning behaviour of major invertebrate species on the Great Barrier Reef, Australia, over the same two month period in consecutive years. Whilst conducting this work, they were able to measure fertilization success in two of the holothurian species observed to be spawning. Similarly, Oliver and Babcock (1992) were able to measure spawning behaviour and fertilization success in the scleractinian coral *Montipora digitata* whilst Brazeau and Lasker (1992) studied the gorgonian coral *Briareum asbestinum.* More recently, Lasker *et al.*. (1996) and Coma and Lasker (1997a,b) have measured fertilization success in two additional coral species.

From these studies (both artificially induced and natural spawning), it has been possible to determine some of the constraints that are imposed upon fertilization success in the field. One of the striking features of the work is the variability of fertilization success. Table 1.1 showing fertilization success in some of the organisms studied to date fails to represent this aspect. In many cases, fertilization can be as high as 100%, even at great distances between spawning individuals (Babcock *et al.*. 1994). However, it often falls well short of this figure and values of 0% have been recorded at the peak of spawning for some species even where spawning individuals are in close proximity (Levitan *et al.*. 1992).

Fertilization ecology has been reviewed recently by Levitan (1995), and many of the important features will be discussed later in the appropriate experimental chapters. However, the salient points regarding the field fertilization

success of broadcast spawning marine invertebrates are that high fertilization success appears to rely on synchrony of spawning, reasonably high fecundity, proximity to spawning individuals, population density, water flow and turbulence and gamete longevity. Fertilization success is not assured however, and is highly variable in nature.

1.6 The Biology of *Arenicola marina*

1.6.1 General Biology

The infaunal polychaete *Arenicola marina* (L.)(Annelida: Polychaeta: Arenicolidae: *Arenicola*), commonly known as the lugworm, is an integral part of the fauna of sandy and muddy beaches and outer estuaries of Northern Europe. The genus Arenicolidae contains 3 other species common to British shores, *Arenicolides ecaudata*, *Arenicola cristata* and the closely related *Arenicola defodiens* (Cadman and Nelson-Smith). *A. defodiens* has only recently been recognised as a species distinct from *A. marina* (Cadman and Nelson-Smith 1990, 1993).

Arenicola marina is a relatively large animal, reaching up to 25cm in length at certain localities, although there are often great differences of size and colour between animals from different sites (Newell 1948, Duncan 1960). It is unclear to what extent these differences are genetic or environmental in origin (Auckland 1993). Gamble and Ashworth (1900) and Ashworth (1904) give detailed descriptions of the gross morphology of the lugworm, and these works form the basis of the following account:

The lugworm consists of an anterior "head" section, "thoracic" trunk section and posterior "tail" region. The "head" region consists of a small trilobate prostomium on the dorsal surface of a peristomium which contains the papillae - covered eversible proboscis used in feeding and burrowing. The thoracic section comprises the main trunk region of the lugworm. It is composed of segments that are themselves externally divided by a variable number of annulation rings. Each segment has one large annulus which bears the parapodia and chaetae (the last 13

also bearing dendritically branched gills), and a variable number of achaetigerous annulations. The posterior caudal section is very much narrower than the trunk and is free from annulations.

The lugworm is of great ecological importance as a food source for wading birds, fish and other predators (de Vlas 1979). In some areas such as the Dutch Wadden Sea *Arenicola marina* makes up almost a third of the benthic biomass (Beukema 1974). It is also important in enriching "sterile" sediments through mucus production, faecal casting and the reworking of nutrients in the sediment (Cadée 1976, Asmus 1986). In addition, it is increasingly important economically as bait for fishing. This has led to extensive over-exploitation of natural populations, damaging both the worm stock and surrounding habitat (Jackson and James 1979, McLusky *et al.*. 1983, Olive 1993). Beukema (1995) even reports the widespread use of damaging mechanical harvesting techniques in the Dutch Wadden Sea. Restrictions to digging are now becoming increasingly common at various sites around the UK

Arenicola marina forms a semi-permanent burrow in the sediment. The form of the burrow is subject to debate, but tends to be either "U" or "L" shaped to a depth of around 30cm depending on the size of the animal (Thamdrup 1935, Wells 1945, Jacobsen 1967). The burrow can be divided into a head shaft, characterised at the surface of the sediment by a marked depression in the sand, and a tail shaft marked by the familiar coiled faecal casts. Irrigation of the burrow is achieved through rhythmic contractions of the body wall circular muscle. This draws oxygenated surface water down through the tail shaft, over the animal and out via the head shaft (Newell 1948, Wells 1949a,b, Kruger 1959, Mangum 1976).

The irrigatory current also helps in feeding by loosening the sand around the head shaft, drawing the detritus rich sediments into the burrow where they are ingested by the worm (Wells 1966, Jacobsen 1967). Some controversy exists over the exact nature of the food source. In older literature, reviewed by Wells (1945) it was commonly held that the lugworm fed upon detritus, and Hunt (1925) proposed that the diet consisted of diatoms, detritus and microbes. Kruger (1959) suggested that lugworms demonstrate a kind of filter feeding mechanism. He maintained that

the respiratory irrigation current deposited diatoms and other food particles as it passed through the sediment-filled head shaft. However, it has recently been shown that through the maintenance of a water current, microbial growth is stimulated in the fresh sediments drawn into the burrow. Although all is ingested (detrital material, diatoms, microbes, sand), it is the microbial components which are primarily digested by the worm (reviewed by Zebe and Schiedek 1996).

1.6.2 Reproductive Biology

Arenicola marina is a gonochoristic, annual iteroparous (polytelic) polychaete which has had its reproduction described by a number of authors (Pirlot 1933, Newell 1948, Howie 1959, Duncan 1960, Olive and Clark 1978, Howie 1984). There appear to be slight differences in the breeding season between different populations of *A. marina*. In general, the spawning season at most localities in the UK and northern Europe is during late Autumn and early Winter (Duncan 1960, Howie 1984).

Most populations of *Arenicola marina* around the UK appear to have an epidemic "spawning crisis" over a few days during Autumn (Newell 1948, Duncan 1960, Howie 1984). It has been reported that some populations may have two discrete spawning periods, Autumn and Spring (reviewed in Howie 1984). However, some of the confusion in the literature could be due to the recent description of a new species of lugworm, *Arenicola defodiens*. This species was originally termed the "laminarian" variety of *Arenicola marina* (as opposed to the "littoral variety") by Gamble and Ashworth (1900). This distinction was rejected by Wells (1948) and not referred to in later works. It has now been fully accepted as a new species after detailed ecological, morphological and genetic studies by Cadman and Nelson Smith (1990, 1993).

Despite the confusion created by the mis-identification of *Arenicola defodiens* as *A. marina*, a number of differences still persist in the breeding cycles of different populations of *A. marina*. Populations that are geographically quite close have very different spawning times. These differences manifest themselves not only

as differences in actual spawning time, but also in the nature of the spawning. For example, at the East and West Sands, St Andrews, the population spawns over a few days during late October or early November, whilst at the Eden Estuary only a few hundred metres away the population spawns slightly later over a period of 2-3 weeks. In addition, the populations at Fairlie Sands and the Clyde estuary appear to have a second season during the spring. This second spawning may be a different component of the population following its own cycle of reproduction (Howie 1984).

Epidemic spawning during Autumn is reported from 9 sites around the UK, and from 1 site on the northern coast of continental Europe. This is certainly the situation in the populations studied in this thesis (Williams *et al.*. 1997).

1.6.3 Gametogenesis

Gametogenesis in Autumn spawning *Arenicola marina* takes place over a prolonged period from early Spring through to the time of spawning in late Autumn (De Wilde and Berghuis 1979b). Gamete proliferation takes place in the gonads. These are minute structures located behind the coelomic funnel at the base of each of the 6 pairs of nephromixia (Ashworth 1904, Newell 1948). The gametocytes are immediately released in to the coelomic fluid, where they undergo the remainder of gamete development (see Howie 1984 for review).

Oogenesis

Upon release from the gonads, the oocytes are around 13μm in diameter (De Wilde and Berghuis 1979a). Egg proliferation by the gonads in early spring is quite slow, but rapidly increases during late spring, with production slowing by summer as the full complement of eggs in the coelomic fluid is reached (De Wilde and Berghuis 1979b).

The oocytes undergo solitary vitellogenesis in the coelomic fluid, a process which commences around the middle of June (Howie 1961a, De Wilde and

Berghuis 1979a,b). By August or September around 90% of the coelomic oocytes have reached full size of around 180μm (Howie 1984). The fully developed eggs are unable to be fertilized at this stage, however, and are arrested at prophase of the first meiotic division. They require a maturation step to initiate germinal vesicle breakdown (GVBD) and advancement to metaphase of the first meiotic division before fertilization is possible (Howie 1961a, Meijer 1979a, Watson and Bentley 1997).

Oocyte Maturation and Spawning

It was known from early work (Howie 1962, 1966, reviewed in Bentley and Pacey 1992) that the injection of homogenised prostomia from ripe donor females into the coelomic cavity of gravid receptor females led to GVBD and spawning. This response was usually only seen around the time of natural spawning of the parent population. Meijer and Durchon (1977) and Meijer (1979a,b) conducted a series of investigations into the nature of this response. They concluded that the maturation and spawning of the oocytes is caused by a chemical located in the prostomia and released into the coelomic cavity. However, it is now apparent that they were working on the closely related species *Arenicola defodiens* and not *Arenicola marina* (Watson and Bentley 1997).

Rather than acting directly on the oocytes in a one-step maturation process, the prostomial extract initiates the production in the coelomic fluid of a secondary factor. It is this secondary factor, termed the coelomic maturation factor (CMF), which acts upon the oocytes to bring about re-initiation of meiosis, and maturation of the oocytes (Watson 1996, Watson and Bentley 1997, 1998 in press). The exact mode of action and identity of the CMF are presently unknown, but it appears to be a heat labile protein larger than 30 kilodaltons (Watson 1996, Watson and Bentley 1997, 1998).

Spawning appears to be a consequence of maturation (Howie 1961a,b). Only mature oocytes are spawned, and these appear to be selected out by the ciliated funnels of the nephromixia. Howie (1961a) even found that injection of mature

oocytes into a female with only immature oocytes will lead to the expulsion of the injected oocytes a short while later. Exact reasons for this are presently unknown, by it may be because of the shape of the matured oocytes (Howie 1984).

Spermatogenesis

As with the oocytes, following release from the gonads, sperm development takes place in the coelomic cavity (Gamble and Ashworth 1900, Ashworth 1904, Howie 1959, Howie and McCleneghan 1965, Olive 1972a,b). The sperm are shed from the testes at the eight cell stage, and undergo spermatogenesis within the coelomic cavity (Ashworth 1904). Individual spermatozoa do not appear until just prior to spawning. Instead, following release from the testes during late April or May, the spermatocytes develop simultaneously as a cluster of up to several hundred sperm heads attached to a nucleated cytoplasmic structure called a cytophore (Ashworth 1904, Newell 1948, Howie 1959, Pacey and Bentley 1992). This is called the sperm morula, and in this condition the sperm become fully differentiated (Pacey and Bentley 1992).

The number of sperm morulae present in the coelomic cavity operate a negative feedback mechanism on the activity of the testes. When the coelomic cavity is full of developing morulae, the testes slow down and stop production of more spermatocytes. Removal (by syringe) of samples of the developing morulae re-initiates mitotic proliferation of the testes (Howie and McCleneghan 1965, Olive 1972a).

Sperm Maturation and Spawning

Like oocyte maturation, sperm maturation is initiated by a "hormone" which originates in the prostomia (Howie 1961a,b,c, Olive 1972a,b). Unlike the oocytes, however, sperm maturation is a one-step process, the prostomial hormone acting directly upon the sperm morulae. Bentley (1985) termed this "hormone" the sperm maturation factor (SMF), and it was later discovered to be the 20 carbon fatty acid

8,11,14-eicosatrienoic acid (Pacey and Bentley 1992, Bentley and Pacey 1992, Bentley and Hardege 1996).

The fatty acid acts to break down the sperm morulae resulting in free spermatozoa within the coelomic cavity. However, in this state they are immotile and require a second step (or activation step) to become fully motile. The trigger for motility appears to be an elevation of intracellular pH from 7.3 (the pH of coelomic fluid) to 8.2 (the pH of buffered seawater) (Pacey *et al.*. 1994a). Activation of the sperm is therefore achieved simply through spawning via the nephromixia into the water column and the subsequent dilution by seawater. Sperm then demonstrate a characteristc intermittent swimming behaviour (Pacey *et al.*. 1994b).

1.6.4 Spawning Behaviour and Fertilization

The spawning period is characterised by the appearance of milky-white sperm "puddles" on the surface of the sediment at low water (Kyle 1896, Pirlot 1933, Newell 1948, Howie 1959, Duncan 1960). Males and females exhibit different spawning behaviours during this period.

In both sexes, spawning is preceded by release of the appropriate maturation factors, and coelomic maturation of the gametes. Observations of spawning behaviour in males injected with 8,11,14-eicosatrienoic acid in the laboratory reveals that they thrash about violently during spawning (Pacey and Bentley 1992). In the field, this results in the expulsion from the tail shaft of thin streams of sperm onto the surface of the sediment (Williams, personal observation). The sperm then coalesces to form a thick puddle with an oily consistency. In this state the sperm is mostly inactive, and becomes fully active upon dilution with seawater (Pacey *et al.*. 1994a).

Some early workers (Ashworth 1904, Newell 1948) reported that females spawning in aquaria release eggs onto the surface of the sand. They concluded that the lack of field evidence to support this was because of the fact that they are a similar colour to the sand, making them difficult to spot. However, it is now

generally accepted that the female retains the spawned eggs within the burrow after spawning, and that this is the site of fertilization (Farke and Berghuis 1979a).

Fertilization is achieved by the female irrigating the burrow as the tide floods during the spawning season. The flood tide carries the diluted sperm puddles. It has been shown that irrigation of the burrow is at its maximum during the early part of the tidal flood cycle (Baumfalk 1979). There is also evidence to suggest that a "pheromone" present in male spawning water stimulates more vigorous irrigatory behaviour (Hardege *et al.*. 1996). These factors should maximise the amount of water and hence sperm passing through the female burrow.

1.6.5 Larval development and recruitment

The fertilized ova undergo cleavage and develop to blastula in approximately 24 hours (Farke and Berghuis 1979a, personal observations). The larvae remain within the burrow for several days, and are released at the 3 chaetigerous segment stage. The actual fate of these larvae is unclear, and some early reports suggested the existence of a planktonic larval phase (Benham, 1893, Thorson 1946, Wells 1949). It is now apparent that the developing larvae adhere to sand grains following release from the female burrow, and that they settle rapidly without a planktonic phase (Farke and Berghuis 1979a,b).

Following release from the burrow, the larvae appear to be swept upshore to juvenile beds (Farke and Berghuis 1979a,b). Here they undergo a period of development which varies according to the local conditions. Recruitment to the mature adult stock generally takes about 2 years, but can be as little as one year in some localities (Farke and Berghuis 1979a,b, Howie 1984).

1.6.6 The Control of Reproduction in *Arenicola marina*

Synchronisation of the reproductive cycle is of primary importance in epidemic free spawning invertebrates. It is essential that all members of a lugworm

population mature and spawn in synchrony to maximise the chances of successful fertilization. The precise environmental cues controlling reproduction are as yet unknown. Indeed, given that different reproductive cycles persist in different populations, there is likely to be some controversy over this issue.

Temperature, as with many marine invertebrates appears to influence the timing of spawning of a lugworm population. Early reports linked drops in temperature with the onset of spawning, the spawning being coincident with the first frosts of the year (Howie 1959). Latterly, spawning has been shown to be inhibited above around 13-15°C (Farke and Berghuis 1979a). However, many workers have found that animals removed from the field and isolated from the normal conditions experienced at that site (temperature, photoperiod, tidal cycle) will still spawn at the same time as their field counterparts (Howie 1963, Farke and Berghuis 1979a, reviewed by Howie 1984). It would thus appear that the timetable of gamete development and spawning is entrained at an earlier stage, and is not influenced by transient drops in temperature.

1.7 The Biology of *Nereis virens*

1.7.1 General Biology

The infaunal polychaete *Nereis virens* (Annelida: Polychaeta: Nereidae), known colloquially as the "King Ragworm" is a well known member of the intertidal and shallow sub-tidal communities of northern temperate sandy and muddy beaches and outer estuaries of Europe, North America and Asia (Pettibone 1963). The Nereidae are, in general, errant polychaetes, highly motile within the sediment and capable of swimming.

Nereis virens is ecologically important as a major predator of interstitial infauna. The worm possesses an eversible pharynx and a large set of jaws which it uses to seize and ingest other invertebrates (e.g. *Nephtys hombergi*), although it is catholic in its diet and will also ingest detrital material (Kay 1972). It is itself preyed upon by benthonic fish, wading birds and decapod Crustacea. Considerable variation exists in the literature regarding the size of the worms, and anything between 38cm and 1m is reported (Khlebovich 1963, Sveshnikov 1965, reviewed in Creaser and Clifford 1982). *N. virens*, like *Arenicola marina* is also important economically and is heavily exploited by fishermen as a bait species. In certain localities this has led to the decline of the natural population (Olive 1993). In addition it is grown commercially for bait and also for fishmeal to supply the finfish industry.

1.7.2 Reproductive Biology

Nereis virens is typical of nereid polychaetes in demonstrating a semelparous (Cole 1954, Gadgil and Bossert 1970, -monotelic of Clark and Olive 1973) reproductive strategy (Brafield and Chapman 1967, Bass and Brafield 1972). Generally, individuals will reproduce in their 2nd, 3rd or 4th year depending on a number of factors including nutritional state. Gametogenesis takes place through Autumn and Winter and the population spawns the following spring (Brafield and

Chapman 1967, Bass and Brafield 1972, Snow and Marsden 1974, Creaser and Clifford 1982, Desrosiers *et al.*. 1994).

As with many nereids, in *Nereis virens* there are morphological changes associated with reproduction, termed epitoky. In some polychaete families, for example the Syllidae and Eunicidae, epitoky involves the specialist adaptation of reproductive parts (stolons) which may become separate from the adult. Such stolonisation (termed schizogamy by Schroeder and Hermans 1975) does not occur in *N. virens*. Its morphological changes are much less pronounced throughout the adult body and it is therefore called epigamy. Gravid individuals are recognisable by their deep green, often iridescent, colour. The males in particular are easily distinguishable because of the milky white coelomic sperm which is visible through the sides of the body wall, between the parapodia.

The morphological changes are much more profound in males than in females, and manifest themselves as muscular degeneration, parapodial enlargement and vascularisation, atrophication of the digestive tract and body wall histolysis (Bass and Brafield 1972). In addition, caudal regeneration of lost segments ceases (Olive 1995). It is presumed that the parapodial enlargement of males is linked with the need to swarm during spawning.

Epitokous male king ragworms demonstrate a swimming behaviour known as heteronereid swimming. This differs from atokous swimming in that the body undulations have a lower amplitude and higher frequency. This uses the enlarged parapodia to great effect in propelling the animals forward, and is reviewed by Bass and Brafield (1972). Males have been observed to perform heteronereid movement in the laboratory for up to one hour. Females do not show heteronereid movement for such an extended period of time and normally will only swim in this fashion for a matter of minutes before reverting back to normal swimming.

Males also show a further morphological change during the reproductive growth, namely the development of the anal rosette apparatus (Bass and Brafield 1972). The hindmost segment of the male (the pygidium) develops 4 pores through which seminal fluid is released. This differs from the female ragworm and several

other nereids (Pettibone 1963) which release gametes through rents in the body wall. It is assumed that the further development of the male is associated with swarming behaviour during spawning (see below).

1.7.3 Gametogenesis

Oogenesis

The oocytes of *Nereis virens* develop freely within the coelomic cavity, although the origin of the oocytes is presently unknown because of the lack of clearly differentiated ovaries (Dhainaut 1984). Small eggs are present within the coelomic cavity of maturing individuals for much of the year (Brafield and Chapman 1967). These eggs undergo solitary vitellogenesis (Raven 1961) and enter a rapid growth phase through the winter to reach a maximum size of around 180 - 200μm immediately prior to spawning. The eggs are arrested at prophase of the first meiotic division, and do not require a maturation step to become fertilizable.

Spermatogenesis

Unlike oocytes, Brafield and Chapman (1967) found that sperm did not appear in the coelomic fluid until Autumn. The sperm develop as tetrads, four sperm joined together at the sperm head. They remain in this condition until late winter, solitary sperm appearing in the coelom a few weeks prior to spawning (Brafield and Chapman 1967, Bass and Brafield 1972). Like *Arenicola marina* the sperm are immotile until diluted by seawater.

1.7.4 Spawning Behaviour

Some controversy exists over the spawning behaviour of *Nereis virens*. Spawning is characterised by individuals swimming out of the burrow at high tide, releasing gametes in a mass of swarming animals. However, it is unclear exactly

which sex swarms. The specific behaviour appears to be a characteristic of local populations. There are reports of male only swarms in southern England, Denmark and Canada (Bass and Brafield 1972), whilst male and female swarms have been observed in the west of Scotland (Clark 1960) and the White Sea (Sveshnikov 1955).

The most detailed account of spawning to date (Bass and Brafield 1972) is of field and laboratory experiments in which only the males swarmed. Here, swarming involved the males leaving their burrows, and swimming just below the surface of the water with vigorous thrashing movements (heteronereid behaviour). This was accompanied by the release of seminal fluid in thin streams from the anal rosette apparatus in the pygidium. In contrast, the female remained within the burrow and deposited the eggs on the surface of the sediment at the mouth of the burrow. They also found that animals (particularly females) could spawn several times before being "spent".

Dean (1978) reports that non-reproductive swarming takes place in a North American population at Maine. This is attributed to a winter night high tide seaward migration, presumably to exploit a richer region of the shore. No gravid animals were observed. This migratory behaviour is also described by Desrosiers *et al.*. (1994), who suggest that it is the migration of 3 year old juveniles downshore to the adult population.

Once spawning has taken place the animals will die, although not all at once. Males can be seen washed up on the strandline on the tides following the spawning. Creaser and Clifford (1982) record males re-burrowing after swarming, but that the burrows formed are very shallow and non-permanent. It is presumed that the majority of males fail to re-burrow themselves. Non-swarming spent females may remain within the burrow for a month or so afterwards before dying and decaying in the sediment (Bass and Brafield 1972).

1.7.5 Fertilization and Larval Development

Fertilization takes place either within the water column (in populations where both sexes swarm) or at the entrance of the female burrow on the surface of the sediment. Unlike *Arenicola marina*, fertilization in *Nereis virens* results in the rapid raising of a very thick jelly coat caused by cortical granule exocytosis (Bass and Brafield 1972), which acts as a block to polyspermy and to protect the egg. This leads to an apparent overall size increase of the oocyte to around 250-300μm, however the fertilization membrane is underneath the jelly layer, and hence the egg itself is still around 180μm in diameter.

The first cleavage takes place after about 5-7 hours depending on temperature, and the trochophore larvae hatch after about 30 hours. On the 6th day after fertilization, the 3 chaetigerous segmented nectochaete larva undergoes a short planktonic phase of less than 24 hours before settling and developing further. There then follows a period of pre-reproductive growth of between 1 and 7 years depending on local conditions (Bass and Brafield 1972).

1.7.6 The Control of Reproduction in *Nereis virens*

The discrete semelparous reproductive strategy of *Nereis virens*, it is suggested, is controlled by a series of "gated" rhythms (Olive 1995). The "decision" to switch between a somatic growth phase and gametic growth is under the influence of temperature and photoperiod (Olive 1995). The switch between long and short days (16 light : 8 dark to 8 light : 16 dark) has been found to induce the acceleration of oocyte maturation in females (S.W. Rees, P.J.W. Olive personal communication).

Not all members of a year class will enter the gametic growth phase at the same time. Laboratory experiments have shown that only those animals which have reached a certain size (or "energy fitness" level) will be stimulated to begin the reproductive cycle (Olive *et al.*. 1986). Indeed, commercially farmed animals can

be induced under ideal (entirely artificial) conditions to reach maturity in only 6 months (P.J.W. Olive personal communication).

Once the gametic growth phase has been entered, the onset of sexual maturity is irreversible. There is a complex endogenous control of reproduction in nereids which is similar throughout the group (Franke and Pfannenstiel 1984). Control and maintenance of somatic growth in *Nereis diversicolor* takes place through the continuous high level of endocrine activity in the supra-oesophageal ganglion (Golding 1972). This also exerts an influence on gametogenesis, and it is thought that the processes are controlled by a single hormone (Golding 1987). It is believed that the hormone responsible for the control of gametic growth is a small peptide (Cardon *et al.*. 1980), although the lack of an appropriate bioassay for endocrine action has hindered further progress.

The hormone is necessary during the early stages of gametogenesis, and de-cerebration of individuals at such early stages leads to the inhibition of further gametic growth (Porchet 1970). However, a decline in the cerebral endocrine activity at a later stage promotes the growth of the cohort of gametes within the coelomic cavity (Dhainaut 1970, reviewed in Bentley and Pacey 1992). Thus the hormone is required to induce initial gamete proliferation and development, but a fall in the hormone level is required to advance gametogenesis.

Implantation of juvenile brains into maturing individuals of *Platynereis dumerilii* halts further gametogenesis and prevents metamorphosis into the epitokous form (Hofmann and Schiedges 1984). This has been further demonstrated in *Nereis diversicolor* by Golding and Yuwono (1994) who show that repeated implantation of juvenile brains into mature specimens leads to gamete resorption, resumption of somatic maintenance and repeated gametogenic cycling. A positive feedback process is in operation to control the level of hormone secreted by the brain. Oocyte growth causes a fall in brain hormone and thus an increased oocyte growth. (Durchon 1952, Porchet 1967, Porchet *et al.*. 1979).

Spawning of *Nereis virens* as with other nereids is partly controlled by environmental conditions. Almost all populations appear to spawn in the early

Spring, and Goerke (1984) reports that spawning only takes place (in North Sea populations) when the local sea temperatures rise above 6-8°C. Other factors are probably also acting to induce spawning. Generally, populations spawn during night time spring tides at high water, although there are also reports of daytime neap tide spawning (Brafield and Chapman 1967, Bass and Brafield 1972). Bass and Brafield (1972) also suggest that spawning and reproductive success is heavily influenced by prevailing conditions - reproductive failures could be because of spawning animals being swept offshore in adverse weather.

Nereids possess an array of sensory appendages, and pheromonal induction of spawning has been demonstrated in several species (Zeeck *et al.*. 1988, Hardege *et al.*. 1990, Zeeck *et al.*. 1992). It is likely that such pheromones exist in *Nereis virens*, but this has not been shown to date.

1.8 The Biology of *Asterias rubens*

1.8.1 General Biology

The common starfish *Asterias rubens* is a familiar part of the subtidal (and occasionally intertidal) fauna around the UK and Northern Europe, and is conspecific with *Asterias vulgaris* along the eastern seaboard of the United States. It thrives on both hard and soft substrata, where it is one of the major scavengers of dead material and predators of bivalves.

The general biology and feeding biology of *Asterias rubens* on rocky substrata are extensively reviewed by Feder and Christensen (1966) and Sloan (1980), and by Anger *et al.*. (1977) for soft substrata. *A. rubens* feeds by everting its cardiac stomach to engulf the prey item or scavenged body. The starfish then either secretes digestive enzymes and draws the liquefied remains into its body, or draws the whole prey item into its body for digestion. *A. rubens* is sensitive to chemicals emanating from the carcasses of dead marine organisms, and is able to track such food items in a water current (Castilla and Crisp 1970, Nickell and Moore 1992, Moore and Howarth 1996). In addition, it is able to locate buried food items such as the bivalves *Cerastoderma edule*, *Macoma balthica* and *Spisula solidissima*, and dig them out of the sediment (Allen 1983).

Asterias rubens plays an important role in the ecology of the marine benthos. It is a major predator of bivalve communities, and is itself preyed upon by the starfish *Luidia sarsi* and *L. ciliaris*. A long term study by Guillou (1996) in the Baie de Douarnenez, Brittany, France showed that when populations of bivalve increased, there was a similar increase in the density of *A. rubens*. This lowered the bivalve population, but also led to a rise in the numbers of *L. sarsi* and *L. ciliaris*. This led to a concomitant decrease in the *A. rubens* population. *A. rubens* is of little direct commercial value. It is likely to have a detrimental effect on the populations of commercially important bivalves such as *Pecten maximus* but reliable quantitative data for this have yet to be gathered.

1.8.2 Reproductive Biology

Asterias rubens is an annual, iteroparous echinoderm in which sexes are separate. The spawning season in northern European waters is generally during Spring, but the time of spawning is different between geographically separated sites, with more northerly populations spawning in the late Spring or early Summer (Nichols and Barker 1984). Gamete development (in English Channel populations) takes place from early Autumn, gathers pace through the Winter and maximal gonadal index is reached around April (Nichols and Barker 1984)

The annual cycle of reproduction in starfish correlates with the cycle of feeding and growth. In general, a period of active feeding precedes growth of the pyloric caeca which in turn precedes gonadal growth. This cycle of feeding and reproductive growth appears to be influenced by photoperiod and changes in photoperiod will lead to a change in the timing of gametogenesis (reviewed in Chia and Walker 1991).

1.8.3 Gametogenesis

Oogenesis

Gamete development in the Asteroidea is reviewed extensively by Chia and Walker (1991). *Asterias rubens*, as is the case with most seastars, possesses one pair of ovaries in each of its five arms. Unlike the polychaetes discussed earlier, gamete development is entirely within the gonads. The gonads consist of a large number of lobes or alveoli, resembling a "bunch of grapes". They are very small through late summer (after spawning), but grow to accommodate the developing oocytes and almost fill each arm just prior to the following spawning time.

Oocytes develop within follicles, and in *Asterias rubens* the early vitellogenic follicles have a single oocyte of around 20-50μm in diameter. These are attended by around 40/50 follicle cells. Following further vitellogenesis and oocyte development, just prior to spawning the follicles have one primary oocyte of

around 90μm in diameter, arrested at prophase of the first meiotic division. This is surrounded by a layer of flattened follicle cells.

In this state, the oocytes are unable to be fertilized and as with *Arenicola marina* they require chemically induced maturation to initiate germinal vesicle breakdown and polar body extrusion (see Meijer and Guerrier 1984). The early work on the nature of oocyte maturation is reviewed by Kanatani (1979). It has been shown that the radial nerves of starfish contain a 22 amino acid polypeptide (Shirai *et al.*. 1985), which acts upon the gonad to initiate production of a second substance, 1-methyladenine (1-MeAde) from the follicle cells (Kanatani *et al.* 1969, reviewed in Kanatani 1979, Giese and Kanatani 1987). When in contact with immature oocytes for a period of time (around 20 minutes), 1-MeAde acts on the oocyte membrane to stimulate the production of a maturation promoting factor (MPF). MPF acts on the oocyte to stimulate oocyte maturation, however at present the mechanisms of production and action of MPF are poorly understood.

Spermatogenesis

As with oogenesis, spermatogenesis in the Asteroidea is reviewed comprehensively by Chia and Walker (1991), and the process of spermatogenesis is essentially the same across most of the species in the class. Following spawning and evacuation of the testes, the starfish resorbs any remaining gametic material. The paired gonads in each arm then enter an aspermatogenic phase in which they are suspended in G1 of the mitotic cell cycle for up to 6 months. The testes contain somatic cells in the spermatogenic epithelium, and these are termed somatic accessory cells (SACs). The SACs maintain a minimal amount of mitosis during this period, and they also form follicle-like sub-divisions of the germinal epithelium which enclose the spermatogonia.

The male starfish enter their gametic phase slightly later in the year than females. The entry into the spermatogenic phase is marked by the transition of the spermatogonia from the amitotic phase to the mitotic phase. The release of the spermatogonia from mitotic arrest leads to their undergoing repeated cellular

divisions to form primary spermatocytes. At this time, there is also a massive re-organisation of the germinal epithelium of the testes to form structures known as spermatogenic columns. The spermatogenic phase in *Asterias vulgaris* (believed to be sympatric with *A. rubens*) has been measured at between 8 days and 2 weeks depending on the condition of the starfish (see Chia and Walker 1991). Following completion of meiosis and sperm differentiation, the spermatozoa do not require a maturation step prior to spawning, but will acquire motility upon dilution in seawater.

1.8.4 Spawning

Both the ovaries and the testes of the starfish open to the exterior through gonoducts and gonopores. The gonoducts run from the gonads to the side of the arm, opening at the gonopores in the angle between two adjacent arms. As stated above, the spawning season of *Asterias rubens* varies according to the particular geographic location of the population (Nichols and Barker 1984b), but is usually during April/May or early June.

During spawning, *A. rubens* adopts a spawning posture typical of many asteroids. Waves of contraction pass along the arms of the starfish, and the central disc is raised several inches off the substratum such that the starfish is on "tip toes" (see review by Kanatani 1979). It is believed that 1-MeAde acts during spawning as well as in oocyte maturation, by stimulating the gonads to contract thereby expelling the gametes, inducing the spawning posture and causing the waves of muscular contraction in the arms to further expel the gametes (Kanatani 1979). This spawning posture may be an attempt to release the gametes higher into the water column so that they are carried away from the parent.

The spawning cue of *Asterias rubens* is presently unknown, however it is apparent that photoperiod plays a role in the overall control of reproduction in *A. rubens* (Chia and Walker 1991). Temperature may also be an important determinant of spawning time.

1.8.5 Fertilization and Larval Development

Fertilization and larval development take place free in the water column. Following fertilization, the oocytes reach blastula after around 20-23 hours, feeding bipinnaria larvae at 3-4 days, brachiolaria at 30 days, and by 70 days the larvae have begun to develop spines and ossicles (Nichols and Barker 1984a). The numbers of bipinnaria larvae in the water column in the English Channel peak during April. The larvae settle during early Summer, and in laboratory trials preferentially settled on the undersides of a range of surfaces presented. They avoided surfaces which had been cleaned of all organic material (Barker and Nichols 1983).

1.9 The Biology of *Echinus esculentus*

1.9.1 General Biology

The common sea urchin *Echinus esculentus* (Echinodermata: Echinoidea: Echinidae) is abundant around the shores of the UK, occasionally found intertidally, but most numerous in the shallow sub-tidal (Moore 1934, Nichols *et al.*. 1985). Nichols *et al.*. (1985) record the distribution of *Echinus esculentus* in the English Channel, and report that it is found from 5m to 250m in depth. More recently, Comely and Ansell (1988) provide a detailed account of the population parameters of several populations of *E. esculentus* around the Scottish West Coast. They found that the population densities and morphometrics at different depths varied considerably over several years.

Echinus esculentus in the photic zone feeds on kelp and algae, whilst those animals below browse on encrusting invertebrates and protochordates (Nichols *et al.*. 1985). Comely and Ansell (1988) found no evidence to suggest that *E. esculentus* feeds on the actively growing tissues of *Laminaria* spp., only on the various epibionts growing on the stipes. However, feeding does take place on broken and disintegrating kelps. Feeding in sea urchins takes place by holding the

test close to the substratum and rasping off the organic matter with 5 heavily chitinised teeth. The loosened material is drawn into the gut via ciliary currents.

Echinus esculentus is one of the larger sea urchin species, commonly reaching test diameters greater than 90mm (Comely and Ansell 1988), and has an array of short spines for protection. It plays a major role in the structuring of the shallow subtidal, where it is the most prominent grazer. Jones and Kain (1967) found that exclusion of large numbers of *E. esculentus* from the top 5m of the shallow subtidal led to a massive increase in algal cover. Additionally, it has some commercial value as an ornament, and populations in southern England have been depleted by souvenir hunters. There is also concern that UK populations may be targeted for food (roe), particularly for export to the far East (Comely and Ansell 1988).

1.9.2. Reproductive Biology

As with *Asterias rubens*, *Echinus esculentus* has an annual, iteroparous reproductive strategy in which the sexes are separate. The reproductive cycle of *E. esculentus* was described by Moore (1934). He examined several populations around the Isle of Man, measuring gonad volume, and investigating the spawning times. It appears that populations around the Isle of Man have a spawning time of late April or early May, with gonadal indices very low in the post-spawning samples taken in June. He stated that spawning time is determined by sea temperature, however, Comely and Ansell (1989) suggest that local conditions of temperature (rather than general temperature increases) are equally important.

Recently, more comprehensive studies of the reproductive cycle of *E. esculentus* have been undertaken by Nichols *et al.*. (1985) and Comely and Ansell (1989). They confirm the annual spawning season, however it appears that in the Scottish populations, spawning takes place during June/July rather than April/May around the Isle of Man (Comely and Ansell 1989). Nichols *et al.*. (1985) also suggest that for an English Channel population there may be two spawning seasons, one major spawning during Spring, followed by a second spawning in July. One of

the features to arise from the work of Comely and Ansell (1989) is that the cycle of gonadal development appears to vary slightly each year.

1.9.3 Gametogenesis

Sperm and eggs are proliferated within the five gonads prominently located hanging in the interambulacral areas under the aboral side of the test. A short gonoduct extends from each gonad, opening via a gonopore located on each of the five genital plates on the top of the urchin. Gonad indices are a minimum following spawning, and remaining gametes are resorbed by the urchin. Gametogenesis increases during mid Autumn, with development rate peaking in November/December and gonadal index reaching a maximum in early Spring as the gametes reach full maturity (egg diameter ~90μm, personal observations) (Comely and Ansell 1989). Neither eggs nor sperm require hormonally induced maturation prior to spawning.

1.9.4 Spawning

It is suggested that spawning in *Echinus esculentus* is preceded by an inshore migration to form aggregations of urchins and thus improve the chance of fertilization (Elmhirst 1922, Stott 1931). However, this was later refuted by other workers, and a comprehensive survey of urchin distribution with depth monthly over several years by Comley and Ansell (1988) showed that distribution was stable throughout the year.

Spawning takes place through the mechanical contraction of the gonads which expels the gametes via the gonopores located on the genital plates on the top of the urchin. Several male urchins have been observed spawning in the field having climbed to the top of kelp stipes (personal observation, G.J. Watson personal communication), and this is also reported in *Paracentrotus lividus* (Minchin 1992) which climbs to the top of rocky outcrops to release gametes. This behaviour is presumably intended to broadcast the sperm over a wider area.

1.9.5 Fertilization and Larval Development

Fertilization takes place in the water column, and leads to the rapid raising of a fertilization membrane. The fertilized oocytes follow a typical echinoderm development, with the ciliated free swimming blastula formed after around 24 hours (depending on temperature). The blastula develops into the planktonic feeding echinopluteus larvae, and remains in the water column for several months, developing the adult skeleton prior to settlement. Metamorphosis is rapid, and young urchins are around 1-2mm in diameter.

1.10 Objectives of this study

The principal objectives of this thesis are to explore the factors which affect fertilization success in several marine invertebrate species. The species chosen for study are the polychaete annelids *Arenicola marina* and *Nereis virens*, the echinoid echinoderm *Echinus esculentus* and the asteroid echinoderm *Asterias rubens*. To date, the polychaete annelids have been largely neglected by fertilization ecologists. This is perhaps a little curious since they are one of the major components of the marine invertebrate biota. Indeed, infaunal organisms have been entirely ignored in fertilization studies thus far, presumably because of the awkwardness that working with animals that inhabit soft sediment presents.

A great deal of work on the ecology of *Arenicola marina* has been published in the last 50 years, and fertilization ecology is one of the few outstanding topics left for study. This thesis will therefore attempt to make an important contribution to our understanding of this part of the life cycle. As well as investigating laboratory studies of fertilization success in this species, studies will be conducted of fertilization success in the field, and some of the factors affecting fertilization success for this iteroparous, intertidal, infaunal organism.

Slightly less is known about *Nereis virens* than *Arenicola marina* and little has been published about its fertilization ecology since the major work of Bass and

Brafield (1972). This thesis will report on the factors affecting fertilization success, and also record field fertilization success for this semelparous (as opposed to the strictly iteroparous species studied previously), intertidal, infaunal polychaete.

The study of the fertilization ecology of echinoderms is fairly advanced compared with other invertebrate groups. It is becoming increasingly relevant for certain echinoid species as exploitation for food is now commonplace, and there are fears for the continued survival of traditionally exploited free spawning stocks such as scallops and oysters. Ensuring good recruitment thus depends upon an understanding of the fertilization ecology of these species. As stated earlier, there are interspecific differences in the fertilization success of various species, and studies will be conducted upon these organisms in order to provide a comparison with the polychaetes, which are the main animals under study.

1.10.1 Specific Aims of This Thesis

1. The nature of the spawning behaviour of *Arenicola marina* will be examined. In particular, the question of when and where during the spawning period the female spawns will be investigated.

2. Attempts will be made to determine the level of fertilization success in the field achieved by *Arenicola marina* during a natural spawning event.

3. The factors affecting fertilization success in the field for *Arenicola marina* will be examined.

4. Field fertilization success will be measured in *Nereis virens* during a natural spawning event.

5. Laboratory studies of fertilization success will be conducted upon *Arenicola marina*, *Nereis virens*, *Asterias rubens* and *Echinus esculentus*. The factors under study include sperm : egg ratio, sperm concentration, sperm : egg contact time and gamete longevity.

6. The chemical mediation of sperm activity by free spawned eggs will be examined for *Arenicola marina* and *Asterias rubens*.

It is hoped that through this work, the fertilization ecology of *Arenicola marina* can be qualitatively and quantitatively examined, and comparisons made between this species, *Nereis virens* and the organisms studied previously. In addition, comparisons will be drawn between the fertilization kinetics of polychaete annelids and the echinoderms studied previously and in this thesis.

Chapter 2

Materials and Methods

2.1 Introduction

This materials and methods section will address only those techniques and procedures that are generally applicable to the studies reported in this thesis. These are principally those techniques associated with specimen collection, maintenance, inducement to spawn and gamete handling. Techniques that pertain specifically to certain experiments will be described separately in the appropriate chapters.

The seasonal nature of the reproductive strategies of many marine invertebrates dicatated that a given species was available for experimentation for no more than two to three months. The principal animal under investigation was *Arenicola marina*, and in order to maximise the availability of experimental animals, different populations of *Arenicola marina* with slightly varying spawning times were used (e.g. Duncan 1960). Additionally, other species were chosen for their suitability for study because of their ease of manipulation, ready availability and comparability with other experimental animals studied elsewhere. This enabled a programme of research to be undertaken, with gravid animals of a variety of species available for approximately 8 months of the year.

Species	Fully Gravid
Arenicola marina	Late October - Late December
*Nereis virens**	Late February - Late April
Asterias rubens	Early April - Late May
Echinus esculentus	Early July - Mid August

Table 2.1 The availability of gravid animals for experimentation (* *Nereis virens* supplied by Seabait Ltd.)

The availability of gravid animals is shown in Table 2.1. These represent only the times during the reproductive cycle when the fertilization and development success of spawned (naturally or artificially induced) gametes was around 100%.

2.2 Collection and maintenance of experimental animals

2.2.1 Collection and maintenance of *Arenicola marina* (L.)

Arenicola marina (Annelida: Polychaeta: Arenicolidae) were collected from a number of locations around the U.K. during the three years of this investigation (see table 2.2). During the first year, only animals from East Sands and Dunbar were used. In the second year, the population at East Sands had been over exploited by bait diggers during the summer prior to the Autumn breeding

Location	Lat/Long	Dates
East Sands, St Andrews, Fife	56°20.3' 2°47.1' W	Mid Oct - Mid Nov
West Sands, St Andrews, Fife	56°21.4' 2°48.1' W	Mid Oct - Mid Nov
Kingsbarns, Fife	56°18.2' 2°39.3' W	Mid Oct - Mid Nov
Red Wharf Bay, Anglesey	53°18.6' 4°12.0' W	Mid Nov - Early Dec
Dunbar, East Lothian	56°00.4' 2°34.2' W	Early Dec - Late Dec

Table 2.2 The collecting sites and dates for *Arenicola marina* during the course of this study. Not all sites were exploited in all years.

season rendering it useless for sampling, hence Kingsbarns, West Sands and Dunbar were used. During the third year, it was found that the site at Kingsbarns had been badly damaged by winter storms which had removed large amounts of sediment from the beach and with it much of the sizeable adult *Arenicola marina* population. In this year it was also found that the population at East Sands had still not fully recovered, and the West Sands' animals were in a poor condition and did not survive well in the laboratory or respond to endocrine manipulation. Reasons for this poor condition are unknown. It was therefore possible to use *Arenicola marina* only from the populations at Red Wharf Bay and Dunbar.

Individual worms were collected by digging with flat pronged forks to minimise the risk of damaging the animals. At East Sands, West Sands and Kingsbarns the density and size of the animals was such that it was possible to select individuals for collection by identifying head and tail shafts (head depression, tail cast). The worms were extracted from the burrow by digging from the head shaft, backwards to the tail, taking shallow sections each time. This minimised the likelihood that the animals would be damaged. At the other sites, the animals were of sufficient density to allow trench digging and removing all the worms from the overturned sand. Animals harvested were placed into buckets and taken back to the laboratory in small amounts of fresh seawater and sand.

Once back in the laboratory, the animals were sexed by close examination under bright illumination. Males were characterised by their milky white tails, and eggs were usually visible through the body walls of females. Where individuals could not be sexed reliably, small quantities of coelomic fluid were withdrawn using a 1ml disposable syringe fitted with a size 25g hypodermic needle, and examined under a compound microscope.

Following sexing, the animals were kept individually in polyethylene pots (10cm x 10cm x 6cm) or polystyrene trays (12cm x 8cm x 7cm) filled with 0.3µm filtered natural seawater. The animals were then placed in a controlled temperature room at 8-10°C close to the ambient temperature for the time of year,

of the local sea temperature conditions and under ambient illumination. They were inspected daily and the water changed regularly. The worms were left for at least 24 hours before use in experiments to allow full defecation of gut contents.

2.2.2 Collection and maintenance of *Nereis virens*

Collection of gravid *Nereis virens* (Sars) (Annelida : Polychaeta: Nereidae) in the field proved to be problematic. It is exploited extensively locally for bait, as in other parts of the country (Olive 1993), and consequently gravid animals were difficult to obtain in great numbers. All experiments were therefore conducted with animals provided by Seabait Ltd., Ashington, Northumberland. The animals were part of the brood stock of the company that were surplus to requirements, and were collected from field sites in the early stages of maturity, and reared under optimal conditions to full maturity. These conditions mimicked the short day photoperiod necessary to induce gamete maturation (see Chapter 1).

Using farmed animals rather than field collected specimens is advantageous because they are of a known stage of reproductive development. Observations of the developing gametes, fertilization and larval development at the farm enabled the animals to be divided into separate brood stocks of known developmental stages. It was thus possible to select only those animals which are at the peak of gamete development for use in experiments. In addition, access to a number of broodstocks all coming into full maturity slightly out of phase with each other enabled the very short experimental season to be extended artificially, allowing more work to be completed. Broodstock failures during the second and third years of this investigation, however, resulted in very few animals being available for use.

Gravid *Nereis virens* were collected by digging from a designated broodstock bed at the worm farm during late February and March. They were then placed individually into plastic pots (10cm x 10cm x 6cm) with seawater from the site and transported back to the laboratory in trays containing a layer of crushed ice. Once back in the laboratory, the animals were transferred to identical pots

with a layer of gravel and small (0.5mm diameter) holes drilled into the walls and lid. They were then placed into single sex flow-through tanks and maintained at 5°C, close to the ambient for the time of year with ambient illumination. Prior to use in experiments, the gametes were tested for fertilization and development success (see later). Only those animals which exhibited 100% fertilization and development of eggs to blastula, or 100% fertilization of eggs at sperm concentrations of approximately 10^5 sperm.ml^{-1} were used.

2.2.3 Collection and maintenance of *Asterias rubens*

Mature *Asterias rubens* (Echinodermata: Asteroidea) were collected using fishing creels from a number of sites in the shallow waters around the East coast of Fife during April and early May of 1996 and 1997. They were transported back to the laboratory in seawater, and placed in large flow through tanks (1m x 3m x 1m) at ambient temperatures and illumination until required.

2.2.4 Collection and maintenance of *Echinus esculentus*

Mature *Echinus esculentus* were collected by snorkeling at Loch Melfort on the West coast of Scotland (O.S. Landranger grid ref NM 8314, lat/long 56°15.2' 5°32.7' W) during July of 1996 and 1997. Several diferent sites around the loch were used to avoid over-exploiting particular areas. The urchins were transported back to the laboratory in an assortment of portable tanks filled with seawater from the loch.

Once in the laboratory they were placed in the same tanks as used for the starfish, and kept in flow through tanks at ambient temperatures until required for experimentation. All experiments were conducted on freshly collected specimens (in the laboratory for less than one week) to avoid using animals whose condition had deteriorated.

2.3 Induction to Spawn and Collection of Gametes

2.3.1 Collection of gametes from *Arenicola marina*

Fully developed gametes (both eggs and sperm) of *Arenicola marina* require an endocrine maturation step before they are capable of fertilization (see Chapter 1 for details). In males, this maturation is a single step process with the release of 8,11,14-eicosatrienoic acid from the prostomium into the coelomic cavity where it acts upon the sperm morulae to induce morula breakdown (Bentley and Pacey 1992). Subsequent shedding of the sperm into seawater induces free swimming (Pacey *et al.* 1994a). In females, a two step process is present, with a maturation hormone (not 8,11,14-eicosatrienoic acid) released into the coelomic fluid where it stimulates production of a "coelomic maturation factor" (CMF) (Watson 1996, Watson and Bentley 1997) which releases the oocytes from prophase arrest of meiosis I to undergo GVBD before being held at metaphase I. Spawning always follows gamete maturation, and only mature gametes are spawned (Howie 1961, Watson 1996).

Oocytes

Mature oocytes were collected through the injection of homogenised prostomia according to Howie (1961). Normally a female will only respond reliably to such endocrine manipulation within about two weeks of the period of natural spawning of its parent population.

Preparation of prostomia

Prostomia were ablated from mature female *Arenicola marina* using watchmakers forceps and bow iridectomy scissors. The donor female was left undisturbed in seawater for a few minutes to relax and evert its prostomia naturally. The prostomium was then gripped with the forceps and cut from the

animal with the scissors. Usually the prostomia was removed cleanly with little extraneous tissue, but when this was not the case the prostomia was cleaned of excess tissue under a binocular dissecting microscope. Three prostomia were placed in a 1.5ml Eppendorf tube with 1ml sterile filtered seawater (0.2µm filtered seawater) (SFSW), and kept at 0°C over ice. The three prostomia were then homogenised over ice using a sonic disintegrator (MSE Soniprep 150). Occasionally prostomia were stored whole for later use at -20°C, and used within about one month of freezing. They showed not loss in activity over this time.

Injection of prostomial homogenate

The 1ml of prostomial homogenate prepared previously was drawn into a 1ml disposable syringe fitted with a size 25g hypodermic needle. Approximately 300µl of homogenate was injected into each female just where the trunk meets the tail. This resulted in each female receiving 1 prostomial equivalent of homogenate, which is sufficient to induce maturation and spawning. The injected animals were placed into 150ml crystallising dishes containing approximately 100ml SFSW. They were left for up to a maximum of 6 hours, to allow sufficient time to spawn.

Collection of oocytes

Once the worm had spawned (usually within 3-4 hours), it was removed from the crystallising dish. The eggs were inspected for germinal vesicle breakdown (GVBD) to ensure that they were actually spawned rather than the result of leakage from the coelomic cavity. Excess water was decanted off and the eggs washed twice by re-suspending in 100ml SFSW. They were then allowed to settle out and a sample transferred to Eppendorf tubes. The density of settled eggs in the tubes was determined by counting microlitre volumes under a compound microscope, allowing precise numbers of eggs to be used in experiments. Eggs not used immediately were stored at 8°C in approximately 100ml SFSW until

required. Eggs showed no decrease in viability over several days (see Chapter 7), but were always used within one day of spawning.

Sperm

Active sperm was collected from male *Arenicola marina* by one of three techniques. Sperm could be collected through the injection of prostomial homogenate (male or female) using the same procedure as described for the female, through the injection of 8,11,14-eicosatrienoic acid or by *in vitro* incubation in 8,11,14-eicosatrienoic acid. The particular technique used depended on the experiment in progress, and this will be indicated where relevant.

1. Sperm maturation through prostomial injection

The same technique as used in females was applied to males for the collection of sperm. This technique was used when there was an abundance of males. Spawning usually followed between 45 minutes and 1hour after injection. Sperm was collected "dry" as it was extruded from the nephromixia, using a 200µl Gilson pipette and deposited into Eppendorf tubes. The sperm samples were then left for a few minutes to allow the sperm to coalesce to its maximum concentration and the excess water pipetted off.

2. Sperm maturation through the injection of 8,11,14-eicosatrienoic acid

Injection of 8,11,14-eicosatrienoic acid (Sigma) was carried out directly into the coelomic cavity to induce sperm maturation. 25mg 8,11,14-eicosatrienoic acid was taken up in approximately 200µl of methanol and dissolved in 25ml SFSW at a final concentration of $1mg.ml^{-1}$. 1ml aliquots were then pipetted into 1.5ml Eppendorf tubes and stored at -20°C until required. The threshold concentration required to induce maturation and spawning in males is $13\mu g.g^{-1}$

body mass of 8,11,14-eicosatrienoic acid (Pacey and Bentley 1992), and this was the concentration used in these experiments. The males were weighed to the nearest gramme, and the required dosage calculated. 1ml aliquots of 8,11,14-eicosatrienoic acid were defrosted, sonicated for 15 seconds to ensure a homogeneous solution and made up to the appropriate dilution. The males were then injected where the trunk meets the tail with 300µl of the appropriate concentration of fatty acid using a disposable 1ml syringe with a size 25g hypodermic syringe. Sperm was collected as before. This technique was used when there was a scarcity of males, or when large numbers of males were required for injection.

3. In vitro *maturation of coelomic sperm*

Sperm can be activated *in vitro* by incubation in appropriate quantities of 8,11,14-eicosatrienoic acid (Pacey and Bentley 1992). Samples of coelomic fluid were withdrawn from the coelomic cavity of gravid males using a disposable 1ml syringe and size 25g hypodermic needle and placed in Eppendorf tubes. Quantities of fluid withdrawn varied according to the size of the animal and amount of sperm required, but generally not more than about 200µl was withdrawn on any one occasion. This avoided damaging the animal and allowed samples to be taken from the same animal several times. Coelomic samples were incubated with equivalent volumes of $1x10^{-4}$ M 8,11,14-eicosatrienoic acid. They were then shaken by hand, and left for 30 minutes at 8°C to mature. The mature sperm typically coalesced into oily droplets at the bottom of the tube, and the excess fluid was removed. This technique was used when large numbers of small samples of sperm were required, and only when the eggs alone were the subject of investigation.

Sperm counts

Sperm was stored "dry" in Eppendorf tubes untill required. Sperm concentration was determined by taking microlitre aliquots of the "dry" stock and diluting by known amounts with natural seawater in another Eppendorf tube. After dilution the sperm was killed by brief immersion of the tube in a hot water bath to allow accurate counting of non-motile sperm. Sperm counts were performed using a Neubauer Haemocytometer and back calculated to give the sperm concentration in the "dry" stock. Four counts of each sperm sample were performed and the mean calculated. A stock solution was then made from samples of the "dry" sperm at 1 x 10^8 sperm.ml^{-1} so that all sperm samples were standardised before use. This basic procedure applies to sperm samples drawn from all species under study.

2.3.2 Collection of gametes from *Nereis virens*

No endocrine manipulation is required to induce maturation of gametes in *Nereis virens* (see Chapter 1). Eggs and sperm taken from the coelomic cavity are ready to fertilize. This means that they can be drawn from the coelom of a mature animal using a disposable 1ml syringe fitted with a size 21g hypodermic needle and used directly in experiments.

Oocytes

Samples of oocytes were withdrawn from the coelomic cavity with a disposable 1ml syringe and size 21g hypodermic needle. Each animal was used on several occassions, so the samples were taken by puncturing near to the tail of the animals and working forward on each occasion. This prolonged the experimental life of the animal and tended to reduce the likelihood that it would spawn spontaeously. The oocyte sample was expelled into approximately 100ml SFSW, and allowed to settle out whereupon it was washed again in the same volume.

Samples of eggs were then transferred to Eppendorf tubes and the density of settled eggs determined to allow precise quantities of eggs to be used in experiments. Stock samples of eggs were kept in 100ml SFSW at 8°C until required.

Sperm

Coelomic sperm was withdrawn in the same manner as were eggs. It was stored "dry" (undiluted) in Eppendorf tubes at 8°C until required. Sperm counts were performed as for *Arenicola marina*, and stock solutions from each male made up at 2×10^8 sperm.ml^{-1}.

2.3.3 Collection of gametes from *Asterias rubens*

As with *Arenicola marina*, oocytes from *Asterias rubens* require a chemical maturation prior to spawning before they are fertilizable. This is required to advance the oocytes from prophase of the first meiotic division through GVBD and polar body formation (Meijer and Guerrier 1984). Sperm does not require this maturation, but can be taken straight from excised gonad. Males will however respond to the same chemical manipulation as females and undergo spawning.

Oocytes

Oocyte maturation was brought about by the action of 1 methyl adenine (1-MeAde) on the ovaries of ripe *Asterias rubens*. This was performed either *in-vivo* or *in-vitro* depending on the requirements of the experiment.

In vivo *maturation of oocytes and induction to spawn*

Stock solutions of 10^{-4} M 1-MeAde were prepared in 100ml SFSW. Fresh starfish were collected from the aquarium and placed in approximately 100ml SFSW in large finger bowls. Between 1 and 2ml of the solution (depending on the size of the animal) was injected into the starfish in one of the upper arms, just before it joins the oral disc. This gave an approximate concentration in the animals of 1×10^{-6} M 1-MeAde. Within 30 minutes this resulted in the adoption of the typical asteroid spawning posture (see Chapter 1), and the commencement of spawning. Mature oocytes were collected from the bowl after the starfish had been removed and washed twice in SFSW and transferred to beaker with approximately 100ml SFSW. Settled egg density was determined as before for the polychaetes, and the stock of eggs stored at 8°C until required.

In-vitro *maturation of oocytes*

Pieces of intact gonad were carefully dissected from the arms of ripe females. The excised gonad was washed of excess eggs by placing in 2 consecutive water baths with 25ml SFSW, and handled carefully to avoid further release of immature oocytes. It was then transferred to a petri dish containing 20ml 1×10^{-6} M 1-MeAde and left at 8°C for at least 30 minutes. During this time the eggs matured and were released from the gonad into the petri dish forming a large pool of eggs around the gonad. They were then washed in SFSW and treated as before. This technique was used in experiments testing sperm activity enabling the same female to be used several times. Where the eggs were under observation, the previous method was used.

Sperm

Sperm can similarly be collected from *Asterias rubens* by injection of 1-MeAde which elicits a spawning response in males. The injection procedure was

identical to that for females, and sperm was collected as it was extruded from the gonopores using a pipette. It was kept "dry" as for the earlier sperm collections, and the counting procedure was similarly identical.

Sperm can also be collected by dissection of gonadal tissue and direct extraction, as it does not require maturation. Pieces of excised testis were placed in a small petri dish containing 10ml SFSW. Gentle probing with a pipette tip led to the extrusion of sperm from the tissue, which was collected "dry" (before significant dilution) using a P-100 Gilson pipette. During this study, this technique was used only when eggs alone were under test, and the previous method was used during investigations of sperm performance.

2.3.4 Collection of gametes from *Echinus esculentus*

Gametes in mature specimens of *Echinus esculentus*, do not require chemical maturation prior to spawning to become fertilizable (see Chapter 1). Gamete extraction can either be through dissection of the gonads, or through artificial induction of spawning. In all experiments here, gamete collection was performed through the induction of spawning.

Freshly collected sea urchins were taken from the aquarium and placed upside down on crystallising dishes, suspended above approximately 100ml SFSW. They were injected through the soft membranes around the mouth with 1-2ml (depending on the size of the animal) 0.5M KCl using a 1ml disposable syringe fitted with a 21g hypodermic needle. Spawning was almost immediate, the sperm and eggs released as streams from each gonopore. Sperm was collected as it settled on the bottom and pipetted into Eppendorf tubes as a "dry" suspension. Eggs collected in a large pool beneath the animals and were pipetted into large volumes of SFSW. All the gametes were treated as for the species described previously to prepare them for use in experiments.

Data Analysis

Fertilization and development success was measured as the percentage of eggs fertilized and/or developed to blastula from at least 3 subsamples of 50 eggs from the main pool of eggs. In order to perform statistical analyses on these proportionate data, it was necessary to carry out an arcsine transformation (see Sokal and Rohlf 1981). The arcsine transformation has the effect of compressing the middle of a range of percentages while stretching out the tails, so normalising the data which are converted to angles between 0° and 90°. Standard error of the mean was calculated using arcsine transformed data, and the positive and negative errors back transformed to proportionate values for graphical representation.

Statistical analyses were carried out on several computer packages. These included Minitab version 11 and Microsoft Excel version 7 on the PC, and StatView on the Macintosh. Tests performed included 2-way analysis of variance (2-way ANOVA), analysis of covariance (ANCOVA) (see Sokal and Rohlf 1981), and multivariate analysis of variance using a general linear model (MANOVA) (Minitab user's guide). Explanations of the use of these tests can be found in the relevant chapters.

Chapter 3

Field Fertilization Success During Two Natural Spawning Events of *Arenicola marina* (Linnaeus)

3.1 Introduction

The assessment of field fertilization success in broadcast spawning marine invertebrates has progressed enormously in recent years. However, until the publication of Williams *et al.* (1997), reported here, the literature consisted solely of reports of fertilization success of epifaunal organisms inhabiting hard substratea. Species favoured in particular by ecologists include echinoderms and corals (Pennington 1985, Levitan *et al* 1991, Babcock and Mundy 1992, Coma and Lasker 1997), although success in broadcast spawning coral reef fishes has also been examined (Petersen 1991). This may be simply because these organisms are easily accessible, occupying a shallow subtidal habitat. Often they have a predictable spawning pattern, or can be easily manipulated in the field through induction to spawn (e.g. injection of KCl in urchins, 1-methyladenine in starfish). In such cases, gametes are released into the water column where they are fertilized, often remote from the site of release. This enables workers to collect eggs following spawning, downstream of the female, and thereby determine fertilization success. In addition, because these organisms inhabit a shallow subtidal habitat, water movements, although often strong and turbulent, are more predictable than in the intertidal zone where wave action causes an extremely high level of turbulent mixing (Denny 1988, Denny and Shibata 1989).

The majority of these studies were concerned with the artificial manipulation of animals and gametes under natural (Pennington 1985, Levitan *et al* 1991, Babcock and Mundy 1992), or simulated field conditions such as flumes (Pennington 1985). The methodology employed throughout these examples was mostly quite straightforward. In artificially stimulated spawning events, gametes (eggs) were either collected *in situ* downstream of the (induced) spawning event, or collected prior to the field experiment and suspended in mesh bags downstream of spawning males (directly measuring fertilization in the field). Other experiments measured fertilization success in the field indirectly by collecting sea water from the site of the spawning event, and

incubating eggs *in vitro* with the collected seawater. Several studies of natural spawning events have also been undertaken, in corals (Babcock *et al* 1992, Coma and Lasker 1997), asteroid echinoderms (Babcock *et al* 1992), holothurians (Babcock *et al* 1992) and hydroids (Yund 1990). In all cases, gametes were collected from the site of spawning at particular time points in the spawning event, fixed immediately to prevent further fertilization or rinsed in clean seawater to remove excess sperm and incubated. Thus, the recorded fertilization success is a measure of fertilization success of eggs at a particular point in the water column between the site of release and the site of capture.

As shown through modelling (Vogel *et al.* 1982) and the experimental studies mentioned above, the level of fertilization success at particular points in the water column will change over time, from the commencement of spawning until spawning ceases perhaps several hours later. Many of these studies (e.g. Coma and Lasker 1997) misuse the term "fertilization rate" to describe the final recorded fertilization success of the organisms under study. Whilst fertilization success varies at different points in the water column, it is incorrect to state that the rate of fertilization changes when there is no temporal element to the study, as in the majority of the studies undertaken to date.

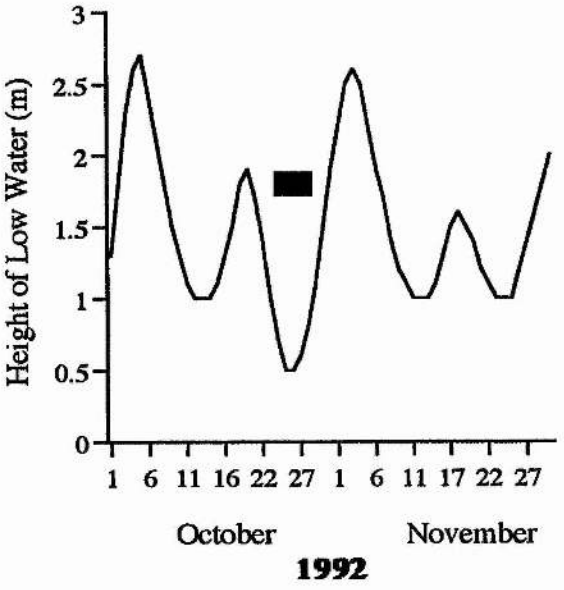
In all experiments conducted to date which measure such "instantaneous" fertilization success in the field, the final record of fertilization success may not in fact be an accurate assessment of the total fertilization success of the individual under study. Rather, it can be regarded as a measure of the fertilization success of eggs at a particular point and time after spawning in the water column. It is therefore only possible to make predictions of the fertilization success of the individual since the proportion of fertilized eggs may change downstream of the collection point, and will not take account of any eggs carried elsewhere in the current, or which settle to the substratum upstream of the collection point.

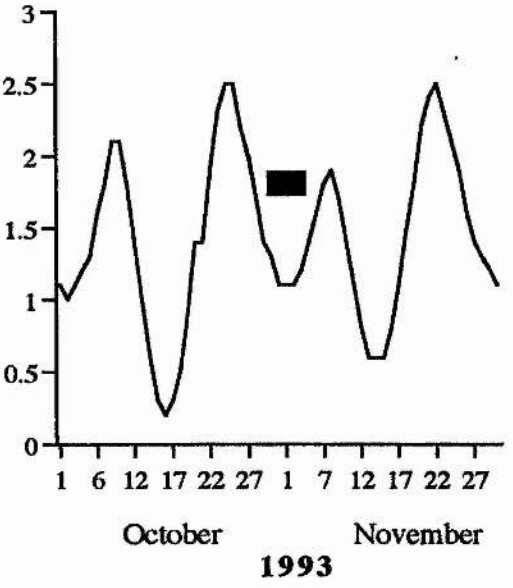
Fertilization in infaunal broadcast spawners is very different to the epifaunal condition. The most obvious difference is the lack of a permanent substratum, and therefore the transient nature of the soft shore habitat.

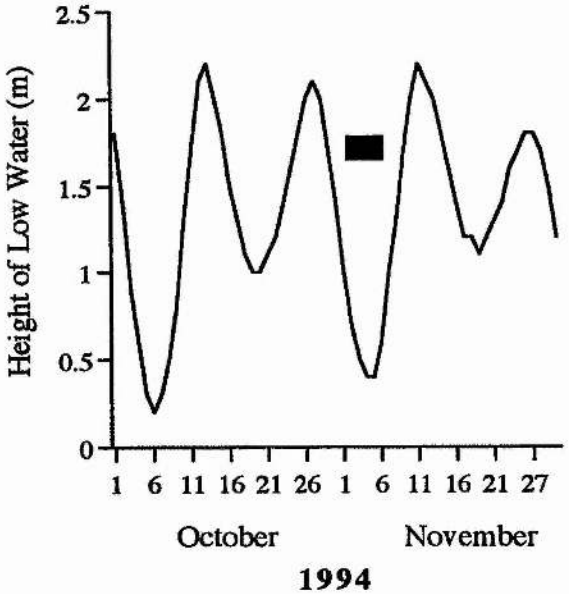
Presumably, it is this fact, along with the lack of a suitable experimental animal that has tempered the research into the fertilization ecology of this habitat. Broadcast spawning among infauna and inhabitants of soft substrata is rare compared to epifaunal organisms. It occurs widely in bivalves such as scallops or oysters which inhabit surface sediments, but among dominant infaunal groups such as polychaetes, it occurs only in larger individuals. Reproduction in smaller interstitial polychaetes is characterised by either copulation or internal fertilization, or the production of spermatophores (Schroeder and Hermans 1975). Among many of the larger free-living polychaetes that broadcast their gametes, fertilization is ensured through behavioural and physiological adaptations. In groups such as the nereids, this manifests itself as swarming where reproductive individuals leave the sediment and swarm together, releasing male and female gametes in close proximity. Often, a "nuptial dance" is performed (e.g. *Platynereis dumerilii*, *Nereis succinea*), with male and female worms induced by pheromones to swim around each other in very tight circles so that the sperm and eggs are released into the same space (Hauenschild 1960, Hardege *et al.*. 1990). Physiological adaptations associated with reproduction (epitoky) are also common among the Polychaeta and are discussed in Chapter 1.

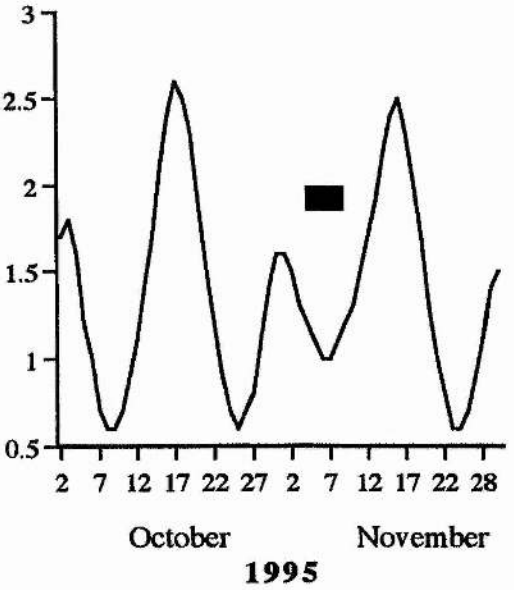
Arenicola marina is an annual, iteroparous polychaete which is common around the shores of the British Isles. The habitat and reproductive cycles of this polychaete are widely described in Chapter 1. Many of the studies of the reproductive cycle of *A. marina* were conducted on the population at the East Sands, St Andrews (Howie 1959, Bentley and Pacey 1992). These studies have provided a wealth of information about the nature of the spawning at this location. This population spawns epidemically during the Autumn, following a period of gametogenic growth between March and September. Spawning has been observed over many years at this site, and it is now possible to predict with reasonable accuracy when the spawning period will occur. Some of the more recent spawning times are shown in Figure 3.1 detailing the tidal heights over several spawning periods. The peaks represent neap tides and the troughs indicate spring tide periods. It can be seen from these graphs that this

Figure 3.1 Spawning period of the populations of *Arenicola marina* at East Sands St Andrews, and Kingsbarns, Fife in relation to the tidal cycle. Where the graphs peak indicate neap tides, and the troughs indicate the peak of spring tides. Spawning usually takes place on the last spring tide of October or the first spring tide of November irrespective of whether it occurs on a new or full moon.









= Spawning Period

population spawns during the final set of spring tides in October, or at the first spring tides in November irrespective of whether the moon is full or new. Figure 3.1 shows only four spawning periods, but observations since 1986 are in accordance with these data (Bentley and Pacey 1992, M.G. Bentley pers.comm). In addition, Howie (1959) reports that this population spawned over the same period during the 1950s, and factors influencing spawning time will be discussed in Chapter 8.

The predictability of spawning at this site enables preparations to be made to quantify the level of fertilization success in the field. The assessment of fertilization success in *Arenicola marina* requires a different methodology than that for the free-spawning echinoderms and corals described previously. Farke and Berghuis (1979a) suggest that female *A. marina* retain their eggs following spawning within the burrow. Their laboratory experiments indicate that the female burrow is the site of fertilization and early development. Males release sperm onto the surface of the sediment at low water, where they coalesce to form sperm puddles with an "oily" consistency (see Pacey and Bentley 1992). Fertilization is apparently brought about through the mobilisation of the sperm puddles on the flood tide, and the female irrigating the burrow as described in Chapter 1. In this respect, *A. marina* can be regarded as a male only broadcaster, with the female "brooding" her eggs in the burrow although there are no physiological or behavioural adaptations to facilitate this and hence she is not a true brooder. It is therefore clearly impossible to measure fertilization success by sampling eggs in the water column as has been carried out previously.

This chapter consists of two distinct sections each with its own methods and results sections, but with a common discussion. This avoids the repetition that would occur if they were separated into entirely different chapters. The first section deals with the 1994 spawning period at the East Sands, St Andrews and the second with the 1995 season at Kingsbarns, a beach located a few miles along the coast from the East Sands.

Figure 3.2 Sperm puddles produced by male *Arenicola marina* during the spawning period at East Sands, St andrews (reproduced from Bentley and Pacey 1992).

Part 1: Field fertilization success at the East Sands, St Andrews

3.2 Materials and Methods

The main aims of these studies were to investigate the fertilization success of female *Arenicola marina* during a natural spawning event under field conditions. Due to the spawning behaviour and habitat of the animals, this necessitated the construction of experimental apparatus to allow the placement of female worms into the field. The animals had to be maintained in good health, be permitted to interact with the environment, and be retained inside the apparatus along with any spawned eggs until the experiment was terminated. In order to achieve this, the apparatus shown in Figure 3.3 was constructed.

3.2.1 Field apparatus

Individual worms were housed in artificial "burrows" constructed from 40cm lengths of 16mm bore size electrical conduit tubing (Marshall Tufflex Ltd.). These were bent into the characteristic "U" shape of the *Arenicola marina* burrow, and housed in a frame of stainless steel screw-thread rod (Radio Spares) with a combination of electrical conduit fittings (Marshall Tufflex Ltd.). Five tubes were placed into each frame, and these were bolted to two 15cm x 15cm x 1.5cm heavy slate tiles with steel brackets. The steel brackets were constructed from 1m lengths of 2cm wide x 2mm thick steel strip which had been painted to avoid excessive corrosion. The steel strip was cut into 16cm lengths, and bent into an "L" shape at 12 cm along the length. One 3mm hole was drilled at the top of the longest (12cm) length to take the screwthread rod holding the tubes in place, whilst the shorter (4cm) length had a 5mm hole drilled in it through which the whole frame was bolted to the tile base. Stainless steel nuts, bolts and washers were used in all cases. At each end of the tube, removable caps were fitted. These were electrical conduit tubing fittings which are normally used to

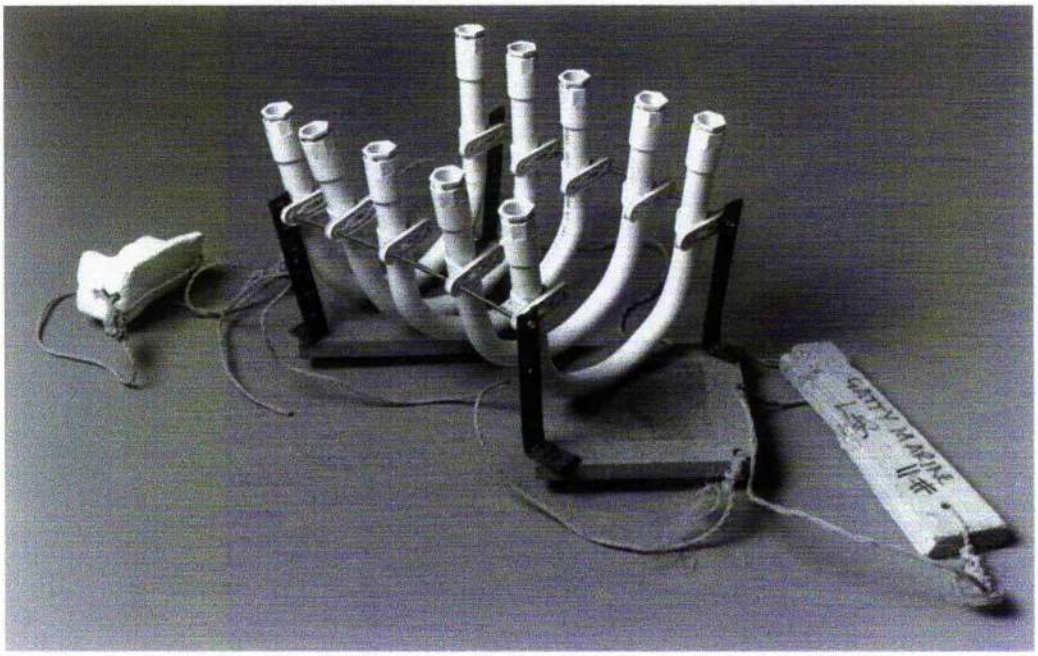


Figure 3.3 The experimental apparatus used to determine field fertilization success in *Arenicola marina*. Artificial burrows were constructed from 25cm lengths of electrical conduit tubing bent to form a "U" shape. These were held in a stainless steel frame and bolted to heavy tiles for anchorage.

join sections of wiring (Marshall Tufflex Ltd.). They had a screw-in top section which was used to sandwich in 2.4cm diameter discs of coarse (3mm) plastic mesh which when in place allowed normal exchange of water and sediment whilst preventing the worms from escaping. To each of the frames, a small float was attached to facilitate recovery in the event that the entire frame became covered by sand. The floats were cut from either a polystyrene block, or lengths of pine, and tied to the tiles with nylon rope.

Field tests

During tests prior to the anticipated spawning period of 1994 (see Figure 3.1), maturing lugworms were collected from East Sands, and placed individually into each tube, and transplanted into the field for a period of 4 days. The apparatus was examined daily to ensure it was still in place, and it was noted that there were worm casts from one opening of each tube. The presumed position in the tubes of the worms is shown in Figure 3.4. At the end of the four days, the apparatus was removed and the worms taken out.

Because of the amount of sediment within the tube, it was impossible to simply tip the worm and tube contents out. The worms were therefore removed from the tubes using hydrostatic pressure. The "stoppers" which contained the mesh preventing worms from escaping were removed from both ends of the tube, and a hose connected to one opening. The other opening was placed over a clean glass beaker. The hose was connected to a tap carrying 0.34µm filtered seawater, and the tap slowly turned on. By carefully regulating the amount of water flowing (by hand) it was possible to force the worm and the contents of the tube out and into the beaker, without causing damage to the worm.

Following removal, all worms were examined and found to be in good condition, showing no visible effect of spending time in the tubes. Along with the worms, a large amount of sediment came out of the tubes, and closer examination revealed that the worms had actually formed their own burrow from the sediment within the conduit tubing. In many cases, a tail shaft was clearly

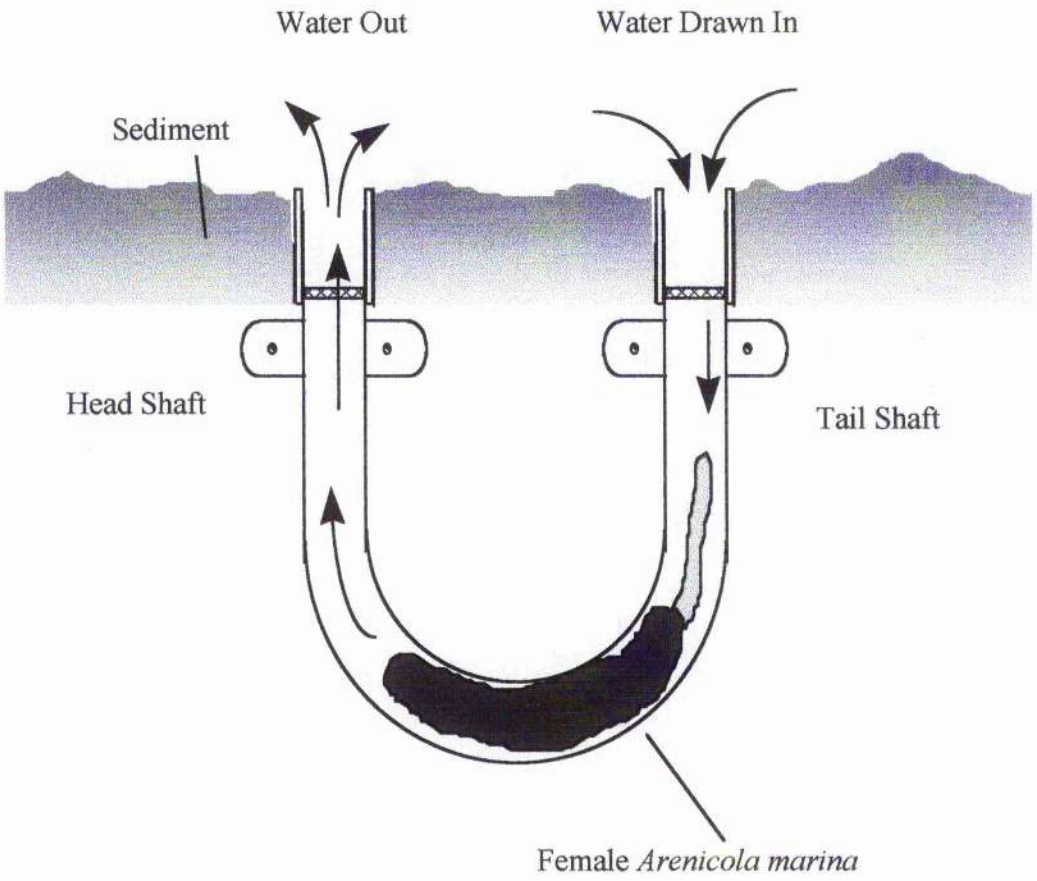


Figure 3.4 Schematic diagram of the (presumed) position of female *Arenicola marina* within the experimental apparatus. The apparatus is buried in the sediment such that the top of the artificial burrow is continuous with the surface of the sediment

visible when the stoppers were removed. It can therefore be argued that the experimental apparatus forms a good simulation of the natural circumstance of the animals. The animals appeared to be casting and hence feeding and behaving normally.

3.2.2 Experimental Design

It was predicted from tidal charts and data on previous years spawning observations that the population of *Arenicola marina* at East Sands, St Andrews would spawn during the period of spring tides commencing the 1st November 1994. During October 1994, worms were collected by digging from East Sands, as stated in Chapter 2. The worms were sexed and kept individually at 8-10°C. Eight frames of five tubes were constructed as above, although because of premature spawning and some mortality, only four tubes in each rack were used. Female worms were placed individually into tubes within the eight racks, and deployed in the field. They were placed into the sediment in four groups of two at 1m above chart datum on 1/11/94. The frames were buried such that the tops of the tubes were almost continuous with the surrounding sediment (because of small amounts of erosion over the tidal cycle, it was impossible to maintain absolute continuity with the sediment). The frames were checked twice daily at low tide to ensure they were still intact, and to check for evidence of spawning.

The design of the experiment was intended to take account of the likely patchy distribution of sperm puddles in the field, and the uncertainties of the spawning response of transplanted females. In an ideal situation, all females transplanted would spawn, and each frame with 4 females would act as a block with 4 replicates. This would enable analyses of variance to be performed by taking each frame as a factor in the analysis, along with local sperm puddle density. However, to maximise the chances of having sufficient females spawn in an area of the beach, it was decided to transplant the frames as four groups of two, each group (block) of two frames within 4/5m of each other and at least 10m from another block. In this way, it would be possible to compare

fertilization successes between groups of frames where there may have been only one or two worms spawning in each.

The racks were removed from the beach after 4 days, on the evening tide of 4th November 1994, after no further male spawning was observed on the morning tide (no sperm puddles seen). They were carried into the laboratory, and disassembled to remove the tubes. The contents of the tubes (sand/worms/gametes) were then removed as stated earlier, and collected in a glass beaker. The worm was removed immediately in case of further spawning or leakage of unspawned eggs. The remainder of the tube contents (sand, oocytes) were re-suspended in fresh 0.34μm filtered seawater, and the (lighter) egg fraction decanted to a clean 200ml crystallising dish. The sand fraction was re-checked for eggs, and if eggs were still present was re-suspended and eggs freshly decanted. This was repeated until all eggs had been removed from the sediment. 100 eggs from each tube were then assessed for fertilization success. This was carried out as described in Chapter 2, the main criterion being evidence of cleavage.

Sperm puddle density

Sperm puddle density was determined by counting all puddles located within a 10m radius of each of the eight racks. In addition, the distance from the rack, and the direction (degrees from North) was also recorded. This was in order to examine the effect of position of the puddle on the fertilization success of the nearest rack. At all times, great care was taken to leave the sperm puddles undisturbed to prevent dilution of puddles before tidal inundation.

3.3 Results

3.3.1 Spawning time of *Arenicola marina*

Figure 3.1 shows the observed times of the spawning period of *Arenicola marina* at East Sands, St Andrews, between 1991 and 1994. Spawning was always during a cycle of spring tides (troughs) rather than neap tides (peaks). The earliest date noted for the commencement of spawning was the 22 October 1991, and the latest the 1 November 1994 (although in the following year, the population at Kingsbarns which has previously spawned at the same time as the East Sands population commenced spawning on 6 November 1995 - see below). The duration of the spawning period was usually 3 to 4 days. An interesting feature of the spawning behaviour is that sperm puddles were only observed on the morning tides during the spawning season. The beach was thoroughly searched every night time low tide with powerful hand held lamps, and no sperm puddles were ever observed despite the beach being searched in all areas (not just the experimental area) until it was covered by the flood tide.

It seems clear therefore that the spawning time of *A. marina* at this location is predictable as being for a period of 3 or 4 days at either the last spring tide of October or the first spring tide of November. It is interesting to note that the particular phase of the moon does not affect the spawning time. In some years, the population spawns on the full moon, and in others on the new moon. These data are a sample of 10 years of spawning observed at East Sands, all of which show the same pattern. Indeed, the timing of the spawning seasons for the years shown in Figure 3.1 also agree with observations by Howie (1959) for the same location.

3.3.2 Sperm puddle density

Figure 3.5 shows the sperm puddle density as observed over the spawning period. Density is extremely low throughout the period with puddles

observed in the experimental area on only two of the four days. However, it does appear to reach a peak in the middle of the spawning period. Spawning took place over the entire beach, outside of the area in which the experiment was conducted, and puddle densities were similarly low. However, because of the (apparently) very low sperm puddle density, no conclusions could be drawn about the position of the sperm puddles relative to the spawning females.

3.3.3 Spawning response of females

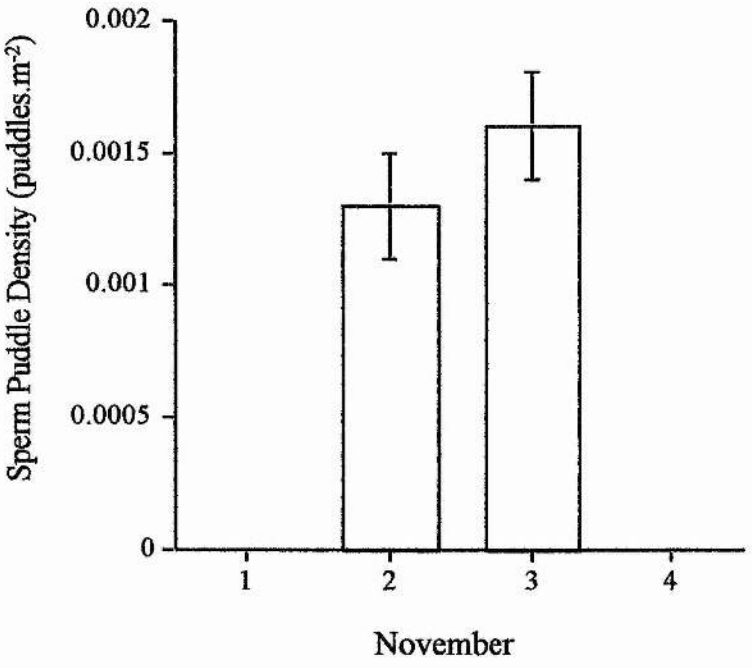
Of the 32 female *Arenicola marina* transplanted into the field over the spawning period, less than 50% actually spawned. Reasons for this poor response will be discussed later.

3.3.4 Fertilization success

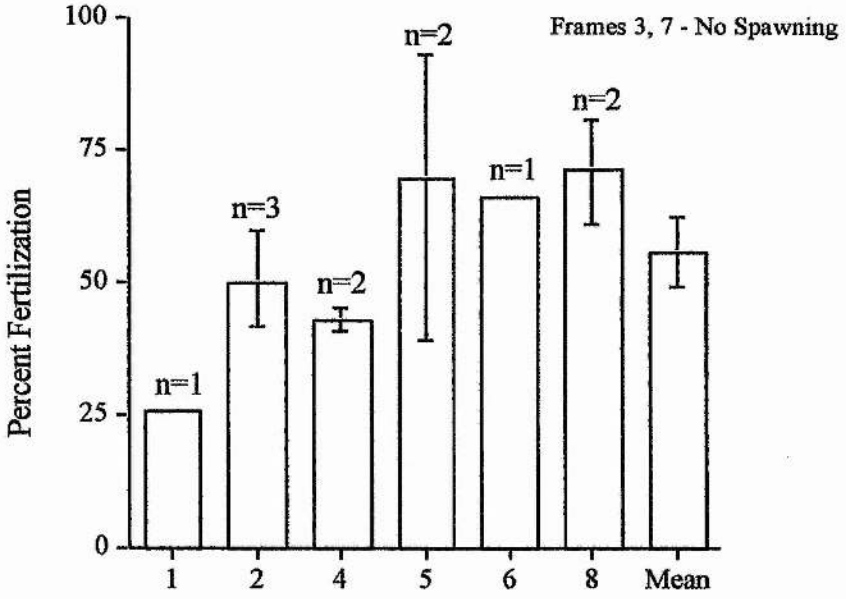
Fertilization success of those females that spawned in the tubes during the experiment is shown in Figure 3.6. Many of the eggs were at an advanced stage of development (post-blastula), but there were a range of developmental stages. It is apparent that the level of fertilization success is very variable, as is the spawning response of transplanted females. In racks 3 and 7, no females spawned and in none of the racks did all of the transplanted worms spawn. The maximum value of fertilization success recorded of any individual female was 93%, fertilization. In no case was fertilization around 100%, and most values fell within the range of 40-60%. All tubes contained at least some fertilized eggs, and the lowest recorded fertilization success was 20%. Meaningful statistical analysis of these results was impossible to perform because so few worms actually spawned in the field. Allied to this, very few sperm puddles were observed, and no trends were discernible between local sperm puddle density and fertilization success.

Figure 3.5 Spawning intensity (sperm puddles per m^2) of male *Arenicola marina* during the November 1994 spawning season at East Sands, St Andrews. Puddle density was measured by counting the number of sperm puddles within 8 circular areas of 10m radius within the spawning area on each day of the spawning period.

Figure 3.6 Fertilization success of female *Arenicola marina* transplanted into the field during the 1994 spawning period. Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.



Date During Spawning Period



Frame Number

Part 2: Field Fertilization Success During a Natural Spawning Event at Kingsbarns, Fife

3.4 Introduction

Originally, it was intended to repeat the previous field experiment during the following spawning season (1995) using the same population at the East Sands. However, during the summer of 1995, the East Sands was heavily exploited by bait diggers over a number of weeks. This greatly reduced the population density from that seen in the previous year, and although no quantitative values are available, sampling for experimental animals proved to be almost impossible with only 2 or 3 gravid females able to be collected during a low tide.

The field experiments were conducted during 1995, therefore, on the population of *Arenicola marina* at Kingsbarns, Fife. This site is approximately 14 kilometres south of the East Sands, subject to a similar exposure level and the beach consists of sand of a similar "coarseness" to the East Sands. In addition, during the previous year's spawning period at the East Sands, the population at Kingsbarns was also observed spawning epidemically (G.J. Watson, personal communication). It was assumed therefore that this population would follow the long term trend of that at the East Sands, and spawn on the last spring tides of October or the first springs of November.

3.5 Materials and Methods

Following consultation of the Admiralty tide tables, it was predicted that the spawning period of the population at Kingsbarns would commence on the spring tides of 24th October. However this proved to be erroneous, and the actual spawning season commenced on the 6th November, the latest spawning time yet recorded. This was extremely fortunate because storm force winds

battered the East coast of Fife during the originally predicted spawning time, and all experimental equipment was washed out of the beach and had to be recovered across a wide area of rocky shore adjacent to Kingsbarns beach.

3.5.1 Success over the entire spawning period

Mature female *Arenicola marina* were collected by digging from the beach at Kingsbarns during a two week period prior to spawning, and stored as described in Chapter 2. 48 females were selected for transplantation into the field to measure the fertilization success over the entire spawning season. These were placed individually into one of 4 tubes contained within 12 experimental racks of the same design as used previously (see above). The experimental racks were transplanted into the field on the morning tide of 6 November 1995, using the same protocol as before of placing them such that the top of the tubes were continuous (as far as possible given the small amount of erosion that occurred) with the surface of the sediment. They were placed randomly in three groups of four racks within an area of the beach not previously dug during sampling for experimental animals. The experimental area was at approximately 1.3m above chart datum. The racks were checked twice daily as with the experiment at East Sands in order to ensure they were still in place and to check for spawning. They were left in the field for five days, and removed on the evening tide of the 10th November. The racks were removed despite the fact that sperm puddles were still observed, because the evening tide was the last tide in the cycle of spring tides to uncover the spawning area for 2 weeks. Following removal, the racks were taken back to the laboratory, worms extracted and fertilization success assessed as before.

Sperm puddle density

Sperm puddle density was determined in the same way as before for, and the distance and direction of all puddles within a 10m radius recorded.

Sperm dilution rate

Williams *et al* (1997) report on the dilution rate of sperm over one tide at the East Sands, St Andrews during the 1993 spawning season by taking 25ml water samples and counting sperm using a haemocytometer. This was repeated at Kingsbarns during 1995, but with some modifications. Two transects were set up in the spawning area, between low water and the end of the *Arenicola marina* zone. Water samples were collected along each transect in 3 replicate 7ml bijou bottles (Sterilin). Samples were collected at the end of low water and every 10 minutes thereafter until the spawning area was covered with water (approximately 10 sampling points). In addition, one station was set up randomly on each transect roughly in the centre of the spawning area, and three replicate water samples taken every minute until the water depth had reached approximately 25cm (usually 8 minutes). Once back in the laboratory, the water samples were filtered with an 80μm mesh to remove larger debris, and 2ml water placed in a clean 7ml bijou bottle (Sterilin). 1ml 5% neutral buffered glutaraldehyde was added to the water sample to fix any sperm present for later examination. Samples were examined for the presence of sperm with a compound microscope and, if present, sperm concentration was determined using a haemocytometer.

3.5.2 Daily fertilization success

Fertilization success was examined over a single tidal cycle, as well as over the entire spawning period. To this end, six further experimental racks were constructed using the same specifications as those for the previous experiments, but with only three tubes per rack. 72 gravid *Arenicola marina* females were collected from Kingsbarns prior to the commencement of the spawning season, and kept as described in Chapter 2. Male spawning in the populations at Kingsbarns and at the East Sands was only observed on the morning tides, and never on the evening low water period. This enabled those

females transplanted into the field on the evening tide to acclimatise for one high tide before being exposed to sperm puddles for one low tide period. They could then be removed and replaced on the low tide of the following evening.

18 gravid females were placed individually into three tubes in each of six experimental racks. These were placed in the field on the evening of 5 November, the first day of the 1995 spawning period. The transplanted worms were removed to the laboratory on the evening of the 6 November and replaced with a further 18 gravid females housed in 6 identical racks. Once retrieved, the racks were removed to the laboratory that evening and the worms immediately removed as described above. Fertilization success was then assessed for each of the worms transplanted. This procedure was repeated each day until 9 November.

3.5.3 1996 Spawning Season

During Winter 1995/1996, a number of heavy storms hit the East coast of Fife, and the beach at Kingsbarns was particularly affected. Large areas of sand were washed away, and much of the beach profile in the area used in the 1995 experiment was changed. Originally, it had been intended to carry out a repeat of the 1995 experiment at Kingsbarns and to this end monthly cast counts of *Arenicola marina* were carried out from May until September in order to determine the population density. However, during sampling in September and October prior to the spawning season, it was found that all animals collected were immature, rendering the beach useless for field experiments. They did not possess the remnants of spawned gametes (coelomocytes, degenerating gametes), so it is unlikely that the population had spawned exceptionally early. Although the exact reasons for this are unknown, it is possible that the adult population had been badly affected by the previous winter's storms, the majority being washed away or buried under excess sand, leaving the upper shore juvenile worms to colonise downwards.

3.6 Results

3.6.1 Spawning response of transplanted females

Weekly fertilization success experiment

As with the previous experiment at East Sands, fewer than 50% of transplanted females spawned (given by the "n" numbers in figure 3.8). Despite this apparently poor response, the sample size of those females that did spawn and from which it was possible to calculate fertilization success is far larger than any previous observation recorded in the literature for a naturally spawning population. The reasons for this low spawning response will be discussed later.

Daily fertilization success

The best spawning response of those females transplanted was on the first day of the spawning period, when 10 of the 18 transplanted worms spawned (given by the "n" numbers in figure 3.8). The spawning response dropped markedly after this such that on the final day, only one of the transplanted worms spawned.

3.6.2 Sperm puddle density

Sperm puddle density was an order of magnitude greater than at East Sands (figure 3.7). Density was low on the 6 November, when the racks are first placed in the field, and climbed towards a maximum density on the 10 November. Further assessment of the number of sperm puddles was impossible because the area in which the experiments were situated and in which the majority of the sperm puddles were observed was not uncovered by subsequent tides. Again, as with East Sands, no spawning was observed at night, despite careful searching with lamps both within the experimental area and across most of the beach.

3.6.3 Sperm dilution rate

Upon examination of the water samples collected and fixed during the spawning season, it was found that very few contained any sperm. Where sperm were found, they were at such a low density that counting with a haemocytometer was inadequate, with only one sperm being encountered for every four or five samples placed on the slide. Therefore, despite the exhaustive sampling programme, no data could be obtained from this part of the survey either from the fixed stations or the sampling of the tide as it moved up the shore. Retrospectively, the sampling programme should have collected larger volumes of seawater and concentrated the sperm through sedimentation or filtration onto membranes.

3.6.4 Field fertilization success

Fertilization success over the spawning period

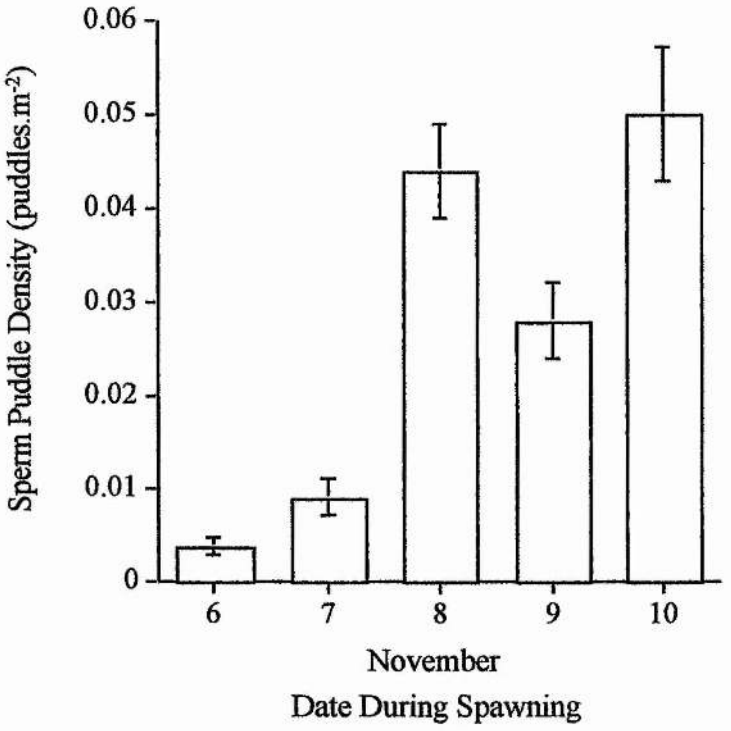
Fertilization success at Kingsbarns (Figure 3.9) was found to be generally very much higher than at the East Sands the previous year. Success ranged from 63.5% to 100%, and the mean fertilization success of all the transplanted females that spawned was 93.9%.

Daily fertilization success

The fertilization success of those animals transplanted and exposed to sperm puddles for one low tide period is shown in Figure 3.8. Fertilization success was at its lowest on the first day of the spawning period (6 November), and the highest value is the 95% recorded in the one individual that spawned on the 9 November.

Figure 3.7 Spawning intensity (sperm puddles per m^2) of male *Arenicola marina* during the November 1995 spawning season at Kingsbarns, Fife. Puddle density was measured by counting the number of sperm puddles within 12 circular areas of 10m radius within the spawning area on each day of the spawning period.

Figure 3.8 Fertilization success of female *Arenicola marina* transplanted into the field and removed on each day during the spawning period. 18 worms were placed into the sediment on the evening tide in anticipation of the males spawning the following morning. They were removed the following evening and replaced with fresh animals. N numbers indicate the number of females (out of 18) that spawned. Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.


Sperm Puddle Density (puddles.m-2)
0.06
0.05
0.04
0.03
0.02
0.01
0
6
7
8
9
10
November
Date During Spawning

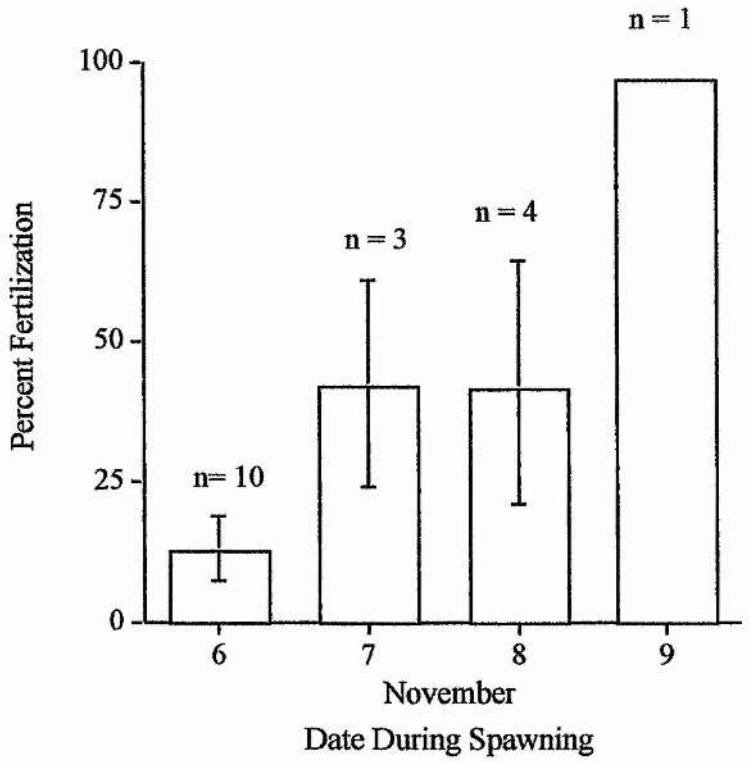
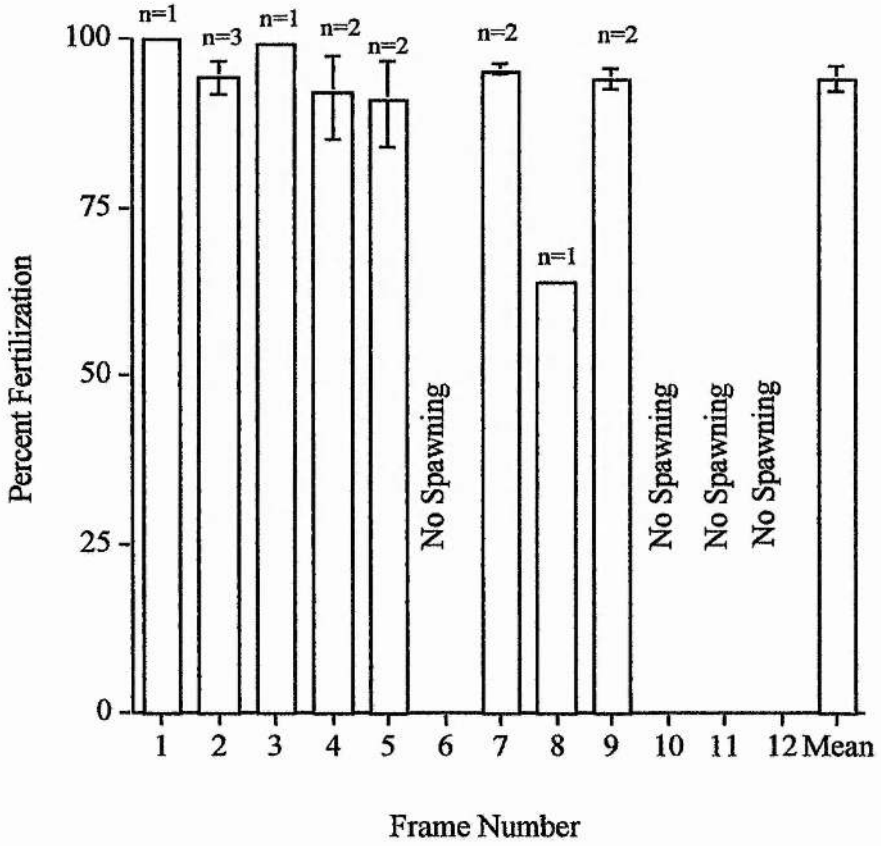

Percent Fertilization
100
75
50
25
0
n= 10
n = 3
n = 4
n = 1
6
7
8
9
November
Date During Spawning

Figure 3.9 Fertilization success of female *Arenicola marina* transplanted into the field during the 1995 spawning period and left for the entire spawning period. Spawning did not occur in three of the transplanted frames. Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.


n=1
n=3
n=1
n=2
n=2
n=2
n=2
n=1
100
75
50
25
0
Percent Fertilization
No Spawning
No Spawning
No Spawning
No Spawning
1
2
3
4
5
6
7
8
9
10
11
12
Mean
Frame Number

3.7 Discussion

Arenicola marina may be considered as a model organism for the study of the fertilization ecology of an epidemic, free-spawning, infaunal, intertidal species. At some locations such as the East Sands St Andrews and Kingsbarns, Fife, the populations appear to have a highly predictable spawning season. The prediction of spawning time is based on long term observations of the previous spawning behaviour (e.g. Howie 1959, Duncan 1960, Bentley and Pacey 1992) and consultation of tidal charts. However, the predictability of spawning is not as precise as other species such as the Pacific Palolo worm (Caspers 1961, 1984) or a range of species on the Great Barrier Reef (Babcock *et al.*. 1986), and often is predictable as one of two or three sets of spring tides during October or November.

As stated in Chapter 1 the spawning time of *Arenicola marina* differs between populations by several weeks, even when the populations are reasonably close together as at the East Sands and Dunbar. The precise spawning cues of a population are presently unknown, but may be a combination of temperature, tidal cycle, photoperiod and endogenous factors. Because there is such a large amount of variation in spawning time between different populations (Duncan 1960) the particular cues (e.g. exact temperatures, photoperiods etc) are unlikely to be universally identical. These factors are discussed in detail in Chapter 5. There is evidence to suggest that once initial spawning is triggered by environmental cues or endogenous rhythms, spawning is further enhanced in the rest of the population by pheromones released along with the gametes, leading to a "cascade" effect (Hardege *et al* 1996, Hardege and Bentley 1997). Such mediation of spawning to maximise the synchrony of gamete release is in evidence in broadcast spawning coral species (Coll *et al* 1995) and nereid polychaetes (Hardege *et al* 1994, Zeeck *et al* 1996), although at present this area of research is still relatively new.

The apparatus used during the field experiments appears to have had little detectable effect upon the behaviour and general health of the female

lugworms transplanted into the field. During tests prior to the first spawning period, it was observed that the tubes quickly became inundated with sand (over one or two tides), and many of the worms produced faecal casts from the tail openings of the tube. These worms had been kept in the laboratory for several days prior to transplantation, and had fully expelled their gut contents. It is reasonable to assume, therefore, that the worms were feeding and thus behaving normally. Worms recovered from the field after 4 days *in situ* showed no visible deleterious effects. This apparatus therefore provides an environment that closely mimics the natural condition under which *Arenicola marina* lives.

In both sets of experiments, developing fertilized eggs were found in the burrows of female lugworms. This confirms observations by Farke and Berghuis (1979a) that the female burrow is indeed the site of fertilization and early development of eggs of *Arenicola marina.* It is curious that the males were observed to release sperm only during the morning low tide period. The advantages of such behaviour are not immediately apparent. Indeed, spawning during daylight hours may make the worms more vulnerable to visual predators such as wading birds, although the threat from other predators such as shore crabs would be reduced. Additionally, one should also consider the possible damaging effects of UV radiation on sperm released onto the sediment during daylight hours (although at dawn this may be fairly low). The cues that could induce such behaviour are unknown.

The spawning response of females was found to be fairly low, and less than half the worms transplanted actually spawned during the field experiments. It is possible that such behaviour is common in the field, but it is also possible that the experimental situation contributed to this poor response. The apparatus may have in some way prevented the normal spawning behaviour of the transplanted female by interrupting the perception of environmental stimuli. It is also possible that the animals failed to spawn because they were stressed by the experimental conditions, although this is less likely because stress through physical handling or water changing in the laboratory often causes animals to spawn. Where tubes were found to contain fertilized eggs, however, it can be

confidently concluded that normal spawning occurred since the oocytes are required to undergo endocrine maturation prior to spawning and fertilization. Eggs which have "leaked" from damaged females are clearly distinguished by the presence of a large germinal vesicle, and are impossible to fertilize in this state (Watson and Bentley 1997).

The spawning response of the daily fertilization success experiment is interesting because it is initially higher than the other field experiments, but on the final day of the experiment has dropped so that only one of the 18 transplanted females actually spawned. If this is a common feature of the reproduction of *Arenicola marina* it suggests that the majority of the females will spawn over the early part of the spawning period. The eggs will then remain in the burrow, becoming fertilized over the following two or three days of the spawning period.

Fertilization success in both spawning periods and especially the first experiments at the East Sands was highly variable. Some females were found to have a fertilization success approaching 100%, and others 0%. This variable fertilization success along with the lack of success in recording sperm in the water column in the second experiment suggests that sperm may be very patchily distributed on the flood tide. In many cases, particularly in the first experiment, there was also a large amount of variation between females within the same rack. Local variation in sperm distribution cannot fully explain this and it is possible that there was a temporal variation in the spawning of some individuals, with some spawning slightly earlier in the season and therefore the eggs being exposed cumulatively to more sperm. This phenomenon also prevented extensive analysis of the relationship between the local sperm puddle densities and fertilization success. It was originally hoped that a relationship could be found between the sperm puddle distribution and the observed fertilization success. However, the variability of fertilization success between animals located in the same place, and the uniformly high success overall meant that no general trend was apparent. Other factors could have complicated this, including irrigation rates of the females and time of spawning.

Comparison of the fertilization successes during the 1994 spawning season at the East Sands (Figure 3.6) and the 1995 Kingsbarns data (Figure 3.8) reveals great differences. Fertilization success is consistently very much higher at Kingsbarns, with an overall mean fertilization success of 93.9% compared with 55% at the East Sands. This can be explained by examining the sperm puddle densities for each site during the spawning season (Figures 3.5 and 3.7). At the East Sands, sperm puddles were only noted in the experimental area on the middle two days of the spawning period, and density over the area never exceeded 0.002 puddles per square metre. In contrast, the following year at Kingsbarns, sperm puddle density was more than an order of magnitude greater on each day of the spawning period. Density was at its lowest on the first day after the racks were transplanted, rising to a peak on the final day on which the spawning area was uncovered by the tide. Spawning may have continued after this tide, but since the area was not uncovered at low water, the experiment had to be terminated. It seems apparent, therefore, that the higher sperm puddle density at Kingsbarns leads to a concomitant increase in mean fertilization success when compared to the previous year's observations at the East Sands

It has recently been pointed out (Levitan and Petersen 1995) that availability of sperm may be the principal factor limiting the fertilization success of free spawning invertebrates. Experimental evidence from Yund (1990), Levitan *et al* (1991) and Babcock *et al* (1994) suggests that sperm dilution and distance from spawning males directly influences the reproductive success of free spawning females. Whilst the spawning behaviour of *Arenicola marina* (males produce sperm puddles, females retain eggs within the burrow) and its intertidal habitat make it a very different situation to the subtidal broadcast spawners described previously, evidence presented here suggests that sperm limitation is indeed a factor to be considered. Figure 4 of Williams *et al* (1997) showing the sperm dilution rate during the flood tide at the East Sands in the 1993 spawning period, indicate that sperm are available for only a short time after the spawning area has been covered by the tide. In addition, the water samples taken during 1995 to determine rates of dilution, and which ultimately found very few sperm, shows that sperm may be very patchily distributed in the

water column. It is not clear why so many sperm were found in 1993 compared with the 1995 samples, but it may be caused by differences in the sampling technique. As no counts of sperm puddles were made in 1993, it is not known if the sperm puddle density was similarly greater.

As stated above, following fertilization the eggs remain in the burrow until they have developed into swimming trochophore larvae. Whilst it is possible that some eggs could have been expelled sooner than this, observations of females kept within glass tubes in the laboratory, and which have spawned, have shown that very few eggs are lost from the tube in the few days following fertilization. Migration from the burrow therefore appears to be mainly through larval motility rather than female pumping action (Farke and Berghuis 1979a,b). If there were a loss of eggs from the burrow during the course of the experiment, it is unlikely to have affected the fertilization success recorded for the tube concerned. Larval development to the stage where controlled motility is possible takes approximately one week, by which time newly hatched larvae and stages to three chaetigerous segments are able to swim through ciliary movements (Farke and Berghuis 1979a). Thus there is unlikely to be a preferential loss of fertilized over non fertilized eggs from the tube during the period of the experiments (4 days).

One factor that could have enhanced the fertilization success is the rate of burrow irrigation. Baumfalk (1979) demonstrated through an elaborate experimental design that the irrigatory rate of the burrow (both males and females) was at its greatest as the tide began to flood, far higher than the rate when water flow is static, or later during the tide period. According to Williams *et al* (1997) this is likely to be the point at which the greatest concentration of sperm is in the water column. In addition, Hardege and Bentley (1997) report an increase in irrigation rate of female *Arenicola marina* when exposed to male spawning water. These factors would enhance the chances of bringing sperm into the burrow, and therefore of achieving fertilization.

Apart from the final day in the spawning period, the fertilization success of those females transplanted for one tidal cycle is well below that observed over

the entire spawning period. While it is possible that the bulk of the fertilizations could have taken place in all the transplanted animals on this final day, the observations of embryos at several stages of development indicates that many of the eggs were fertilized much earlier. The exact time during the spawning period that the female spawned is not known for the worms transplanted for the entire period. However, the observations from the worms transplanted each day indicates that the incidence of spawning fell from over 50% on the first day to only 1 out of 18 on the final day. This leads to the hypothesis that the female lugworms tend to spawn during the early part of the spawning period and retains the eggs in the burrow. Fertilization then takes place each day during the spawning period, with small numbers of eggs becoming fertilized each time the female irrigates with sperm enriched water. In this respect she takes advantage of multiple male spawnings, and gains a high level of fertilization from what may be several days of low sperm exposure. This is a radically different strategy to those employed by free spawning invertebrates whose fertilization success has been studied to date. It implies that the eggs must be longer lived than previously seen for free spawners, and in this respect is similar to the strategy described for the ascidian *Diplosoma listerianum* (Bishop 1998).

In conclusion, it is possible to say that the fertilization success of a naturally spawning population of *Arenicola marina* is a variable phenomenon, with some individuals achieving success levels of around 100% while others fail to have any eggs fertilized. The observations reported here and in other studies (Babcock and Mundy 1992, Levitan *et al* 1992) appear to contradict the view of Thorson (1946) that almost all eggs of marine benthic invertebrates are fertilized. Rather, it would seem that a highly variable fertilization success exists for most of the free spawning animals so far studied. The main influences upon fertilization success appear to be population density, spatial proximity to spawning individuals (Levitan *et al* 1991) and water currents (Denny and Shibata 1989, Babcock *et al* 1994).

Chapter 4

Fertilization Success During a Simulated Spawning Event of *Arenicola marina* : The Influence of Sperm Puddle Density

4.1 Introduction

The previous chapter revealed that the fertilization success during the entire 1995 spawning period at Kingsbarns was much greater than that recorded at the East Sands during 1994. This difference is perhaps best attributed to the differences in sperm puddle density recorded, density at Kingsbarns in 1995 being very much higher than that at the East Sands in 1994. However, as was stated in Chapter 3, analysis of the relationship between sperm puddle density and fertilization success of individual frames failed to reveal any trends in the data, because of the generally high fertilization success and even distribution of sperm puddles, and the poor spawning response of the transplanted females.

Sperm limitation has been implicated as the principal factor governing the fertilization success of free spawning invertebrates (Levitan 1995, Levitan and Petersen 1995). Unfortunately, the link between sperm availability in the environment and fertilization success of *Arenicola marina* was not proven. To be able to investigate and demonstrate a link, it is necessary to control sperm puddle density, something that is impossible during a natural spawning event. In the literature, this has been achieved for free spawning shallow subtidal species by releasing variable quantities of sperm into the water column, or manipulating the density of individuals which have been induced to spawn (e.g. Pennington 1985, Levitan *et al.*. 1991).

Manipulating the density of spawning male *Arenicola marina* through the transplantation of males into the field to spawn is impractical because of the nature of male spawning. The equipment used to transplant females into the field would interfere with the spawning process of the male since the mesh preventing the escape of the animals would also affect the deposition of sperm puddles. Instead, investigations described in this Chapter aim to determine the effect of sperm puddle density by simulating the production of sperm puddles. Areas of the beach were seeded with variable sperm densities in an attempt to determine the extent to which sperm limitation (through sperm puddle density) determines the fertilization success of lugworm populations. By conducting the

experiment outside of the normal spawning time of the population resident on the experimental beach, it is possible to control absolutely the amount of sperm available to the spawning females. In addition, the incidence of spawning of transplanted females can be greatly increased by manipulating the endocrine controlled maturation processes (injection of female prostomia), a procedure impossible in the natural spawning events because of the large number of animals that would have been required.

4.2 Materials and Methods

Seventy two gravid female and approximately sixty gravid male *Arenicola marina* were collected from Red Wharf Bay, Anglesey, North Wales (grid ref OS-114 545805) during early December 1996. They were maintained individually at 8-10°C as described in Chapter 2. The field apparatus as described in Chapter 3 was re-assembled, with four tubes per frame, and nine frames in total in preparation for the field experiment.

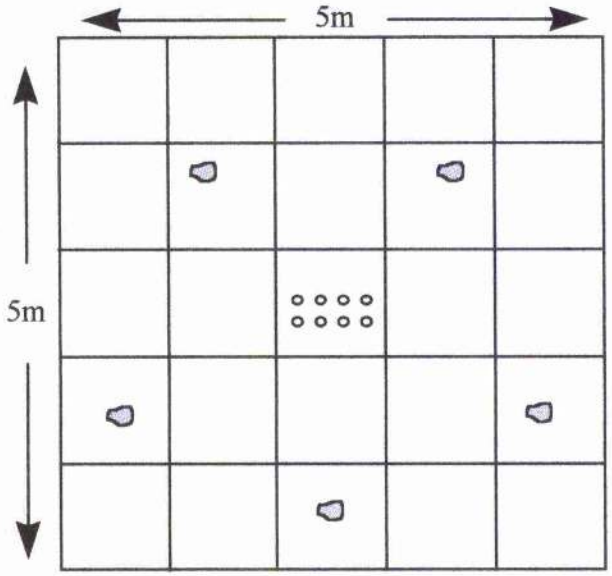
At 10pm on the evening of 5 December 1997, 36 of the 72 gravid females were decerebrated and the prostomia homogenised as described in Chapter 2 to give a final prostomial homogenate concentration of 3 prostomial equivalents. per ml (3 pr.eq.ml^{-1}). Each of the remaining 36 females was injected with 1 prostomial equivalent (0.33ml homogenate), again as described in Chapter 2. The injected females were then placed individually into one of four tubes in each of the nine frames. The frames were transplanted into the field a short time later at 3.30 am on Friday 6 December, the site chosen for the experiment being an area of the beach at West Sands, St Andrews (grid ref OS-59 503185) just above low water. The experimental site was approximately 200m long (along the length of the beach) by 25m wide (upshore/downshore). They were placed in three groups of three frames, each frame being at least 15m apart, and each group of three at least 25m apart. They were then left for one high tide period to spawn (spawning usually occurs within 6 hours of injection, Watson and Bentley 1997)

Whilst the frames were in the field, a solution of 0.1mg.ml^{-1} 8,11,14-eicosatrienoic acid was prepared from the stock solution as described in Chapter 1. Each of the 60 gravid males was then weighed to enable the dosage of fatty acid to be calculated (approximately). The males were injected with the appropriate quantity of fatty acid (to give a concentration in the body of ~13μg.g^{-1} body weight) at approximately midday on Friday 6 December, and left for around two hours to spawn in 150ml glass crystallising dishes. Two worms were placed in each dish in a minimal amount of water (to enable the normal vigorous spawning movements - Pacey and Bentley 1992) in order to maximise the concentration of sperm. Once all the worms had finished spawning, the sperm was pooled together in a 250ml glass beaker. The volume of the sperm suspension was then made up to 150ml with 0.2μm filtered seawater, which was the volume of sperm suspension required for the experiment. This gave a final concentration of approximately 10^8 sperm.ml^{-1} (determined using a haemocytometer).

On the afternoon low tide of Friday 6 December (1650 GMT), the sperm suspension was transported to the experimental site. A 5m x 5m area around each frame was marked out with a (30m) tape measure, and three frames were randomly assigned to be exposed to "low" sperm density, three to "mid" sperm density and three to "high" sperm density. The low sperm density was 0.2 sperm puddles.m^{-2}, the mid 0.6 puddles.m^{-2}, and the high was at a density of 1 puddle.m^{-2}. Artificial sperm puddles were then created by pipetting 1ml of the sperm suspension onto the sediment using a Gilson pipette. The arrangement of sperm puddles in low (5 sperm puddles), mid (15 sperm puddles) and high density (25 sperm puddles) is shown in Figure 4.1. Once all the sperm puddles had been created, the frames were left for one high tide period.

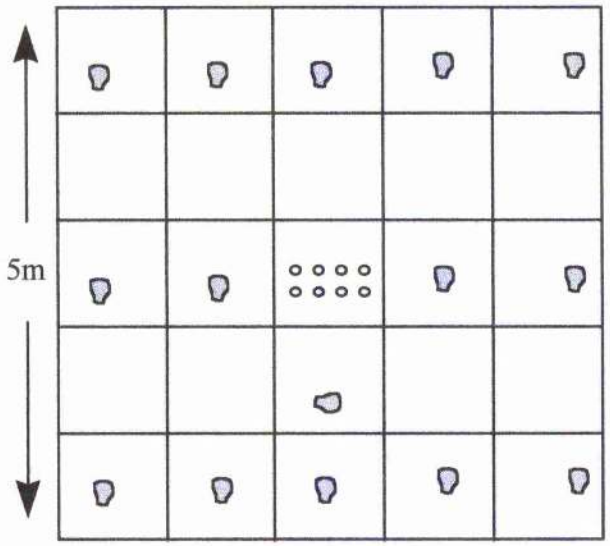
When the frames were exposed on the next period of low water (~4 a.m., Saturday 7th December) they were dug out of the beach and returned to the laboratory. On arrival, the worms were immediately removed from the tubes as described in Chapter 3, and the egg fraction decanted to clean 150ml

Figure 4.1 Schematic diagram showing the layout of the field experiment in terms of the position of the sperm puddles relative to the experimental frames in each of the treatments. Sperm puddles were pipetted at three densities as shown, and no sperm puddles were pipetted into the immediate vicinity of the frames (i.e. the central 1m x 1m area). Three frames, each with four females that had been induced to spawn were exposed to each of the sperm puddle denities.



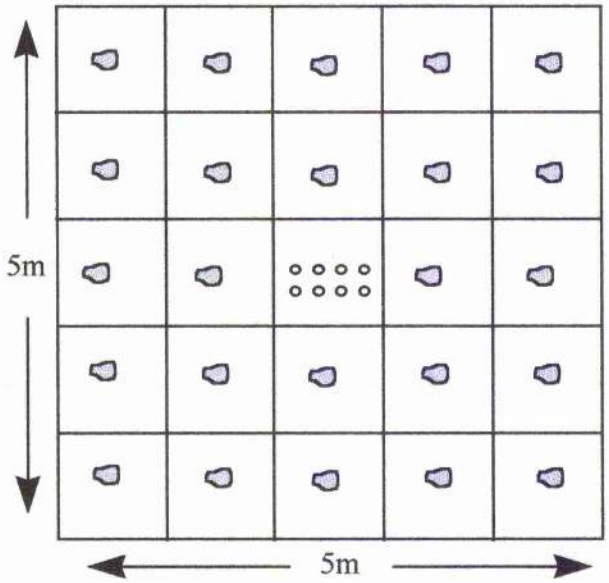
Low Sperm Puddle Density

0.2 puddles.m^{-2}



Mid Sperm Puddle Density

0.6 puddles.m^{-2}



High Sperm Puddle Density

1 puddle.m^{-2}

= sperm puddle

crystallising dishes. 150 eggs were then examined from each dish and the number of fertilized eggs (eggs that had undergone cleavage) calculated.

Statistical Analysis

Two way analysis of variance (2-way ANOVA) was performed on the arcsine transformed percentage data (see Sokal and Rohlf 1981). Each frame was treated as an independent factor in the analysis, with sperm puddle density being the other independent variable.

4.3 Results

Almost all females which had been transplanted into the field spawned successfully (spawning occurred in 90% of females). Figure 4.2 shows the fertilization success of eggs collected from the tubes of frames transplanted at low, mid and high sperm puddle density. Generally, fertilization success was much lower than that recorded during the natural spawning events reported in Chapter 3 despite the much higher sperm puddle densities used. The highest mean fertilization success was recorded in those frames which had been exposed the "high" sperm puddle density, and the lowest was in those exposed to low sperm puddle density.

Two way ANOVA of the data revealed that there were significant differences in fertilization success between each of the treatments, however the variation between frames within a treatment was not significant (see table 4.1). No interaction effect was found between the factors.

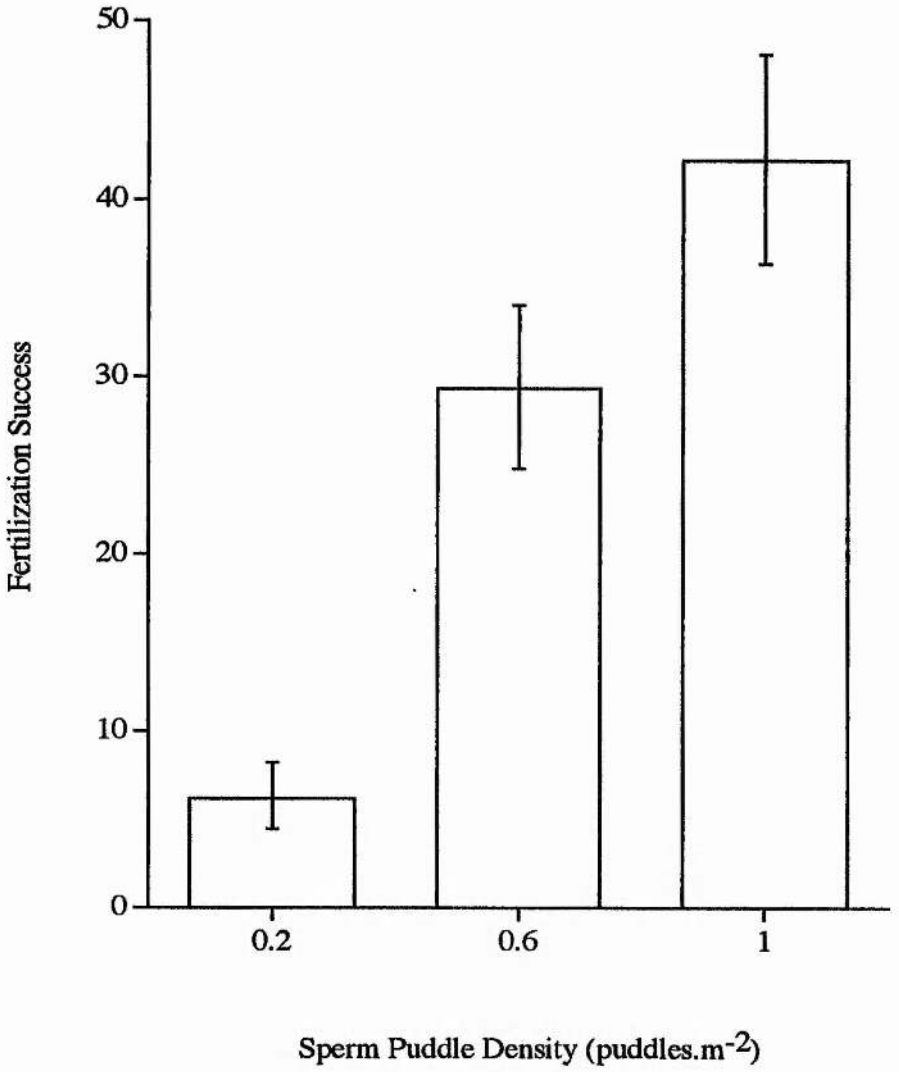


Figure 4.2. Fertilization success recorded from female *Arenicola marina* injected to induce spawning, transplanted into the field and exposed to three sperm puddle densities. Standard errors were calculated from the srcsine transformed percentage data

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Frames	15.38881	1	15.38881	0.201349	0.658989	4.413863
Densities	3614.066	2	1807.033	23.64344	9.2E-06	3.554561
Interaction	278.2475	2	139.1237	1.820312	0.190557	3.554561
Within	1375.713	18	76.42852			
Total	5283.415	23				

Table 4.1. Results of a 2-way ANOVA on the data for fertilization success at each of the sperm densities examined.

Fertilization success varied widely within treatments as was found in the natural spawning event reported in Chapter 3. At the low sperm density, fertilization success varied between a low of 0.7% and a high of 18%, however, most values were below 10%. At the middle sperm density, fertilization success ranged between 6% and 64%, and the high sperm density resulted in values of fertilization between 20.6% and 74%.

4.4 Discussion

The fertilization success reported in this Chapter is very much lower than that reported in Chapter 3 during natural spawning events. This is in spite of the more than 10-fold increase in sperm puddle density. Higher sperm puddle densities were used in this experiment to allow for the drop in sperm concentration. Sperm was collected from a number of males that were kept in small amounts of water. The spawning behaviour of the males (vigorous thrashing movements) ensured that the sperm was well mixed with seawater, and in an active state as a result of the change in pH. Consequently, it did not resemble the typical coalesced sperm puddle seen during the natural spawning events, and may therefore have had different dispersion properties. To compensate, the resulting suspension was diluted to a level where sufficient volume was present to allow the experiment to proceed as described.

The results indicate that sperm puddle density, and hence the density of male *Arenicola marina* in the field has a significant effect upon fertilization success of the female. Figure 4.2 showing the fertilization success at each of the sperm puddle densities indicates that the greatest increase in fertilization success occurs between 0.2 and 0.6 puddles.m^{-2}. The increase in fertilization success between 0.6 and 1 puddle.m^{-2} is much less. Although sperm was still clearly limited, other factors such as the pumping rate of the female lugworms may have further limited the increase of fertilization success.

In Chapter 3, only one worm transplanted for a single exposure to male spawning achieved a success that was approaching the level found for the entire spawning period (figures 3.8 and 3.9). There is a finite quantity of water that can enter the female burrow when she irrigates. Zebe and Schiedek (1996) review some of the physiological adaptations of *Arenicola marina* (see also Riisgard *et al.* 1996). They cite mean rates of irrigation of the burrow as varying between 8 and 26ml.g^{-1}.h^{-1}. However, Baumfalk (1979) found that the rate of irrigation was at its maximum immediately following inundation by the flood tide. It has also been reported that irrigation activity of females is enhanced by substances present in the male coelomic fluid, which may be spawned along with the sperm (Hardege *et al.* 1996). However, precise rates of water flow are not available.

No data are available regarding the size of the females transplanted into the field. Male size, however, (based on measurements prior to injection with fatty acid) was in the range of 5 - 10 grams wet weight (following defaecation). these males were collected from the same site as the females in North Wales, and there is unlikely to be a major difference between the sexes. This is smaller than the size of the animals from the East Sands, St Andrews (Auckland 1993), and comparable to those from Kingsbarns (personal observations, although no data available). Consequently, the volume of water irrigated by the females in this experiment is likely to have been lower than in the first field experiment at East Sands during 1994, but similar to Kingsbarns during 1995. However, the values cited by Zebe and Schiedek (1996) (see above) indicate that only a few millilitres of seawater are likely to enter the female burrow in the first few minutes of tidal

inundation. This places more emphasis on the possibility of a pheromonal modulation of pumping activity in *Arenicola marina* (Hardege and Bentley 1997).

Fertilization success may therefore have been constrained not only by the drop in sperm concentration between the treatments, but also by the ability of the female to irrigate her burrow sufficiently before dilution of the sperm became too great. In this respect, unavoidable artifacts from the experimental equipment may have been introduced. In the previous chapter, the frames that were in the field for several days became fully inundated with sand such that the female was able to create a burrow within the plastic tube. Her irrigation may have been slightly more efficient than in this experiment where, although a large amount of sand was present, there appeared to be slightly less.

Results described in Chapter 3 also contended that high fertilization success was the result of several exposures to sperm. Unless the female is particularly close to a point source of sperm, she may not achieve 100% fertilization success on one tide, and may require exposure to sperm over several tides to irrigate her burrow with sufficient sperm rich water to attain a high fertilization success.

Density Dependent Reproductive Success

Density dependent mating success was suspected as far back as Belding (1912), with regard to scallop production. As stated by Levitan *et al.* (1992), the most prevalent views of density dependent effects among ecologists are that they have a negative effect on the population through resource limitation. Increased density of individuals leads to reduced gamete production and this is certainly true of terrestrial, internally fertilizing species. Levitan (1989, 1991b, 1995) showed that population density acts in several ways for free spawning marine invertebrates. First, high population density can reduce body size and fecundity of individuals and second, it can also enhance fertilization success. In terms of overall zygote production, he found that the mating success of females was

density independent - the decrease in fecundity was compensated by an increase in fertilization success. This factor has been widely demonstrated in flowering plants where pollen limitation substitutes for sperm limitation (e.g. Bierzychudek 1981).

Levitan *et al.* (1992) report on the density of spawning individuals of the sea urchin *Strongylocentrotus franciscanus*, and the resultant effect upon fertilization success. Their experiments examined two spawning group sizes of male and female urchins transplanted either at low density (non aggregated) or high density (aggregated). Unsurprisingly, they found that the fertilization success of the large aggregated group was greatest. Aggregation of spawning individuals was more important than size of the spawning group. Similar (though less comprehensive) results were found by Coma and Lasker (1997a,b) who measured fertilization success during natural spawning events for the Caribbean gorgonian *Pseudoplexaura porosa*, and related the results to the spatial separation between male and female colonies. These observations on the importance of density of spawning individuals are confirmed by the results presented here.

Further studies on the influence of population effects upon fertilization success are provided by Yund and McCartney (1994), Yund (1995) and Atkinson and Yund (1996). They studied fertilization success in colonial ascidians from the male perspective, looking at gene flow through sperm dispersal. The earlier studies (Yund and McCartney 1994, Yund 1995) indicated that overall fertilization success is dependent upon male density, and increasing male density led to a rise in female fertilization success. They concluded that increased male density led to an increase in competition for fertilizations in common with the findings of Grosberg (1987), but that this was partially offset by an overall rise in fertilization success. Atkinson and Yund (1996) varied female density as well as males and found that increases in both male and female numbers did not affect fertilization success. They attributed this to maintenance of the sperm : egg ratio (see Benzie and Dixon 1994, Chapter 6 of this thesis) which resulted in the proportion of eggs being fertilized remaining the same.

This study investigated the effect of density of male spawning in a $25m^2$ area, and found a significant effect. However, proximity to spawning individuals may be equally important. Pennington (1985) and Yund (1991) demonstrate that fertilization success in epifaunal free-spawners is diminished with increasing distance downstream from spawning males. Similarly, Atkinson and Yund (1996) show that individuals on the fringe of an experimental grouping effect fewer fertilizations than those near the centre of an aggregation. The situation in *Arenicola marina* is different in that it is an intertidal species which does not experience a constant current flow as the tide floods. The question therefore arises of whether position of sperm puddle is equally as important to fertilization success as overall density of sperm puddles. For example, does a sperm puddle immediately seaward of the female burrow lead to more fertilization than a sperm puddle landward? Or does the turbulent backwash of the incoming tide mean that there is no real difference? Originally it was intended to study this factor, but this was prevented by the lack of mature individuals late in December 1996.

The implications of density dependent fertilization success to the ecology of *Arenicola marina* require careful consideration. Populations of *A. marina* differ in size and density of individuals between different locations (Auckland 1993), but according to Levitan (1991, 1995) there should be a trade-off of fecundity against proportion of gametes fertilized. Natural fluctuations in population numbers have been found for the lugworm, with numbers of adults reaching a maximum during the summer and declining to a minimum in late winter (Olive 1993). To date, all studies of the impact of bait collecting on lugworm populations have been conducted on very dense populations of fairly small individuals where collection of individuals through digging trenches is possible (e.g. McLusky *et al.* 1983). Exploited areas of these populations recover in a few months. The fertilization experiments reported here have been conducted on less dense populations, where collection of animals is through identification of the head and tail shafts of individual worms. This raises the possibility that over exploitation of a population in the run up to spawning may

remove sufficient males to depress fertilization success of the female worms, although further work would be required to investigate this.

In summary, the high level of spawning synchrony seen in the populations of *Arenicola marina* studied here results in a maximal amount of sperm being deposited on the beach at any one time. This maximises the probability of the female irrigating her burrow with sufficient sperm rich water to achieve fertilization, but she may require several exposures to male spawning on successive days to fertilize a large proportion of her eggs.

Chapter 5

Fertilization Success in a Naturally Spawning Population of *Nereis virens* (Sars)

5.1 Introduction

Fertilization success in the field has been examined in a number of marine invertebrates, and this is reviewed briefly in the preceding chapters. These and other studies have led to the establishment of fertilization ecology as a discipline in its own right.

Previous studies of field fertilization success in marine invertebrates have been conducted solely upon species that have an iteroparous reproductive strategy, reproducing several times before death. Semelparity is a characteristic of several polychaete species, and is particularly prevalent in the Nereidae (see Chapter 1). This study represents the first investigation into the fertilization ecology of a semelparous broadcast spawning invertebrate, the King Ragworm *Nereis virens*.

Nereis virens (Sars) (Annelida: Polychaeta: Nereidae) is a common inhabitant of muddy and sandy shores of northern Europe, Asia and the eastern coast of North America (Pettibone 1963). The habitat and life history of this species is described in detail in Chapter 1. Spawning is usually characterised by a discrete spawning event where the whole population spawns over a few days during spring (Bass and Brafield 1972). During spawning, the reproductive individuals leave the burrow at high water and form a spawning "swarm", releasing gametes near the surface of the water column. It is believed that one of the requirements for spawning is a rise in sea temperatures above 6-8°C, which acts as a threshold below which gravid individuals will not spawn, even when appropriate pheromonal cues are present (Goerke 1984). The spawning behaviour of this species is subject to a certain amount of controversy regarding which sex swarms. It has been reported that spawning of some populations (Thames estuary, Denmark and Canada Bass and Brafield 1972) consists of male only swarms, with males leaving their burrows during high tide and releasing thin streams of seminal fluid from the anal rosette apparatus. This is also reported by Desrosiers *et al* (1994), who hypothesise (without empirical data) that males actively search for the females, who remain in their burrow. In these

populations females remain in the burrow and release eggs onto the surface of the sediment. In other populations, however, such as the Clyde estuary (Clark 1960) and the White Sea (Sveshnikov 1955), both sexes have been reported swarming and releasing gametes.

The population studied in the field experiments reported here is that at Pettycur Bay, Burntisland, Fife, Scotland, situated on the northerly shore of the Firth of Forth. It is believed that at this site only males swarm during spawning, although this has yet to be confirmed. 2 weeks prior to the field experiments, on the spring tides of 11 - 14 March 1997 many males over a large area of the beach were observed to leave their burrows during daytime low water of spring tides. The males were swimming vigorously through the surface sediment and puddles, releasing sperm in streams from the anal rosette apparatus (natural spawning behaviour) as shown in figure 5.1a. Often the sperm trail covered a distance of several metres. This occurred over 3 consecutive days, decreasing in frequency each successive day. No females were observed during this period. This male spawning behaviour was not observed subsequently during the experimental study reported here, and this behaviour is not reported elsewhere in the literature. Many of the emergent worms were predated heavily by herring gulls and crows. The worms which were attacked, however, ruptured whilst being predated and this had the effect of scattering pools of sperm over a wide area. In addition to this, numerous pools of *Nereis virens* oocytes were observed at the entrances to burrows. Close inspection revealed that many pools comprised fertilized eggs (thick jelly coat raised).

The exact spawning behaviour of this population is unknown, because no observations were made during the high tide period. The experiments conducted on this population were therefore based upon the assumption that normally the males leave their burrows and swarm, either during the high tide period as reported elsewhere in the literature (Bass and Brafield 1972, Snow and Marsden 1974, Desrosiers *et al* 1994), or during low tide as seen at this site. The main aim of this study was to attempt to record the level of fertilization success

Figure 5.1 Male *Nereis virens* spawning on the sediment during low water at Pettycur Bay, Burntisland, Fife during March 1997. Numerous males were observed leaving their burrows at low water and spawning in shallow pools of surface water. Some males were attempting to re-burrow into the sediment, but only to very shallow depths.

achieved in the field by *Nereis virens*, and to answer some of the questions regarding the nature of the spawning in this polychaete.

5.2 Materials and Methods

5.2.1 Experimental Animals

As stated in Chapter 2, the field collection of sufficient numbers of gravid *Nereis virens* was extremely difficult, and often the only "mature" animals collected were "spent" individuals that had succeeded in re-burying themselves in the surface sediments. Gravid animals were therefore collected from Seabait Ltd., Northumberland, UK., and kept under the conditions described in Chapter 2.

5.2.2 Experimental Design

All field experiments were carried out at Pettycur Bay, Burntisland, Fife, Scotland (NT 2386) which is a sheltered bay on the North shore of the Forth Estuary. The bay consists of a large area of mud/muddy sand flats on the southeastern part of the bay, becoming sandier and coarser to the north and west. The muddy areas are dug regularly by bait diggers, and immature specimens are readily found. The experimental site was an area of foreshore, 1.5-2m above chart datum where on the previous period of spring tides (9-13 March), some male *Nereis virens* had been observed leaving their burrows and releasing sperm at the surface of the sediment at low tide (see above).

Field experiments were carried out during 2 consecutive periods of spring tides in March and April 1997. The experiments were conducted for 4 consecutive days during the first period (24 - 27 March) and for 5 consecutive days during the second period (7 - 11 April). Fertilization success was examined by transplanting approximately 1000 eggs into the field inside "bags" made from

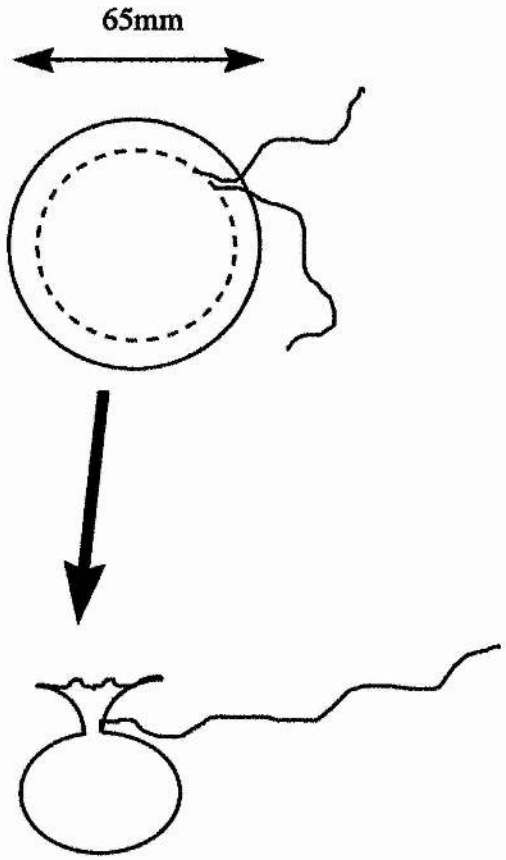


Figure 5.2 The construction of bags to retain eggs from 65mm discs of Nitex mesh and monofilament line

100μm Nitex plankton mesh (see fig 5.2) which retained the 180μm (approx.) diameter eggs. The bags were made by cutting 65mm discs from the mesh, and sewing the edges with 0.2mm, 5lb breaking strain monofilament nylon to create a drawstring bag. They were then anchored in the field to 1.5m bamboo canes driven into the sediment to a depth of at least 70cm to prevent them being washed away by the incoming tide.

Bags were deployed either at ground level by tying to the base of a pole (to simulate eggs deposited at the surface of the burrow), or to polystyrene floats tied to the poles by 4m of 0.3mm monofilament line. The bags were tied to the floats with a short length of the monofilament so that they remained in the top 30cm of the water column throughout the high tide period (simulating swarming females) (see fig 5.3).

Samples of eggs were collected from 5 females and were tested for developmental potential. All the eggs utilised came from females that showed 100% development to blastula during tests. The eggs were pooled, and between 1000 and 2000 eggs were pipetted into each of 24 nitex bags. The bags were taken to the field site at low water, and 12 bags were tied to the bases of 12 poles and 12 to floats which were themselves attached to 12 different poles. All the poles were located randomly into the sediment throughout the spawning area. The bags were then left until the following low tide, when they were removed and replaced by new bags containing unfertilized eggs. The removed bags were transplanted back to the laboratory in 7ml bijou sample bottles (Sterilin) with 5ml fresh seawater taken from the laboratory (0.2μm filtered).

Once back in the laboratory, the bags were cut open, and 150 eggs from each bag examined for fertilization and evidence of cleavage. The time between exposure of the bags to the air, removal from the field, transport to the laboratory and commencement of processing was at least 7 hours, which is sufficient for those eggs which had fertilized successfully during the previous period of high tide to have undergone at least one cleavage. In most cases, the time elapsed was sufficient for the eggs to have undergone several cleavages, and some eggs had reached the blastula stage of development.

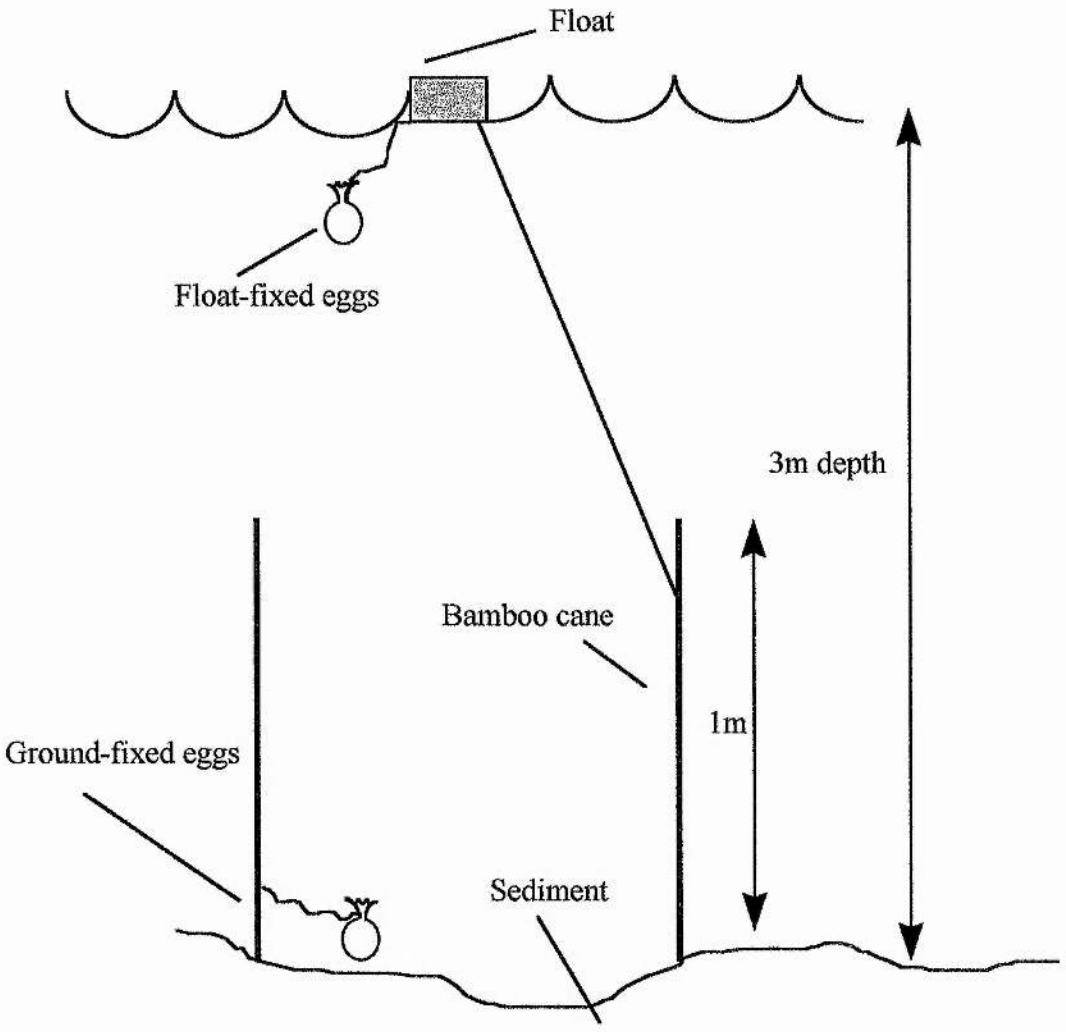


Figure 5.3 Schematic representation of the arrangement of the experimental apparatus in the field

5.3 Results

In almost all cases, all bags deployed in the field were successfully recovered on the following tide. One float deployed bag was lost on 10 April, as were two ground deployed bags on 9 and 11 April. The bags appeared to have a negligible effect on the eggs contained therein. In some cases, there was limited degeneration of unfertilized eggs, the fertilized eggs being afforded protection by the thick jelly coat. However, the distinction between fertilized eggs that had undergone cleavage, and unfertilized eggs was clear, and the bag did not appear to affect the development of the eggs adversely.

First Field Experiment

Figure 5.4 shows the observed fertilization success of transplanted eggs of *Nereis virens* during the first periods of spring tides studied during March 1997. It is apparent that fertilization success in the first set of spring tides is very much greater than that recorded in the second period during April (Figure 5.5). Peak values of around 80-90% are recorded over the first period of spring tides studied, falling to less than 25% on the last tide. In contrast, the second experiment which was conducted over five days on the following period of spring tides shows a rise to a peak of around only 20% on the second day of the cycle, falling to zero on the fifth. Because of problems encountered during sampling, no data exist for the morning high water on 8 April.

A number of trends can be determined by examining the graphs. In general, fertilization success during the first field experiment (figure 5.4) was highest over the first 24 hour cycle examined, with only the daytime deployed float fixed bags having low fertilization values. Peak values fell by the second 24 hours (26 March), but daytime float fixed bags had a much higher fertilization success. Overall fertilization success continued to fall in all bags deployed on the 27 March.

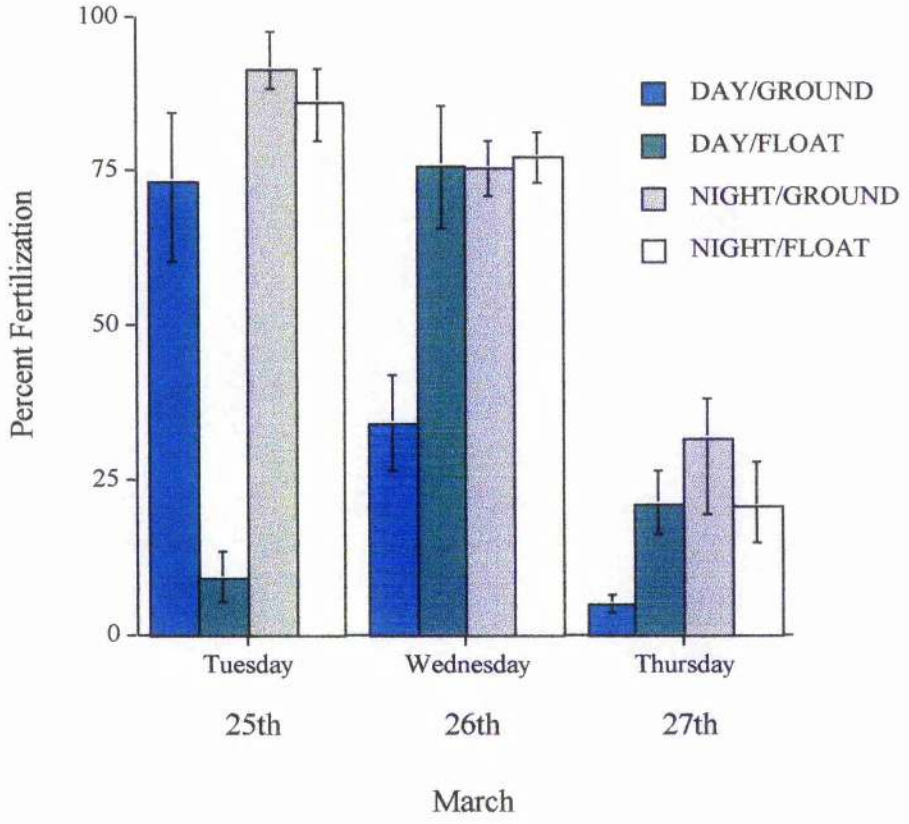


Figure 5.4 Fertilization success of *Nereis virens* oocytes transplanted into the field during spring tides in March 1997 following observations of spawning. Oocytes were transplanted either in the evening or morning, and were either fixed at ground level or attached to floats int he water column. Significant differences were found between treatments (MANOVA $p<0.01$). Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.

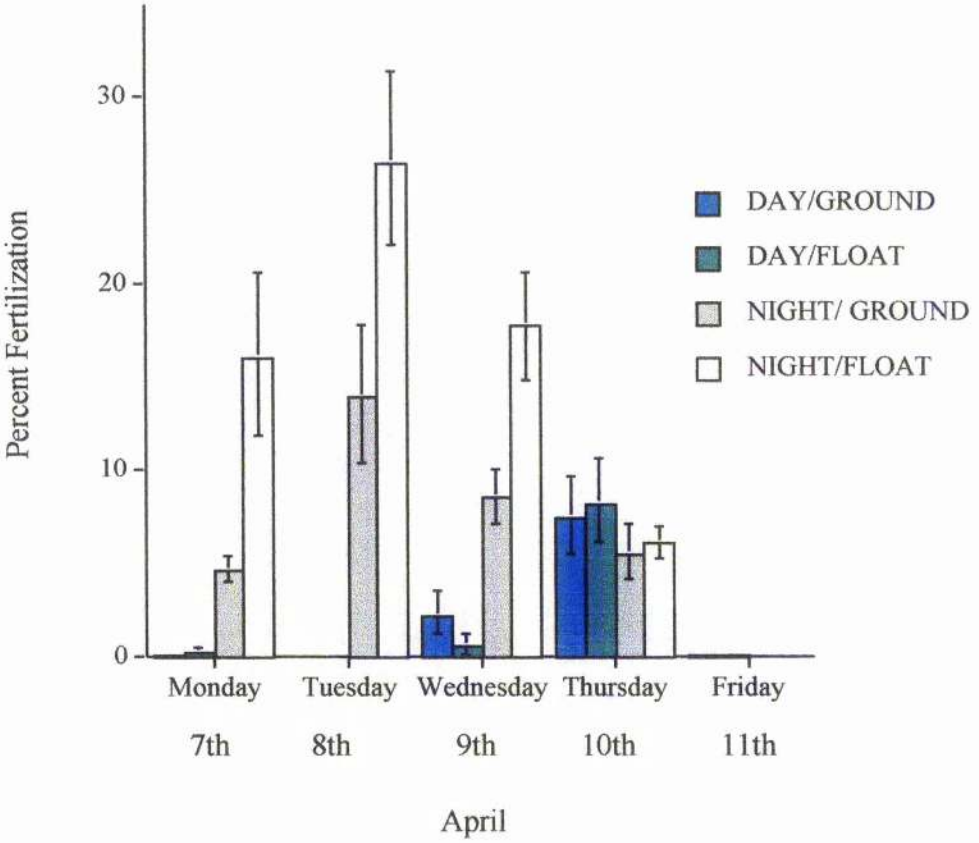


Figure 5.5 Fertilization success of *Nereis virens* oocytes transplanted into the field during spring tides in April 1997 following observations of spawning. Oocytes were transplanted either in the evening or morning, and were either fixed at ground level or attached to floats int he water column. Significant differences were found between treatments (MANOVA $p<0.01$). Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.

Data Analysis

The data were analysed by applying a MANOVA test using a general linear model (GLM) because of the unbalanced nature of the data (unequal observations where some bags were lost). Fertilization success was the dependent variable, and the two factors were morning or evening deployed and float or ground fixed egg bags. Analysis indicated that there were significant differences between the treatments ($p < 0.01$), and the results of the MANOVAs are shown in tables 5.1 and 5.2. In addition, between treatment interaction effects were inferred between night/day deployed bags and day of the spawning period, ground/float fixed bags and day of the spawning period, and night/day deployed, ground/float fixed bags and day of the spawning period in the first experiment. In the second experiment there were interaction effects between all combinations except for ground/float fixed bags and day during the spawning period.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Float/Ground	1	302.5	302.5	302.5	0.8	0.373
A.M./P.M.	1	4597.4	4597.4	4597.4	12.18	0.001
Error	79	29829.3	29829.3	377.6		
Total	81	34729.1				

Table 5.1. Results of a MANOVA test performed on the arcsine transformed percentage data obtained from the first field experiment.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Float/Ground	1	293.1	322.1	322.1	2.88	0.092
A.M./P.M.	1	10824.4	10824.4	10824.4	96.73	0.000
Error	120	13428.4	13428.4	111.9		
Total	122	24545.9				

Table 5.2. Results of a MANOVA test performed on the arcsine transformed percentage data obtained from the second field expeirment

Following the GLM analysis, two-way analysis of variance (ANOVA) of evening tide/morning tide and float/ground deployed bags was carried out to

determine the source of the variation. This was performed because the expectation is that significant differences would appear from GLM analysis since comparisons were made between treatments carried out several days apart. Much more information can be gained by performing several ANOVA tests on specific data points, and the results of these analyses are shown in tables 5.3 to 5.13. Post-hoc Tukey analysis was performed on the results of the 2 way ANOVA, and these are shown in table 5.14 for the first field experiment and 5.15 for the second.

There were significant differences between treatments and in most cases a strong interaction effect was inferred. The results of post-hoc Tukey tests to determine the sources of the variation showed that for the first field experiment most of the significant differences occurred between daytime ground and float deployed egg bags (DG and DF), and the night time deployed ground fixed bags (NG) (table 5.1). Fewer significant differences occurred between daytime ground fixed bags (DG) and night time float fixed bags (NF), and other differences were fairly sporadic. In general this is well represented by figure 5.4 where it is clear that there was no overall trend between treatments in the levels of fertilization success which remained uniformly high in most cases.

Second Field Experiment

The second field experiment recorded a much lower overall fertilization success, but had more clearly defined trends (figure 5.5). On all days except one there was a significantly greater fertilization success over the night time high tides (Tukey figures, table 5.4). In addition, on all tides except one the night time float fixed (NF) bags had a significantly greater fertilization success than the night time deployed ground fixed bags (NG). No such trend was found for the daytime deployed bags (DG,DF), presumably because the overall fertilization success is so low. In general, the results show that there was a rise in success from the first day of the experiment (7 April), peaking during the night time high

tide on the 8th, and success declined to a minimum on the evening tide of the 11 April.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
A.M./P.M.	9065.579	1	9065.579	36.86919	8.81E-07	4.149086
Ground/Float	4144.784	1	4144.784	16.8566	0.00026	4.149086
Interaction	2931.862	1	2931.862	11.92371	0.001581	4.149086
Within	7868.318	32	245.8849			
Total	24010.54	35				

Table 5.3. Results of a 2-way ANOVA performed on the fertilization success of eggs transplanted into the field either on the morning tide of 25/3/97 or the evening tide of 25/3/97 and either in float or ground fixed bags.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
A.M./P.M.	2714.003	1	2714.003	18.38721	0.000193	4.195982
Ground/Float	643.5078	1	643.5078	4.359729	0.046015	4.195982
Interaction	1148.403	1	1148.403	7.780363	0.009393	4.195982
Within	4132.876	28	147.6027			
Total	8638.79	31				

Tuesday P.M. - Wednesday A.M. first

Table 5.4. Results of a 2-way ANOVA performed on the fertilization success of eggs transplanted into the field either on the evening tide of 25/3/97 or the morning tide of 26/3/97 and either in float or ground fixed bags.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
A.M./P.M.	911.645	1	911.645	7.310444	0.011525	4.195982
Ground/Float	1017.005	1	1017.005	8.155322	0.008	4.195982
Interaction	748.845	1	748.845	6.004957	0.020773	4.195982
Within	3491.725	28	124.7045			
Total	6169.22	31				

Table 5.5. Results of a 2-way ANOVA performed on the fertilization success of eggs transplanted into the field either on the morning tide of 26/3/97 or the evening tide of 26/3/97 and either in float or ground fixed bags.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
A.M./P.M.	11617.25	1	11617.25	177.2136	1.36E-14	4.149086
Ground/Float	501.0136	1	501.0136	7.642637	0.00938	4.149086
Interaction	420.9336	1	420.9336	6.421069	0.016372	4.149086
Within	2097.762	32	65.55507			
Total	14636.96	35				

Table 5.6. Results of a 2-way ANOVA performed on the fertilization success of eggs transplanted into the field either on the evening tide of 26/3/97 or the morning tide of 27/3/97 and either in float or ground fixed bags.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
A.M./P.M.	888.3112	1	888.3112	4.619534	0.040398	4.195982
Ground/Float	151.38	1	151.38	0.78723	0.382497	4.195982
Interaction	776.18	1	776.18	4.036412	0.054258	4.195982
Within	5384.247	28	192.2946			
Total	7200.119	31				

Table 5.7. Results of a 2-way ANOVA performed on the fertilization success of eggs transplanted into the field either on the morning tide of 27/3/97 or the evening tide of 27/3/97 and either in float or ground fixed bags.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
A.M./P.M.	3570.125	1	3570.125	39.4019	8.71E-07	4.195982
Ground/Float	374.0112	1	374.0112	4.127797	0.051772	4.195982
Interaction	7.22	1	7.22	0.079684	0.779802	4.195982
Within	2537.023	28	90.60795			
Total	6488.379	31				

Table 5.8. Results of a 2-way ANOVA performed on the fertilization success of eggs transplanted into the field either on the morning tide of 7/4/97 or the evening tide of 7/4/97 and either in float or ground fixed bags.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
A.M./P.M	4114.013	1	4114.013	49.83173	2.68E-07	4.259675
Ground/Float	13.72	1	13.72	0.166186	0.687138	4.259675
Interaction	270.3214	1	270.3214	3.274318	0.082915	4.259675
Within	1981.394	24	82.5581			
Total	6379.449	27				

Table 5.9. Results of a 2-way ANOVA performed on the fertilization success of eggs transplanted into the field either on the evening tide of 8/4/97 or the morning tide of 9/4/97 and either in float or ground fixed bags.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
A.M./P.M.	2752.206	1	2752.206	49.05518	3.05E-07	4.259675
Ground/Float	27.60143	1	27.60143	0.491966	0.489797	4.259675
Interaction	323.68	1	323.68	5.769256	0.024407	4.259675
Within	1346.503	24	56.10429			
Total	4449.99	27				

Table 5.10. Results of a 2-way ANOVA performed on the fertilization success of eggs transplanted into the field either on the morning tide of 9/4/97 or the evening tide of 9/4/97 and either in float or ground fixed bags.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
A.M./P.M.	260.4903	1	260.4903	6.358982	0.017648	4.195982
Ground/Float	204.5253	1	204.5253	4.992788	0.033615	4.195982
Interaction	98.35031	1	98.35031	2.400887	0.132497	4.195982
Within	1146.996	28	40.96415			
Total	1710.362	31				

Table 5.11. Results of a 2-way ANOVA performed on the fertilization success of eggs transplanted into the field either on the evening tide of 9/4/97 or the morning tide of 10/4/97 and either in float or ground fixed bags.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
A.M./P.M.	4.805	1	4.805	0.12392	0.727457	4.195982
Ground/Float	3.51125	1	3.51125	0.090554	0.765697	4.195982
Interaction	6.30125	1	6.30125	0.162508	0.68992	4.195982
Within	1085.703	28	38.77509			
Total	1100.32	31				

Table 5.12. Results of a 2-way ANOVA performed on the fertilization success of eggs transplanted into the field either on the morning tide of 10/4/97 or the evening tide of 10/4/97 and either in float or ground fixed bags.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
A.M./P.M.	4628.534	1	4628.534	123.1689	1.67E-12	4.149086
Ground/Float	3.24	1	3.24	0.086219	0.770936	4.149086
Interaction	0.217778	1	0.217778	0.005795	0.939792	4.149086
Within	1202.52	32	37.57875			
Total	5834.512	35				

Table 5.13. Results of a 2-way ANOVA performed on the fertilization success of eggs transplanted into the field either on the evening tide of 10/4/97 or the morning tide of 11/4/97 and either in float or ground fixed bags.

		DF			NG			NF		
	Date	25/3	26/3	27/3	25/3	26/3	27/3	25/3	26/3	27/3
	25/3	41.8*	-	-	17.8	-	-	12.7	-	-
DG	26/3	-	26.4*	-	38.9*	24.7*	-	34.8*	27.2*	-
	27/3	-	-	12.7	-	47.6*	22.2*	-	-	15.9
	25/3	-	-	-	54.5*	-	-	54.5*	-	-
DF	26/3	-	-	-	12.6	1.6	-	8.4	0.9	-
	27/3	-	-	-	-	34.9*	8.7	-	30.1*	2.4
	25/3	-	-	-	-	-	-	5.2	-	-
NG	26/3	-	-	-	-	-	-	-	1.1	-
	27/3	-	-	-	-	-	-	-	-	6.3

Table 5.14 Post-hoc Tukey test values for field experiment 1. Test was conducted upon the results of a 2-way ANOVA of day and night deployed and ground and float fixed egg bags. Single asterisk (*) indicates significance at $p<0.05$ (DF= daytime deployed, float fixed bags; DG= daytime deployed, ground fixed bags; NF= night time deployed, float fixed bags; NG= night time deployed, ground fixed bags).

		DF					NG					NF				
		7/4	8/4	9/4	10/4	11/4	7/4	8/4	9/4	10/4	11/4	7/4	8/4	9/4	10/4	11/4
	7/4	0.58	-	-	-	-	10.3*	-	-	-		20.6**	-	-	-	
	8/4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DG	9/4	-	-	5.7	-	-	-	12.3*	7*	-	-	-	21.3**	16**	-	-
	10/4	-	-	-	1.5	-	-	-	1.6	0.3	-	-	-	10.3**	0.6	-
	11/4	-	-	-	-	0.9	-	-	-	13.9**	-	-	-	-	13.7**	-
	7/4	-	-	-	-	-	9.8*	-	-	-	-	20**	-	-	-	-
	8/4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DF	9/4	-	-	-	-	-	-	18**	12.7**	-	-	-	26.9	21.7**	-	-
	10/4	-	-	-	-	-	-	-	0.1	1.8	-	-	-	8.9*	2.1	-
	11/4	-	-	-	-	-	-	-	-	13**	-	-	-	-	12.7**	-
	7/4	-	-	-	-	-	-	-	-	-	-	10.3*	-	-	-	-
	8/4	-	-	-	-	-	-	-	-	-	-	-	8.9	-	-	-
NG	9/4	-	-	-	-	-	-	-	-	-	-	-	-	9**	-	-
	10/4	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	-

Table 5.15 Post-hoc Tukey test values for field experiment 2. Test was conducted upon the results of a 2-way ANOVA of day and night deployed and ground and float fixed egg bags. Single asterisk (*) indicates significance at p=0.05, double asterisk (**) indicates significance at p<0.01 (DF= daytime deployed, float fixed bags; DG= daytime deployed, ground fixed bags; NF= night time deployed, float fixed bags; NG= night time deployed, ground fixed bags).

Post-hoc Tukey analysis of the results of the 2-way ANOVA was performed and these results are shown in table 5.15. These show that most of the significant differences occurred between bags transplanted during the day and those transplanted in the night. They also show significant differences between ground and float fixed bags. These results are borne out by figure 5.5.

5.4 Discussion

Field fertilization success in marine invertebrates during natural spawning events is still a relatively new field of research. As stated above, much of the work has been conducted upon a narrow range of organisms, namely the type of broadcast spawners listed in Chapter 1 (Table 1.1). One of the clear features to emerge from these studies, however, is the highly variable nature of field fertilization success in marine invertebrates. Success is seen to vary between 0 and 100%, and theories have been proposed and tested to explain this. As stated in Chapter 3, the main controlling factors from these studies appear to be the density of spawning individuals (Levitan *et al* 1991), current velocity (Babcock and Mundy 1994), turbulence (Denny and Shibata 1989), current direction (Babcock and Mundy 1994) and the degree of spawning synchrony. Together these factors influence the rate of dilution of the gametes, and in particular the sperm, the limitation of which is increasingly seen as the main controller of success (Levitan and Petersen 1995, Levitan 1995).

Nereis virens has a markedly different reproductive strategy from those animals studied previously. As a semelparous species it reproduces only once. Hence in the 6-8 months prior to spawning all its energies are directed towards reproduction to the neglect of normal somatic growth, and all regernerative ability is lost. In addition, somatic changes occur prior to reproduction, which facilitate the process of spawning (see Chapter 1). One might expect, therefore, a situation to have arisen where the fertilization strategy leads to a level of

fertilization success approaching 100%. This leads to the prediction that all breeding individuals in the population should spawn in a highly synchronised fashion, and Goerke (1984) suggests that the main spawning cue is temperature, all animals spawning for only a short period over a few days when the temperature rises above 6-8°C. This would maximise the concentration of sperm in the water column and make sperm - egg interaction more likely. This certainly seems to be the case for other populations reported in the literature, (Sveshnikov 1955, Creaser and Clifford 1982), and raising the temperature from 5°C to 22°C results in spawning among mature individuals (Bass and Brafield 1972).

In contrast to Goerke's (1984) view, it appears from this study that there may in fact be several spawning periods during the annual breeding season. This means that components of the population spawn independently. Immediate post-spawning mortality (among males at least) is high (Bass and Brafield 1972) and it is unlikely that those males spawning on an earlier spring tide will spawn again on later tides. There are reports that males sometimes re-burrow following a period of swarming, but they burrow to only a very shallow depth rather than making a semi-permanent burrow as seen in females (Creaser and Clifford 1982). Such re-burrowing was observed at Burntisland, where males could be found a few centimetres under the surface of the sediment. Females that remain within the burrow may spawn several times during a given period of spring tides, although they too may be unlikely to retain sufficient eggs to spawn again on the subsequent spring tides.

The observation of male *Nereis virens* leaving their burrows during daytime low tides on the spring tide prior to the first field experiment does not preclude the possibility that they may also have been spawning during high water. This is a behaviour that is not reported elsewhere in the literature, and it may represent only a fraction of the spawning population. Unfortunately, the evidence for male spawning times presented here is indirect based upon the measurement of fertilization. These field experiments indicate that there are possibly up to three spawning periods within the reproductive season at Pettycur

Bay, in accordance with Bass and Brafield's (1972) observations for the Thames Estuary, South-East England.

Fertilization success appears to be uniformly high during the first field experiment (see Figure 5.4) and thus few trends are discernible through statistical analysis of the data (see table 5.1, 5.2). In contrast, the second field experiment produced very much lower fertilization successes and clearer trends. The main reasons for this could be that the size of the spawning population at each of the periods of spring tides may be different, some members spawning earlier and some later. Lunar influences on the timing of spawning, as seen in other nereid polychaetes (e.g. Hauenschild 1960, Caspers 1984) are likely. Semi-lunar as well as lunar periodicity may be involved, and Bass and Brafield (1972) reported mass spawning on neap as well as spring tides in the same population over the same breeding season.

A clear trend seen from the results of the second field experiment is the higher fertilization success that occurred during the night time high tide (depth over experimental area approximately 3m) than during the day (Figure 5.5, Table 5.2). This again implies that there is a greater incidence of male swarming during this period. An interesting feature of the second experiment is that during the night high tides (except for the final night), fertilization success is significantly greater for the float deployed egg bags than for those deployed at ground level (Table 5.4). This may be as expected since the bags are located high in the water column close to the site of sperm release of swarming males. However, most accounts of female spawning (e.g. Bass and Brafield 1972) and in this study, observations of pools of eggs at the entrances of burrows indicate that the eggs are released at the sediment-water interface. The eggs are therefore released by the female remote from the site of male spawning, higher in the water column, and are thus not exposed to sperm at its maximum concentration. This is in contrast to much of the previous literature, which emphasises the importance of spawning synchrony and the aggregation of spawning individuals. This results in the release of the gametes in close proximity (Pennington 1985, Yund 1990, Levitan *et al.* 1991, Babcock *et al.* 1994).

There appears to be a great deal of local adaptation in female spawning behaviour. At some sites it has been reported that females and males swarm, for example the White Sea (Sveshnikov 1955) and the Firth of Clyde (Clark 1960), whereas at the Thames estuary, Denmark and Canada (Bass and Brafield 1972) male only swarms have been reported. Leaving the burrow and swarming results in exposure to predation by fish, birds and macroinvertebrates that are abundant on intertidal flats during high water. As stated above, females are capable of spawning several times before being spent. However, her chances of leaving the burrow, swarming, partly spawning, returning to the sediment, burrowing and repeating this behaviour over several tides are probably quite low. In a low energy environment, as at Pettycur (this study), and the Thames estuary (Bass and Brafield 1972) it may be expected that the rate of dilution of sperm from swarming males would be lower than at more turbulent locations (e.g. populations on exposed beaches rather than estuarine mud-flats). As seen in the first field experiment, there is little difference in fertilization success between ground and float deployed egg bags. Hence under these conditions, the costs (increased predation risk) of emergence from the burrow may outweigh the benefits (increased fertilization success). That way she may release quantities of eggs over several tides, achieving moderate levels of success each day (although success can be very high as seen in the first field experiment).

Significant differences in fertilization success do exist in the second experiment, however, between those eggs deployed on floats and those deployed at ground level. There is a greater fertilization success when the eggs are held high in the water column than when they are at the (presumed) site of release. As stated above, egg puddles have been noted at this site, and it is the predominant strategy reported in the literature (Bass and Brafield 1972). Since the recorded fertilization success is so low, in comparison to the first field experiment, it can be concluded that the quantity of sperm available is lower and thus there is a lower density of spawning. In this situation, it may therefore be more "profitable" for the female to swarm to maximise her fertilization success. The actual behaviour of the females during this experiment is unknown, but it is extremely unlikely that it differs from the first experiment when egg pools were

observed. However, female swarming may have evolved as the predominant strategy in those populations in the White Sea (Sveshnikov 1955) and the Firth of Clyde (Clark 1960) in response to local conditions (for example exposure) which may limit the availability of sperm at ground level. In the population at Pettycur Bay, the reduced fertilization success at ground level during the second field experiment may be a consequence of not spawning with the major component of the population. However, insufficient data exist on the spawning strategy of *Nereis virens* under different environmental conditions, and few firm conclusions can be drawn.

Eggs released onto the sediment on one tide may still be fertilized on a succeeding tide. Spawned eggs of *Nereis virens* are viable over at least 40 hours (Chapter 7), hence a higher fertilization success may be achieved through the accumulation of fertilizations over several tides, although predation of unfertilized eggs will obviously increase over the period. Developmental success also falls with increasing gamete age (Chapter 7). This strategy is demonstrated by *Arenicola marina* where the female retains her spawned eggs within the burrow and the male broadcast spawns onto the sediment at low water. Daily fertilization success is lower than that for the whole spawning period indicating that final fertilization success is a result of more and more spawned eggs becoming fertilized each day as the season progresses (Chapters 3 and 4). *A. marina* eggs are similarly long lived, remaining viable for 5 days post-spawning (Chapter 7).

One of the criticisms that may be levelled at the experimental design employed in this chapter is that there is no way of knowing if the eggs deployed on floats were fertilized at ground level on the incoming tide, or high in the water column. This could have been controlled for if both the float and ground deployed bags were attached to the same bamboo cane. This would have allowed pairwise comparisons of the fertilization success in each situation. One of the principal reasons for not doing this was the need to avoid entanglement of the monofilament line. However, minor modifications of the design would

prevent such an occurrence, and future experiments should be performed with this in mind.

It is reasonable to suggest that the fertilization strategies of the polychaetes studied here are different to the free spawning invertebrates studied previously. This will be discussed in Chapter 8, which will examine the relationship between factors that influence fertilization success (field spawning behaviour, sperm : egg ratio, gamete longevity, gamete contact time, chemoattraction) to establish a fertilization strategy for each of the organisms studied.

Chapter 6

Fertilization Success in Marine Invertebrates : The Relative Influence of Sperm Concentration, Sperm:Egg Ratio and Sperm - Egg Contact Time

6.1 Introduction

In comparison to field studies, laboratory investigations of fertilization success have a long history. Lillie (1915) quantitatively examined the parameters necessary to ensure fertilization success in the sea urchin *Arbacia*. Since then there have been a number of studies that have investigated this phenomenon. Rothschild and Swann (1951) became the first to propose mathematical models predicting the likelihood of fertilization taking place, and these have been improved upon by Vogel *et al.* (1982). Recent interest in this field follows the proliferation of shellfish aquaculture, where the need to establish optimum fertilization parameters is paramount (e.g. Gruffydd and Beaumont 1970, Clotteau and Dubé 1993, André and Lindegarth 1994).

There has also been an increased interest in the field fertilization success of marine animals, and a number of field studies have been conducted since 1985 (see Chapter 3 for a review). This has led to further studies of the laboratory fertilization success in an attempt to explain some of the observations in the field. The most comprehensive studies include work by Levitan *et al.* (1991) and Benzie and Dixon (1994), although the majority of the work has been restricted to the Echinodermata.

The main aims of this research are to investigate some of the factors that affect fertilization and development success, and to attempt to place them in an ecological context. These include sperm concentration, sperm:egg ratio and sperm – egg contact time.

6.2 Materials and Methods

6.2.1 Collection of gametes

Gravid specimens of *Arenicola marina*, *Nereis virens*, *Asterias rubens* and *Echinus esculentus* were collected and maintained as described in Chapter 2.

In the laboratory, *A. marina*, *E. esculentus* and *A. rubens* were induced to spawn whilst gametes were harvested directly from the coelomic cavity of *N. virens* and tested for fertilizability and developmental competence (see Chapter 2).

Once collected, the gametes were prepared for use in experiments. Stock sperm suspensions appropriate to the particular experiment being conducted were prepared in autoclaved glassware with 0.2µm twice filtered (sterile filtered) seawater (SFSW). All sperm counts were performed with a Neubauer Improved Haemocytometer (Sigma), and four replicate counts (2 counts per replicate - 2 grids located on the haemocytometer) were performed for each suspension in order to achieve a mean value. Eggs were washed and gently re-suspended in 0.2µm SFSW and allowed to settle to the bottom of a beaker. They were then diluted 10-fold, and allowed to re-settle. 10µl of these settled eggs were then collected using a P20 Gilson pipette, and transferred to a microscope slide. The end of the pipette tip had been clipped to prevent damage to the eggs by taking them up through too small an aperture. The number of eggs in 10µl was then determined, and repeated a further 4 times to obtain a mean number of settled eggs per 10µl. This enabled known quantities of eggs to be pipetted and used in each of the experiments. The gametes were then maintained at 8-10°C until required, and all experiments carried out within 4 hours of the spawning event.

6.2.2 Experimental Design

Several preliminary experiments were performed to determine the most effective experimental design to be employed. There exists a trade-off between the number of treatments, number of replicates and number of inter-individual crosses that it is feasible to perform. Ideally, one would perform multiple crosses between single males and females with three replicates for each cross. However, this restricts the number of treatments possible, and as the questions asked become more complex the design becomes unwieldy. The results of the preliminary work indicated that there was no difference between these two designs. It was therefore decided to use gametes pooled from 5 individuals with

three replicates performed at each treatment, thereby enabling more complex experiments to be performed with sufficient replication. This basic design was followed for all experiments where individual gamete fitness was not being examined (e.g. gamete viability over time, performance of sperm incubated in egg water).

6.2.3 Sperm Concentration Experiments

Fertilization and development success was measured for *Arenicola marina*, *Nereis virens*, *Asterias rubens* and *Echinus esculentus* across a range of sperm concentrations. Six concentrations were used, at 10-fold dilutions from 2.5×10^7 sperm.ml^{-1} to 2.5×10^2 sperm.ml^{-1}, and three replicates were performed at each of the concentrations giving a total of 18 fertilizations. The fertilizations were performed in 25ml glass petri dishes containing 20ml SFSW. The sperm and egg suspensions were prepared as described above, using eggs pooled from 5 females. Sperm concentrations were calculated, and equal aliquots from each male were added to three 25ml glass petri dishes to form three 10ml sperm suspensions each at a concentration of 5×10^8 sperm.ml^{-1}. Serial dilutions were then performed of these stock suspensions and pipetted into the experimental dishes in order to achieve the appropriate sperm concentrations. Three replicates were performed for each concentration of sperm used. Immediately following this, 1000 eggs were pipetted into each of the petri dishes, and they were removed to a controlled temperature room set at 8 - 10°C to develop.

Fertilization success was assessed after approximately 18 - 24 hours. Preliminary observations not reported here indicated that this was sufficient time for the majority of embryos to have undergone several divisions and to have reached the early blastula stage of development. Successful fertilization was scored by the examination of 150 eggs from each petri dish for evidence of a raised fertilization membrane and cleavage. In addition, it was noted whether the egg had undergone normal division, or had failed to develop and was degenerating. Abnormal development in this instance was taken to be arrested

development after polar body formation, or eggs in a degenerative state having undergone only a few cleavages. Observations of these eggs over longer periods of time revealed only further degeneration, and no development. The stock of unfertilized eggs from which the test eggs had been drawn were also examined for fertilization which would indicate contamination. For each species used in these experiments, unfertilized eggs retained a normal appearance for at least 24 hours, making it a simple matter to identify those eggs in the fertilization tests which had failed to develop normally.

6.2.4 Sperm:Egg Ratio Experiments

The fertilization success at varying sperm:egg ratios was examined across several different sperm concentrations. For each of the species examined (*Arenicola marina*, *Nereis virens*, *Asterias rubens* and *Echinus esculentus*), fresh sperm and eggs were collected from five males and five females using the methods described previously. Stock sperm suspensions were made up with 0.2µl SFSW for each of the males used, whilst the eggs were diluted and pooled to form a suspension which, when allowed to settle out, enabled a known quantity of eggs to be pipetted. These were stored at 8 - 10°C until required, and gametes were always used within 2 hours of spawning.

The changes in fertilization success with varying sperm:egg ratio were examined across 4 different sperm concentrations, and 4 sperm:egg ratios. The concentrations tested were 10^3 sperm.ml^{-1}, 10^4 sperm.ml^{-1}, 10^5 sperm.ml^{-1} and 10^6 sperm.ml^{-1}, and the ratios 250,000 : 1 (100,000 : 1 at 2.5 x 10^3 sperm.ml^{-1}), 25000 : 1, 2500 : 1 and 250:1. Because of the requirement that the sperm:egg ratios across all the sperm concentrations be equal, and that at least 50 eggs from each beaker be examined, it was necessary to vary the volume of sperm suspension used. For example, at 250,000 sperm : 1 egg, a minimum volume of 5ml of 2.5 x 10^6 sperm.ml^{-1} was required in which to place 50 eggs, yet 500ml of suspension at 2.5 x 10^4 sperm.ml^{-1} is needed in order to obtain the same ratio. Additionally, reasons of practicality dictated that the maximum ratio at 2.5 x 10^3

sperm.ml^{-1} be 100,000 : 1 since a ratio of 250,000 : 1 would require a volume of 5 litres and insufficient suitable glassware was available to fully perform these ratios. The same volumes were used for all ratios within a particular concentration.

Sperm concentrations were calculated and equal aliquots from each male added to the appropriate volume of SFSW in the relevant beaker to make up the correct sperm concentration. Three replicates were used at each ratio within each sperm concentration. Once all the beakers were prepared (approx. 5 minutes), the appropriate number of eggs were added to each beaker. The experiment was then left for 24 hours at 8-10°C, and fertilization and development success scored as for the sperm concentration experiment above.

6.2.5 Sperm - Egg Contact time Experiments

The influence upon fertilization success of the length of time eggs were allowed to remain in contact with a sperm suspension was examined in *Nereis virens*, *Asterias rubens* and *Echinus esculentus*. Unfortunately, as a result of problems encountered during the 1996 spawning season insufficient *Arenicola marina* were available upon which to conduct this experiment.

Fresh sperm and eggs were collected for each of the species as described above. Eggs from five females were collected, diluted and pooled together in a 1l beaker of 0.2µm SFSW, and the number of settled eggs per microlitre determined. Sperm was similarly collected from 5 males, counted, and stored as 5 stock suspensions. Subsamples of the sperm were pooled immediately prior to the experiment by pipetting appropriate aliquots into the fertilization dishes, such that the 5 subsamples together made up the sperm concentration under test.

Fertilization chambers were constructed to fit inside 30ml petri dishes. These allowed excess sperm to be removed as required, and retained all eggs placed inside. They were made from 150ml plastic volumetric beakers, from which the bases had been cut and the rough edges sanded smooth. This gave a

base diameter of approximately 4cm which fitted neatly into 30ml glass petri dishes. 7cm diameter discs of 60μm nitex mesh (sufficient to retain 100% of the eggs (approximate mean diameter 80-90μm) of *Echinus esculentus*, the smallest of the species under examination) were then cut and placed over the base of each of the plastic beakers. They were secured in place with plastic rings cut from identical volumetric beakers and placed over the mesh/base of the beaker, such that the periphery of the mesh disc was secured between the ring and the outside wall of the beaker. The ring was pushed onto the beaker until the mesh became just taught, and care was taken not to stretch it. This enabled the mesh to be washed, removed and replaced as required.

The influence of sperm - egg contact time was examined at 4 different sperm concentrations, 2.5×10^3, 2.5×10^4, 2.5×10^5 and 2.5×10^6 sperm.ml^{-1}, and across 8 contact times, 5 seconds, 30 seconds, 1 minute, 2 minutes, 5 minutes, 10 minutes, 30 minutes and 1 hour. Three replicate fertilizations were conducted for each treatment. The fertilizations were performed in autoclaved 30ml glass petri dishes that contained 20ml 0.2μm SFSW, giving a total of 24 dishes at each sperm concentration (8 time points x 3 replicates at each time). A further 24 petri dishes that did not contain water were located adjacent to the fertilization dishes in preparation for growing the fertilized eggs through several cell cleavages. On the other side, 6 x 6 litre glass tanks were arranged in series to act as washes for removing the sperm. They were filled with 0.34μm filtered seawater, which was replaced after each sperm concentration had been tested.

Aliquots of each of the 5 sperm samples were added to each of the petri dishes to give the correct sperm concentration to be tested. A fertilization chamber as detailed above was then placed into each of the dishes such that the mesh was on the bottom of the petri dish and the sperm suspension rose inside the beaker. Approximately 1000 eggs were then pipetted into the fertilization chambers, and left for the appropriate time period. When the time had expired, the three fertilization chambers for that time point were removed from the petri dishes, with the nitex mesh retaining all the eggs. They were then immediately immersed in the first of the 6 litre tanks, and gently raised and lowered several

times. The beakers had a volume of 150ml, and so the minimal volume of sperm suspension retained in the material of the mesh was immediately diluted at least 100 fold. Repetition of this, and proceeding through each of the other 5 tanks ensured complete removal of all free sperm (periodically tested by examination under a microscope). Care was taken during the washes to prevent damage to the eggs. The fertilization chamber was then inverted over one of the incubation petri dishes and the eggs gently washed into the dish with 25ml 0.2μm SFSW. The lids were placed on the dishes, and the eggs incubated for 18 hours at 8-10°C. Fertilization success was then scored by assessing 150 eggs from each incubation dish for evidence of cleavage.

6.3 Results

6.3.1 Sperm Concentration

Figures 6.1 – 6.4 show the effect of varying the sperm concentration (plotted as $\log_{10}$) upon the resultant fertilization success for each of the species studied here. Each of the graphs shows a similar profile with fertilization success being very low over the lowest sperm concentrations, rising rapidly towards a plateau approaching 100% at 10^6 – 10^7 sperm.ml^{-1}. In each of the species studied, the greatest rise in fertilization success took place between 10^4 and 10^5 sperm.ml^{-1}. Although the graphs are similar, some differences do exist between the species. The fertilization success of *Nereis virens* oocytes (figure 6.2) increased more rapidly over the lower sperm concentrations, particularly in comparison to *Arenicola marina* (figure 6.1). The closest similarities in graph profile exist between *Asterias rubens* (figure 6.3) and *Echinus esculentus* (figure 6.4).

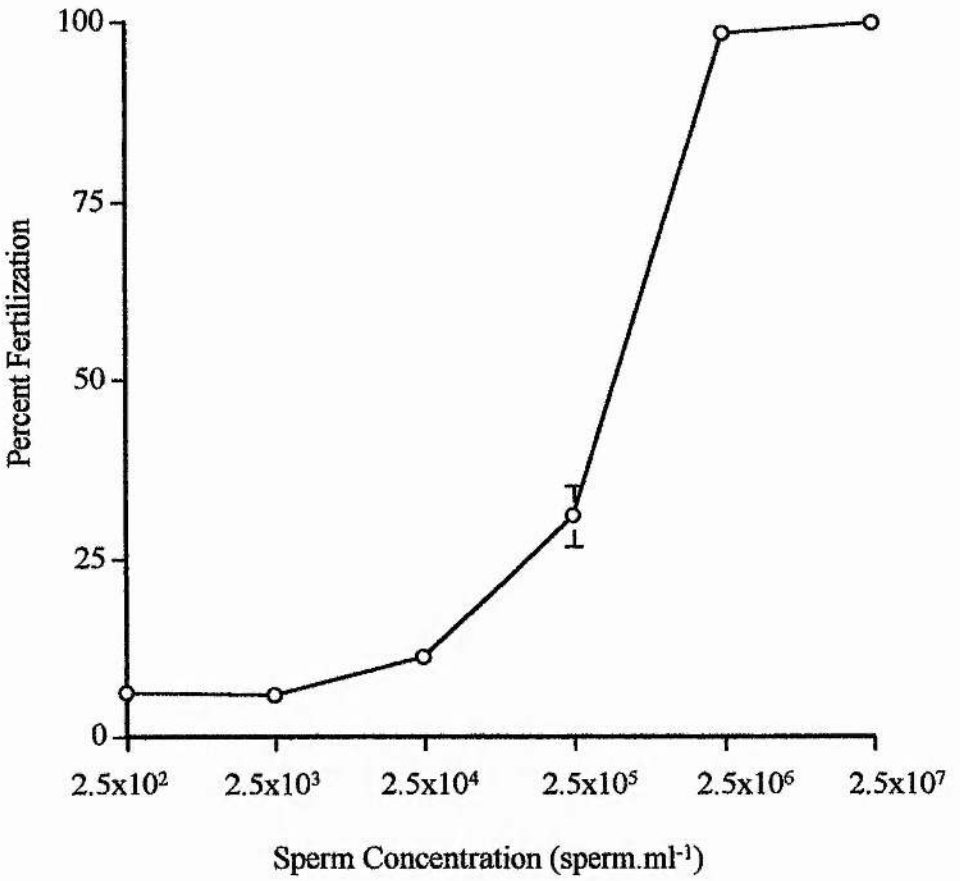


Figure 6.1 Fertilization success of *Arenicola marina* oocytes exposed to a range of sperm concentrations. Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.

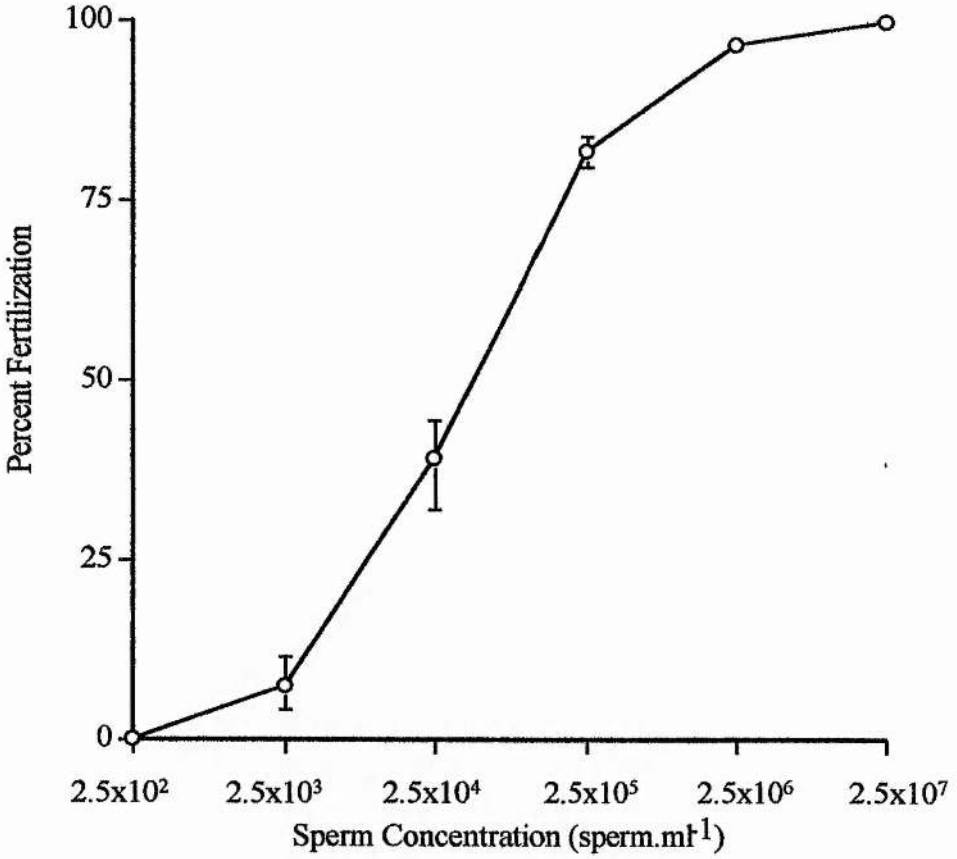


Figure 6.2 Fertilization success of *Nereis virens* oocytes exposed to a range of sperm concentrations. Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.

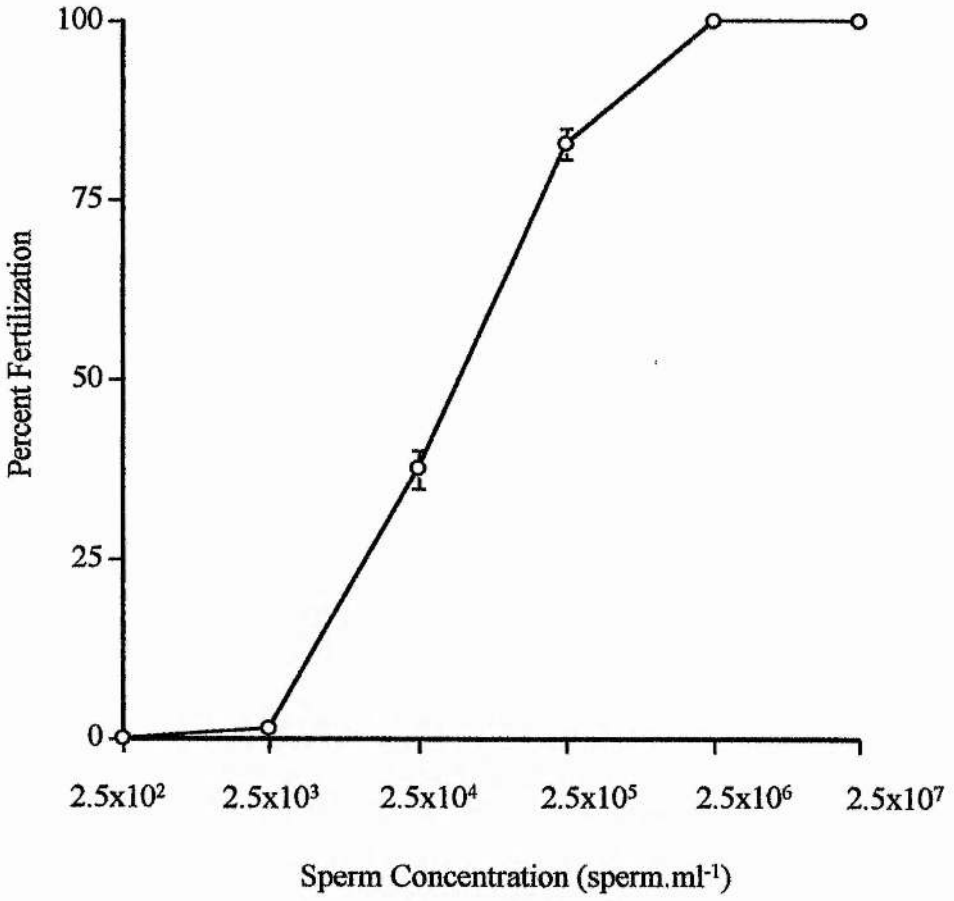


Figure 6.3 Fertilization success of *Asterias rubens* oocytes exposed to a range of sperm concentrations. Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.

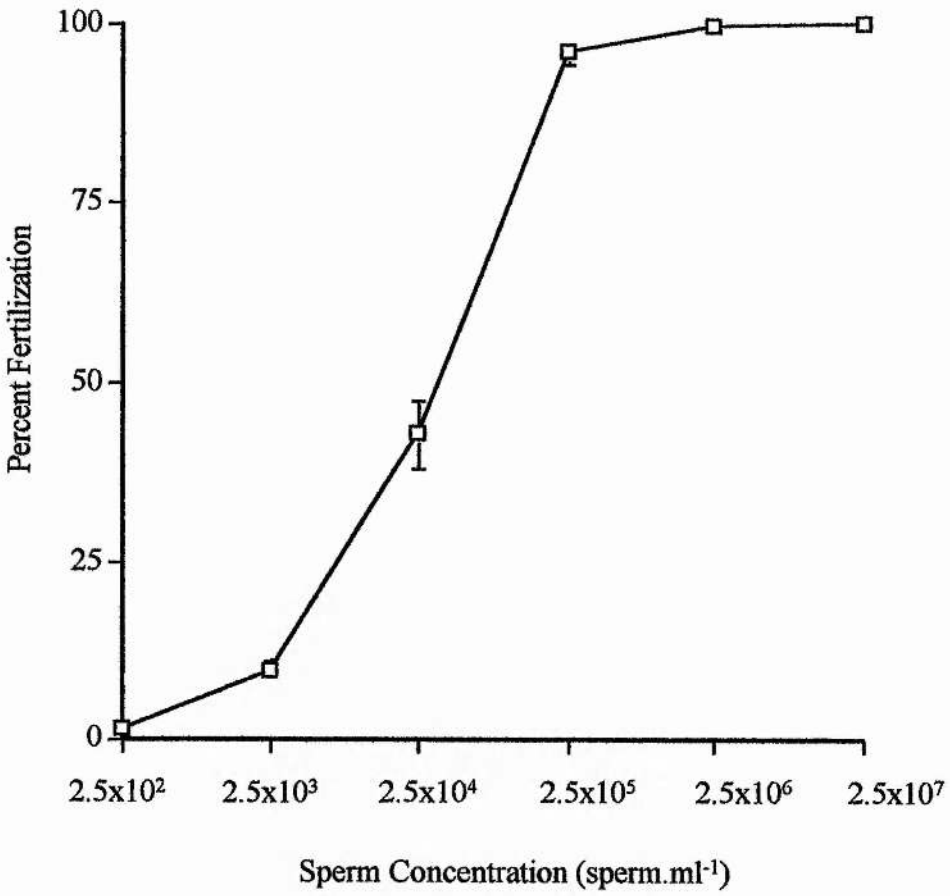


Figure 6.4 Fertilization success of *Echinus esculentus* oocytes exposed to a range of sperm concentrations. Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.

6.3.2 Sperm:egg Ratio

Fertilization success

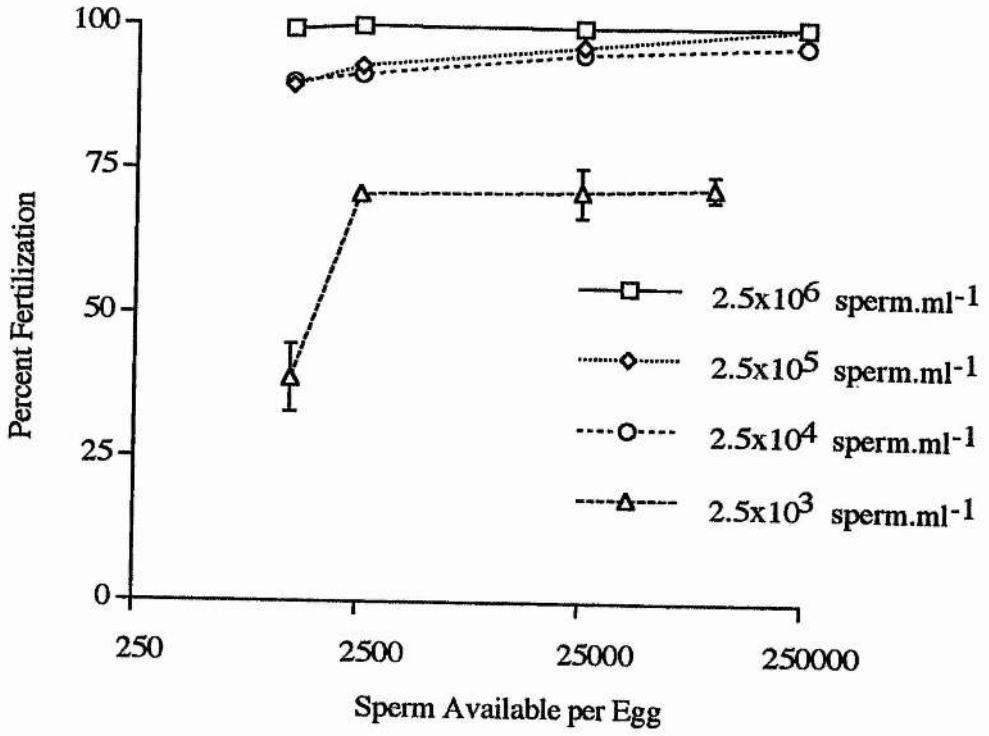
The combined effects of variations in sperm concentration and sperm:egg ratio upon fertilization success can be seen in figures 6.5 (*Arenicola marina*), 6.7 (*Nereis virens*), 6.9 (*Asterias rubens*) and 6.11 (*Echinus esculentus*). Figure 6.5 shows that fertilization success of *A. marina* oocytes at each sperm:egg ratio tested was generally high (>80%) at 10^4 sperm.ml^{-1}, 10^5 sperm.ml^{-1} and 10^6 sperm.ml^{-1}. Fertilization success was much less when sperm concentration was further diluted to 10^3 sperm.ml^{-1}. Increases in sperm:egg ratio, however, resulted in a rise in fertilization success (however small) for each of the sperm concentrations tested. Analysis of covariance (ANCOVA) of these results revealed that for *A. marina*, sperm concentration and the covariate of sperm:egg ratio had a significant effect upon fertilization success ($P<0.001$ for each factor).

Sperm:egg ratio was found to have no significant effect upon fertilization success for the ratios used in *Nereis virens* (ANCOVA, $p=0.237$), however there was a significant effect of variation in sperm concentration (ANCOVA, $p<0.001$). Figure 6.7 shows that fertilization success in *N. virens* was uniformly high across all the sperm:egg ratios, but that higher sperm concentrations resulted in an overall higher fertilization success.

Statistical analysis (ANCOVA) demonstrated that both sperm concentration ($p<0.001$) and sperm:egg ratio ($p<0.001$) significantly affected fertilization success in the echinoderms *Asterias rubens* and *Echinus esculentus*. The results of these analyses are shown in tables 6.1 to 6.4. Fertilization success in the echinoderms was generally lower than the polychaetes at the sperm concentrations and sperm:egg ratios tested. Analysis of the differences in fertilization success between these species (ANCOVA) gave a significant result at the 95% confidence level ($p= 0.017$).

Figure 6.5 The influence of sperm : egg ratio and sperm concentration on the resultant fertilization success of *Arenicola marina* oocytes. Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.

Figure 6.6 The proportion of fertilized eggs of *Arenicola marina*, fertilized at the sperm concentrations and sperm : egg ratios shown in figure 6.5, which fail to develop normally to blastula. Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.


100
75
50
25
0
Percent Fertilization
250
2500
25000
250000
Sperm Available per Egg
2.5x10^6 sperm.ml^-1
2.5x10^5 sperm.ml^-1
2.5x10^4 sperm.ml^-1
2.5x10^3 sperm.ml^-1

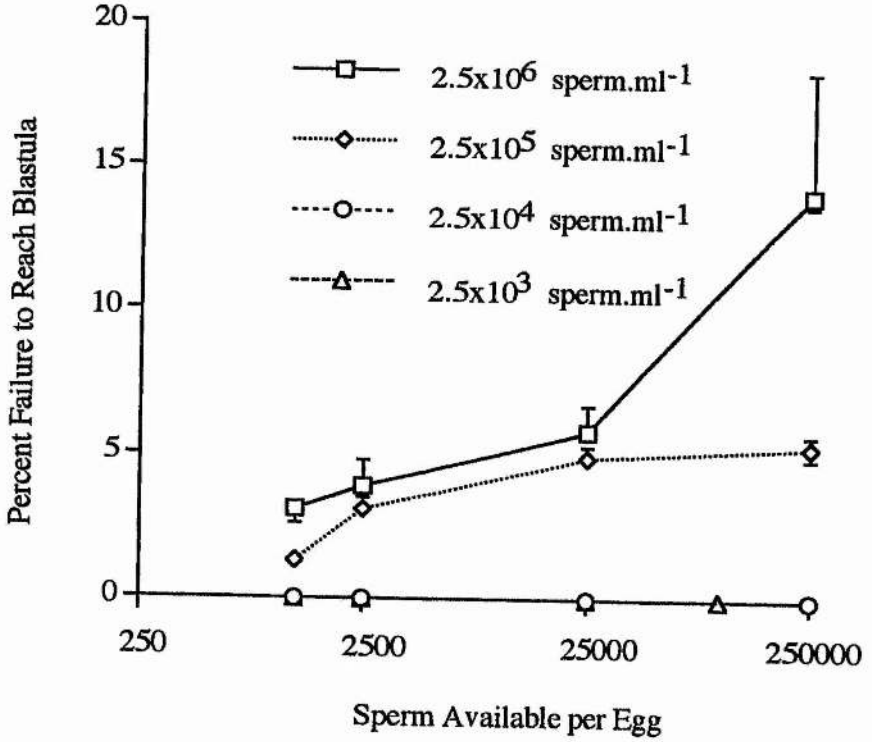
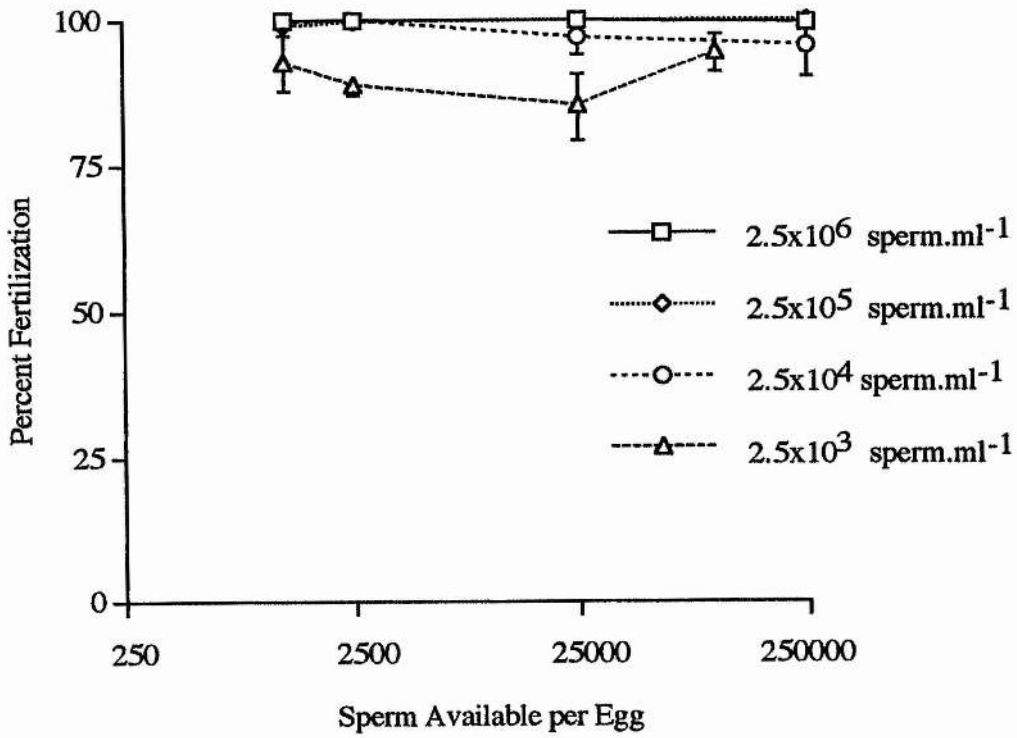

20
15
10
5
0
Percent Failure to Reach Blastula
250
2500
25000
250000
Sperm Available per Egg
2.5x10^6 sperm.ml^-1
2.5x10^5 sperm.ml^-1
2.5x10^4 sperm.ml^-1
2.5x10^3 sperm.ml^-1

Figure 6.7 The influence of sperm : egg ratio and sperm concentration on the resultant fertilization success of *Nereis virens* oocytes. Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.

Figure 6.8 The proportion of fertilized eggs of *Nereis virens*, fertilized at the sperm concentrations and sperm : egg ratios shown in figure 6.5, which fail to develop normally to blastula. Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.


100
75
50
25
0
Percent Fertilization
250
2500
25000
250000
Sperm Available per Egg
2.5x10^6 sperm.ml^-1
2.5x10^5 sperm.ml^-1
2.5x10^4 sperm.ml^-1
2.5x10^3 sperm.ml^-1

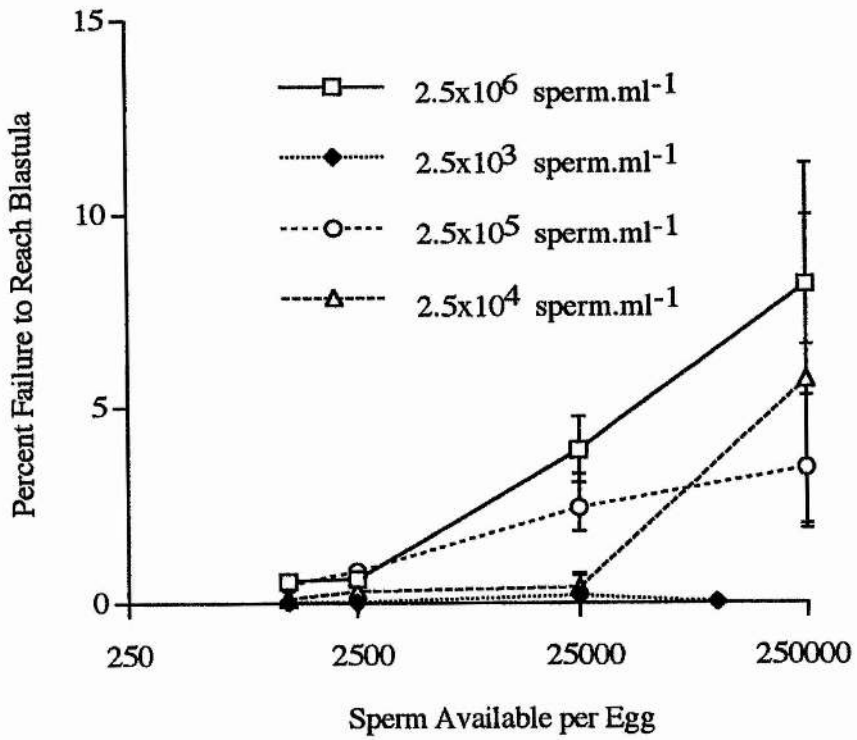
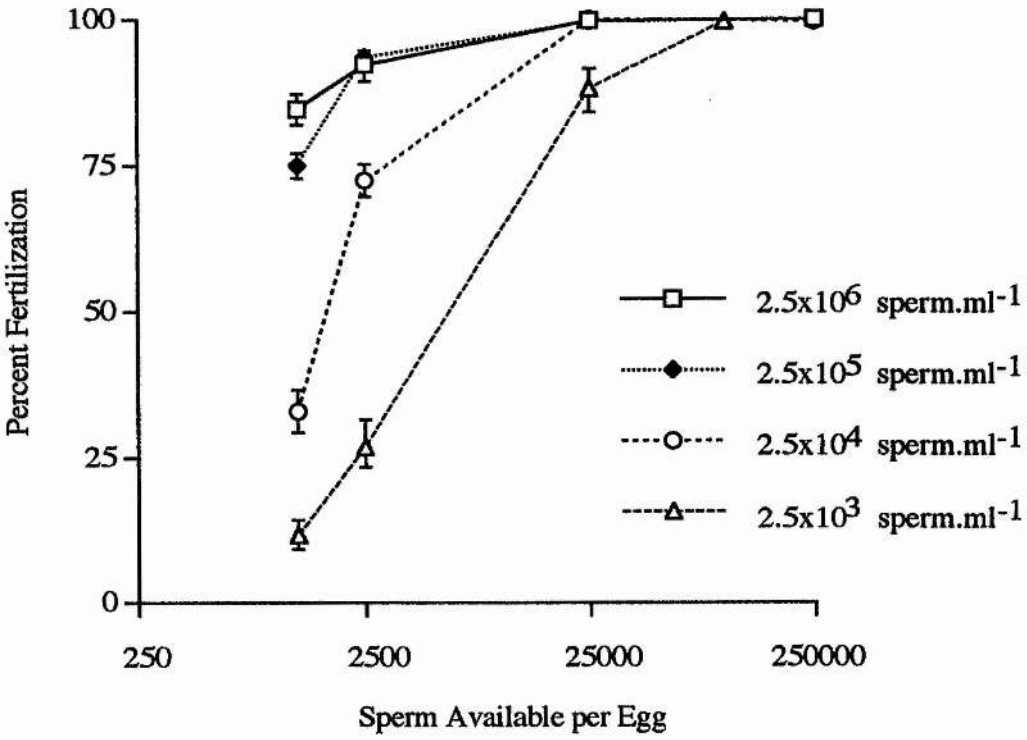

15
10
5
0
Percent Failure to Reach Blastula
250
2500
25000
250000
Sperm Available per Egg
2.5x10^6 sperm.ml^-1
2.5x10^3 sperm.ml^-1
2.5x10^5 sperm.ml^-1
2.5x10^4 sperm.ml^-1

Figure 6.9 The influence of sperm : egg ratio and sperm concentration on the resultant fertilization success of *Asterias rubens* oocytes. Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.

Figure 6.10 The proportion of fertilized eggs of *Asterias rubens*, fertilized at the sperm concentrations and sperm : egg ratios shown in figure 6.5, which fail to develop normally to blastula. Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.


100
75
50
25
0
Percent Fertilization
250
2500
25000
250000
Sperm Available per Egg
2.5x10^6 sperm.ml^-1
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2.5x10^3 sperm.ml^-1

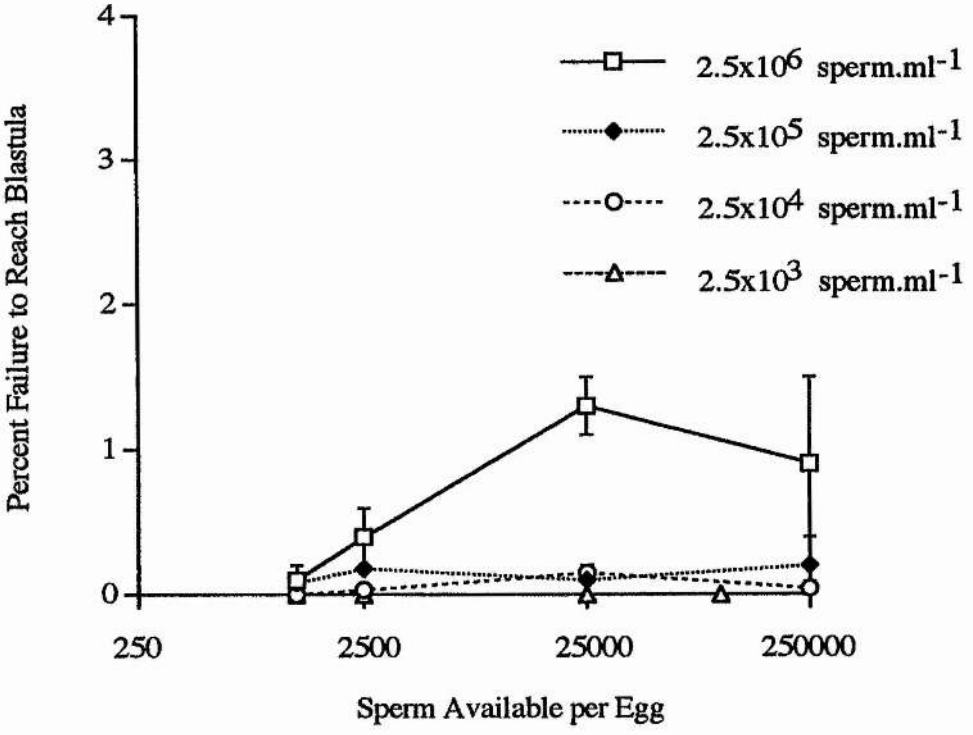
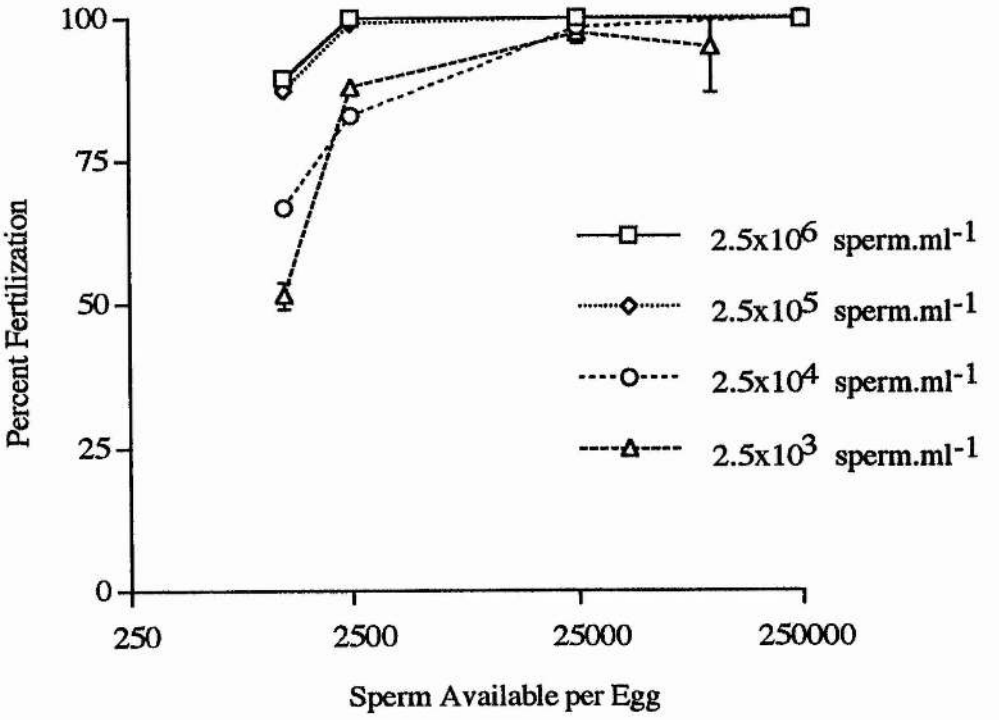

4
3
2
1
0
Percent Failure to Reach Blastula
250
2500
25000
250000
Sperm Available per Egg
2.5x10^6 sperm.ml^-1
2.5x10^5 sperm.ml^-1
2.5x10^4 sperm.ml^-1
2.5x10^3 sperm.ml^-1

Figure 6.11 The influence of sperm : egg ratio and sperm concentration on the resultant fertilization success of *Echinus esculentus* oocytes. Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.

Figure 6.12 The proportion of fertilized eggs of *Echinus esculentus*, fertilized at the sperm concentrations and sperm : egg ratios shown in figure 6.5, which fail to develop normally to blastula. Data shown are mean values from the arcsine transformed percentage fertilization success data +/- standard error of the mean. All data were back transformed for presentation.


100
75
50
25
0
Percent Fertilization
250
2500
25000
250000
Sperm Available per Egg
2.5x10^6 sperm.ml^-1
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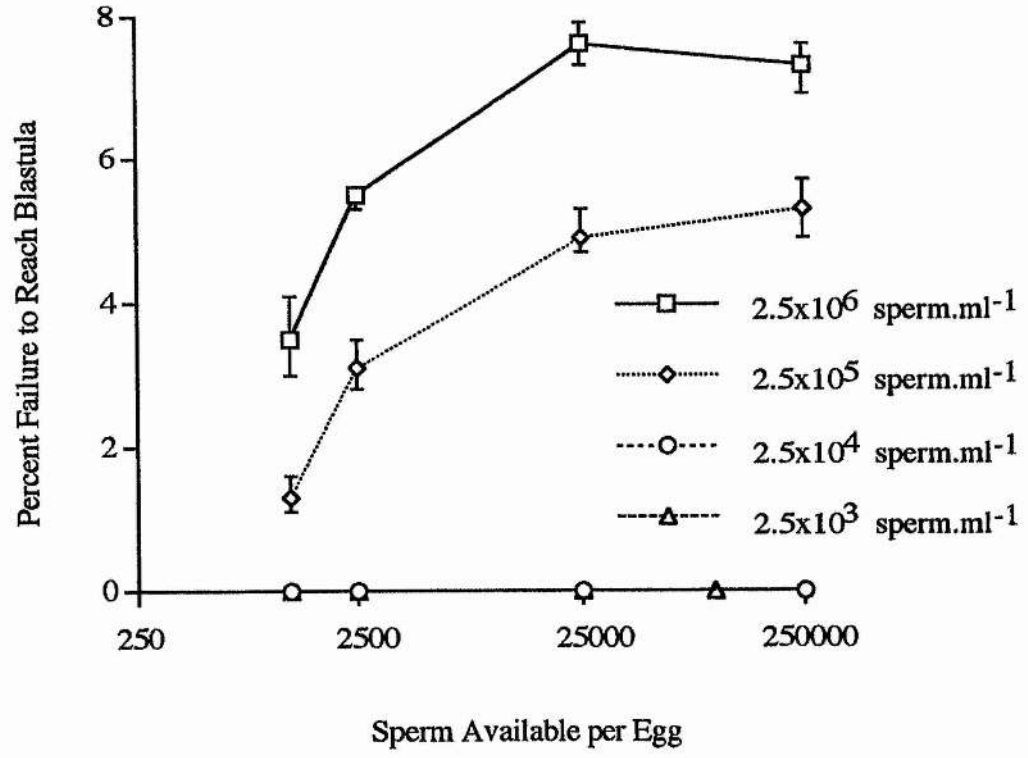
2.5x10^4 sperm.ml^-1
2.5x10^3 sperm.ml^-1


8
6
4
2
0
Percent Failure to Reach Blastula
250
2500
25000
250000
Sperm Available per Egg
2.5x10^6 sperm.ml^-1
2.5x10^5 sperm.ml^-1
2.5x10^4 sperm.ml^-1
2.5x10^3 sperm.ml^-1

Source	DF	Adj SS	MS	F	P
Covariates	1	701.4	701.4	17.50	0.000
Sperm Conc.	3	18161.9	6054.0	151.01	0.000
Error	103	4129.4	40.1		
Total	107	22992.7			

Covariate	Coef	StDev	T	P
S/E ratio	0.005838	0.00140	4.183	0.000

Table 6.1. Results of an ANCOVA test on the results of the sperm-egg ratio experiment for *Arenicola marina*. Covariance was measured at 4 sperm/egg ratios and across 4 sperm concentrations.

Source	DF	Adj SS	MS	F	P
Covariates	1	49.4	49.4	1.41	0.237
Sperm conc	3	3970.7	1323.6	37.87	0.000
Error	103	3600.0	35.0		
Total	107	7620.0			

Covariate	Coef	StDev	T	P
S/E ratio	0.001549	0.00130	1.189	0.237

Table 6.2. Results of an ANCOVA test on the results of the sperm-egg ratio experiment for *Nereis virens*. Covariance was measured at 4 sperm/egg ratios and across 4 sperm concentrations.

Source	DF	Adj SS	MS	F	P
Covariates	1	6411.3	6411.3	73.76	0.000
Sperm conc.	3	7079.0	2359.7	27.15	0.000
Error	103	8953.2	86.9		
Total	107	22443.6			

Covariate	Coef	StDev	T	P
S/E ratio	0.01765	0.00206	8.588	0.000

Table 6.3. Results of an ANCOVA test on the results of the sperm-egg ratio experiment for *Asterias rubens*. Covariance was measured at 4 sperm/egg ratios and across 4 sperm concentrations.

Source	DF	Adj SS	MS	F	P
Covariates	1	6671.1	6671.1	79.94	0.000
Sperm conc.	3	6482.0	2160.7	25.89	0.000
Error	103	8595.1	83.4		
Total	107	21748.2			

Covariate	Coef	StDev	T	P
S/E ratio	0.01800	0.00201	8.941	0.000

Table 6.4. Results of an ANCOVA test on the results of the sperm-egg ratio experiment for *Echinus esculentus*. Covariance was measured at 4 sperm/egg ratios and across 4 sperm concentrations.

Developmental Competence

Whilst higher sperm : egg ratios and sperm concentrations appeared to ensure a high fertilization success they also led to a higher incidence of post fertilization abnormalities. Figures 6.6, 6.8, 6.10 and 6.12 show respectively the percentage of fertilized eggs, which failed to develop in the above experiment for *Arenicola marina*, *Nereis virens*, *Asterias rubens* and *Echinus esculentus*. It is clear that for each of the species, there was a general trend towards lower development success with increasing sperm concentration and sperm:egg ratio. It is interesting to note that the polychaete oocytes (and in particular those of *A. marina*) were more susceptible to poor development with increasing sperm:egg ratios and sperm concentration than the echinoderm species. The maximum incidence of development failure in *A. marina* was 14% at 10^6 sperm.ml^{-1} and 250,000 sperm : 1 egg, as compared to approximately 7% for *N. virens* and *E. esculentus* under the same conditions. Abnormal development was generally very low for *A. rubens* compared to the other species, reaching a maximum of less than 2%.

6.3.3 Sperm – Egg Contact Time

The length of time sperm and eggs are permitted to interact had a significant effect upon fertilization success. Figures 6.13, 6.14 and 6.15 show the fertilization success across 4 sperm concentrations of *Nereis virens*, *Asterias rubens* and *Echinus esculentus* respectively. In each case, fertilization success increased rapidly over the first few time points, and close inspection of the graphs reveals that in many cases the increase was exponential for the first minute after addition of eggs. The rate of increase in success fell rapidly after 10 minutes, and for the echinoderms further increases in fertilization success were small.

Figure 6.13 shows that for *N. virens*, there was an overall higher fertilization success at the lower sperm concentrations than was found for the

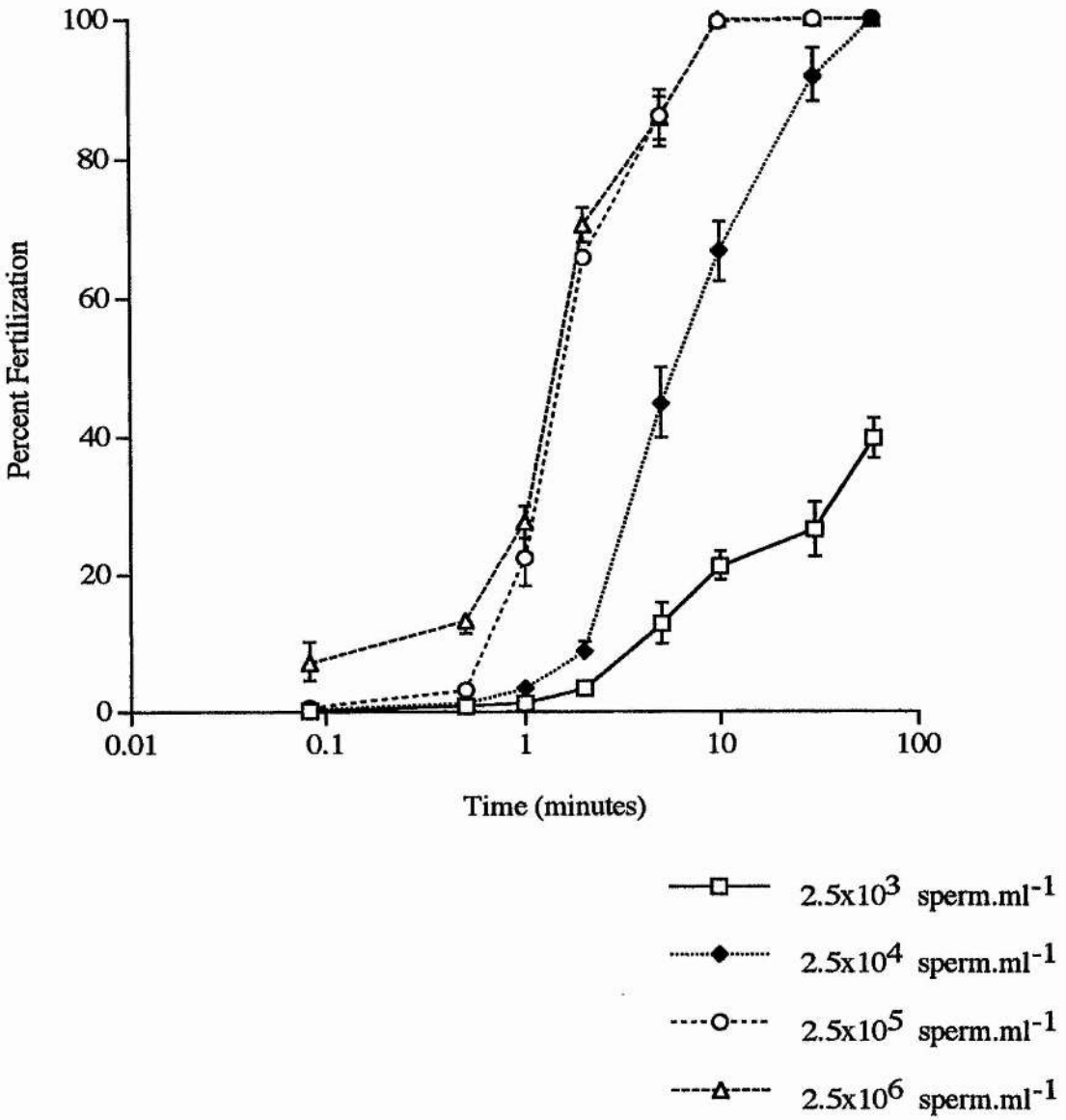


Figure 6.13 Fertilization success of *Nereis virens* oocytes across a range of sperm concentrations and sperm - egg contact times. Data shown are mean values from the arcsine transformed percentage data +/- standard error of the mean. All data were back transformed for presentation.

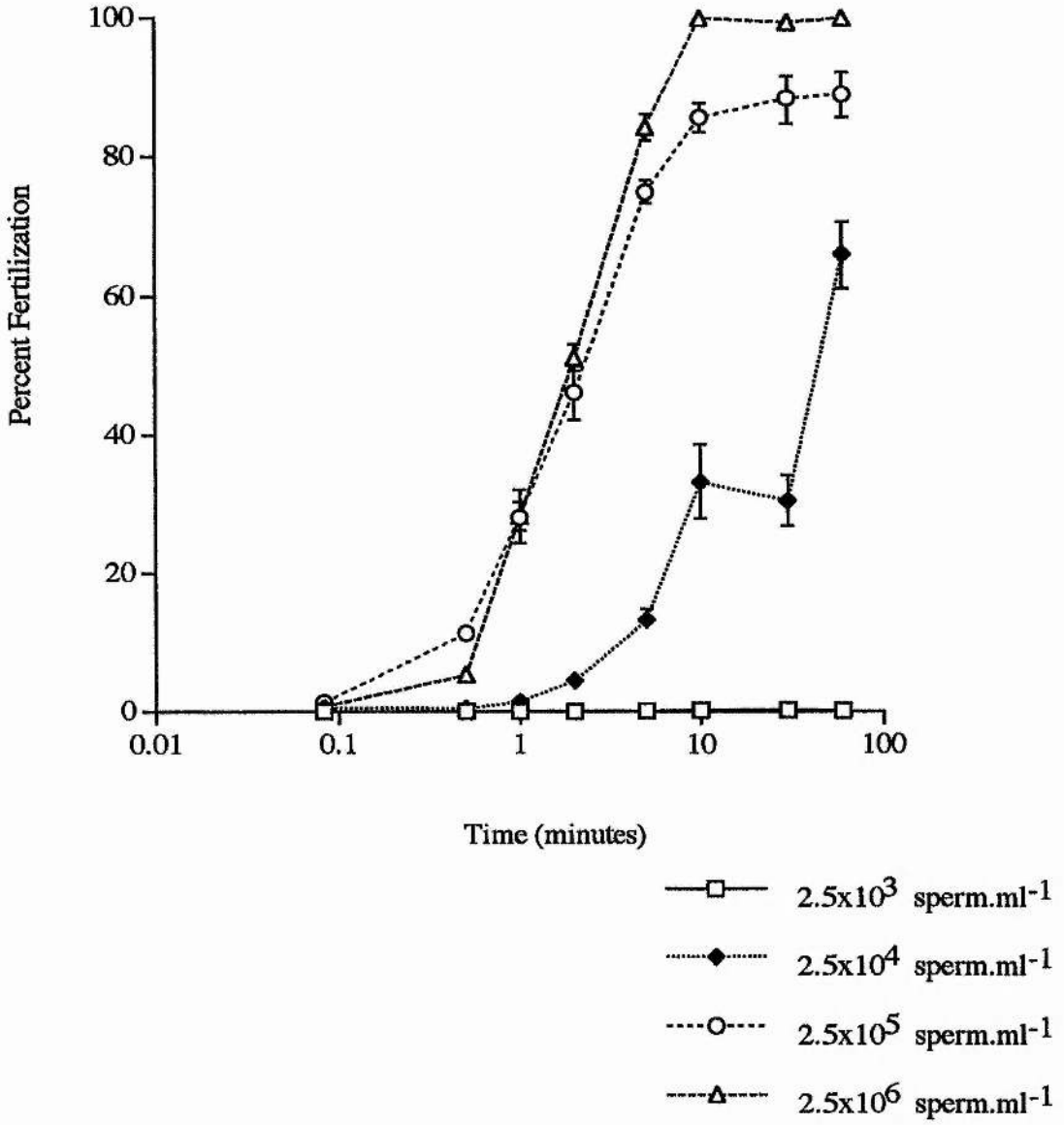


Figure 6.14 Fertilization success of *Asterias rubens* oocytes across a range of sperm concentrations and sperm - egg contact times. Data shown are mean values from the arcsine transformed percentage data +/- standard error of the mean. All data were back transformed for presentation.

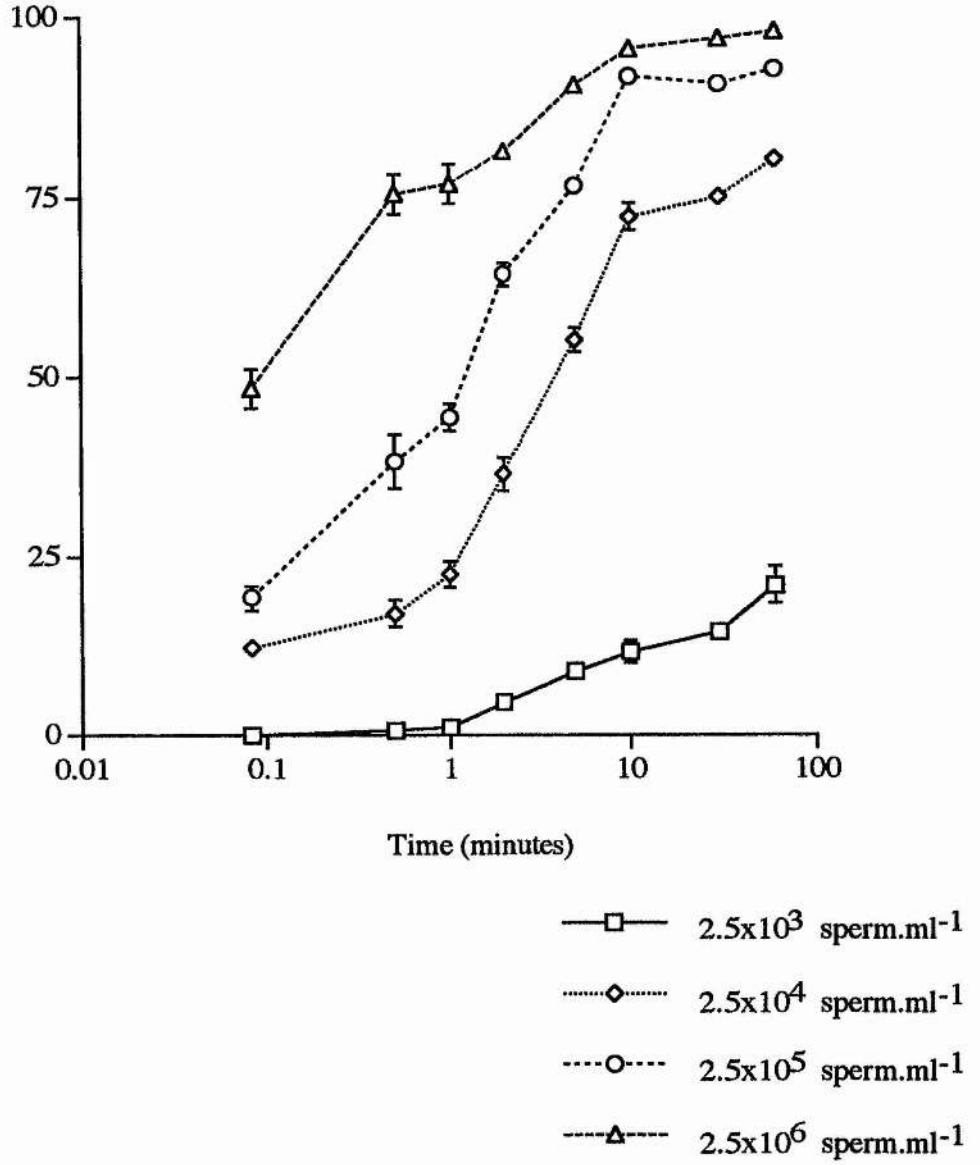


Figure 6.15 Fertilization success of *Echinus esculentus* oocytes across a range of sperm concentrations and sperm - egg contact times. Data shown are mean values from the arcsine transformed percentage data +/- standard error of the mean. All data were back transformed for presentation.

Source	DF	SS	MS	F	P
Time	7	147243	21035	310.71	0.000
Sperm conc	3	50856	16952	250.40	0.000
Error	277	18753	68		
Total	287	216852			

Table 6.5. Analysis of covariance for the variation in fertilization success with sperm concentration and sperm-egg contact time in *Nereis virens*.

Source	DF	Adj SS	MS	F	P
Covariates	1	20809	20809	564.53	0.000
Sperm conc	1	29881	29881	810.68	0.000
Error	141	5197	37		
Total	143	55887			

Covariate	Coef	StDev	T	P
time	13.54	0.570	23.76	0.000

Table 6.6. Analysis of covariance for the variation in fertilization success with sperm concentration and sperm-egg contact time in *Asterias rubens*.

Source	DF	Adj SS	MS	F	P
Covariates	1	49739	49739	1388.95	0.000
spermcon	3	115160	38387	1071.93	0.000
Error	283	10134	36		
Total	287	175034			

Covariate	Coef	StDev	T	P
log(time	14.81	0.397	37.27	0.000

Table 6.7. Analysis of covariance for the variation in fertilization success with sperm concentration and sperm-egg contact time in *Echinus esculentus*.

echinoderms. The profile of the plots for the effect of contact time at 10^6 sperm.ml^{-1} and 10^5 sperm.ml^{-1} differ only slightly over the first few minutes, with the success at 10^5 sperm.ml^{-1} lagging slightly behind that at 10^6 sperm.ml^{-1}. They both reach a plateau of 100% success 10 minutes after the addition of eggs to the fertilization dishes. Following a rapid rise, fertilization success at 10^4 sperm.ml^{-1} reached 100% after 1 hour, whilst portions of those eggs incubated at 10^3 sperm.ml^{-1} continued to fertilize at a slow rate even up to 1 hour after addition to the fertilization chamber.

Fertilization success of *Asterias rubens* oocytes (figure 6.14) was found to be slightly lower overall than for *Echinus esculentus* (figure 6.15). Success at 10^3 sperm.ml^{-1} reached only 2% after 1 hour, and the maximum recorded successes were 62%, 82% and 100% for 10^4 sperm.ml^{-1}, 10^5 sperm.ml^{-1} and 10^6 sperm.ml^{-1} respectively. In comparison, 18% of *E. esculentus* oocytes were fertilized after 1 hour at 10^3 sperm.ml^{-1}, and 75% at 10^4 sperm.ml^{-1}. Statistical analysis (ANCOVA) of these results showed that there was a significant effect upon fertilization success of both sperm concentration and sperm – egg contact time ($p<0.001$ for each factor). The results of the ANCOVA analyses are shown in tables 6.5 to 6.7.

6.4 Discussion

It is apparent from the data presented here that fertilization success in marine invertebrates depends upon several common variables. Sperm limitation is clearly an important constraint upon the fertilization success of free spawning organisms. This has been shown to be the case for several species of echinoderm and corals (Pennington 1985, Babcock and Mundy 1992, Babcock *et al.* 1994, Brazeau and Lasker 1997) and was also demonstrated in Chapter 4 for *Arenicola marina*.

For each of the species examined, sperm concentration was the most influential factor governing the proportion of oocytes that are fertilized. Lillie

(1915), working on the sea urchin *Arbacia punctulata*, demonstrated that the fertilizing "power" of sperm was limited by its concentration, and this was later confirmed by Rothschild and Swann (1951). More recently, this has been further demonstrated for other urchin species (Pennington 1985, Levitan *et al.* 1991), asteroids (Babcock and Mundy 1992) and bivalve molluscs (Clotteau and Dubé 1993, André and Lindegarth 1995). In each of these studies, the profiles of the graphs produced when fertilization success is plotted against $\log_{10}$ of the sperm concentration (or dilution factor), are identical to those presented here in figures 6.1, 6.2, 6.3 and 6.4. Close examination of the figures presented here do not reveal any major differences between the polychaete and echinoderm species studied, with the largest increases in fertilization success taking place across the intermediate sperm concentrations, and they do not appear to deviate greatly from previously published data (see previous references).

Sperm:egg Ratio

Experiments investigating sperm limitation by varying the ratio between the number of sperm and number of eggs reveal more about the conditions necessary for a large proportion of the eggs to be fertilized than simply changing sperm concentration. The experiments conducted here show some differences between the sensitivity (measured as proportion of eggs fertilized) of polychaete and echinoderm oocytes to low sperm availability. A large proportion of the polychaete oocytes, and in particular those of *Nereis virens* (figure 6.7), are fertilized at low sperm:egg ratios. *Arenicola marina* oocytes appear more affected by sperm limitation, and fertilization success is much lower at 10^2 sperm.ml^{-1}, particularly at the lower sperm:egg ratios (figure 6.5). In comparison, fertilization success in the asteroid *Asterias rubens* (figure 6.9) and the echinoid *Echinus esculentus* (figure 6.11) is heavily dependent upon both the sperm concentration and the number of sperm available per egg.

There appear to be some discrepancies between the sperm:egg ratio experiments and the first set of experiments on sperm concentration alone which

involved the serial dilution of sperm, whilst egg concentration remained the same. In the first set of experiments, the proportion of fertilized oocytes fell rapidly towards zero with decreasing sperm concentration. However, very high fertilization successes that far exceeded these values were recorded at similarly low sperm concentrations in the second experiment that also varied the number of eggs present in the fertilization vessel. These discrepancies serve to demonstrate that it is the number of sperm available per egg and not simply the sperm concentration that governs the proportion of eggs that will be fertilized at a given sperm concentration. In the first experiment, there were between 1000 and 1500 eggs per fertilization vessel, and this number was maintained throughout all the concentrations of sperm used. If we then consider that experiment in terms of sperm:egg ratio, it is clear that the ratio changes from greater than 10,000 : 1 down to less than 1 : 1, far exceeding the ranges used in the second experiment. This does not fully explain the discrepancies, but another factor that should be considered is the variation in volume of sperm suspension used. At the lower sperm densities in experiment 2, it was necessary to use ever larger volumes of sperm suspension in order to maintain the sperm:egg ratios required. Pipetting oocytes into 2 litres of sperm suspension at 10^3 sperm.ml^{-1} is quite different to pipetting eggs into the 20ml of the same concentration. As the oocytes disperse and sink in the larger volume, there are a greater number of potential interactions with sperm that may occur before the bottom is reached than in the small volume. Thus, large volumes of sperm suspension at a low concentration have the potential to fertilize a large proportion of eggs, provided that the sperm:egg ratio is sufficiently high.

We can conclude, therefore, that although sperm concentration has a significant effect upon fertilization success, sperm:egg ratio is equally important in determining the level of fertilization success attained by the echinoderms studied here. The effects of sperm:egg ratio were not as evident for the polychaetes, but this may be a result of the ranges used. Fertilization success decreases slightly at the lower sperm:egg ratios, and had the experiment explored still lower ratios, these effects may have become more evident. A better indication that sperm:egg ratio is important to the fertilization success of the

polychaetes can be see by comparing the effects of sperm concentration (figures 6.1 and 6.2) with the combined effects of sperm:egg ratio and sperm concentration (figures 6.5 and 6.7). Where fertilization success falls at the lower sperm concentrations in the first experiment, the comparatively high ratios in the second experiment ensure that large proportions of the eggs are fertilized.

These results which indicate that sperm:egg ratio has a significant effect upon the fertilization success of marine invertebrates deviate slightly from previously published work. Lillie (1915) showed that egg concentration has a much lesser effect on fertilization success in the sea urchin *Arbacia punctulata* than does sperm concentration. Levitan *et al.* (1991) investigated the effect of sperm:egg ratio (defined as egg concentration) and sperm concentration upon the resultant fertilization success for the echinoid *Strongylocentrotus franciscanus*. Their results indicated that sperm:egg ratio had little effect upon fertilization success, except at the lowest sperm concentrations/highest egg concentrations. They speculated that egg concentration would be unlikely to have a significant effect in the field because the rate of dilution of eggs is likely to be so rapid, that only immediately after release from the female would the egg concentration be sufficient to affect the fertilization success. This was further studied by Benzie and Dixon (1994) working on the starfish *Acanthaster planci* who found that sperm:egg ratio had a significant effect upon fertilization. However, they came to the same conclusion as Levitan *et al.* (1991) that the rate at which eggs are extruded in the field compared to sperm would mean that sperm:egg ratio probably would not have an effect. Levitan (1995) reviews much of the recent work.

Mathematical considerations

The experiments conducted here show clearly that sperm:egg ratio affects the fertilization success of echinoderms, but that over the same ratios it is less important for the polychaetes. The implications to the field situation of these results are difficult to ascertain. Several workers have modelled the probability

of sperm – egg collision and the likelihood of fertilization occurring. Rothschild and Swann (1951) produced a mathematical expression to predict the rate of fertilization in the sea urchin *Psammechinus miliaris*. They used the same model principles that apply to the movement of gas molecules, and defined the rate of sperm - egg collisions (Z) as follows (equation 1):

$$Z = v.\sigma_0.S$$

where v is the average sperm velocity, σ_0 the cross sectional area of the oocyte and S the sperm concentration. Latterly, Vogel *et al.* (1982) developed a more sophisticated model for the prediction of fertilization success in the sea urchin *Paracentrotus lividus*. They pointed out that actual sperm - egg interactions differ from the gaseous molecule system used as a basis for the model of Rothschild and Swann (1951). Sperm - egg encounter is not a brief collision, but leads (in most instances) to a permanent attachment. They also recognised that sperm and eggs have a limited lifespan.

Vogel *et al.* (1982) proposed three models for the prediction of fertilization success, based on three different assumptions. The second model, "Don Ottavio", is the one that corresponds most closely to observed data, and assumes that sperm will stick to the first egg they encounter whether or not fertilization takes place. This model predicts the proportion of fertilized eggs (φ) as follows (equation 2):

$$\varphi_\infty = 1 - \exp\left[\beta S_0/\beta_0 E_0 \left(1 - e^{-\beta_0 E_0 \tau}\right)\right]$$

where β is the rate constant of fertilization, and is the product of the mean spermatozoon speed (v) and the fertilization cross sectional area of the egg (σ) ($\beta = v\sigma$). β_0 is the rate constant of sperm - egg encounter and is the product of the mean spermatozoon speed (v) and the total cross sectional area of the egg (σ_0) ($\beta_0 = v\sigma_0$). S_0 is the sperm concentration, E_0 is the egg concentration and τ is the length of time a spermatozoon remains viable. This model appears to be fairly robust in that it explained 91% of the variation in fertilization success of the sea urchin *Strongylocentrotus purpuratus* (Levitan *et al.* 1991).

It is apparent from this model that sperm:egg ratio is a component that should be considered in the fertilization kinetics of marine invertebrates ($\beta S_0/\beta_0 E_0$). As mentioned above, subsequent work on this aspect (e.g. Levitan *et al.* 1991, Benzie and Dixon 1994) found that the ratio of sperm to eggs did have a small effect on the resultant fertilization success (particularly Benzie and Dixon, 1994), however the applicability of these findings to the natural situation is questioned. Several workers (Pennington 1985, Denny and Shibata 1989, Denny et al 1992, Thomas 1994a, b Benzie *et al.* 1994), have examined the dispersion rate of gametes in the field and in the laboratory. Following release, gametes are diluted and dispersed extremely rapidly over quite short distances. Exceptions to this can be found in semi-enclosed environments such as spawning in surge channels (Denny *et al.* 1992), where the reflection of gametes from the sides and bottom of the channel can lead to a locally high overall sperm concentration and high fertilization success. Additionally, Babcock *et al.* (1994) found that given suitable hydrodynamic conditions, the fertilization success of individual starfish spawning many metres apart could be over 90%.

Despite these exceptions, the perception that the ratio of sperm to eggs is important only in the first few moments is probably accurate for free spawning organisms such as sea urchins. The situation is likely to be quite different for the polychaete *Arenicola marina* (see chapters 3 and 4). The female spawns her eggs into the burrow where they are retained (without being brooded *per se*). There is thus a high local concentration of eggs, which is not diminished over many hours (or days). The sperm is released onto the surface of the sediment at low water, where it is diluted by the incoming tide and drawn into the female burrow by irrigation. The dilution of this sperm is rapid, and as reported in Chapter 3, a comprehensive sampling programme during the 1995 spawning season failed to record appreciable quantities in the water column. The pumping rate of the female as she irrigates the burrow is quite low (Baumfalk 1979), so it is clear that sperm concentration is likely to be a limiting factor, and that the effect of sperm:egg ratio upon fertilization success may be important. It is therefore interesting to note that over the ratios used in the experiments

conducted here, fertilization success of *A. marina* is much higher than the echinoderms and just lower than *Nereis virens*.

In order to explain these differences in fertilization success between the species, it is necessary to consider again the model of Vogel *et al.* (1982) (equation 2 above). The cross sectional area of the oocyte (σ_0) and the fertilization cross section (σ) are important to the calculation of the two rate constants β_0 and β, which are central to the model. The oocyte cross section area was also considered important by Rothschild and Swann (1951) (equation 1 above), who found that fertilization success was lower for oocytes from which the external jelly coat had been removed. Levitan (1993) proposed sperm limitation to be a driving force in the evolution of egg size. He compared the gamete attributes of three sea urchin species, and applied the models of Vogel *et al.* (1982) to the results. It was found that the species of urchin with the largest oocytes (*Strongylocentrotus droebachiensis*) had a greater fertilization success at low sperm densities that the related species (*S. purpuratus*, *S. franciscanus*) which had smaller oocytes. Although there were found to be differences in the relative fertilizability of the oocytes of the three species, the main factor was deemed to be egg size. This led to the conclusion that a direct relationship exists between egg size and fertilization success (see Levitan 1995 for review).

The oocyte sizes of the species studied here are not the same. The smallest oocytes are those of *Echinus esculentus* with a diameter of approximately 85µm (personal observations), followed by *Asterias rubens* (90 - 100µm), *Arenicola marina* (180µm) (personal observations, Watson and Bentley 1997) and *Nereis virens* (180 - 200µm). It is perhaps not surprising therefore that the polychaetes, which have oocytes diameters almost twice those of the echinoderms, should achieve a much greater fertilization success at low sperm densities. Levitan (1993) emphasises the link between sperm limitation and egg size by pointing out that at high sperm densities, it is advantageous to have greater numbers of smaller eggs since a high yield of zygotes will result from a hypothetical unit of egg material. In sperm limited conditions however (when

fertilization success is less than 100%), species which produce fewer, larger eggs yield more zygotes per unit of egg material (see figure 6 of Levitan 1993).

Other factors within the model of Vogel *et al.* (1982) which may account for the differences between species include gamete longevity and sperm swimming speed. The longevity of unfertilized gametes is considered separately in the next Chapter. Sperm swimming speed was not measured in this study for any of the species examined, and this prevents the estimation of β and β_0 for use in the model (see above). It will have a direct effect on the two rate constants and thus upon the proportion of eggs fertilized per unit time. Both Gray (1955) and Levitan *et al.* (1991) measured the sperm swimming speed of several sea urchin species. They found that the sperm swimming rate varied greatly between species and also between individuals of the same species. It would thus be necessary to directly measure sperm swimming speed for the species studied here in order to fully apply the models to this system.

Another factor which may influence the probability of sperm – egg interaction is the presence (or absence) of sperm attractants from the egg (see reviews by Cosson 1990, Yoshino *et al.* 1990). Indirect evidence to date suggests that such attractants are produced by oocytes of *Arenicola marina*, *Asterias rubens* and *Echinus esculentus* (see Chapter 7). It was not examined in *Nereis virens* because of the lack of sufficient animals during the 1997 spawning season. Sperm attractants may act in two main ways. First, assuming that the diffusion of such molecules from the oocyte occurs at a constant rate, these would have the effect of enlarging the "target" area of the sperm (increasing σ_0). Chemotaxis of sperm acts only over short distances of a few hundred microns, so such molecules would therefore guide nearby sperm to the egg through a short concentration gradient (e.g. Ward *et al.* 1985). Second, sperm attractant molecules may have a physiological effect on the sperm. Some of these effects are discussed in the next chapter, but in summary they act by directing the swimming sperm towards the source of the chemical (i.e. the oocyte), and also increase the metabolic rate of the sperm.

Developmental Competence

As can be seen in figures 6.6, 6.8, 6.10 and 6.12, fertilization success at high sperm concentrations and sperm:egg ratios is countered by an increased incidence of abnormal development. This is unlikely to be a result of physical factors such as oxygen deprivation, as in the situation where there are 10^5 sperm.ml^{-1} developmental competence increases with an increase in egg concentration. One of the main factors that can lead to poor development following fertilization is polyspermy, where more than one sperm pronucleus enters the oocyte. Byrd and Collins (1975) studied the incidence of polyspermy in the sea urchin *Strongylocentrotus purpuratus* in relation to sperm:egg ratio. They found that fertilizations were generally monospermic up to about 1000 sperm : 1 egg. Similarly, Clotteau and Dubé (1993) examined the fertilization parameters of the surf clam *Spisula solidissima*. They found a greater incidence of polyspermy at high sperm concentrations and sperm:egg ratios, and state that in order to obtain 95% monospermic fertilization, sperm:egg ratios need to be maintained in the range of 50 : 1 to 100 : 1.

The incidence of polyspermy in the experiments of Clotteau and Dubé (1993) exceeds the observations of abnormal development in the work conducted here. This is also true of the oyster *Crassostrea virginica* (Alliegro and Wright 1983), where multiple sperm entry occurs in greater than 50% of oocytes at sperm:egg ratios above 10,000 : 1. One reason for this may be the mechanisms present within the oocyte for preventing polyspermy. In echinoderms, the block to polyspermy is well described, and entails a fast, temporary depolarisation of the outer membrane which prevents further sperm fusion, followed by a rapid exocytosis of cortical granules (for a review of early work see Longo 1987). Transient depolarisation of the oocyte membrane has been described for the mussel *Mytilus edulis* (Togo *et al.* 1995), however the permanent block that occurs in sea urchins has yet to be described for the molluscs. No literature is yet available for the Polychaeta. Given that the block to polyspermy is so well developed in the sea urchin oocyte, it is interesting to note that the incidence of

abnormal development is much lower for *Echinus esculentus* (figure 6.12) than for any of the other species studied here.

Sperm - Egg Contact Time

The length of time which eggs and sperm are allowed to interact has a significant effect upon fertilization success. Equation 2 (above) predicts the proportion of eggs that would be fertilized based on a measure of τ, the lifespan of the sperm. However, Vogel *et al.* (1982) recognised that a finite length of time may exist, during which the sperm and egg suspensions are in contact. They therefore substituted τ for t, the length of time sperm and eggs are in contact, to predict φ_t, the proportion of eggs fertilized during a specific length of time. From the above equation, it is therefore apparent that sperm – egg contact time will have a marked effect on the fertilization success, as indeed it is demonstrated here (figures 6.13, 6.14, 6.15).

The most significant changes in fertilization success occur in the first few minutes after addition of the eggs. Levitan *et al.* (1991) found similar results for the sea urchin *Strongylocentrotus purpuratus*, although in a less comprehensive study. These results indicate that in order to achieve a high fertilization success, a body of eggs would need to be in contact with a sperm suspension for several minutes, and the length of time required increases as sperm concentration decreases. The important factor to note here is that fertilization success reaches a plateau for each of the species before the length of time that the sperm is active is exceeded. This will be discussed in greater detail in Chapter 7, where the longevity of unfertilized gametes in also studied. Additionally, in keeping with the data from the sperm:egg ratio experiments, and the equations above, we see that the oocytes of *Nereis virens* are fertilized at a higher rate than those of the echinoderms.

Wider Considerations

As stated above, in the natural situation it is likely that the dispersion rate of eggs and sperm from free spawning echinoderms means that sperm:egg ratio will affect fertilization success only during the first few moments of spawning. However, there are instances such as spawning in tidepools (Epel 1991) where the spawning event will occur in an enclosed environment analogous to a beaker. Epel (1991) postulates that this will confer a selective pressure towards a strong block to polyspermy. Similarly, Levitan (1993, 1996a,b) uses data gathered from field and laboratory experiments (see Levitan 1995) to demonstrate the selective pressures inherent in a free spawning fertilization strategy. He proposes that sperm limitation leads to the "preferential" fertilization of larger oocytes, and that sperm limitation can shift the optimum size of an oocyte dramatically. In the species studied here it is apparent that the polychaetes with their larger oocytes are more resistant to lower sperm densities, although given the huge size differences and that they are derived from different phyla, direct comparisons are problematic.

The conditions under which these species spawn in the field are, however, likely to lead to similar selective pressures as discussed by Levitan (1996a,b). *Arenicola marina* females in the field will often achieve fertilization success approaching 100%, but this represents the fertilizations accumulated over several days of exposure to sperm, the daily success being well below this value (Chapter 3). This sperm limitation is better demonstrated in Chapter 4. Fertilization success of *Nereis virens* can be high under certain circumstances (Chapter 5), and as discussed earlier there is some controversy surrounding the fertilization strategy of this species. There appears to be a degree of behavioural adaptation of spawning strategy to suit the local conditions, however one can still conclude that sperm limitation is likely to occur in this species since fertilization success never reached 100%. The field fertilization successes of *Asterias rubens* and *Echinus esculentus* were not examined here, however the conditions under which spawning takes place are similar to the echinoderms studied to date (e.g.

Pennington 1985, Levitan *et al.* 1991, Babcock *et al.* 1994). Therefore, sperm limitation is again likely to be important.

Behavioural adaptations to enhance reproductive success may also occur. The swarming behaviour of *Nereis virens* is a classic example of spawning synchrony, which maximises the concentration of gametes in an area. Such spawning synchrony is also clearly present in the populations of *Arenicola marina* studied in this thesis (Chapter 3). Less information is available about the spawning strategies of *Asterias rubens* and *Echinus esculentus*. Work by Moore (1934), and particularly Comely and Ansell (1988, 1989) suggest a late spring/early summer breeding season of *E. esculentus*. However, there is no evidence to suggest that there is an aggregation of individuals prior to spawning as is observed in a number of starfish (e.g. Run *et al.* 1988) and other echinoderms (Levitan 1988, Young *et al.* 1992, Tyler *et al.* 1992). Such behaviour has been shown to lead to an increased probability of sperm – egg encounter and fertilization success (Levitan 1991b, Levitan *et al.* 1992).

One important factor to be considered is that fertilization in the laboratory is very different from fertilization in the field. Epel (1991) demonstrates a dramatic drop in fertilization success with the introduction of turbulence to a fertilizing beaker. Mead and Denny (1995) studied the effect of turbulence and shear stress upon fertilization success and found similar results (see Mead and Epel 1995 for a brief review). Similarly, the models of Vogel *et al.* (1982) do not take turbulent flow into account. Denny and Shibata (1989) demonstrate that the introduction of hydrodynamic principles into the model of Vogel *et al.* (1982) greatly reduces the probability of fertilization success.

Chapter 7

Fertilization Success in Marine Invertebrates: The Influence of Gamete Age

7.1 Introduction

The length of time that sperm and eggs are viable will affect the reproductive success of a species. The prevailing view of workers such as Levitan *et al.* (1991) is that the dilution of gametes, particularly sperm, below fertilizable concentrations occurs before the viable life of the gametes has expired. Consequently, gamete longevity is not thought to be important to the overall fertilization ecology of a free spawning species. This may, however, only be true for the shallow subtidal free spawning echinoderms upon which the bulk of the fertilization ecology studies have focused. A different situation exists, for example, in the brooding starfish *Leptasterias polaris* in which the sperm are deposited on the bottom of the substratum without being dispersed and remain quiescent until activated by female spawning (Hamel and Mercier 1995). Sperm longevity also appears to be important in some internally fertilizing ascidians. In these species, females filter large volumes of seawater during normal behaviour, and this can result in the collection of sufficient sperm to facilitate fertilization many hours after male spawning, when the sperm has become extremely dilute (Bishop 1998).

The aim of the studies reported in this chapter is to investigate the longevity of eggs and sperm of the polychaetes *Arenicola marina* and *Nereis virens* and the asteroid *Asterias rubens*. The findings will be discussed in an ecological context. The mediation of sperm activity by egg compounds will also be examined to a small extent for *Arenicola marina* and *Asterias rubens*.

7.2 Materials and Methods

Mature specimens of *Arenicola marina*, *Nereis virens* and *Asterias rubens* were collected and maintained as described in Chapter 2. Preliminary experiments on the competence of eggs and sperm of *Arenicola marina* were performed in order to determine the timescale over which the experiments

should be conducted and also the efficacy of the design for use in subsequent experiments. Two basic designs were tested, one utilising gametes from individual males and females and one using pooled sperm and eggs, in much the same way that the designs were tested in the previous chapter (Chapter 6). In the case where individual male and female worms were used, two male and two female worms were induced to spawn, and each male was crossed with each of the females at each of the time points, with three replicates performed for each cross. The second design employed, and ultimately the design that was applied for all of the species tested used pooled gametes to fertilize the eggs or sperm under test.

7.2.1 Oocyte Age

Mature oocytes were harvested from gravid females as described in Chapter 2 by induction of spawning (*Arenicola marina*, *Asterias rubens*) or by extracting from the coelomic cavity with a hypodermic needle and syringe (*Nereis virens*). Actual numbers of females used varied according to availability, and 5 lugworm and starfish females were used whilst only 3 ragworm females were sufficiently ripe (i.e. gave the requisite level of fertilization in pre-experiment tests - see Chapter 2). Once collected, eggs from each female were allowed to settle and re-suspended twice in 0.2μm twice filtered seawater (SFSW). They concentration of each sample of settled oocytes was then determined as described in Chapter 6, and approximately 10^6 eggs from each female were transferred to separate autoclaved beakers containing 1l SFSW. Sampling 1ml of the mixed egg suspensions yielded between 500 and 1000 eggs. The beakers were then stored at 8 - 10°C for the duration of the experiment, and the water carefully changed every 4 – 8 hours with fresh SFSW to maintain oxygen tensions.

Fertilization Assay

In order to discern small drops in gamete fitness, it was necessary to fertilize the eggs with the minimum quantity of sperm required to achieve 100% fertilization. The previous chapter indicated that sperm concentrations above 10^4 sperm.ml^{-1} were required in the static petri dish, however the incidence of abnormal development and polyspermy also increased above this level. These values were obtained by pipetting 1000 - 1500 eggs into a pre-prepared sperm suspension. It was found that by pipetting a lower concentration of sperm directly over a small number of eggs settled on the bottom of a vessel, almost 100% fertilization and development success could regularly be achieved. The fertilization assay was therefore conducted by pipetting 1ml of $2x10^4$ sperm.ml^{-1} directly over the eggs in the fertilization chamber. This gave almost 100% fertilization, and the incidence of abnormal development (possibly attributable to polyspermy) were rare. Any fall in fertilization success (and hence gamete fitness) was therefore immediately apparent.

Gamete Handling

At each of the time points, a fresh sperm suspension was prepared from at least 3 males (5 for both *Arenicola marina* and *Asterias rubens*). In order to prevent the excessive wastage of males, small (100 - 150μm) aliquots of coelomic sperm were drawn from male *Arenicola marina* and incubated for 1 hour in an equivalent volume of 1 x 10^{-4} M 8, 11, 14-eicosatrienoic acid (see Chapter 2 for full details). Excess fluid was pipetted off and the sperm was diluted with SFSW. The sperm from each of the 5 males was examined under the microscope to ensure morulae breakdown had occurred to give free swimming sperm, and sperm concentrations were determined using a haemocytometer. Sperm from each of the males was then pooled to give a final concentration of 10ml of 5 x 10^5 sperm.ml^{-1}. Care had to be taken with the repeated sampling of coelomic sperm from *A. marina* and each male was used

only 3 times as further sampling led to large amounts of blood appearing in the coelomic cavity.

Similarly, for male *Asterias rubens* small pieces of testis were excised from one arm of each of 5 males, and sperm was extracted as described in Chapter 2. Each of the 5 pairs of testes were sampled only once. Following collection, the sperm were treated as for *Arenicola marina.* Sperm were collected from *Nereis virens* males directly from the coelomic cavity as described in Chapter 2, and stock solutions of 10ml of 5×10^5 sperm.ml^{-1} were pooled from at least 3 males. The fertilizing capacity of the ragworm sperm was tested as described in Chapter 2, and only those males that met the previously stated criteria were selected. Use of these techniques permitted each male to be used several times through the course of the experiment.

Experimental Design

Fertilizations were carried out at the following time points post-spawning shown in table 7.1.

Arenicola marina	*Nereis virens*	*Asterias rubens*
0hr	0hr	0hr
6hr	4hr	2hr
12hr	10hr	3hr
24hr	16hr	5hr
36hr	24hr	7hr
48hr	36hr	10hr
60hr	48hr	14hr
72hr	60hr	20hr
84hr	72hr	26hr
96hr	96hr	---
108hr	120hr	---
120hr	---	---
132hr	---	---

Table 7.1 Time points at which the longevity of unfertilized oocytes were examined

Fertilizations were conducted in new (sterile) 25ml inert polypropylene petri dishes (Sterilin). Three replicate fertilizations were conducted for each of the 5 females at all of the time points used. At each time point, 500 - 1000

oocytes were pipetted in 1ml seawater from the storage beakers into the appropriate petri dish which contained 18ml SFSW. 1ml of the sperm suspension pooled from each of the males was then added to the petri dish as described above, such that the final volume of the dish was 20ml and the final sperm concentration approximately 2.5 x 10^4 sperm.ml^{-1}. The petri dishes were then left at 8 - 10°C for 24 hours. Fertilization success was assessed by examining 150 eggs from each of the petri dishes. Oocytes were recorded as fertilized with normal development, fertilized with abnormal development, or unfertilized. Abnormal development was defined as embryos failing to develop, or degenerating. Allowing such eggs to continue to develop reveals only further degeneration.

7.2.2 Sperm Longevity at 10^5 sperm.ml^{-1} and 10^9sperm.ml^{-1}

Gamete Handling

Fresh sperm were collected "dry" from each of 5 male *Arenicola marina*, 5 *Asterias rubens* and 3 *Nereis virens*. Spawning was induced in *A. marina* by injecting with prostomial homogenate, and in *A. rubens* by injection with 1-methyladenine (1-MeAde). Gametes were harvested directly from the coelomic cavity of *N. virens* (Chapter 2). The concentrations of each of the sperm samples were then determined using a Neubauer Haemocytometer, and stock suspensions of 20ml 5 x 10^9 sperm.ml^{-1} prepared in autoclaved glass petri dishes. Aliquots of each of the sperm suspensions were taken and serially diluted down to 200ml of sperm suspension at a concentration of 5 x 10^5 sperm.ml^{-1}. These were then stored at 8 - 10°C for the duration of the experiment.

Oocytes were collected from at least 3 females of each of the species as described above, with the exception of *Asterias rubens*. Large intact pieces of *A. rubens* ovary from a single arm were dissected out, washed and incubated for 30 minutes in 20ml of 1 x 10^{-6} M 1-methyladenine made up in SFSW (see Chapter 2). This helped prevent wastage of starfish as each female could be used several times. Once collected, the oocytes were washed by re-suspension in SFSW, and

the density determined. Fresh oocytes were collected from *Arenicola marina* every 24 hours, and from *A. rubens* and *Nereis virens* at each fertilization point.

Experimental Design

Fertilizations were carried out at each of the following time points:

Arenicola marina	*Nereis virens*	*Asterias rubens*
0hr	0hr	0hr
8hr	4hr	1hr
20hr	8hr	2hr
28hr	12hr	3hr
40hr	16hr	5hr
52hr	24hr	7hr
64hr	32hr	10hr
86hr	40hr	14hr
---	---	20hr
---	---	26hr

Table 7.2 Time points over which sperm longevity was investigated

Fertilizations were conducted in new 25ml plastic petri dishes (Sterilin). At each time point, 18ml SFSW was pipetted into the fertilization dishes, and 1ml of approximately 500 – 1000 fresh oocytes added to each dish. 1ml of the 5 x 10^5 sperm.ml^{-1} suspension was pipetted into the appropriate petri dish to give a final concentration of 2.5 x 10^4 sperm.ml^{-1}. 100µl samples of the 5 x 10^9

sperm.ml^{-1} suspensions were taken and serially diluted to a concentration of 5 x 10^5 sperm.ml^{-1}, 1ml of which was pipetted into the appropriate petri dishes. Three replicate fertilizations were performed for each male and treatment used. The fertilization dishes were then left at 8 - 10°C for at least 18 hours. Fertilization success was scored by sampling 150 eggs from each petri dish for evidence of cleavage. Poor development was not recorded.

7.2.3 The Effect of Egg Derived Compounds upon Sperm Longevity

The effect of egg compounds upon sperm longevity was investigated in *Arenicola marina* and *Asterias rubens*. Eggs were collected 12 hours prior to the commencement of the experiment, from each of 5 females by the injection of appropriate spawning hormones, as described above. The oocytes were gently washed twice in SFSW, pooled and the density determined. Approximately 3 x 10^6 eggs were then carefully pipetted into an autoclaved beaker containing 300ml SFSW. The suspension was then incubated for 12 hours at 8 - 10°C. At the end of the incubation period, the upper 200ml of water was slowly decanted from the eggs to avoid re-suspending them, and passed through a 60μm Nitex mesh. No eggs were observed on the mesh. The water was then passed through a 0.2μm syringe filter (Millipore) to remove any particulate matter.

Fresh oocytes were collected, again from 5 females as described above, pooled and the concentrations calculated. Eggs from *Asterias rubens* were used for only 3-4 hours before being replaced by fresh samples; however oocytes collected from *Arenicola marina* were used throughout the experiment, and were stored as described in section 7.2.1. The egg concentration was then determined and the volume of water containing 500 – 1000 eggs calculated.

Sperm were collected "dry" from each of 5 males by the injection of spawning hormones (see above). Samples of sperm were taken, diluted with SFSW and the concentrations calculated. One aliquot of sperm from each male was then taken and pipetted individually into 5 crystallising dishes containing 40ml of the egg water prepared above to give a final concentration of 5 x 10^5

sperm.ml^{-1}. As a control, further aliquots of sperm were taken from each sample and pipetted into crystallising dishes with 40ml 0.2μm filtered seawater that had been left for 12 hours at 8 - 10°C.

Three replicate fertilizations were performed for each male at all treatments and time points. Fertilizations and assessment of fertilization success was determined exactly as described above.

7.3 Results

7.3.1 Oocyte Age

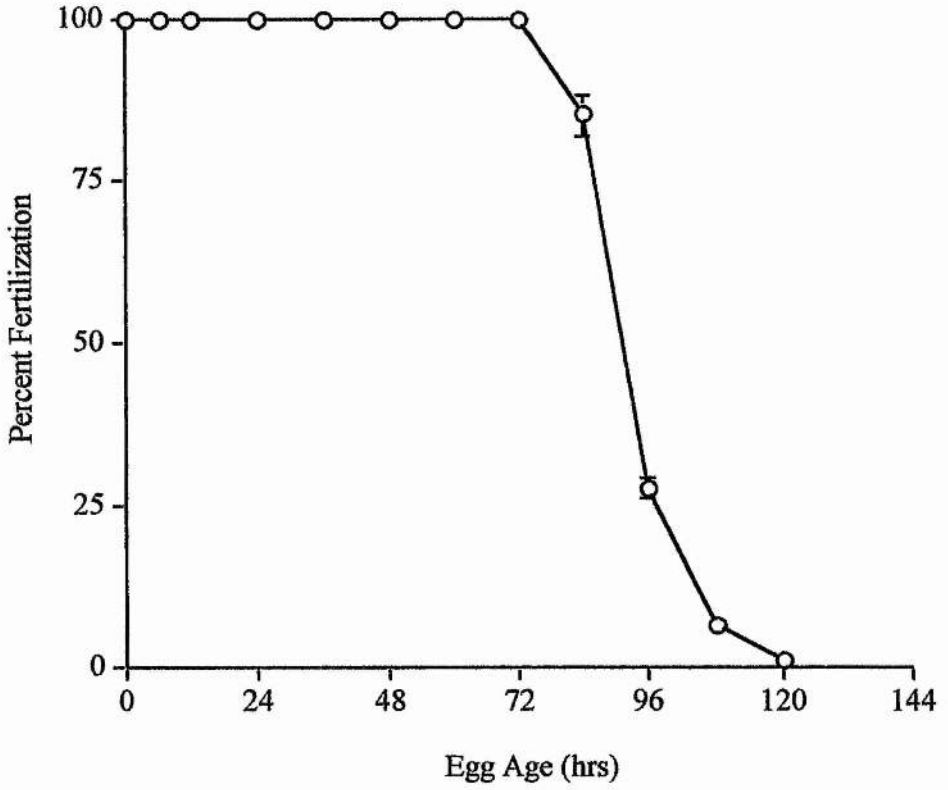
Figure 7.1 shows the percent fertilization success of oocytes from *Arenicola marina* aged over 5 days. Fertilization success remained around 100% up to 72 hours, but then began to fall rapidly with only a quarter of oocytes being fertilized at 96 hours and almost none at 120 hours. The development success of these oocytes is shown in figure 7.2. Developmental capacity was not diminished to any great extent until 96 hours post-spawning. This increase in abnormal development before blastula coincided with the biggest drop in fertilization success. Subsequently, abnormal development peaked at 50% at 120 hours, but only 2 oocytes were fertilized. No fertilizations took place at 144 hours.

Most oocytes of *Nereis virens* were able to be fertilized for at least 72 hours after extraction from the coelomic cavity (figure 7.3). However, figure 7.4 shows that the developmental capacity decreased throughout this period so that after 48 hours only about 10% of the oocytes developed to blastula.

In contrast to the polychaetes, the oocytes of *Asterias rubens* showed 100% fertilization for only the first 4 hours after spawning (figure 7.5). Thereafter, fertilization success fell to zero by 24 hours. Cases of abnormal development gradually rose with increasing age (figure 7.6).

Figure 7.1. Percent fertilization success of *Arenicola marina* oocytes aged over 5 days. Data shown are mean values from the percentage fertilization success. Standard errors were calculated from the arcsine transformed percentage data and back transformed for presentation.

Figure 7.2. Percentage of fertilized eggs of *Arenicola marina* aged over 5 days which fail to develop to blastula. Data shown are mean values from the percentage fertilization success. Standard errors were calculated from the arcsine transformed percentage data and back transformed for presentation.


Percent Fertilization
100
75
50
25
0
0
24
48
72
96
120
144
Egg Age (hrs)

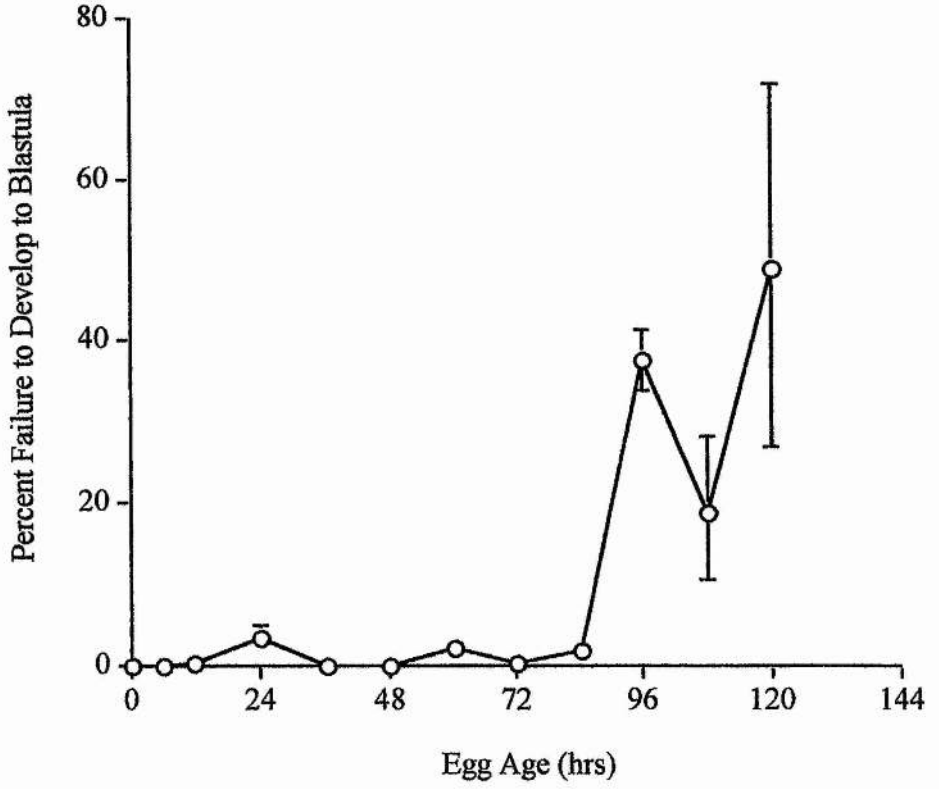
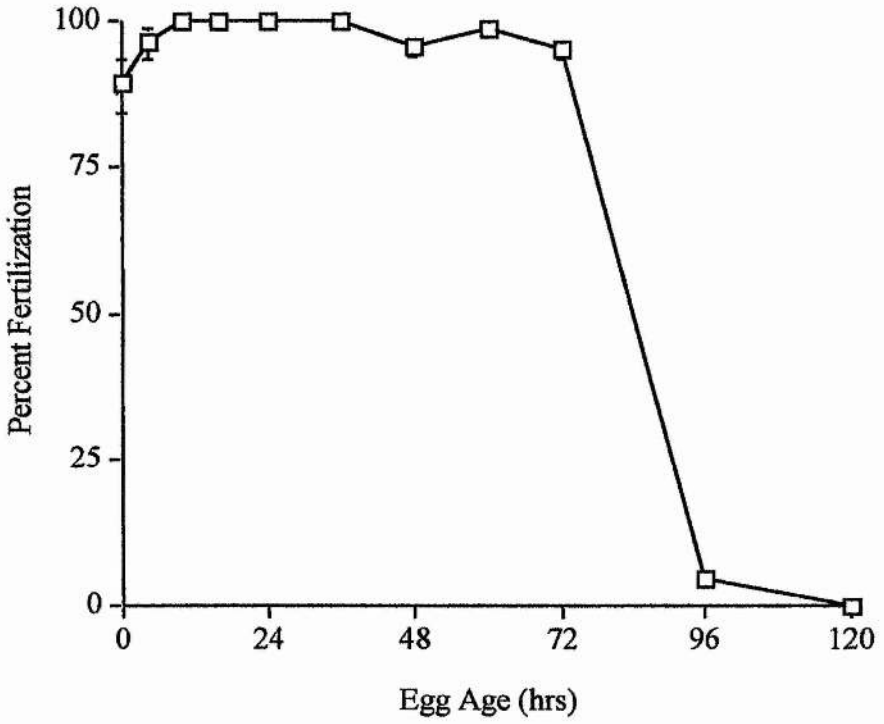

Percent Failure to Develop to Blastula
80
60
40
20
0
0
24
48
72
96
120
144
Egg Age (hrs)

Figure 7.3. Percent fertilization success of *Nereis virens* oocytes aged over 5 days. Data shown are mean values from the percentage fertilization success. Standard errors were calculated from the arcsine transformed percentage data and back transformed for presentation.

Figure 7.4. Percentage of fertilized eggs of *Nereis virens* aged over 5 days which fail to develop to blastula. Data shown are mean values from the percentage fertilization success. Standard errors were calculated from the arcsine transformed percentage data and back transformed for presentation.


Percent Fertilization
100
75
50
25
0
0
24
48
72
96
120
Egg Age (hrs)

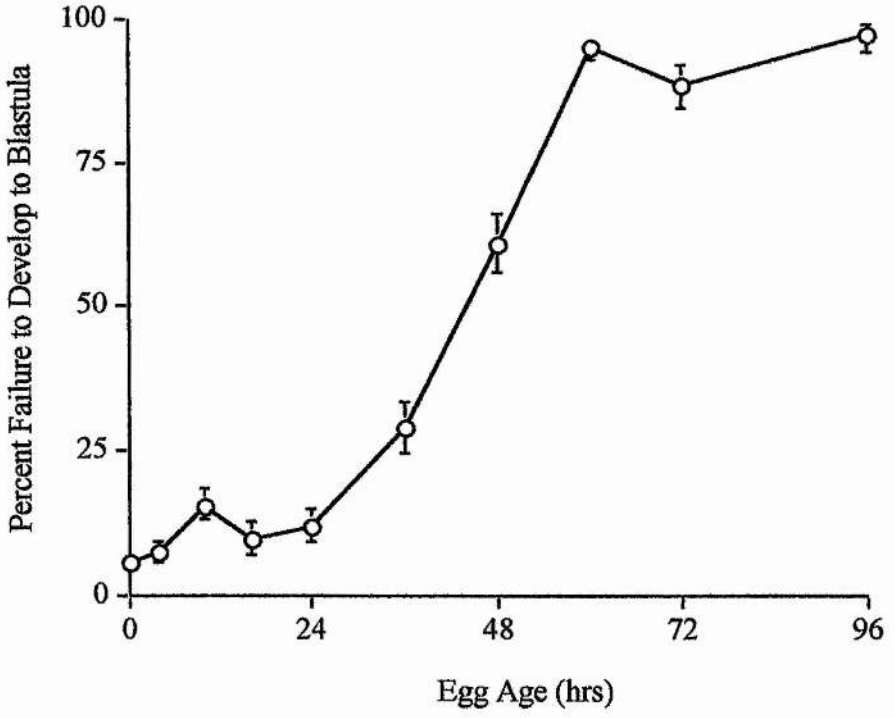
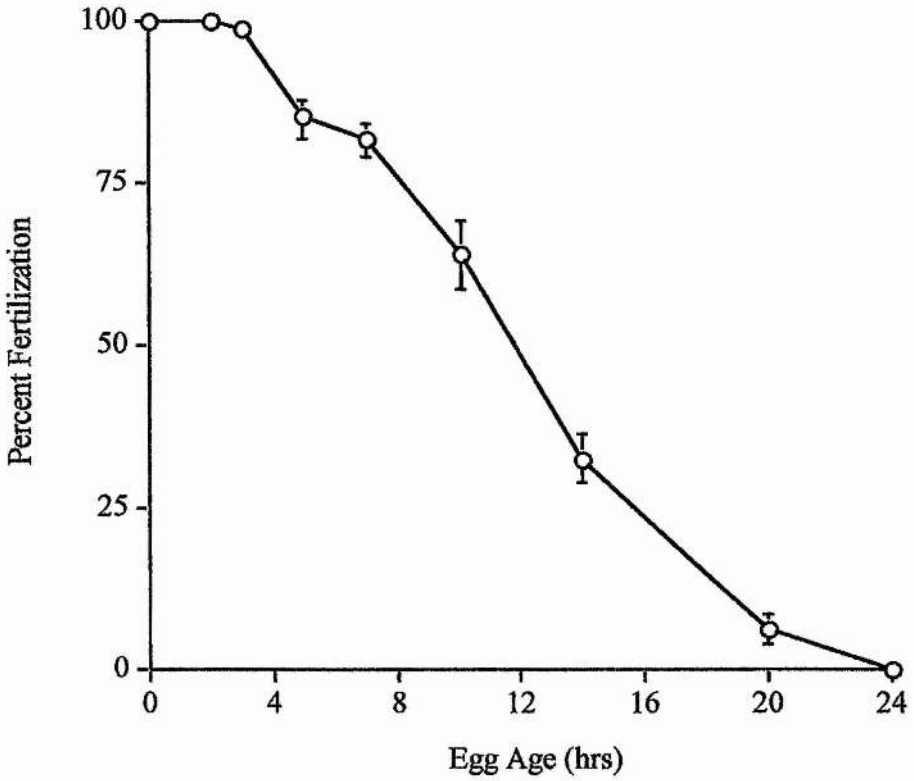
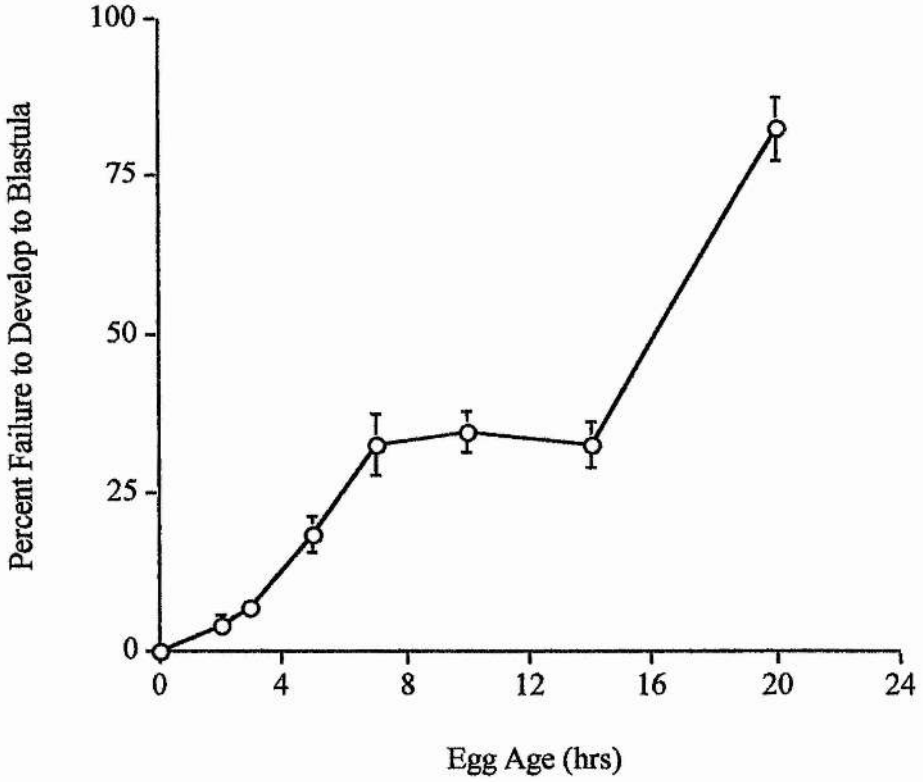

Percent Failure to Develop to Blastula
100
75
50
25
0
0
24
48
72
96
Egg Age (hrs)

Figure 7.5. Percent fertilization success of *Asterias rubens* oocytes aged over 24 hours. Data shown are mean values from the percentage fertilization success. Standard errors were calculated from the arcsine transformed percentage data and back transformed for presentation.

Figure 7.6. Percentage of fertilized eggs of *Asterias rubens* aged over 24 hours which fail to develop to blastula. Data shown are mean values from the percentage fertilization success. Standard errors were calculated from the arcsine transformed percentage data and back transformed for presentation.


Percent Fertilization
100
75
50
25
0
0
4
8
12
16
20
24
Egg Age (hrs)


Percent Failure to Develop to Blastula
100
75
50
25
0
0
4
8
12
16
20
24
Egg Age (hrs)

7.3.2 Sperm Age

Arenicola marina sperm stored at 10^4 sperm.ml^{-1} retained a high fertilization capacity up to 60 hours post spawning, before dropping to almost zero at 86 hours (figure 7.7). On the same graph is shown the fertilization success of sperm stored at 10^9 sperm.ml^{-1}. Success remained high throughout the experiment, and continued beyond the time shown in the figure. When examined, sperm stored at 10^9 sperm.ml^{-1} had coalesced into oily droplets at the bottom of the petri dish, and in this state it was quiescent.

Nereis virens sperm stored at the higher concentration is similarly long lived. However, sperm stored at 10^5 sperm.ml^{-1} rapidly lost the ability to fertilize, falling to almost zero after 24 hours (figure 7.8). In contrast, the fertilizing capacity of *Asterias rubens* sperm stored at 10^9 sperm.ml^{-1} and 10^5 sperm.ml^{-1} was found to be similar (figure 7.9). At each of the time points, success was slightly greater for the sperm stored at the higher concentration, but both successes fell to approximately zero after 26 hours.

7.3.3 Sperm Stored in Egg Water

It is apparent that egg water had a marked effect upon the fertilizing capacity of sperm for both *Arenicola marina* (figure 7.10) and *Asterias rubens* (figure 7.11). The fertilization capacity of *A. marina* sperm stored in egg water fell to zero after 28 hours while that stored in SFSW remained at 100% (data same as in figure 7.7). The fall in fertilization success of *A. rubens* sperm was even more dramatic, dropping to zero after 8 hours. The fertilization success of sperm stored in SFSW is comparable to the data presented in figure 7.9.

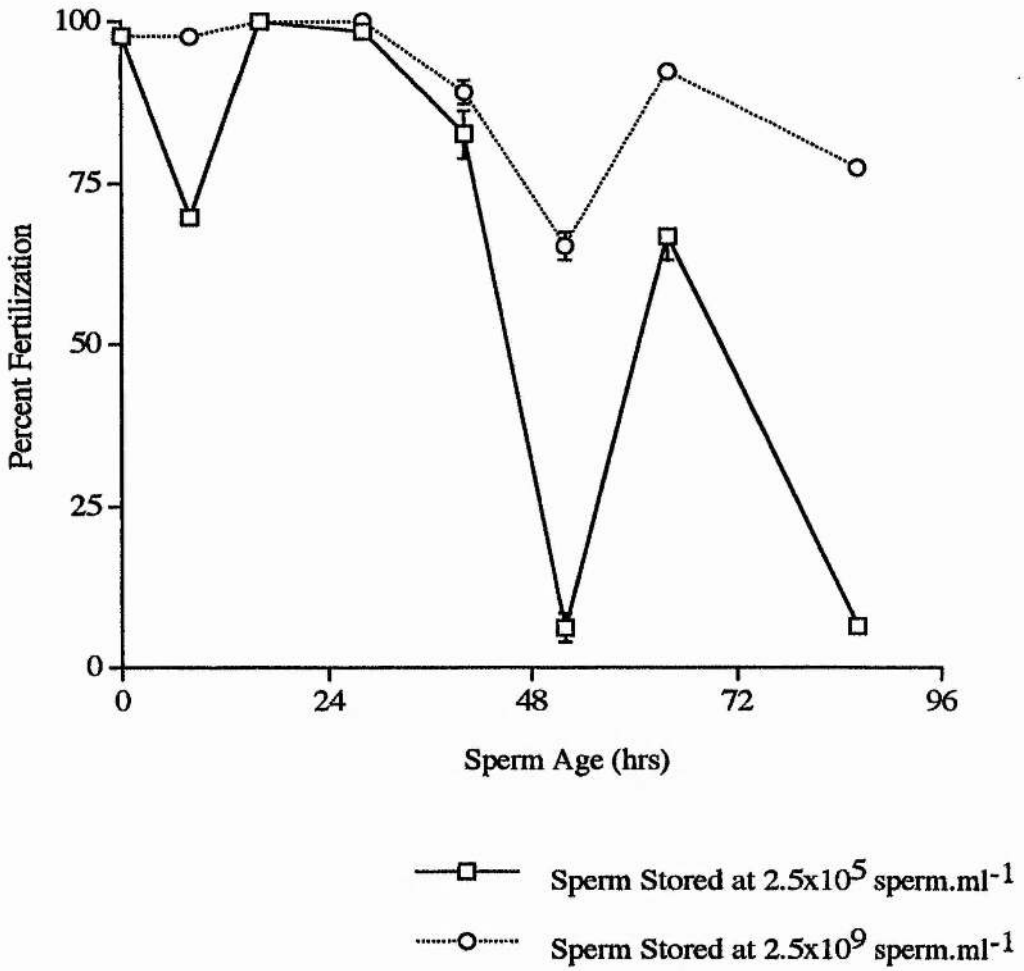


Fig 7.7. Fertilizing capacity of *Arenicola marina* sperm stored and aged at 2 different concentrations. Standard errors are calculated from the arcsine transformed percentage data

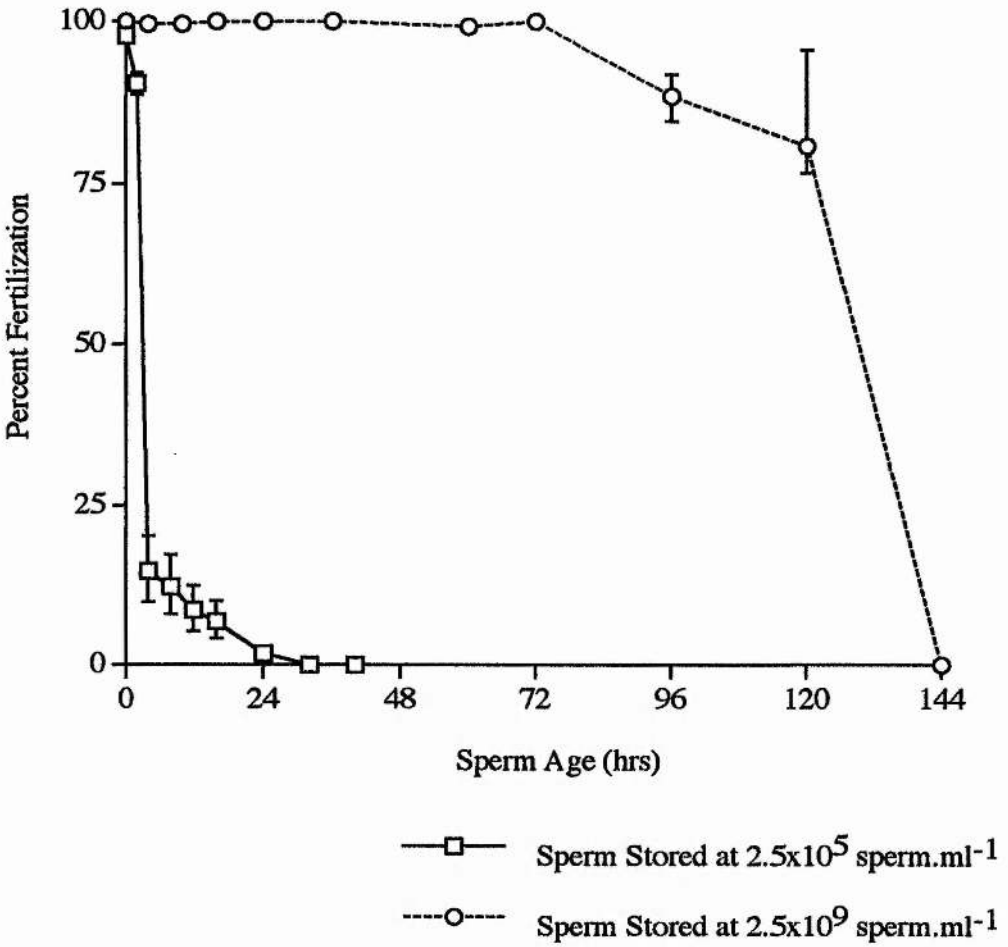


Fig 7.8. Fertilizing capacity of *Nereis virens* sperm stored and aged at 2 different concentrations. Standard errors are calculated from the arcsine transformed percentage data

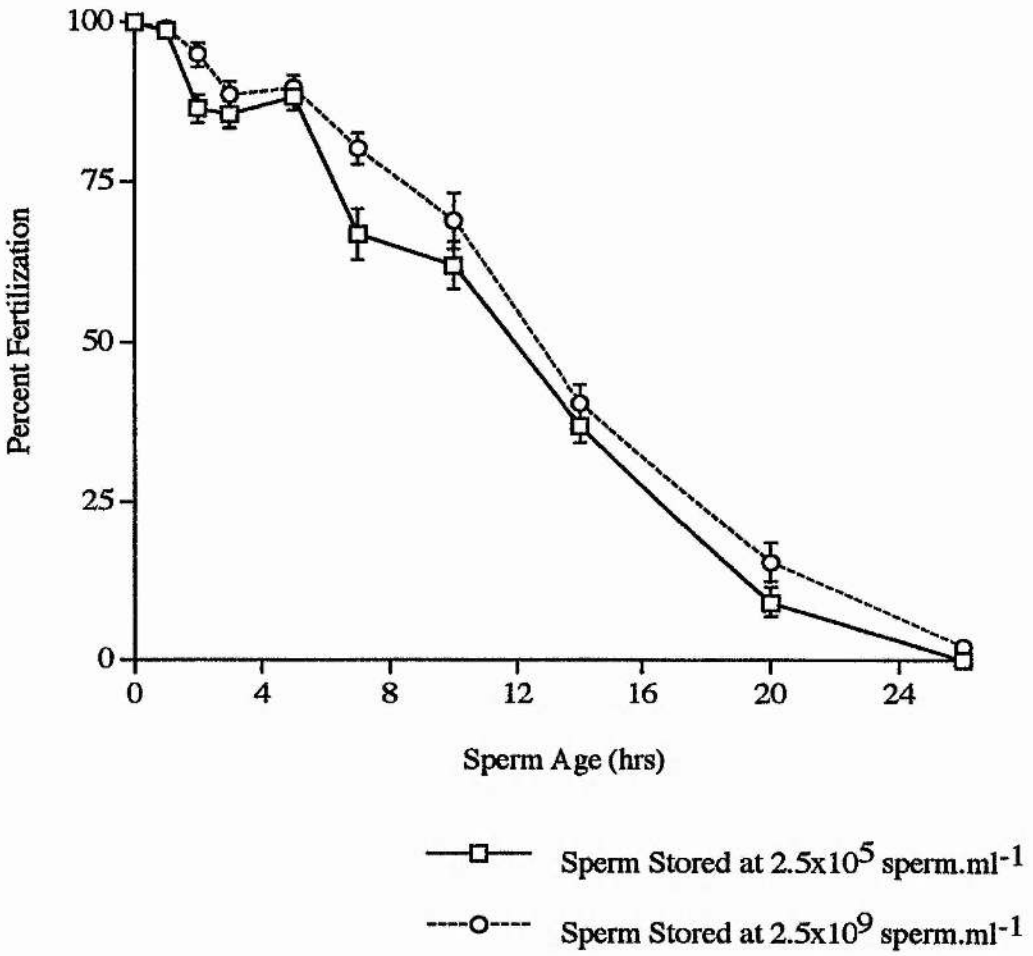


Fig 7.9. Fertilizing capacity of *Asterias rubens* sperm stored and aged at 2 different concentrations. Standard errors are calculated from the arcsine transformed percentage data

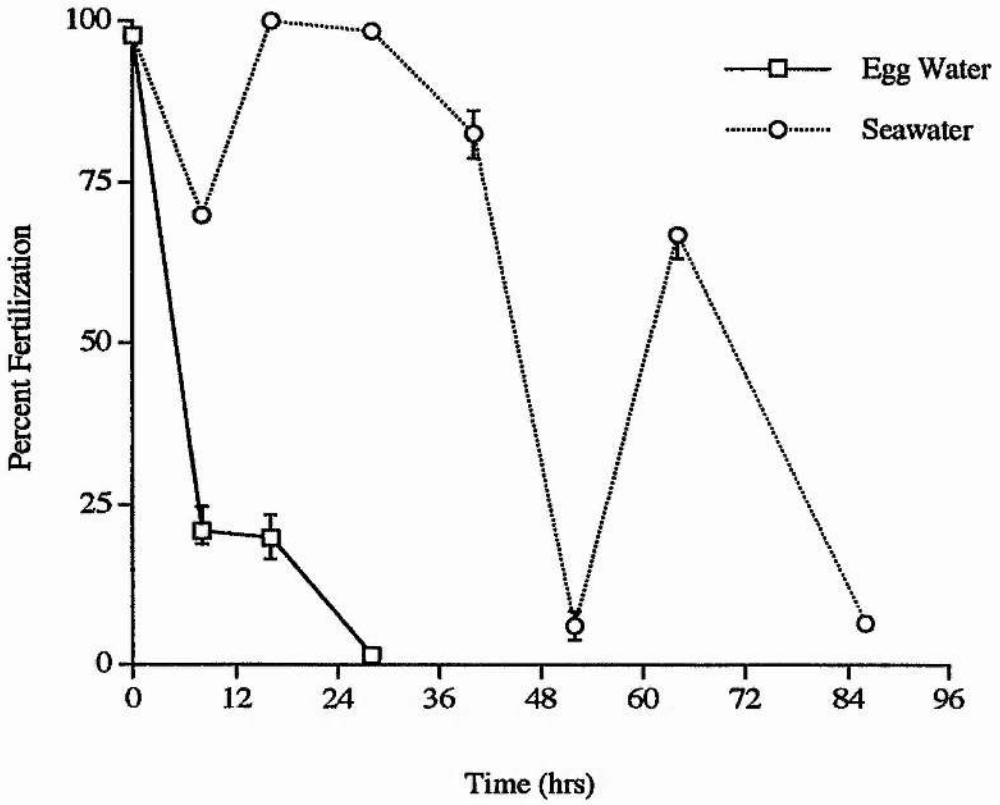


Fig 7.10 Sperm longevity in *Arenicola marina* . Sperm was stored at 10^5 sperm.ml^{-1} in either fresh TFSW or egg water. Standard errors were calculated from the arcsine transformed percentage data.

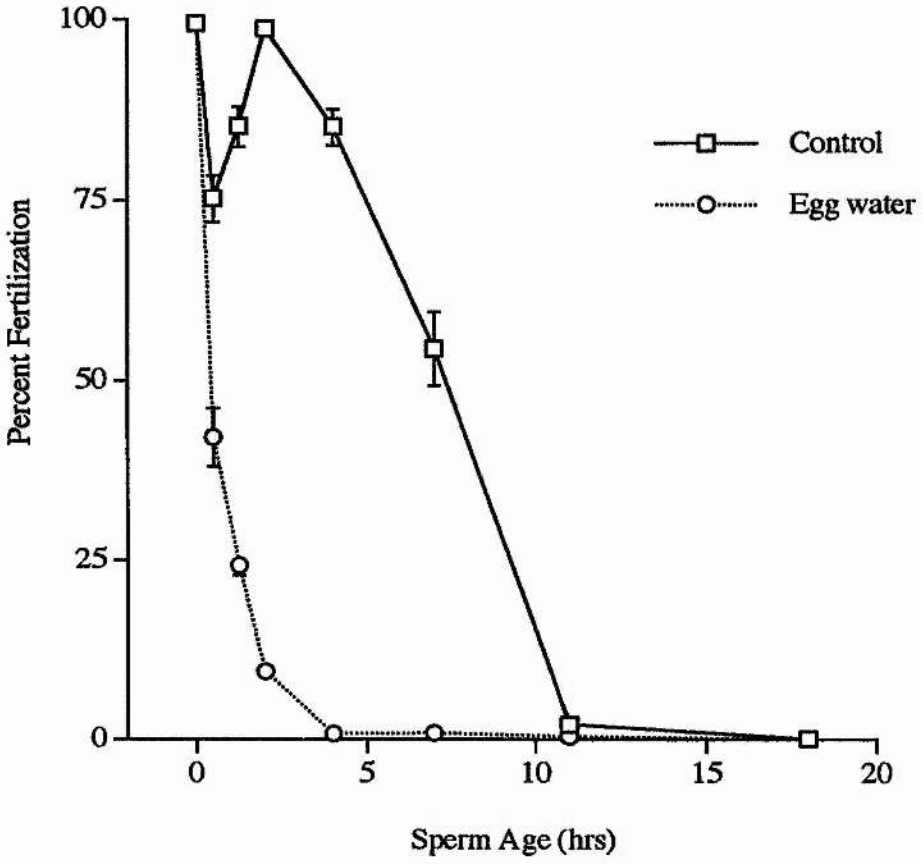


Figure 7.11. Sperm longevity of *Asterias rubens*. Sperm was stored at 10^5 sperm.ml^{-1} in either fresh TFSW or egg conditioned water. Standard errors were calculated from the arcsine transformed percentage data.

7.4 Discussion

The longevity of unfertilized oocytes has a marked impact upon fertilization success. This chapter demonstrates that there is a finite period following spawning in which the oocyte is capable not only of being fertilized but also successfully developing to at least blastula. The choice of blastula/early gastrula stage as a cut-off point in the experiments was an arbitrary decision based on the likelihood of artefacts associated with nurturing large batches of larvae influencing the results. Further development may have been impeded for some embryos as a result of oxygen tensions or biotic factors (e.g. infection). The blastula/early gastrula stage, which lasts for a number of hours, also provided a convenient window of time in which to examine and score the success (or otherwise) of early embryonic development, without the need for fixation of the samples.

Very little data exist in the literature on the longevity of unfertilized gametes. That there is a limited lifespan of gametes was recognised by several early workers (e.g. Lillie 1915). More recent work has quantified the length of time which gametes are viable, but as Benzie and Dixon (1994) point out, this has not been discussed in an ecological context. It is generally reported that sperm become senescent more rapidly than eggs in free-spawning invertebrates, their longevity being measured from minutes to one or two hours (see Levitan 1995, table 7.3 below). This extends across a range of invertebrate groups including chelicerates (Brown and Knouse 1973), echinoids (Pennington 1985, Levitan *et al.* 1991), asteroids (Benzie and Dixon 1994), bivalve molluscs (André and Lindegarth 1993) and hydroids (Yund 1990).

Havenhand (1991) however, showed that sperm from a solitary ascidian (*Ascidia mentula*) can successfully fertilize oocytes for up to 48 hours after release, and that eggs are similarly longer lived. This longevity was subsequently confirmed for three other ascidian species (Bolton and Havenhand 1996, Bishop 1998). The situation in these ascidians is slightly different to other free spawning invertebrates in that the eggs are retained by the female and

fertilization is facilitated by the female collecting the sperm and brooding the eggs. Fertilization of eggs occurs at sperm densities that are low compared to studies by Levitan *et al.* (1991) and Benzie and Dixon (1994), high fertilization success reported at sperm densities of around 10 sperm.ml $^{-1}$ (Bishop 1998). The sperm of the ascidian *Diplosoma listerianum* is proposed to be long lived at low density, possibly by remaining quiescent for periods of time (Bishop 1998).

Oocyte Longevity

There are marked differences in oocyte longevity of the species studied here. *Arenicola marina* oocytes remain fertilizable and capable of developing to blastula for more than 96 hours (figures 7.1 and 7.2). Oocytes from *Nereis virens* remain fertilizable for a similar length of time, but the probability of producing viable offspring decreases to almost zero after 48 hours. In contrast, the fertilization capacity of *Asterias rubens* oocytes falls to zero after 24 hours, while cases of abnormal development rise steadily throughout. Most of the comprehensive studies of the effect of gamete age upon fertilization success fail to record the length of time that eggs are viable. They simply state that the fall in fertilization success is attributable to a drop in sperm viability, and that eggs remain fertilizable for a short time after the sperm has senesced (see references above). The oocyte longevity of *A. rubens* is comparable to the qualitative observation by Benzie and Dixon (1994) that oocytes of the starfish *Acanthaster planci* are fertilizable for "a few hours". The independent effect of egg age was investigated by André and Lindegarth (1993) for the bivalve *Cerastoderma edule*. They reported that eggs can give rise to normally developing embryos for less than 5 hours post spawning.

Arenicola marina oocytes have a remarkable longevity compared to the free-spawning species mentioned above. In this respect, they are comparable to the eggs of the ascidian *Ascidia mentula*, where fertilization and development proceeds normally for 96 hours after extraction (Havenhand 1991).

Such longevity is not reported elsewhere for externally fertilizing marine invertebrates, and little is known of the factors which determine longevity of oocytes. Internal energy stores will most likely be involved, and the oocytes of both *Arenicola marina* and *Nereis virens* are considerably larger than *Asterias rubens* oocytes (see Chapter 6). Following extrusion from the body cavity, the oocytes will be exposed to a range of pathogenic bacteria and toxic compounds. Toomey and Epel (1993) describe embryos of the infaunal echiuran *Urechis caupo* which possess a well developed system for removing toxic compounds, which is absent in free-spawned sea urchin embryos. In addition, antibiotic and antifungal resistance is described for the mollusc *Aplysia kurodai* (Iijima *et al.* 1995). It is likely that the oocytes of *A. marina* will have well developed defence mechanisms to enable them to remain viable over a longer period of time in the sediment.

Sperm Longevity

As stated, most of the work conducted to date has centred upon the longevity of free spawned sperm. The results presented here for the polychaetes (figures 7.7, 7.8) are in accordance with the respiratory dilution effect described by Chia and Bickell (1983), and reported in several species of echinoderm (Levitan *et al.* 1991, Benzie and Dixon 1994). The longevity of the sperm suspension is related to its concentration, and this has been attributed to the rate of consumption of oxygen. Concentrated sperm respire at a lower rate than dilute sperm, termed the respiratory dilution effect (Chia and Bickell 1983). This has been clearly demonstrated for the echinoid *Strongylocentrotus franciscanus* (Levitan *et al.* 1991) and the asteroid *Acanthaster planci* (Benzie and Dixon 1994). *Asterias rubens* sperm was only slightly longer lived at the higher sperm concentration (figure 7.9). However, the dilute (10^5 sperm.ml^{-1}) sperm samples for all the species studied here were considerably longer lived than some of the species reported elsewhere at comparable concentrations (see table 7.1). The sperm longevity of the species presented in this table is less than 5 hours, while sperm from *Arenicola marina* remains viable for more than 72

hours (though at a reduced fertilization success after 48 hours). Sperm from *Nereis virens* and *Asterias rubens* have a similar longevity, failing to fertilize any eggs within 24 hours.

Species	Max Viable Time	Temperature	Reference
Cerastoderma edule (Bivalve mollusc)	5 hours	20°C	André and Lindegarth 1995
Acanthaster planci (Asteroid echinoderm)	5 hours	28°C	Benzie and Dixon 1994
Strongylocentrotus droebachiensis (Echinoid echinoderm)	2 hours	14°C	Pennington 1985
Strongylocentrotus franciscanus (Echinoid echinoderm)	2.5 hours	12°C	Levitan *et al.* 1991

Table 7.3. Length of time that dilute sperm remain viable **at concentrations comparable to this chapter** in some of the species for which sperm longevity has been extensively studied. Longevity times at concentrations much greater than the 10^5 sperm.ml^{-1} used here are not included.

Following release from the male, sperm from many species require an activation step which in free spawning invertebrates is provided by dilution in seawater. Dilution raises the pH of the sperm to that of seawater, and this has been shown to activate the spermatozoa of *Arenicola marina* (Pacey *et al.* 1994a). Dilution also leads to a raised respiration rate and a more active

swimming behaviour. As there is a finite "respiratory life" of a spermatozoon, those stored at low density have a shorter half life than more concentrated sperm (Chia and Bickell 1993). Studies on the swimming behaviour of *A. marina* sperm have shown that it has intermittent periods of quiescence, which may be modulated by light radiation (Pacey *et al.* 1994b). This has also been described for tunicates (Brokaw 1984) and some sea urchins (Gibbons 1980). Bishop (1998) attributes the longevity of ascidian sperm to its intermittent swimming behaviour which results in the conservation of energy stores, and such a mechanism could conceivably facilitate sperm longevity of *A. marina*.

Temperature may also influence sperm longevity by changing respiration rate. In the experiments here sperm were stored at 8-10°C, close to ambient for the time of year. This is cooler than the studies presented in table 7.3.

Impact of Egg Conditioned Water on Sperm Activity

Some of the literature on chemotaxis was reviewed in Chapter 1 and will not be repeated here. Much of the work has described the chemo-orientation and swimming behaviour of the sperm which results in aggregation of sperm around either the egg, or the sperm attractant molecules (see Cosson 1990). Sperm attracting compounds such as speract and resact also raise the respiratory rate of sperm in sea urchins (Suzuki and Garbers 1984). Incubation of *Arenicola marina* sperm and *Asterias rubens* sperm in egg conditioned water dramatically reduces their respective half lives. Sperm fertilizing capacity in *Arenicola marina* drops to zero after little more than a day, while assays with *Asterias rubens* sperm result in almost no fertilization after 4 hours. These results are similar to data obtained by Bolton and Havenhand (1996) for the solitary ascidians *Ciona intestinalis* and *Ascidiella aspersa*, whose sperm is usually active for more than 12 hours. The chemical nature of the compounds is presently unknown, but Bolton and Havenhand (1996) found some species specificity in that activity of sperm incubated in homospecific egg water of one

of the species was significantly greater than that incubated in egg water from the other, although this was not reciprocated.

It seems likely that the mode of action of the egg derived compounds studied here is through the raising of activity and respiration levels of the sperm, similar to that described by Suzuki and Garbers (1984). Bolton and Havenhand (1996) noted an increased incidence of swimming activity of ascidian sperm incubated in egg water as opposed to seawater. In this study, attempts were made to measure the respiratory rate of *Arenicola marina* sperm incubated in egg water and seawater using a Clark electrode in a 5ml test chamber with a small magnetic stirrer. Preliminary results indicated that respiration rate was higher for the sperm incubated in egg water, but problems were encountered in getting consistent results from day to day. Therefore, these preliminary results are not presented here.

Ecological Considerations

Longevity of oocytes is likely to be related to the mode of reproduction of a species. The data gathered from the field indicates that the oocytes of *Arenicola marina* may be spawned early in the spawning period. Fertilization success is limited by sperm puddle density, and not all oocytes are fertilized after one exposure to sperm. Consequently, longer lived oocytes will confer an advantage in that they will be capable of fertilizing and developing up to 5 days after spawning. By comparison, spawning behaviour in *Nereis virens* and *Asterias rubens* means that eggs and sperm are more likely to be dispersed by water currents before their longevity has expired, in accordance with the opinion of Levitan (1995). Thus there is no "need" for gametes to be fertilizable for more than a few hours.

Arenicola marina sperm longevity at low densities may also be an adaptation to its fertilization strategy. Sperm are released onto the surface of the sediment at low water, where they coalesces into dense puddles. It may be several hours before they are dispersed by the tide, so the lack of activity in high

concentration, which is further suppressed by light levels (Pacey *et al.* 1994b), prevents the spermatozoa from expending too much energy while they are remote from the eggs. Following dilution and passage into the female burrow, there may be a further delay before sperm - egg interaction, as the rate of irrigation of the female burrow is low (see Chapter 4 for discussion). Consequently, interaction between sperm and egg in *Arenicola marina* may occur many hours after either has been spawned, in contrast to most other free spawning invertebrates.

Egg compounds may facilitate the fertilization success in two ways. First, they increase the "target area" of the oocyte which, as described in Chapter 6, improve the chances of sperm - egg interaction. Second, they modify sperm swimming behaviour and increase the overall level of activity (Suzuki and Garbers 1984, reviewed by Cosson 1990). In relation to the fertilization strategy, this means that the sperm will most active when in close proximity to an oocyte. The hydrodynamic factors may act to bring the sperm and eggs into brief contact, and the chemicals emanating from the egg stimulate the sperm to take advantage. The lack of turbulent mixing in the female burrow of *Arenicola marina*, and the comparatively long viable life of the sperm may mean that compounds released by the egg, and which enhance sperm swimming are of particular importance in increasing the probability of encounters between sperm and egg. In this situation, sperm-egg chemotaxis may account for a large proportion of the fertilizations which take place.

Chapter 8

General Discussion

8.1 Introduction

The main aims of this study were to investigate aspects of the fertilization ecology of marine invertebrates. Investigations were carried out to determine the level of *in situ* fertilization success attained during natural spawning events of two polychaete species, *Arenicola marina* and *Nereis virens*. In this respect, this thesis represents the first study of field fertilization success among polychaetes, and is also the first record of fertilization success in species that inhabit the intertidal zone. These data were supplemented by laboratory investigations of the factors that affect fertilization success, and comparative data for the echinoderms *Asterias rubens* and *Echinus esculentus*. This Chapter will briefly review some of the conclusions that can be made from this and other studies, and will attempt to draw parallels that are generally applicable to marine invertebrates that reproduce by broadcasting their gametes.

8.2 Field Fertilization Success in *Arenicola marina*

A major hindrance to the study of fertilization success of epidemic spawners is the ability (or otherwise) to predict the time that a population spawns. A certain degree of spawning predictability is demonstrated by corals of various species on the Great Barrier Reef (e.g. Harrison *et al.*. 1984) and this has meant that most studies of fertilization success during natural spawning events have been conducted on these species. Polychaete annelids provide several examples of species which spawn in a predictable manner. For example, the spawning of the Pacific palolo worm (*Eunice viridis*) and the European nereid *Nereis succinea* can be observed on specific nights of the year depending on the lunar phase (Caspers 1984, Hardege *et al.*. 1990). The populations of *Arenicola marina* at Kingsbarns and East Sands, St Andrews on the east coast of Fife, Scotland also possess such spawning predictability (figure 3.1). Spawning has been reported in these populations as far back as the 1950s (Howie 1984) and usually takes place on the last spring tide of October or the first spring tide of

November regardless of the phase of the moon (new or full). Without such a consistent spawning cycle, preparing and conducting field studies to measure fertilization success would have been a much more daunting prospect.

There is undoubtedly a link between male spawning intensity (number of sperm puddles per m^2) and female fertilization success in the two populations of *Arenicola marina* studied here. Success at East Sands, St Andrews was very much lower than at Kingsbarns, where male spawning intensity was very much greater, and fertilization success was almost 100%. That fertilization success is dependent upon sperm puddle density was demonstrated further in Chapter 4. These results complement the findings of other workers that density and proximity of spawning males determines the level of female fertilization success (Levitan *et al.*. 1991, Yund and McCartney 1994, Yund 1995).

Spawning synchrony among lugworm populations is reported from a number of locations (Duncan 1960). This serves to maximise the male spawning intensity, and hence the female fertilization success. However, such population-wide spawning synchrony may not occur at all localities. There are often marked differences in density and size of adults and spawning time between lugworm populations, even when they are geographically quite close. For example, the population at the Eden Estuary, Fife is only 2 kilometres from the population at East Sands, yet is typically a more densely populated site composed of very much smaller individuals. Spawning in this population takes place over a number of weeks as opposed to a few days (Auckland 1993, Watson 1996), and when it occurs, is limited to small areas of the estuary (personal observations), where different components of the population spawn in a localised synchrony. This prevented assessment of fertilization success in the population because it was impossible to predict the exact area where spawning might occur.

The reasons for the differences between lugworm populations may be attributed to the different habitats. Populations at East Sands and West Sands, St Andrews, and Kingsbarns inhabit exposed beaches, and breeding adults tend to be large (15 - 25cm in length, personal observations). By contrast, the dense population of small adults in the estuary inhabit a very sheltered environment,

and Johnson *et al.*. (1978) report up to 35 lugworm casts per square metre, far exceeding those at East Sands and Kingsbarns (Auckland 1993, personal observations). Similar densities are also reported in the very sheltered Dutch Wadden Sea (Beukema and de Vlas 1979). As a result, selection for spawning synchrony in the whole population is likely to be stronger at the more exposed locations, where sperm dilution is likely to be enhanced by the higher levels of turbulence experienced at wave swept sites. The tide in the estuary floods gradually, with little or no wave action, and as a result sperm dilution is likely to be slower than on the exposed beach. Therefore, the requirement for such well synchronised spawning throughout the population would be reduced.

8.3 Field Fertilization Success in *Nereis virens*

The results presented here provide some insight into the spawning strategy of *Nereis virens*, although an exact account of the spawning behaviour remains to be elucidated. Previous accounts of the spawning behaviour indicate that at some localities both male and female ragworms swarm in the water column (Clark 1960, Sveshnikov 1955), while at others only the male swarms and the female remains in the burrow (Bass and Brafield 1972, Desrosiers *et al.*. 1994). The population studied here at Pettycur Bay, Burntisland, Fife appears to have at least two spawning crises, and conforms to the latter accounts in that only males were observed leaving the burrows and spawning, while pools of eggs were located on the surface of the sediment indicating that the females remain within the burrow.

The results presented suggest that when fertilization success is generally low, higher levels of fertilization success are attained by eggs that are located high in the water column. It therefore seems peculiar that females are seldom reported to swarm in order to deposit their eggs where they have the highest probability of being fertilized. The persistence of the male swarm may be explained by considering that females are capable of spawning over several tides. Repeated spawning through the spawning period is likely to increase the chance

that at least one spawned batch of eggs is successfully fertilized. However, in vacating the sediment to spawn, the female risks predation and lowers her chances of re-burrowing to spawn at another time during the spawning period (though being semelparous, each spawn involves releasing oocytes from the same cohort of gametes). The differences in strategy that exist between some of the sites examined in the literature are more difficult to explain. It is possible that the particular strategy exhibited by a population is an adaptation to the local site, and acts through sperm limitation. For example, at more exposed locations sperm released by males would disperse rapidly, and possibly be swept well away from the site of the female spawning. At more sheltered locations (such as the site studies here), sperm may remain in sufficient concentration to effect fertilization of eggs lower in the water column. However, insufficient data are available on the spawning strategy of *Nereis virens* from different sites to enable general conclusions to be made.

8.4 General Considerations

Both *Arenicola marina* and *Nereis virens* are exploited commercially for bait. In many areas this has led to the establishment of protected areas (Olive 1993), not only to prevent over exploitation of the target species, but also to prevent excessive damage to the habitat and conjoint species. With regard to the fertilization success of exploited populations, Chapter 4 would indicate that, for *A. marina* at least, a decreased population density would result in a lower male spawning intensity, and therefore reduced fertilization success.

Whether such a phenomenon would cause recruitment failures and long-term decline of the population depends on several factors, not least the size of the shallow subtidal component of the population, and its contribution to larval production. In the case of *Arenicola marina*, male spawning in the subtidal is likely to result in a rapid dilution of the gametes, as they will not coalesce into dense puddles as is the case with the intertidal population. Also, spawning of subtidal specimens may not be as synchronised as their intertidal counterparts.

The fate of larvae following fertilization will also determine to what extent over-exploitation of a population can lead to a long term problem. Larvae of *Arenicola marina* are swept upshore following release from the female burrow, where they rapidly adhere to sand grains and settle in nursery areas. The scope for rafting of larvae from remote, unexploited locations therefore appears slight. However, as Johannesson (1988) showed, this may not necessarily be a barrier to dispersal. Olive (1993) proposed that immigration of adult worms into over-exploited areas accounted for some of the recovery of a population of lugworms on tidal flats in Northumberland, U.K. He suggested that such migration was commonplace, and should be considered when planning a management strategy for an exploited area. For example, could protection of a sufficiently large area(s) of beach (harvest refugia) yield sufficient worms to re-colonise adjacent exploited areas? Such immigration may be less prevalent in small bays isolated by rocky outcrops as at East Sands and Kingsbarns, and different management strategies may be required between different habitats.

The results obtained for both *Arenicola marina* and *Nereis virens* compare favourably with field studies conducted on the shallow subtidal echinoderms and corals studied to date. When spawning density is high, fertilization success of *A. marina* approaches 100%. However, this is the result of several days of exposure to male spawning. Such repeated exposure of eggs to sperm does not occur in any of the studies on natural spawning success conducted elsewhere. Therefore, direct comparisons between them are not necessarily valid. In this respect, fertilization success in *A. marina* is more akin to the strategy demonstrated by brooding ascidians (e.g. Bishop 1998).

One of the general conclusions that can be applied to the species studied here and those investigated elsewhere is that fertilization among free spawning invertebrates is far from certain. Often, fertilization success varies considerably between individuals of the same population, and this is demonstrated in table 1.1, the daily fertilization success data for *Arenicola marina* (Chapter 3) and the successes observed for transplanted *Nereis virens* oocytes (Chapter 5). Successful fertilization of oocytes is dependent upon density and proximity of

individuals that are spawning in synchrony, and a range of hydrodynamic effects. Given ideal water movements, fertilization success can be very high between distant individuals (Babcock *et al.*. 1994), or vanishingly small within a metre of a spawning male (Pennington 1985, Levitan *et al.*. 1992).

Fertilization Success : Proportionate v Numerate Data

One of the problems associated with the comparison of fertilization success between different species is the way in which success is recorded. Almost without exception, fertilization success in the recent literature has been measured as the proportion of eggs fertilized from a subsample of eggs (e.g. Levitan *et al.*. 1991, 1992, Babcock *et al.*. 1994), and in this respect this thesis is no different. However, the basic properties of breeding individuals, such as fecundity, may differ by several orders of magnitude, and comparisons of percentages are, in ecological terms, inappropriate. A highly fecund broadcast spawning invertebrate, such as the crown of thorns starfish *Acanthaster planci* (Babcock *et al.*. 1994), may have a low fertilization success in percentage terms but still produce an order of magnitude more larvae than the much less fecund sea urchin *Strongylocentrotus purpuratus* (Levitan *et al.* 1991), even if fertilization success is 100%. There is thus a need to clarify the difference between fertilization success in proportionate terms and fertilization success in ecological terms - the total reproductive output (larval production) of an individual. Levitan (1991b) made the point that reproductive output of an individual is related to its size and, hence, fecundity, but this needs to be addressed more universally in the literature.

With respect to the work in this thesis, while fertilization success may be comparable between *Arenicola marina* and *Nereis virens* in percentage terms (20% - 80%), *N. virens* produces in the region of 10^9 eggs per female compared to 10^5 - 10^6 eggs per female of *A. marina*. Therefore the reproductive output of a female ragworm, even at low fertilization success, is in no way comparable to *A. marina*. Fertilization success should perhaps be viewed in terms of fertilization

efficiency rather than absolute success. This would avoid misleading statements in relation to the comparison of success between species. The reproductive success of an individual will then be a product of its fertilization efficiency, and its fecundity. Other factors, such as polyspermic fertilization would then have to be taken into account when describing reproductive success.

8.5 Laboratory Studies of Fertilization Success

The results obtained in the laboratory investigations of fertilization success confirm earlier work that sperm concentration and gamete age have a significant effect upon fertilization success. They also show that egg concentration and sperm : egg ratio are more important than previously reported (e.g. Levitan *et al.*. 1991, Benzie and Dixon 1994), and that there are differences between species which may be attributed to differences between the gametes such as egg size. The length of time that eggs are in contact with a body of sperm rich water also affects fertilization success more significantly than reported by Levitan *et al.*. (1991). This indicates that there is a finite rate at which oocytes are fertilized that varies with sperm concentration. For this reason, it should be made clear that there is a distinction between fertilization rate and fertilization success. Most of the papers published to date (particularly on field fertilization success) refer to the proportion of eggs fertilized as the fertilization rate. This implies a temporal component which is invalid in these studies, and the "amount" of fertilization observed is more accurately termed the fertilization success.

Fertilization success also depends upon interactions between sperm and egg through the production of sperm attracting substances. Such compounds act by increasing sperm metabolic rate and swimming speed, directing sperm towards the source of the compound (i.e. the egg) and increasing the "target area" for the sperm (i.e. increasing egg size). Although no chemical characterisation of these compounds has yet taken place for the species studied here, it is apparent that sperm of *Arenicola marina* and *Asterias rubens* are

affected by substances derived from conspecific oocytes. In these species, the longevity of sperm incubated in water which contains egg material is reduced dramatically.

Egg size

Laboratory studies of fertilization kinetics may also provide further insight into the evolutionary aspects of egg size and fertilization success in broadcast spawning marine invertebrates. Traditionally, the evolution of egg size in marine invertebrates with planktonic larvae has been explained in terms of the "energy fitness" of the subsequent larvae. Since there is a finite reproductive allocation, a trade-off exists between the fecundity of the parent and investment in each individual oocyte (Thorson 1950, Smith and Fretwell 1974, Hart 1995). Additionally the eggs of many species are larger than the minimum size required for successful development, indicating a fitness cost of smaller eggs (see Hart 1995). There is thus an optimal offspring size that satisfies both offspring and parental fitness (Levitan 1993).

It is apparent from the results presented in this thesis and elsewhere (Vogel *et al.*. 1982, Levitan 1993) that fertilization success also acts as a selective pressure for egg size. Large eggs present a larger "target" for passing sperm. However, it would be too simplistic to assume a direct relationship between egg size and overall reproductive success, particularly because there are energetically inexpensive means of enlarging a target area (e.g. jelly coats or sperm attractants - Cosson 1990). It has been shown (Levitan 1993) that in sympatric species of sea urchin, those with larger oocytes achieve a greater fertilization success in a situation where sperm is limited. Where sperm is not limited however, as demonstrated in Chapter 6, the development success falls with increasing sperm concentration. While no definite conclusions could be drawn between the species studied here, the influence of egg size on the incidence of polyspermic fertilizations should be considered. The Don Ottavio model of Vogel *et al.*. (1982) assumes that all fertilization is monospermic

irrespective of sperm concentration. Styan (1998) recognised that a finite time period existed between fertilization of an oocyte and activation of the polyspermic block, and that given suitable sperm concentration and egg size further sperm entry could take place before the block was established. He reasoned that this time period should be incorporated into the Vogel *et al.*. (1982) Don Ottavio model, and proposed a "polyspermy adjusted" model. In such a model, the time to activate a polyspermic block is estimated by fitting the modified Don Ottavio model to kinetics curve data (Styan 1998). This model differs from the original Don Ottavio model in that it examines overall zygote production, and concludes that larger oocytes have a greater probability of yielding polyspermic embryos.

Egg size can therefore be explained not only in terms of energy fitness of the parent and offspring, but also as a trade off between the need to maximise probability of fertilization success and avoidance of polyspermy.

8.6 The Reproductive Strategy of *Arenicola marina*

The results presented here on field and laboratory fertilization success, coupled with previous investigations of endocrine and environmental control of reproduction make it possible to postulate the fertilization strategy of *Arenicola marina.*

8.6.1 Environmental Control of Reproduction

The cycle of gametogenesis in *Arenicola marina* is well described and is reviewed in Chapter 1. By early autumn, gametes are fully developed, with the eggs held in meiotic arrest at prophase I, and the sperm joined together by the sperm head around a nucleated cytophore. Hormones are required to induce maturation and spawning of the gametes. Temperature is important in regulating the time of spawning of *A. marina*. Spawning can be inhibited by maintaining

animals above 15°C, and will only take place following a drop in temperature (Farke and Berghuis 1979a). This provides a clue as to the possible control of spawning in *A. marina*. The environmental control of reproduction in *Nereis virens* was discussed by Olive (1995) who proposed that the reproductive cycle is controlled by a series of "gated rhythms". The gates are the switch between summer conditions (long days, warm temperatures) to winter (short days), and the rise in temperature in the spring to induce spawning. Animals that are sufficiently developed with suitable energy levels will enter gametic growth, effectively passing through the first gate. Those that are not will continue pre-reproductive growth and may enter the gametic phase in the following year.

The spawning period of *Arenicola marina* may be controlled in a similar way. Synchrony of gametogenesis in the population is likely to be maintained by environmental conditions of temperature and food availability, with those animals that are mature and have sufficient energy reserves entering the gametic growth cycle in spring. By late summer/early autumn the gametes are fully developed, but will not be spawned. Once environmental temperatures have dropped sufficiently, the lugworms are ready to spawn. Temperature therefore provides the first barrier to spawning. Whether this is because the active substance is only produced by the prostomium in response to environmental temperature, or its action to stimulate coelomic maturation factor production is prevented is presently unknown.

At this point, there appears to be a second barrier to spawning governed by lunar phase. The populations studied here spawn on spring tides regardless of the phase of the moon, and this can also be observed in animals collected from the field prior to spawning and maintained in the laboratory. The mode of action of this cue is presently unknown, but it serves to synchronise the period of spawning to a few days in the year. It can be hypothesised that the populations of *Arenicola marina* at the East Sands, St Andrews and Kingsbarns spawn on the first spring tide after the sea temperature has dropped below a threshold level, regardless of whether the tides are caused by the new or full moon.

8.6.2 Fertilization Strategy

Fertilization takes place within the female burrow, dilute sperm having been drawn into the burrow by the irrigatory actions of the female. The level of fertilization success attained by females over the entire spawning period exceeds that found on a daily basis (Chapter 3), which indicates that repeated exposure to sperm is necessary to achieve high levels of fertilization success. Both sperm and eggs of *Arenicola marina* are long lived compared to the other species studied here and work conducted elsewhere. Eggs are viable for the entire spawning period, and dilute sperm retains some fertilizing capacity for 48 hours. This leads to the hypothesis that the female releases her eggs early in the spawning period, and they are exposed each day to varying amounts of sperm. The final fertilization success of the female is the result of cumulative increases in the proportion of eggs fertilized as the spawning period progresses. Fertilization is enhanced by sperm longevity, since there is a potential delay of many hours between sperm release and sperm - egg interaction. Successful fertilization may also be assured by the release of compounds from the egg which attract sperm. Following fertilization, the embryos develop for a few days within the female burrow, before being released through their ciliary swimming behaviour and the irrigatory activity of the female (Farke and Berghuis 1979a).

8.7 Areas for Future Research

The data presented in this thesis have enabled a detailed understanding to be gained of the optimal fertilization parameters for marine invertebrates. These data also provide information on the field fertilization success of *Arenicola marina* and *Nereis virens* that enable hypotheses to be formulated about the nature of spawning in these two polychaetes. However, a number of new questions arise in the light of the findings presented here:

i) It has been demonstrated that sperm puddle density affects fertilization success in *Arenicola marina*. To what extent, however, does position of sperm puddle in relation to the female burrow determine the amount of fertilization that can take place? This should be investigated to determine whether sperm puddles seaward or landward of the burrow effect most fertilization.

ii) To what extent do environmental conditions affect fertilization success? In all field studies presented here, weather conditions were good with little wind and very little wave action. Increased turbulence would undoubtedly lead to a more rapid sperm dilution rate. This also leads to the question of whether or not *Arenicola marina* are sensitive to weather conditions? In 1995 and 1996, spawning was predicted wrongly, both predictions coinciding with a period of low barometric pressure and high onshore winds. In 1995, spawning took place on the following spring tide, during a period of clement weather.

iii) The fertilization strategy of *Nereis virens* has yet to be fully described, with some reports describing male only swarms (apparently the case for the population studied here) while others indicate that both male and female worms swarm.

iv) The fertilization kinetics of the species studied here should be examined using the mathematical models described in Chapter 6. This requires the measurement of sperm swimming velocity for each of the species. This would enable comparisons to be made between predicted and observed values, and also between the species examined here and the work of Levitan *et al.*. (1991). It is hoped that all the relevant data will be gathered shortly, and that such models will be applied successfully.

v) The role of sperm attractants requires further consideration. Chemical characterisation of the compound(s) should begin by determining the type of chemicals involved, whether a protein, lipid steroid etc. Molecular sizes could be determined by ultrafiltration, and purification could be attempted by HPLC. The sperm longevity experiments are unsuitable as a bioassay for such

compounds since they will take many hours. Further refinement of the sperm respirometry mentioned in Chapter 7 could lead to the development of a bioassay. However, best results may be obtained using established protocols of recording changes in sperm swimming behaviour when presented with an active compound, and aggregation around a point source (e.g. Miller 1985).

Further investigations into the areas suggested above would begin to answer some of the outstanding fundamental questions on the fertilization ecology of marine invertebrates.

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