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'Fertilization Kinetics in Marine Invertebrates'

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ABSTRACT

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FERTILIZATION KINETICS IN MARINE INVERTEBRATES

By Maria Clare Baker

Allee (1931) first reported that reproductive success in free-spawning marine invertebrates was dependent on population density. However, little attention was paid to the 'Allee' effect until Pennington's (1985) pivotal paper describing the consequences of sperm dilution in echinoid fertilization. This thesis is a study of the factors affecting fertilization success in five species of commercial shellfish. Commercial shellfish are particularly prone to the deleterious 'Allee' effect. These shellfish are subject to natural and anthropogenic disturbances and disease, and certain species have been severely over-exploited. Some shellfisheries have declined to such an extent that natural recovery is almost impossible (Roberts and Hawkins, 1999).

This thesis examined pre-larval effects on reproductive success in terms of fertilization kinetics. A series of laboratory experiments were conducted to determine fertilization success in the abalone, *Haliotis tuberculata*; the oyster, *Crassostrea gigas*; the clam, *Tapes decussatus*; the limpet, *Patella vulgata* and the echinoid, *Psammechinus miliaris*. For all of these species, fertilization success was found to be reliant upon a number of factors such as sperm dilution, gamete age, sperm-egg ratio, sperm-egg contact time and temperature. These data indicate that sperm limitation may severely compromise reproductive success in commercially exploited populations of all these species.

The laboratory-derived data were used with models of sperm dispersal and field experiments to determine minimum stocking densities that would be required to support successful recruitment in adult populations, in an attempt to combat the 'Allee' effect.

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For my parents, Marian and Douglas Baker
With Love

CHAPTER ONE *– GENERAL INTRODUCTION*

1 Preface

During the course of the late 19th, and 20th centuries, studies of the reproductive biology of marine invertebrates have collectively produced a vast wealth of knowledge. Aspects of study in this field have included reproductive behaviour, timing and gross morphology; fertilization processes at the molecular level; the hormonal control of reproduction; gamete physiology; developmental biology and population genetics and gene flow. A substantial amount of progress has been made over the past two decades in the field of larval ecology. Nevertheless, it would appear that there remain some areas of reproductive biology that are far less well understood. Such areas include the complex role of fertilization ecology in free-spawning marine invertebrates. Work in this area is only recently beginning to progress (Levitan, 1995). Any variability in such a fundamental process in the life-history of free-spawning species has important consequences. It is therefore surprising that fertilization success has, to a large degree, been overlooked, particularly in the field of fisheries management.

There is a central problem inherent in external fertilization. Following a spawning event, there may only be a remote chance of spermatozoa finding and then subsequently fertilizing an egg. In a turbulent sea, this problem is magnified as gametes are often rapidly dispersed and gamete viability may be short-lived. It is time and space that co-ordinate preparatory events to ensure gametes meet and fuse.

Many free-spawning invertebrates exhibit a number of mechanisms that serve to increase the probability of sperm and egg collision, the most universal of which is synchronous spawning of both sexes. Environmental factors that may trigger this synchrony include temperature, lunar phases, day length, abundance of food, tidal flux and physical shock (Giese and Kanatani, 1987). Also, the induction of spawning in one sex by the release of gametes of the other is known for a variety of free-spawning marine invertebrates (e.g. ascidians, Miller, 1982; echinoderms, Miller, 1989). Another behavioural mechanism to increase gamete contact probability is that of adult aggregation from pairs or larger groups during spawning (Mortensen, 1931; Ankel, 1936; von Medem, 1945; Komatsu, 1983; Run et al., 1988; Tyler et al., 1992; Young et al., 1992). In order to obtain a high fertilization success rate, maintenance of an adequate population density is also of paramount importance (Allee, 1931).

Successful fertilization may be affected by four major factors: attributes of (1) the gametes; (2) the individual; (3) the population; (4) the environment (Levitan, 1995). Table 1 provides a list of the individual components from each of these categories.

Table 1.1 (after Levitan, 1995)

Factors Influencing Fertilization

Gamete	**Individual**	**Population**	**Environmental**
Sperm	Behaviour	Density	Topographical
Morphology	Aggregation	Size	complexity
Behaviour	Synchrony	Distribution	Flow
Velocity	Spawning posture	Size structure	Advective velocity
Longevity	Spawning rate	Age structure	Turbulence
Egg	Morphology	Sex ratio	Water depth
Size	size		Water quality
Jelly coat	Reproductive output		Temperature
Chemotaxis	Age		Salinity
Sperm receptors	Energy allocation		pH
General			
Age			
Compatibility			

Although the possibility of limitations imposed upon fertilization have been recognised for many years, attempts to quantify these processes have only occurred in the last decade or so. Through the publication of these recent studies, the importance of fertilization success to the ultimate success of recruitment of a species is being recognised.

1.1Early Reports of Fertilization Success Limitation in the Marine Environment

Belding (1910), reporting on the scallop fishery of Massachusetts, noted the limitations in achieving successful fertilization when individuals are widely spaced as sperm chemotaxis is thought to operate over relatively short distances. Spärck (1927) commented on the likelihood that the size and density of an oyster stock influences reproductive success to some extent. Gross and Smyth (1946) re-emphasised this point in their account on declining oyster populations. They expanded on Spärck's comments and proposed that the immobility of oysters confounded the problem of successful fertilization. In 1931, Allee published a book entitled 'Animal Aggregations' in which

he recognised the importance of such aggregative behaviour in free-spawners with respect to fertilization success. He noted that a reduction in population density results in a decline in reproductive success. Mortensen (1931) suggested 'gamete wastage' as a major contributing factor to the low numbers of settling echinoid offspring compared with the number of eggs produced. This early work bought attention to the fact that successful fertilization is dependent upon spawning behaviours i.e. aggregation of a species and synchronous gamete release. Conversely, Thorson (1946) suggested that high levels of fertilization result as animals generally spawn synchronously in aggregated groups and that low juvenile recruitment results from predation on embryos and larvae. His assumption that fertilization success was generally high became accepted in subsequent literature for many years.

1.2 Laboratory Studies of Fertilization Success

1.2.1 Influence of Gamete Concentration and Sperm-Egg Ratio

Laboratory studies of fertilization success of marine invertebrates (primarily sea urchins) have aided our understanding of the processes involved in this highly complex operation. Some of this landmark work includes that of Lillie (1915) who was the first to successfully conduct quantitative studies in this field. He found that decreasing sperm concentration led to a decrease in percent fertilization success in the echinoid *Arbacia* and that fertilization is insensitive to egg concentration. Although this early work and subsequent work (e.g. Brown and Knouse, 1973; Kikuchi and Uki, 1974; Vogel et al., 1982; Pennington, 1985; Levitan et al., 1991; Clavier, 1992; Clotteau and Dube, 1993; Benzie and Dixon, 1994; Andre and Lindegarth, 1995; Babcock and Keesing, 1999; Williams, 1999; Baker and Tyler, in press) have shown influence of sperm dilution on rate and level of fertilization, controversy remains as to whether sperm dilution or sperm-egg ratio is the influential factor (see Lessios and Cunningham, 1990, 1994; McClary, 1992). Denny (1988) investigated this aspect and stated the importance of sperm-egg ratio only when gametes are at similar concentrations. Levitan et al. (1991) predicted that egg concentration of the sea urchin *Strongylocentrotus franciscanus* would significantly influence the percentage of eggs fertilized only at the point immediately following female spawning, when egg concentration is relatively high. In the seastar *Acanthaster planci*, Benzie and Dixon (1994) highlight the importance of sperm-egg ratios prior to dispersal of eggs into the water column, but conclude that the

ratios have little impact after broadcast spawning has occurred. It would appear from these studies that different species, with different gamete attributes, may result in variable importance of the sperm-egg ratio, although they support the idea that higher encounter rates of sperm to eggs increase the significance of sperm-egg ratio.

1.2.2 Gamete Age

Laboratory studies have also shown that gamete age has some bearing on fertilization success. The first work was that of Lillie (1915) who, by chance, showed a decrease in fertilization success with increasing age of sperm in the sea urchin, *Arbacia.* Lillie supposed that the individual spermatozoa in suspension tend to lose their fertilizing capacity and become ineffective despite their continued motility. Sperm from different invertebrate species have been shown to generally live < a few hours (sea urchins, Pennington 1985; Levitan et al., 1991; ascidians, Havenhand, 1991; seastars, Benzie and Dixon, 1994; cockles, Andre and Lindegarth, 1995; abalone, Babcock and Keesing, 1999; Baker and Tyler, in press). Pennington (1985) found that eggs of *Strongylocentrotus droebachiensis* lived >90 minutes compared with sperm that lived < 30 minutes. Sperm were generally thought to be much more sensitive than eggs to age effects. Levitan et al. (1991) suggested that sperm age experiments may be conducted with relative ease, as one variable may be assumed constant. Nevertheless, there are exceptions to this general theorem. For example, Andre and Lindegarth (1995) found viability of both eggs and sperm from *Cerastoderma edule* declined with age with no fertilization occurring after 4 to 8 hours. The egg and sperm lifespans were similar to that found for other invertebrates (e.g. Yund, 1990; Oliver and Babcock, 1992; Benzie and Dixon, 1994; Levy and Couturier, 1996; Encena et al., 1998; Babcock and Keesing, 1999; Baker and Tyler, in press). Much longer-lived sperm and eggs have been found in some species, namely *Arenicola marina, Nereis virens, Asterias rubens* (Williams, 1999); *Ascidia mentula* (Havenhand, 1991); *Limulus polyphemus* (Brown and Knouse, 1973); *Nacella concinna* (Powell, in press).

Table 2 gives an overview of gamete longevity in a number of different free-spawning invertebrate phyla.

Table 1.2

Gamete Longevity

Species	*Max. sperm longevity*	*Sperm concentration*	*Max. egg longevity*	*Authors (in publication date order)*
Limulus polyphemus (horseshoe crab)	96 h +	10%	not tested	Brown & Knouse (1973)
Haliotis rufescens (gastropod)	~1 h	not stated	~6 h	Ebert & Hamilton (1983)
Strongylocentrotus droebachiensis (sea urchin)	0.3 h	10^6 sperm.ml^{-1}	1.5 h +	Pennington (1985)
Hydractinia echinata (hydroid)	4 h	not stated	not tested	Yund (1990)
Strongylocentrotus franciscanus (sea urchin)	2.5 h	10^6 sperm.ml^{-1}	not tested	Levitan et al. (1991)
Ascidia mentula (ascidian)	48 h	not stated	144 h	Havenhand (1991)
Platygyra sinensis (coral)	~5 h	10^5 sperm.ml^{-1}	not tested	Oliver & Babcock (1992)
Acanthaster planci (sea star)	7 h +	10^5 sperm.ml^{-1}	7 h +	Benzie & Dixon (1994)
Cerastoderma edule (bivalve)	4 – 8 h	10^5 sperm.ml^{-1}	4 - 8 h	André & Lindegarth (1995)
Mytilus edulis (bivalve)	5 h +	10^6 sperm.ml^{-1}	not tested	Levy & Couturier (1996)
Haliotis asinina (gastropod)	5 h	10^5 sperm.ml^{-1}	not tested	Encena et al. (1998)
Haliotis laevigata (gastropod)	4 h +	10^6 sperm.ml^{-1}	7 h +	Babcock & Keesing (1999)
Arenicola marina (polychaete)	~85 h	2.5 x 10^5 sperm.ml^{-1}	120 h	Williams (1999)
Nereis virens (polychaete)	~24 h	2.5 x 10^5 sperm.ml^{-1}	72 h	Williams (1999)
Asterias rubens (sea star)	24 h	2.5 x 10^5 sperm.ml^{-1}	24 h	Williams (1999)
Nacella concinna (gastropod)	70 h	3.6 x 10^6 sperm.ml^{-1}	not tested	Powell et al. (in press)
Haliotis tuberculata (gastropod)	2.5 h	10^6 sperm.ml^{-1}	~ 2.5 h	Baker & Tyler (in press)

The concentration of sperm and its effect upon sperm longevity was first reported by Gemmill (1900) and again by Lillie (1915). Gemmill (1900) supposed that the greater activity of the spermatozoa, and consequent earlier exhaustion in the more dilute suspensions to be responsible, together with the dilution of the spermatic fluid by which he thought spermatozoa were nourished. Later termed "respiratory dilution effect" (reviewed by Chia and Bickell, 1983), it was observed that the concentration of sperm influences its longevity as the amount of oxygen fixed by a spermatozoa over its lifetime is set, and the rate of fixation is increased in dilute sperm. A spermatozoon in a concentrated medium can maintain a slower metabolic activity level over a longer life span than a spermatozoon in a dilute medium. The longevity of sea urchin sperm is extended when sperm are stored at high concentrations (Pennington, 1985). Under conditions of low flow rates, the importance of the 'respiratory dilution effect' may come into effect as, in nature, we are more likely to find highly concentrated sperm clouds in areas of little or no flow. In the natural environment where broadcast spawning occurs, turbulence acts to dilute quickly the sperm cloud, rendering sperm longevity relatively unimportant in fertilization as sperm will be diluted to below levels required for successful fertilization. Nevertheless, when conducting laboratory-based experimentation, sperm age and concentration becomes of paramount importance and may have substantial effects upon results.

Bolton and Havenhand (1996) examined the chemical mediation of sperm activity and longevity in the solitary ascidians *Ciona intestinalis* and *Ascidiella aspersa*. Previous authors have shown that increases in both sperm activity and respiration are characteristic of the chemotactic response of sea urchin sperm (Ward et al., 1985; Suzuki and Garbers, 1984). Miller (1982) observed that sperm chemotaxis is widespread amongst solitary ascidians. Bolton and Havenhand (1996) hypothesised that sperm longevity may be reduced as a result of depleting energy reserves by the induction of increased sperm activity with exposure to compounds associated with the egg. They proposed that the activation of non-motile sperm by compounds associated with the eggs may provide a mechanism by which the energy reserves of a sperm can be conserved in the absence of eggs, thereby maintaining sperm viability for extended periods. They discussed the possible important ecological implications of this function in the sense of enhancement of fertilization success and organism dispersal in ascidians in highly populated, low energy environments. They pointed out that until such work

has been conducted on other organisms in different environmental conditions, their stated conclusions are speculative only.

1.2.3 Sperm-Egg Contact Time

Rothschild and Swann (1951) demonstrated that the amount of time sperm and egg were in contact had some bearing upon fertilization success in the sea urchin *Psammechinus miliaris*. Their findings were substantiated by Levitan et al. (1991) who found that the amount of time an egg spent at a particular sperm concentration had a significant effect on fertilization success in *Strongylocentrotus franciscanus*, particularly within small time intervals, which are most likely to be important in the natural environment. They suggest that this factor should be incorporated into fertilization models in the future. As sperm and egg plumes expand into the water column, the rate of dilution influences both the concentration of sperm and eggs and the time spent at a particular concentration. In their multi-factorial experiments, sperm concentration had the greatest effect on fertilization with sperm-egg contact time and sperm age being the next two most important factors. Williams (1999) noted a significant effect of sperm-egg contact time upon fertilization success in the polychaete annelid *Nereis virens*, the seastar *Asterias rubens* and the sea urchin *Echinus esculentus*.

1.2.4 Influence of Egg Size and Egg Quality on Fertilization Success

Consideration must be given to both egg size and quality and their effects on fertilization success. Effective egg size may be a function of the egg itself, a jelly coat, accessory cell attachment and chemical attractants. Exploration of the influence of effective egg size on fertilization success has been carried and it was reported that fertilization rates increased proportionally with an increase in cross-sectional area of eggs of ascidians via follicle cells. Jelly coats were removed from eggs of the echinoid *Dendraster excentricus* and it was found that this decreased the likelihood of fertilization in proportion to target egg size. (reviewed by Levitan, 1995). The correlation of larger egg sizes with higher fertilization rates has been documented among *Strongylocentrotus* species (Levitan, 1993; 1998). In his work, Levitan suggests an evolution hypothesis of trade-off between egg size, fecundity and fertilization potential, although some of his conclusions were disputed (see section *1.4* for further discussion). A recent study has also shown increases in fertilization success in larger eggs of larger

females compared with the smaller eggs of smaller females of the intertidal ascidian *Pyura stolonifera*. When sperm is limited, the relative individual sizes of females in a population could have some bearing upon the success of fertilization and hence recruitment in low density populations of this species.

Sperm chemotaxis has been demonstrated in species from four marine invertebrate phyla: Cnidaria (Miller, 1966, 1978, 1979); Mollusca (Miller, 1977); Urochordata (Miller, 1975); and Echinodermata (Miller, 1985; Ward et al., 1985). The adaptive significance of sperm chemotaxis is believed to lie in the increased probability of gamete contact, even when gamete concentration is low (Miller, 1982). Sperm chemotaxis acts only at a short distance of ~100-200μm, but effectively increases the target size of the egg, thereby increasing the likelihood of collision and hence of possible fertilization. To date, no experiments have been conducted on the effectiveness of chemotaxis in the field.

Laboratory measurements suggest that sea urchin eggs behave as if only a small fraction of their surface area is receptive to fertilization. The reason for this reduced cross section remains unclear. Vogel et al. (1982) suggest that the apparent fertilizable area of an egg is only about 1% of the overall area for *Paracentrotus lividus*. More recent experiments with *Strongylocentrotus franciscanus* (Levitan et al., 1991) suggest an apparent fraction of 3%. Similar studies on any other free-spawning marine invertebrates are not apparent in the literature. However, a study on the freshwater bivalve *Unio elongatulus* has shown that sperm entry is restricted to a small crater at the vegetal pole of the egg (Focarelli et al. 1988). Also, eggs of the (algal) broadcast spawner *Fucus serratus* have been shown to have 2.5 x 10^9 receptor molecules per egg (Evans et al. 1980). It could be that this limited fertilizable surface area on eggs is common to many species or it may be a phylogenetic characteristic. Observations of the eggs of the Californian abalone, *Haliotis rufescens*, suggest that the entire surface of the vitelline envelope can participate in sperm attachment (Vacquier, pers. comm.). Babcock and Keesing (1999) used Vogel's model (1992) to predict the percentage of egg surface area available for fertilization in *Haliotis laevigata* and calculated 1 % available.

It may be seen that effective egg size in any form appears to have an effect on fertilization success but the magnitude of their individual and combined effects under various conditions need attention. Egg characteristics are of fundamental importance in

our understanding of gamete evolution in those animals that display a free-spawning reproductive strategy (for discussion see Levitan, 1992; Podolsky and Strathmann, 1996; Levitan, 1996; 1998; Styan, 1998).

Hultin and Hagström (1956) experimented on fertilization rates on the sea urchin *Paracentrotus lividus* and found substantial variability in fertilization rates of individual eggs from both the same female and different individuals sampled at the same and different space and time intervals. They concluded that variability in egg quality makes it necessary to check the fertilization rate of eggs intended to be used for investigations in which material of a high uniformity is desirable. This potential variability in fertilization was suggested to be a function of one or more of: genotype, maturation, size or disease (Hultin and Hagström, 1956).

1.2.5 Sperm Velocity

The swimming speeds of sperm are typically ~0.1mm s^{-1} (Denny and Shibata 1989). Levitan et al. (1991) noted the variation in sperm swimming speed within and among individuals (0.05 to 0.30 mm s^{-1}) in *Strongylocentrotus franciscanus* and in 1993 he noted that mean sperm velocity varied between 0.088 and 0.145 mm s^{-1} among the three *Strongylocentrotus* species tested. Vogel et al. (1982) estimated average speed of sea urchin sperm to be 0.04mm s^{-1}. Gray (1955) had already looked at a range of sperm velocities from sea urchin species (0.12 to 0.19 mm s^{-1}). In turbulent environments however, the sperm velocity factor is negligible with respect to fertilization success. Nevertheless, it could be that short bursts of sperm activity in close proximity to an egg are important. Knowledge of sperm velocity is also useful in determining the percentage fertilizable area of an egg surface using Vogel's model (1992).

A recent study by Levitan (2000) is the first to present direct evidence of how sperm velocity can influence fertilization success. A 0.1 mm s^{-1} decrease in velocity is correlated with an order of magnitude increase in the number of sperm needed to achieve 50% fertilization in the sea urchin *Lytechinus variegatus*. In addition to variable sperm velocity, Levitan also describes the inverse relationship of this trait with that of sperm longevity. He suggests that this relationship may be an adaptation for varying environmental conditions, to optimise fertilization success along a continuum from sperm limitation to sperm competition.

1.2.6 Temperature and Salinity Effects on Fertilization Success

Temperatures above and below ambient have been shown to result in reduced fertilization with associated increases in polyspermy and abnormal cleavages in many free-spawning marine invertebrates (Loosanoff, 1937; Hagstrom and Hagstrom, 1959; Lonning, 1959; Moore, 1959; Davis and Calabrese, 1964; Calabrese, 1969; Kinne, 1970; Rupp, 1973; Kingston, 1974; Andronikov, 1975; Greenwood and Bennett, 1981; Clotteau and Dube, 1993; Davenport, 1995; Powell, in prep.) For example, Rupp (1973) studied effects of temperature upon fertilization and early cleavage of 5 tropical echinoderms. Reduced fertilization success and abnormal early cleavage were noted, with cleavage being more sensitive to increased temperature than fertilization. Greenwood and Bennett (1981) studied the effects of variations in temperature-salinity combinations on fertilization success of the sea urchin *Parechinus angulosus*. The purpose of their study was to assess possible effects of the construction of a nuclear power station on the west coast of South Africa. Knowledge was required of the effects of warm cooling-water effluent on the ecology of a naturally cold-temperature system. They showed that fertilization is reduced to zero at a temperature of 25°C, some 10°C above normal (an expected increase with discharge from the power station). Decreased sperm viability was the primary cause of fertilization failure. Conversely, the eggs of this species were more susceptible to salinity fluctuations. A wide range of temperatures from 6 to 24°C and salinities ranging from 20 to 35 had no detrimental effect upon fertilization in the surf clam *Spisula solidissima* (Clotteau and Dube, 1993), although optimum fertilization occurred in conditions of 8 to 20°C and salinities of 23 to 30. Powell (in prep.) conducted experiments to identify the effects of temperature and salinity on the fertilization success of the Antarctic clam, *Laternula eliptica* and the limpet *Nacella concinna*. She found optimum conditions for fertilization lay at around 0°C and salinity 33 for *L. eliptica* and 2°C and salinity 33 for *N. concinna*.

1.2.7 Effects of Anthropogenic Pollutants on Fertilization Success

Connell et al. (1991) showed effects of titanium dioxide waste on fertilization of the sea urchin *Echinometra mathaei*. Iron hydroxide caused clumping of the sperm, which led to inhibition of fertilization, with deleterious implications for external fertilizers exposed to such pollutants.

Krause (1994) studied sub-lethal responses of *Strongylocentrotus purpuratus* subjected to chronic discharges from a point source of oil production effluent. During this discharge, an *in-situ* caging experiment was performed on animals. Cages, each with 25 animals, were placed in the field at distances from 5 to 1000m away from the source for a period of 8 weeks. After this time, examination of gametogenesis showed a significant negative relationship between relative gonad mass and distance from outfall for both sexes, indicating that those urchins closer to the outfall produced significantly larger gonads. Gamete performance was assessed via a fertilization kinetics bioassay that held the egg concentration constant and varied the sperm concentration. Fertilization success was determined for each scenario and fitted to kinetics models. Results showed significant differences in fertilizability of eggs between cages, and egg fertilization success showed a positive relationship with distance away from the outfall.

Riveros et al. (1996) examined relationships between fertilization of the Pacific sea urchin *Arbacia spatuligera* and environmental variables in polluted coastal waters off the coast of Chile. They measured temperature, salinity, pH, turbidity and O_2 *in-situ*. They also determined concentrations of heavy metals (copper, cadmium and mercury) and organic compounds (oil and grease, lindare, aldrin, dieldrin, DDT, DDE and phenols). They used a bioassay procedure to determine fertilization success under different toxicity levels, and applied multivariate analysis (PCA) over environmental factors, heavy metals and organic compounds. Riveros et al. (1996) concluded that the coastal waters containing the highest levels of oil and grease and the lowest levels of oxygen concentrations, had a significantly detrimental effect on the fertilization success of *A. spatuligera* eggs. A number of other studies have also examined the effects of pollutants on reproduction of free-spawners (e.g. Au et al., 2001a,b).

1.3 Theoretical Modelling of Fertilization Rates

Systematic experiments on fertilization kinetics were first performed by Rothschild and Swann (1951). They estimated fertilization rates in the echinoid *Psammechinus miliaris* and used an equation for sperm-egg contact modelled on random gas molecule collision:

$$Z = \upsilon \sigma_0 S$$

where Z is sperm collisions per unit time, υ is the average sperm velocity, σ_0 is the cross-sectional area of the egg and S is the sperm concentration. On average 1/Z is the time in seconds for a spermatozoan to hit an egg (T). The proportion of all sperm-egg collisions that led to successful fertilization was predicted to be:

$$p = 1 - (T\,\alpha)$$

where α is the slope of the $\log_{10}$-transformed portion of unfertilized eggs as a function of time.

Rothschild and Swann found a *decline* in probability of successful sperm-egg collision with increasing sperm density, above 7 x 10^4 sperm ml^{-1} (by contrast to Lillie 1915; Brown and Knouse 1973, Levitan et al., 1991) and with the removal of the jelly coat surrounding the egg. Rothschild and Swann thought the inverse relationship of collision and density a function of an increase in sperm-sperm interaction and the jelly coat acting as a sperm trap. They also recognised that these jelly coats may act to increase the effective egg diameter (and thus the collision frequency) and that sperm chemotaxis plays some role in attracting the eggs. They discounted the earlier suggestion that egg jelly has an adverse effect on sperm fertilization capability. They found that fertilization is sensitive to the time that eggs spend in a sperm solution, and also noted that sperm velocity does not vary with sperm density. In their paper, Rothschild and Swann describe in detail potential sources of error associated with their experimental practices. One such error was that the proportion of live and dead spermatozoa in a suspension is undetermined. Hancock (1951) used at staining technique to determine a quantitative estimate of live and dead spermatozoa in a sample of bull semen.

Vogel et al. (1982) discuss the work of Rothschild and Swann (1951) and argue that their sperm inactivation methodology may have introduced an artefact to the evaluation procedure in that sperm viability decreases over time which was not accounted for as sperm were inactivated well before the end of their natural life span. They also point out how spermatozoa behaviour differs from that of gas molecules and re-define this model. In 1982, Vogel et al. developed their predictive model of fertilization rates. They made the assumption that sperm attach to the first egg they

contact, whether or not they fertilize it. The result is independent of sperm concentration, unlike that of Rothschild and Swann (1951). Vogel et al. (1982) predicted the proportion of eggs fertilized as:

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

The model incorporates concentration of virgin sperm (S_0, sperm μl^{-1}) and eggs (E_0, eggs μl^{-1}), sperm half life (τ, sec), and two rate constants, β_0 and β.

This model predicts that the proportion of eggs fertilized will increase with sperm concentration (which, it should be noted, conflicts with Rothschild and Swann, 1951 results), sperm velocity, sperm longevity (or the time that eggs are in contact with a sperm suspension), and egg size. Percent fertilization will decrease with increasing egg concentration. In their paper, Vogel et al. (1982) also express their thoughts on chemotaxis and argue against the possibility of chemotactic attraction in sea urchin fertilization. Their model was validated by both Vogel et al. (1982) on *Paracentrotus lividus* and by Levitan et al. (1991) on *Strongylocentrotus franciscanus*. Both studies found a good correlation between predicted and empirical results, suggesting that sperm velocity and egg size influence fertilization rates. Levitan (1993) used Vogel's model on three co-occurring species of *Strongylocentrotus* and the predicted outcome fitted well to the real data. In addition to this, Levitan observed an inverse relationship between egg size and the amount of sperm required to ensure fertilization (unpub.). This study also highlighted an inverse relationship between egg size and sperm velocity and between sperm velocity and sperm longevity. Levitan suggested the latter may be similar to the respiratory dilution effect in that some species may trade an increase in velocity for a decrease in longevity or vice versa. Levitan (1993) put forward the fertilization success hypothesis as an alternative to the fecundity-time hypothesis, proposing that larger egg sizes have evolved as a function of sperm limitation bought about by environmental influences on adult life-history. Laboratory experiments indicate that larger eggs are more likely to be fertilized at a given concentration of sperm. As sperm availability decreases, selection should favour increase in egg size. High sperm availability would select for smaller eggs and also faster sperm because sperm competition would be more likely. When sperm are less abundant, increased sperm

longevity would increase the survival chance of a sperm until it encounters an egg. Levitan's findings supported this hypothesis, finding the three *Strongylocentrotus* species in predicted environmental conditions with respect to their egg size and sperm velocity and longevity. Eckelbarger et al.(1989) support these observations with their own observations of long-lived sperm and relatively large eggs in deep-sea echinoids where environmental conditions would select for these attributes. Podolsky and Strathmann (1996) showed concern about Levitan's predictions of egg size as a function of sperm limitation in free-spawning marine invertebrates. They suggested that in animals where sperm may be limited, a more energy-efficient way of increasing egg size would be by means of chemical attractants, jelly coats or accessory cells or by increasing the number of sperm receptor sites. Styan (1998) put forward a further possible explanation for their quandary. He suggested that larger eggs resulting in an increased rate of sperm-egg collisions can result in increased rates of polyspermy. In nature, eggs are exposed to a wide range of sperm concentrations depending upon local hydrodynamic conditions and avoiding polyspermy may be as important as sperm limitation. Styan suggested that Vogel's model should incorporate polyspermy and he presented a new polyspermy-adjusted model to account for the decrease in fertilization success at high sperm concentrations noted for some species.

1.4 Field Studies of Fertilization Success

1.4.1 Models of Turbulence Effects on Gamete Dispersal

Studies of fertilization success in the laboratory are relatively easy to control. Field studies, on the other hand, are not. The complexity of fluid dynamics in any given water mass make reliable *in-situ* experimentation extremely difficult to achieve. In an attempt to understand fluid flow and its relationship with gamete advection and diffusion, Csanady (1973) developed a series of predictive particle diffusion equations for a turbulent fluid environment. These equations were modified by Denny (1988) for the prediction of gamete concentration as a function of either distance or time from a release point. Further modification of these equations by Babcock et al. (1994) incorporated sperm reflection off the bottom and off the surface of the water, important in shallow water environments. The dispersion coefficient values used in the equations are currently being estimated by studying the spread of dye clouds in order to gain more accurate predictions (reviewed by Levitan 1995).

Denny and Shibata (1989) used theoretical models to predict the effectiveness of external fertilization in turbulent benthic boundary layers of the surf and shallow subtidal zones. Assumptions they made included:

- in turbulent flow, sperm motility is negligible
- males and females spawn synchronously
- each sperm attaches permanently to the first egg is contacts
- the rate of contact is in direct proportion to the product of the concentrations of sperm and ova (as in Vogel et al., 1982).

Given these assumptions, they calculated the % of eggs fertilized as a function of time, mean current velocity, sperm release rate, dispersion coefficients, proximity of female to male and the number of males upstream of a female. They applied their model to data gathered from the sea urchin *Strongylocentrotus purpuratus* and concluded that fertilization was affected by all parameter values and that fertilization on wave-swept shores is likely to be very low (>0.1%). The authors state "the assumptions of the model are generally conservative, in that they are likely to over-predict the fraction of ova fertilized", and contrast this statement with two possibilities of under estimation: (1) the possibility of over-estimated surf zone turbulence, and (2) slow water exchange in surge channels resulting in local accumulation of sperm. They compared results from their theoretical models with empirical data collected by Pennington (1985) and found they corresponded well with his findings of *in-situ* fertilization success in sea-urchins.

Levitan (1995) noted that the estimated figure of >0.1% fertilization success by Denny and Shibata (1989) is likely to be conservative because values used for dispersion coefficients were low. Levitan also noted that their suggestion that, under these conditions, increasing the number of spawning males would have little influence on fertilization success is, in part, a result of only modelling sperm plumes passing over spawning females. Denny and Shibata (1989) suggested that only the nearest male would substantially influence fertilization. Levitan and Young (1995) disputed this point and noted that when eggs drift, an increased number of males would increase the probability of an egg passing directly over a spawning male, thereby resulting in an increase in fertilization.

Young et al. (1992) input laboratory data from serial sperm dilutions from the deep-sea echinoid *Stylocidaris lineata* and estimates of dye advection into Denny's (1988) models. They predicted near 100% fertilization success at a distance of 5m downstream from the sperm source. This high success rate was a function of low turbulence at this deep-sea site.

Following on from their earlier work, Denny et al. (1992) published data on the use of their model in high-energy surge channels. Although surge channels are well mixed, they have a slow water exchange rate with the adjacent mainstream. These environments act to contain any spawned gametes, thereby limiting their dilution and hence, enhancing the efficiency in external fertilizing organisms. By following the model of Denny and Shibata (1989), based primarily on sea urchin data, they suggest that between 80-100% of eggs may be fertilized provided a sufficient population of adult males are present within the channel. They conclude that on a shoreline where surge channels are abundant, external fertilization may be far more effective than on shores lacking surge channels. They point out two possible limitations in their theory. Firstly, the model assumes that the gametes are instantaneously mixed throughout a surge channel. Secondly, the assumptions of gamete behaviour ignore the small-scale hydrodynamics relevant to sperm and eggs and may result in the overestimation of the probability of fertilization. They calculated that gametes in the surf zone experience a flow regime characterised primarily by linear shears rather than by turbulent eddies. This causes a typical urchin egg to rotate on average 104 times per second in the surf zone. It is unclear whether this rotation has an effect upon sperm-egg contact, on dislodging of sperm from the egg, or imparting damage to the gametes. They point out that their calculations may have grossly overestimated fertilization effectiveness in surge channels should the effects of shear stress and egg rotation have detrimental effects to any of the processes involved.

Subsequently, Mead and Denny (1995) used a couette cell to identify the interference of small-scale hydrodynamic conditions with sperm-egg interactions regardless of the concentration of sperm. Turbulence-induced shear stresses of the magnitude found in the surf zone were found to limit fertilization (probably by diminishing sperm-egg contact time and encounter rate) and to increase the likelihood of abnormal early development.

Benzie et al. (1994) used dispersal models to assess the small-scale dispersion of eggs and sperm of the asteroid *Acanthaster planci* in a shallow coral reef habitat. They quantified the relationship between current flows, turbulence and boundary conditions and the dispersion of gametes in the field. By doing this they were able to demonstrate the importance of hydrodynamics in the recruitment process of *A. planci*. They describe a mechanism for self-recruitment - entrapment of gametes in the substratum - as being of importance in the onset of outbreaks of these seastars.

Fertilization models make the assumption that gametes are released into the surrounding medium in a cloud of discrete particles. Thomas (1994a;b) has raised awareness of the potential underestimation of fertilization success by using the existing theoretical models. She determined the viscosity of spawned material for species of polychaete and sea urchin and found that species in more turbulent environments tend to display more viscous spawned material. In habitats where mainstream velocities fall below 0.13m s^{-1}, urchin gametes form large clumps or strings resulting from inter-gamete stickiness and/or viscosity of their surrounding material. Sea urchin gametes were found to be negatively buoyant and have lower dispersion rates than that of freely diffusing fluorescein dye, which is regularly used in determining dispersion rates for use in the models. Thomas (1994a) adds that gamete clumps may sink and become trapped in areas between roughness elements, increasing the possibility of fertilization. Her results add a further element of complexity to gamete mixing models and indicate that gametes from some species may have physical properties that minimise rapid rates of dilution by turbulence. Ignoring such factors as viscosity and buoyancy of gametes, local topography and adult morphology that could trap released gametes, could lead to an underestimation of fertilization success using current predictive modelling techniques. However, the plume model may be accurate for urchins under most natural flow conditions (which are greater than 0.13 ms^{-1}), but the above mentioned factors should be assessed in order to gain a more accurate prediction. Current models, for example, would be inadequate in the prediction of fertilization success in the polychaete *Phragmatopoma californica* as they tend to release gametes in discrete clumps and not in constant plumes (Thomas, 1994b).

Most empirical data collection and theoretical models of reproductive success have been based upon small population sizes and over small spatial scales. Although these studies have demonstrated that fertilization success increases with population size

and degree of aggregation, extrapolation of these results to predict what happens in larger populations is difficult because of the sensitivity of the data to small changes in population size (Levitan et al., 1992). Nevertheless, Levitan and Young (1995) have attempted to develop a predictive theoretical modelling approach for large-scale spawning events in a natural population of the echinoid *Clypeaster rosaceus* under moderate flow conditions. They adapted models of Denny (1988); Denny and Shibata (1989) and Babcock et al. (1994) to estimate transport, diffusion and concentration of released gametes. They used the gamete kinetics model of Vogel et al. (1982) to estimate the proportion of eggs fertilized in the field. Empirical data from both the field and the laboratory were obtained for incorporation into these models. The results emphasised the importance of population size and population density upon fertilization over a large range of individuals (2 to >250,000). A relationship between population size and density was also noted, with the importance of population density increasing with decreasing population size. Levitan and Young (1995) proposed this finding as a possible reason for not always seeing aggregative behaviour among spawning individuals in the natural environment. They suggested that at high population sizes, the benefits of aggregation to fertilization success will decline as the costs increase. Their simulation data suggested that fertilization success was limited by the availability of sperm unless population size was in the hundreds of thousands.

Most studies of fertilization success have been based on data acquired from sea urchins. Andre and Lindegarth (1995) assessed the fertilization efficiency and gamete viability of the free-spawning cockle, *Cerastoderma edule* in laboratory experimentation. In keeping with observations on sea urchins, fertilization success declined markedly with decreasing sperm concentration. Gamete viability decreased with age, with no fertilization occurring after 4 to 8 hours. Empirical data on dilution and dispersion of gametes, in the form of egg sinking velocity and gamete release rates, were used in conjunction with the diffusion model of Denny (1988). Overall, it appeared that their results indicate a relatively low fertilization efficiency for *Cerastoderma edule* when compared to that of other bivalve species. For example, 50% of eggs of *C. edule* were fertilized when exposed to sperm concentrations of 5×10^2 μl^{-1} compared to *Mytilus edulis* where 50% of eggs were fertilized at sperm concentrations of only 10^0 - 10^1 μl^{-1} (Sprung and Bayne, 1984). However, there could have been a slight underestimation in gamete concentration experiments (Andre and Lindegarth, 1995).

Andre and Lindegarth (1995) constructed a simple model which shows that under certain circumstances, high concentrations of sperm may accumulate over dense populations of bivalves, thereby enhancing fertilization success.

1.4.2 Observations of Natural Spawning Events

Observations of natural spawning events in marine broadcast spawning invertebrates are rare. Nevertheless, a number of surveys of fertilization in the field have been conducted which highlight the variability of success and of spawning behaviours in a number of different species (see e.g. Pearse et al., 1988; Brazeau and Lasker, 1992; Sewell and Levitan, 1992; Sewell, 1994; Oliver and Babcock, 1992; Babcock and Mundy, 1992; Babcock et al., 1992; Minchin, 1992; Petersen 1991a; Petersen et al., 1992; Fujiwara et al.,1998).

1.4.3 In-situ Experimentation

Pennington (1985) was the first to study fertilization success in the field. This work determined percent fertilization of eggs of the echinoid *Strongylocentrotus droebachiensis* both in laboratory and field studies. He confirmed the reduction in fertilization capability of the sperm of *Strongylocentrotus droebachiensis* with dilution and with increased age in the laboratory. From this he inferred that sperm dilution effects were largely responsible for the declining fertilization success he observed in field experiments in which he increased the distance between eggs and spawning males. Pennington's work gained support from other workers on sea urchins (Levitan, 1991; Levitan et al., 1992) and also in hydroids (Yund, 1990) and ascidians (Grosberg, 1991).

Brazeau and Lasker (1992) studied reproductive success in the Caribbean octocoral *Briareum asbestinum* and concluded that simple counts of mature eggs present within female colonies prior to the reproductive season provide a poor estimate of reproductive success in this species. Through a series of field experiments they found that female reproductive success was enhanced by the close proximity of males, but that it was generally low in the region examined. Oliver and Babcock (1992) also measured *in-situ* fertilization of broadcast spawning corals and combined results with those from laboratory sperm dilution experiments. They determined that gamete dilution played an important role in limiting the fertilization of gorgonian eggs in the field during natural spawning events. The authors suggested that these gorgonians were under selective

pressure to minimise effects of gamete dilution by adopting synchronous spawning behaviour, by spawning buoyant gamete bundles and by spawning in periods of slack water. Coma and Lasker (1997) ran a set of *in-situ* experiments to investigate small-scale heterogeneity of fertilization success in the gorgonian, *Pseudoplexaura porosa*. They found a large degree of variance in fertilization potential of eggs in water samples collected within seconds and centimetres of each other.

Yund (1990) developed field experiments to study fertilization success in the colonial hydrozoan *Hydractinia echinata*. He found the fertilization rate to be high within 3m of a sperm source but this declined rapidly with no evidence of fertilization beyond 7m. Compared to Pennington's (1985) observations on sea urchins, the sperm dispersal distance is greater and probably a function of reduced sperm dilution in the absence of turbulence at low flow velocities. However, Yund (1990) also suggested differences seen may be related to the differences in reproductive biology of the two species. Viability of sperm of the hydroid greatly exceeded that of the sea urchin (several hours and <30mins respectively). Sperm release rates were also very different between species. Yund (1990) pointed out that Pennington's (1985) results are measurements of instantaneous fertilization rates at each distance rather than the total level of fertilization that would occur if eggs at a given distance from the male experienced sperm over the total time of release. Consequently, actual fertilization rates for urchins in the field may vary as a function of the relative movement patterns of eggs and sperm.

Other field experiments on sea urchins have been conducted. For example, Levitan (1991) studied sperm dilution in the field using *Diadema antillarum*. Fertilization decreased with distance downstream of a sperm source, in a similar way to Pennington's (1985) investigations. In these particular experiments, flow velocities were low ($<0.01 m\ s^{-1}$) and variation in these slow current speeds did not have any significant affect on fertilization. Further experimentation by Levitan (1991) suggested that the distribution of males may have a greater influence on enhancement of fertilization rates than the amount of sperm released by each male. Small individuals with low gamete production but at high population density may have similar *per capita* zygote production as large individuals with greater gamete production at low population density. This emphasises the probable inaccuracy in estimating reproductive output via body size or gamete production without accounting for fertilization success.

Continuing his work with sea urchins, Levitan et al. (1992) assessed the importance of population density upon fertilization in *Strongylocentrotus franciscanus.* They manipulated both population size (4 to 16 individuals) and levels of aggregation (0.5 to 2m of even spacing between individuals). Fertilization increased both with increasing population size and with degree of aggregation. This finding demonstrated the sensitivity of fertilization success to relatively small changes in population parameters. Levitan's (1991) data suggested that as previous knowledge of fertilization success is based upon small population sizes (a function of logistics), sperm limitation may not be as common as once thought in natural larger spawning populations (Levitan and Young, 1995).

In-situ experimentation by Babcock and Mundy (1992) on fertilization kinetics of the Crown of Thorns sea star *Acanthaster planci* showed high levels of fertilization from the sperm of one male even with substantial distance from the sperm source. Forty-seven % fertilization was found in animals separated by 32m and 23% for animals greater than 60m apart. These high levels were, at the time, attributed to the large volume of sperm released by *Acanthaster planci* compared with other marine organisms that showed much lower or non-existent fertilization rates with such distances. However, an increase in the background sperm concentration during experimentation stimulated by surrounding spawning males could have had some impact on the high levels seen. Nevertheless, Babcock et al. (1994) combined fresh empirical data and theoretical models to successfully confirm the long-distance fertilization seen in *Acanthaster planci.* Using five males for the sperm source, an average of >20% fertilization was found 100m from the source. Now that they had confirmed their assumptions, they fitted data from other marine organisms to their models and their findings matched previous empirical conclusions. These authors concluded that high fertilization levels at great distances in *A. planci* are a function of its large body size and high sperm production. This was backed up by fertilization success results from smaller *A. planci* which yielded much reduced success with distance compared to their larger counterparts. Benzie et al. (1994) suggested that *A. planci* required less sperm than other species (i.e. sea urchins) to achieve the same levels of fertilization - their sperm have higher fertilizing capacity.

Lasker et al. (1996) determined *in-situ* rates of fertilization of the Caribbean gorgonian corals *Plexaura kuna* and *Pseudoplexaura porosa.* More recently, Coma and

Lasker (1997) have detailed the effects of spatial distribution and reproductive biology on *in-situ* fertilization rates of *P. porosa*. They found that a weight average of *in-situ* fertilization rates suggested that at least 67% of spawned eggs are fertilized in nature. During highly synchronous spawning events when most of the eggs were extruded into the water column, sperm limitation did not occur. Their results indicated that in dense populations, eggs may have multiple opportunities to be fertilized. The life-history traits displayed by *P. porosa* act to reduce the effects of gamete dilution during spawning events and hence decrease the importance of sperm limitation in their population dynamics. Lasker and Kapela (1997) examined effects of heterogeneous water flow over time and space upon gamete mixing and transport in gorgonian corals. They concluded that the turbulent diffusion of gametes during spawning events occurs on a scale of meters and tens of seconds and acted to reduce fertilization success. Conversely, eddies on scales of tens of meters and minutes, created by the interaction of currents and reef structures, retarded the transport of gametes off the reef and probably enhanced fertilization success. These processes account for the high levels of variance seen in gorgonian egg fertilization.

Although field studies of fertilization success in external fertilizers have mainly been concerned with epifaunal organisms, Williams et al. (1997) developed methodologies to investigate fertilization success in the burrow-dwelling polychaete, *Arenicola marina*. They found fertilization success in this species to be in the order of 0 - 80% with typical values of 40-60%. This was consistent with results obtained in laboratory assays at a sperm concentration of between 10^4 - 10^5 sperm ml^{-1} which showed values of 30-70% fertilization success.

1.5 Estimates of Fertilization Success and their Role in Shellfish Management

Over-fishing of commercial shellfish stocks has become a major problem over the last two decades. There are indications that over-exploitation of some commercial species has led to their extinction. For example, the white abalone, *Haliotis sorenseni*, is in danger of extinction throughout a significant portion of its range and endangered species status was proposed in May 2000 (Roberts and Hawkins, 1999; National Marine Fisheries Service and the National Oceanic and Atmospheric Administration, USA, 2000). Recent surveys undertaken at Scripps Oceanographic Institution indicate that *H. fulgens* and *H. cracherodii* may also follow the same pathway. With the decrease in the

harvest from shellfisheries, the price of shellfish has risen markedly resulting in even greater pressure on the already depleted stocks. One of the main consequences of over-exploitation of fisheries is a reduction in density of stocks. Allee (1931) showed that a reduction in population density resulted in a disproportionately low recruitment rate. Subsequently, the decreased fertilization success owing to density reduction has been referred to as the *Allee effect*. If the population density remains too low in an over-exploited population, the individuals present will continue to spawn but will not achieve fertilization and, as a result, will not contribute to the next generation.

Knowledge of the biology of exploited shellfish is of paramount importance when devising effective management procedures. One important but often ignored aspect if reproductive biology of relevance to stock enhancement is that of fertilization ecology of a particular species and its relation to the environment. The incorporation of the Allee effect in the management of commercial stocks of free-spawning marine invertebrates has recently been emphasised for abalone (Breen and Adkins, 1980; Shepherd, 1986; Shepherd and Brown, 1993; Shepherd and Partington, 1995; Babcock and Keesing, 1999), sea urchins (Quinn et al., 1993), scallops (Caddy, 1988) and giant clams (Munro, 1993). However, experimental efforts toward such implementation still remain limited.

1.6 Aims of this Research

There have been few studies of fertilization kinetics centred on commercial free-spawning marine invertebrates (e.g. Clavier, 1992; Andre and Lindegarth, 1995; Clotteau and Dube, 1993; Styan, 1998; Babcock and Keesing, 1999). The intention of the present study was to examine, both empirically and theoretically, fertilization success in commercially valuable free-spawning shellfish living naturally in turbulent conditions. Both laboratory and field studies were conducted to obtain empirical data in order to validate predictive models. This included information on fertilization success as a function of gamete concentration, gamete age, current flow data and animal spatial distributions. Finally, an attempt was made to determine the minimum density requirement for maintenance of a population of the particular experimental animal.

Where the population density of a species may be affected by a natural or anthropogenic disturbance, an estimation may be made of the minimum density required to successfully re-colonise the area. For many species, particularly broadcast spawners,

effective reproductive output may be enhanced by the establishment of marine refugia in which maintenance of animal densities and sizes of mature adults for optimal fertilization success may be addressed. Marine refugia should also be set up with a sound knowledge of the local hydrodynamic conditions that can act to enhance reproductive output. It is important to have an understanding of which populations are important sources of offspring and which are sinks. Source populations should be targeted for refugia. All aspects of life history should be included in any review of harvest refugia, and fertilization success is a fundamental process that is often ignored. A better understanding of fertilization success will lead to improved shellfisheries management programmes in the future.

This thesis contributes to the limited knowledge of fertilization success in free-spawning marine invertebrates, specifically in terms of fisheries management. Central to these studies is the European abalone (English common name: ormer), *Haliotis tuberculata*. This is a very important species, both in terms of commercial potential and ecological interactions. Chapter 2 describes and discusses the wide range of experiments that have been conducted on this species to determine factors affecting fertilization success. Chapter 3 focuses upon three other species of mollusc, all of which have commercial value, namely the Pacific oyster, *Crassostrea gigas*, the clam, *Tapes decussatus*, and the limpet *Patella vulgata*. Chapter 4 describes experiments conducted upon the sea urchin, *Psammechinus miliaris*. This species was also chosen for its potential as an important commercial species.

CHAPTER TWO – *FERTILIZATION SUCCESS IN* HALIOTIS TUBERCULATA

Fig.2.1 Adult Haliotis tuberculata, *85mm shell length. Photo by Maria Baker 1999*

2.1 Introduction

2.1.1 Abalone Fisheries World-wide

Abalone are archaeogastropod molluscs belonging to the family Haliotidae. Of the 100 or so species of abalone found world-wide, there are currently ~10 commercially harvested species, all of which are of great economic importance. They are one of the highest priced shellfish in the world (Mercer, 1990). As a result, many of the abalone fisheries have been severely over-exploited. For example, *Haliotis midae*, referred to as 'viagra of the sea' because of its supposed aphrodisiac qualities, fetch around £30 per kilo. As a result of this high price, poaching is a real threat to the fishery.

Overfishing of abalone has been a problem in every country where they are harvested (Tegner, 1989). Important abalone fisheries have existed in Australia, China, Japan, Mexico (Pacific coast), New Zealand, South Africa, and the United States of America (California). Most of these fisheries have collapsed in recent years (Fallu, 1991). For example, Mexican abalone catches declined from an annual production of 6000t in 1950 to 400t in 1985. In California, the total abalone catch in 1968 was 2028t but had diminished to 233t in 1990. Over-exploitation has led to an extremely high risk of extinction for the white abalone *H. sorenseni* (Roberts and Hawkins, 1999; Hobday and Tegner, 2000). Natural fisheries of any reasonable scale are only in Australia and Japan today. The main abalone fishery in Australia is based upon *Haliotis rubra*. This wild fishery started in the early 1960s and reached a peak at the end of that decade. The fishery declined shortly after and now remains more or less static, probably because of

the strict management regime. Japan has the longest history of abalone research dating back some 2000 years (Fallu, 1991). The earliest reference to abalone divers was during the reign of Emperor Suinin in 30 AD. Of the four or five species of abalone fished in Japanese waters *Haliotis discus hannai* is the most sought after. The Japanese fishery remains active as a consequence of tight management controls. Other commercial key abalone species are *H. rufescens* in California and Mexico, *H. iris* in New Zealand and *H. midae* in South Africa.

2.1.2 The Guernsey Fishery

Haliotis tuberculata (Figure 2.1) is the only commercial species of abalone in Europe. A second species, *Haliotis lamellosa*, inhabits the Mediterranean Sea but is too small in terms of size to warrant harvesting. A detailed description of *Haliotis tuberculata* is given by Crofts (1929) and Graham (1988). This European species of abalone is commonly known as the ormer. Ormer fishing has been a prominent activity around Guernsey. An estimated 43 tons were harvested in 1967. Over-harvesting around the island (well documented by Stephenson, 1924 and Bossy and Culley, 1976) led to a decline of wild stocks and consequently ormering, as abalone fishing is sometimes called in this region, was officially banned for 3 years between 1974-76 in order for stocks to recover. In addition, a total ban on diving for ormers was imposed in 1973 and is still in existence. The Guernsey fishery is now seasonal from December to April. Fishing activity is restricted to shore gathering in the intertidal zone limiting fishing days to spring tides. In the Channel Islands, the ormer fishery is managed by the Sea Fisheries Committee of the Channel Islands and fishermen need a licence to fish. They also impose bag limits, closed areas, restricted seasons and minimum size limits of 76mm shell length. Demand for the ormer remains high and this has led to greatly increased interest in all aspects of abalone aquaculture.

Over-harvesting has prompted studies of several European fisheries (Forster, 1962; 1967; Forster et al., 1982). Extensive breeding and rearing experiments have been conducted by the Shellfish Research Laboratory, Carna, Galway, Ireland with an emphasis on hatchery and nursery techniques (LaTouche and Moylan, 1984; Arai and Wilkins, 1986; Gaty and Wilson, 1986; LaTouche et al., 1993; Mgaya and Mercer, 1993; Mercer et al., 1993; Mai et al., 1994). Portsmouth Polytechnic have also developed work in this area (Peck, 1983). Tank rearing of *H. tuberculata* was carried

out from 1973-1974 at the Laboratory of Centre Oceanologique de Bretagne in Brest, France (Koike, 1978). A successful ormer hatchery exists in Rocquaine Bay, Guernsey with sea-based on-growing of ormers in cages. A natural population of ormers also exists at this locality. This natural population has been used for the experiments presented in this thesis.

2.1.3 Distribution

H. tuberculata has a wide natural geographic distribution extending from the Channel Islands and the French coasts of the western Channel in the north (Gaillard, 1958) to the Canary Islands, the Cape Verde Islands, the Azores, the western Mediterranean, the North African coast and the West African coasts of Mauritania and Senegal in the south (Nickles, 1950; Parenzan, 1970; Nordsieck, 1975) (Figure 2.2).

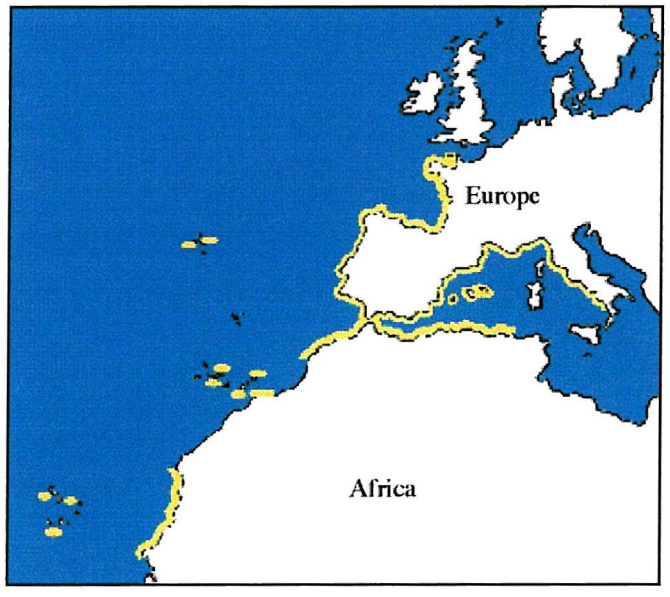


Figure 2.2: Distribution of Haliotis tuberculata *(in yellow). After Gaillard (1958); Nordsiek (1975)*

The greatest densities are found in the Channel Islands at the northern most limit of its geographic range (Bossy and Culley, 1976; Clavier, 1992). The ormer occurs on rocky substrata on the lower shore and in the shallow sub-littoral. Such habitats provide a firm substratum for the animal to adhere to using its large foot, thereby decreasing risk from predation and dislodgement by waves and tidal surges (Crofts, 1929). Exposed rocky habitats also have turbulent waters with high levels of dissolved oxygen, preferred by abalone, and shelter to give protection from direct sunlight and predators. The species is active and has been observed moving at over 5m min^{-1} (Stephenson, 1924).

Individuals only ever move a few hundred meters from their settlement area (Clavier and Chardy, 1989). Apart from the substratum, distribution is also governed by temperature, salinity, hydrodynamics and food (Fretter and Graham, 1962; Poore, 1972; Minchin, 1975; Mottet, 1978; Hayashi, 1980b; Forster et al., 1982; Emberton, 1982; Peck, 1983; Clavier and Chardy, 1989; Peck, 1989; LaTouche et al., 1993). These factors affect different sizes of ormer differentially. A detailed study of factors influencing spatial distributions was carried out by Clavier and Chardy (1989). They concluded that ormer in the vicinity of Saint-Malo are always absent from (1) smooth rocky substrata, (2) areas exposed to a strong current and (3) areas deeper than 8 m below chart datum. As habitat complexity increased and current flow declined, ormer abundance and biomass increased.

2.1.4 Natural Abundance

Estimates of natural abundance of *H. tuberculata* have been made in several areas by a variety of methods. For example, Clavier and Richard (1983; 1986) found a mean density of 0.54 individuals m^{-2} in Saint-Malo, whereas Peck and Culley (1990) found mean densities from 0.05 ind. m^{-2} to 2.35 ind. m^{-2} at 13 sites on Jersey with an overall island-wide mean of 0.79 ind. m^{-2}. Forster (1962) reported a high density of 2.4 ind. m^{-2} at a site in Guernsey. Densities may vary with animal size (Peck and Culley, 1990) and water depth (Clavier and Richard, 1983; Clavier and Chardy, 1989). The size and age distribution of populations has also been assessed for populations in the Channel Islands (Forster, 1967; Hayashi, 1980b; Forster et al., 1982; Peck and Culley, 1990). Peck and Culley (1990) reported a correlation with size distribution and water depth. Larger animals are found in deeper water indicating migration with age. However, this theory was not supported by Findlay and Willerton (1996) for Guernsey ormer populations. Extensive mark-recapture programmes have provided information on natural and fishing mortality. The main predators of adult ormers are octopus and the seastars *Asterias rubens* and *Marthasterias glacialis* (Stephenson, 1924; Crofts, 1929; Foster, 1962). An influx of freshwater or sand can quickly cause mortality to abalone and storm activity may knock animals from rocks, making them more vulnerable to predation.

2.1.5 Reproductive Biology

H. tuberculata grows to a maximum shell length of ~12cm (Stephenson, 1924; Crofts, 1929; 1937). It is an iteroparous species which may live in excess of 15 years (Berthou et al., 1985). *H. tuberculata* is dioecious although some individuals may be hermaphroditic (Girard, 1972; Cochard, 1980). A statistically valid 1:1 ratio of male to females was reported by Hayashi (1980a) for individuals >9cm shell length in Guernsey (403 males: 383 females), but Forster (1962) and Stephenson (1924) found a higher proportion of males in a different population also from Guernsey. Girard (1972) found a preponderance of females among juvenile ormers from France, yet a 1:1 sex ratio in adults. He suggested the possibility of sex change. Male ormers mature earlier and at a smaller size than females (Girard, 1972; Hayashi, 1980a). Minimum size at sexual maturity in the Guernsey populations of the ormer was found to be 40mm and 49mm for males and females respectively (Hayashi, 1980a). These animals would be about 2 to 3 years old. Ormers in other locations have been found to reach sexual maturity at both smaller and larger sizes (Girard, 1972; Berthou et al., 1985; Lopez, 1999).

Temperature is arguably the main exogenous factor that regulates reproductive cycles in marine invertebrates in shallow water (Giese, 1959). Gamete maturation is governed by annual temperature fluctuations (Fretter and Graham, 1964) and both Girard (1972) and Hayashi (1980a) have shown a very strong correlation between gonad development of *H. tuberculata* and water temperature. Discrete cycles of gametogenesis occur in *H. tuberculata.* Each cycle includes a vegetative period and periods of differentiation, cytoplasmic growth, vitellogenesis, spawning and resorption of unspawned gametes. In the Channel Islands populations, from December to February, the ovary contains very few primary oocytes, with resorption of ova taking place (Hayashi, 1980a). February to April sees the onset of oocyte development, reaching a maximum density and size by July.

The only reported wide-scale spawning of haliotids in their natural environment was for *Haliotis kamtschatkana* in British Columbia. Spawning occurred in calm conditions and after several days of very calm weather (Breen and Adkins, 1980). *H. rubra* from Tasmania also spawns during calm weather (Prince et al., 1986). It has been proposed that abalone populations may select periods of low water movement for spawning - an adaptive behaviour to minimise dispersion of gametes and larvae (Hayashi, 1980a; Prince et al., 1987; Clavier and Chardy, 1989). Many haliotids

maintain ripe gonads for long periods (Harrison and Grant, 1971; Mottett, 1978). This could be an adaptation to enable spawning to coincide with short, irregular periods of slack water. Abalone may climb to prominent points on the substratum, including elevated areas of kelp, prior to releasing gametes (Quayle, 1971; Breen and Adkins, 1980).

Release of gametes varies between populations (Hayashi, 1980a; Stephenson, 1924) although all populations around the Channel Islands spawn over a short period in summer. Hayashi (1980a) noted differences in spawning times between Guernsey ormer populations only short distances apart, reflecting local hydrodynamic and biological differences. He hypothesised that spawning seasons were correlated with latitude. In southern populations abalone appeared to spawn twice a year or for a protracted period while in northern populations they spawn only once and for a short period. Crofts (1938) noted spawning in *H. tuberculata* in the Channel Islands between June and November, and Forester (1962) reported spawning in the Channel Islands populations between June and September. Males usually become ripe earlier than females (Girard, 1972; Hayashi, 1980a).

Further evidence of gonad maturation showed a dependence on seasonal variations in the photoperiod (Cochard, 1980). Reproductive cycles have been shown to differ between populations from different locations/latitudes and between years, in terms of timing or duration of spawning, spawning intensity, egg size and fecundity. Intra-specific differences in reproductive events could be a function of both environmental and genetic factors. Spawning over a prolonged period decreases the likelihood of either very weak or very strong recruitment (Paulet et al., 1988) and might be an adaptation to spawning in an unpredictable environment. Spawning in the ormer is thought to be governed by three major factors: (1) the degree of maturation, (2) the intensity of stimuli, and (3) the conditions to which ormers are exposed when stimuli occur (Hayashi, 1980a). Other factors include (1) the presence of conspecific gametes in the surrounding water, (2) neural or hormonal influence and (3) food availability (Shepherd and Laws, 1974; Morse et al., 1977; Mottet, 1978). Catch data suggest that in at least one species of *Haliotis* (*H. discus hannai*) the larger abalones move inshore during the spawning season, presumably to spawn (Ino, 1952). Natural selection has led to the spawning season corresponding to a time when environmental conditions permit good survival of progeny.

Figures 2.3 a and b show the foot being moved to reveal the gonad location of sexually mature *H. tuberculata*.

Figure 2.3a: Male Gonad

Figure 2.3b: Female Gonad

The gonad makes up the outer layer of the conical appendage, surrounding the inner cone-shaped digestive gland. Primary germ cells are located at the tip of the conical appendage and follicles containing eggs are held within the celomic cavity (Girard, 1972). The gonad is largely separate from the rest of the visceral mass. As the gonad matures, the conical appendage becomes larger, heavier and rounder in cross-section. The layer of gonad material may account for 15 to 20% of the total weight of soft tissues just prior to spawning (Webber and Giese, 1969). After spawning, gonads become smaller, flatter in cross-section, colourless and watery in appearance. At this stage, it can be difficult to distinguish between the sexes (Stephenson, 1924; Forster, 1962; pers. obs.). Prior to this it is easy to identify the sexes as the male gonad is generally creamy in colour and the female gonad tends to be dark green or grey-green (Stephenson, 1924; Crofts, 1929). Gametogenic stages were assessed by studying gonad weight variation over time. A gonad index was used which expressed gonad weight as a proportion of total body weight (Hayashi, 1980a; Tutschulte and Connell, 1981). Dry weight was used because the water content of specimens varied seasonally. The Mottett (1978) cross-section methodology was used. Microscopic methods were used to examine the shape and size of gametes for oocyte size/frequencies (Hahn, 1989). Spawning periods were estimated by measuring the abundance of larvae. All assessment methods have advantages and disadvantages that may be reduced by employing more than one method to include quantitative estimates of fecundity and qualitative assessment of gametogenesis. Nevertheless, knowledge of spawning timing and spawning cues remain tentative.

Ormers are broadcast spawners, releasing their gametes directly into the water column (Stephenson, 1924; Crofts, 1929; Girard, 1972). Fertilization takes place externally. Fecundity in *H. tuberculata* is high and has a linear relationship with shell length (Mgaya and Mercer, 1993). Extrusion of gametes is caused by contraction of the large shell muscle which acts to compress the gonad between the foot and shell. Gametes then pass through the kidney into the gill chamber and are carried by currents out through the respiratory pores (see Newman, 1967a). Figures 2.4 a and b show female and male *H. tuberculata* as they are spawning.

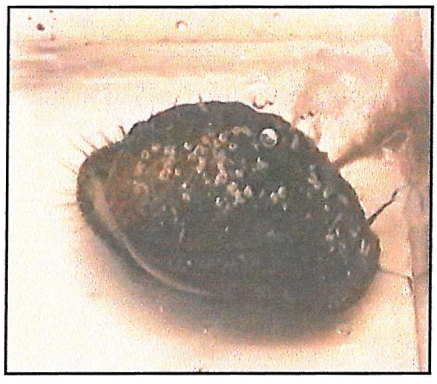


Figure 2.4a: Female Spawning

Figure 2.4b: Male Spawning

The eggs of *H. tuberculata* are negatively buoyant and lecithotrophic. Mature unfertilized spherical eggs have an average diameter of 180μm. They are enclosed within a thick gelatinous coat (Crofts, 1937). Peck (1983) reported that eggs ranged from 170 to 210μm and have a thin jelly coat while Kioke (1978) found a maximum diameter of 210μm, including the membrane. *H. tuberculata* eggs do not have accessory cells which might increase the effective egg size. It is unknown whether the eggs possess chemical attractants. The surface area of an egg that is receptive to fertilization has yet to be determined (but see this thesis, section *2.3*). The eggs are surrounded by a complex extracellular coat that contains three distinct elements: a jelly layer, a vitelline envelope and an egg surface coat (Mozingo et al., 1995). A spermatozoan must penetrate the elevated vitelline envelope before it can fuse with the egg. Abalone sperm cells are classified as primitive sperm and have an enormous acrosomal vesicle containing two proteins that mediate fertilization (Swanson, 1999). The large size of the acrosome is thought to be related to the existence in the corresponding eggs of a vitelline coat plus a thick jelly coat as found in molluscs and echinoderms although reasons for this are not clear (Rosati, 1995).

Soon after fertilization polar bodies are formed. The first cell division commences at ~1.5 hours. Subsequent division is total, unequal and spiral. Micromeres separate at the animal pole (Crofts, 1937). After 4 hours a morula is formed. Megameres can be distinguished at the vegetal pole at this stage. Development proceeds through to the blastula and then to the gastrula stage via epibole (Crofts, 1937; Ino, 1952; Fretter and Graham, 1962; Koike, 1978). During epibole, the micromeres grow out and around the macromeres, eventually forming a squamous epithelial layer in the trochophore larvae. Development to the trochophore stage takes around 12 to 13 hours post-fertilization (at 20 +/- 1°C). The embryo is classified as a trochophore when the stomodeum is formed and cilia along the prottrochal girdle are completely formed. These larvae hatch and swim actively as part of the plankton, becoming veligers and spending a total of about 3.5 to 5 days in the plankton (Koike, 1978). Veliger larvae have a flat apical region and the velum is completely developed with long cilia (Crofts, 1937; Fretter and Graham, 1962). Hatched larvae are positively phototactic. This short planktonic phase leads to restricted larval dispersal (Underwood, 1979: Prince et al., 1987). The 90° torsion of the veliger starts on day 5 and ends at about day 12. By this stage the animal has settled and commences feeding immediately.

2.1.6 Disease and Parasites

Little information exists on the parasites and diseases of ormers. Crofts (1929) found very little evidence of disease, noting that only 2 out of 400 specimens he examined were contaminated. He also found boring molluscs affecting the ormer. Boring sponges, *Cliona celata*, polychaetes, genus *Polydora*, and molluscs may affect ormer adults (Crofts, 1929; Forster, 1967; Peck, 1983; Clavier, 1992). These parasites have only a minor influence on the physiology of the ormer. The Jersey ormer population has been subject to a number of mortalities in recent years attributed to withering foot syndrome (Sendall pers. comm.), a disease first noted in other abalone species. Paralytic shellfish poisoning in *H. tuberculata* from the Galician coast was reported in April 1991. In October 1993 this mollusc fishery was closed because its toxicity was found to be higher than the legally permitted level (Martinez et al., 1993; Bravo et al., 1996). To date, there is no such information available regarding the Channel Islands ormer populations.

2.2 Fertilization Kinetics - Laboratory Experimentation

2.2.1 Introduction

A comprehensive knowledge of the reproductive biology of the abalone is critical in the understanding of abalone ecology, aquaculture and fisheries management. Abalone have been extensively studied in terms of their general biology. Such studies have resulted in a comprehensive knowledge of their evolution, distribution, genetics, physiology of reproduction, larval ecology, larval settlement, feeding, growth, diseases, fishery biology and culture. However, very few data exist on the fertilization success of this animal with respect to its environment.

The main aim of this research is to investigate some of the factors that affect fertilization and development success in *H. tuberculata*, and to view them in a fisheries management context. Predictive models of fertilization success, incorporating laboratory and field data, are produced to give an idea of minimum stocking densities required to re-colonise areas with declining populations of *Haliotis*. An analysis of the use of harvest refugia in preservation of stocks is considered. A number of aspects of *H. tuberculata* fertilization were studied in relation to sperm-egg interactions.

2.2.2 Materials and Methods

2.2.2.1 Collection of animals

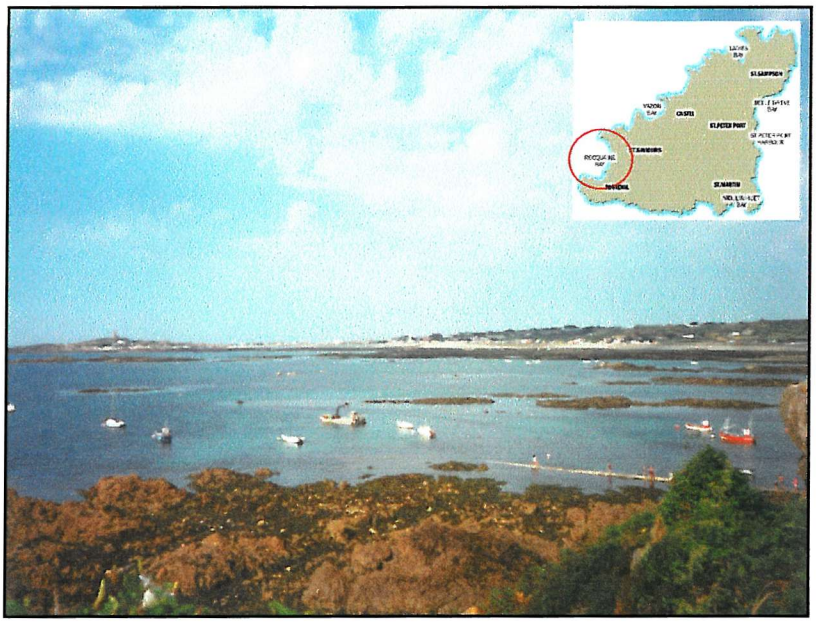


Figure 2.5: Rocquaine Bay, Guernsey

A total of 40 ripe specimens of *Haliotis tuberculata* were collected from Rocquaine Bay, on the southwest coast of Guernsey on 15th July 1998 by diver (Figure 2.5). At this time of year, ripe individuals were spawning naturally in the field. In order to determine sex *in H. tuberculata,* the animals were examined visually by pulling back the foot tissue to expose the gonad on the right side of the shell. Great care was taken during this operation because any slight lesion might severely decrease the fitness of the animal.

Twenty-five male and fifteen female ormer were carefully packed in the red alga *Palmaria palmata* for air transportation back to Southampton. The lapsed time from collection to aquarium was 2.5 hours. In the aquarium, male and female animals were separated and transferred to 60L plastic tanks (held at densities of 0.33 ind. l^{-1}) of well aerated seawater with salinity 33. These tanks were kept at a constant temperature of 15 +/- 1°C during the experimental period. This temperature was set slightly lower than the ambient sea temperature of 16-17°C in an attempt to delay spawning until required. The animals are herbivorous and feed on a variety of red and green algae with a preference for delicate algae such as *Palmaria palmata* and *Ulva lactuca.* This food was provided during the time they were held in the tanks. An excess of light stops animals feeding so illumination was controlled (12 hours dark, 12 hours light) but all animals were kept shaded from direct illumination, which the ormers prefer (Crofts, 1929). Holding tanks were cleaned every other day.

As some problems were experienced in the SOC aquarium in 1998, it was decided that experiments in July 1999 and 2000 be undertaken in Guernsey to avoid such problems and enable animal collection on a daily basis. A diver collected ripe animals from Rocquaine Bay at a depth of around 10-15m. These animals were transferred to holding barrels (Figure 2.6) and re-immersed in a similar area at the same depth so animals continued to condition naturally. This enabled retrieval of animals on a daily basis without further costs incurred by using a diver. The position of the barrels was marked with buoys. Each day these barrels were hauled and around 20 ripe females and 15 ripe males were selected.

Figure 2.6: Holding Barrels with H. tuberculata

2.2.2.2 Collection of gametes

Abalone may be induced to spawn via a number of different techniques that simulate environmental stress. Thermal shock may be used to induce spawning of viable gametes when the animal is fully ripe (Kioke, 1978; Flassch and Aveline, 1984). Chemical stimulation has also been employed, using hydrogen peroxide (H_2O_2) and 2-Mtris(hydroxymethyl)aminomethane buffer in seawater (Morse et al., 1977; Tong and Moss, 1992), maintained at a conditioning temperature of 18°C (Moylan and Mercer, 1993). Kikuchi and Uki (1974) and Hayashi (1982) exposed animals to ultraviolet light-treated seawater. Ultraviolet light breaks up the water molecules and creates small quantities of hydrogen peroxide that stimulates spawning in ripe animals. Hydrogen peroxide causes the animal to produce a prostaglandin-like chemical, which may have a role in the induction of spawning (Hahn, 1989). Genade et al. (1988) used osmotic shock to induce spawning in *H. midae*. Spawning in the Vietnamese abalone, *H. ovina*, was induced by the addition of ESTROFAN containing prostaglandin $F_{2\alpha}$ (Fallu, 1991). In other cases molluscs have been stripped of their gametes via dissection and successful fertilization assays have been conducted in this way (e.g. *Crassostrea gigas*). Strip spawning *H. tuberculata* results in non-viable gametes (Boolootian et al., 1962; pers. obs.) *H. tuberculata* can be conditioned in the laboratory to produce ripe gametes at times other than its normal spawning seasons by regulating maturation via temperature, day length (illumination) and adequate nutrition (Peck, 1983). This fact was

instrumental in my initial choice of experimental animal although limited facilities rendered this manipulation impractical.

The most natural method of spawning inducement, a combination of thermal shock and UV light-irradiated seawater, were used to achieve the most realistic gamete extrusion. In the laboratory, ormers were held in UV light-irradiated and filtered seawater at a temperature of 15°C. In the 1998 experiments, each experimental animal was contained in a separate 3l vessel. The temperature was increased by 1°C every 30 minutes for between 2-4 hours to a maximum of 23°C. As animals began to spawn, they were transferred to water of optimum temperature for fertilization as determined via experimentation (20°C). In 1999, 20 females and 15 males were held in larger tanks (6 to 10 ind. per tank) until the onset of spawning at which time they were transferred to separate 3l containers (Figure 2.7).

Figure 2.7: Conditioning Tanks

Just prior to the onset of spawning, animals became more active and were sometimes seen to contort making it easier to pick out the probable spawners. Spawning appeared to be almost exclusively tied to the onset of evening, irrespective of the time of attempted induction. On occasions, animals were set to condition early in the morning, but still failed to produce gametes until the evening. (This was also found by Kikuchi and Uki (1974) in spawning induction of *H. discuss hanai*). Spawned sperm were kept as concentrated as possible (to minimise ageing effects) and dilutions were made immediately prior to fertilization. Eggs were washed gently and re-suspended in UV light-irradiated, filtered to 1.6μm seawater (UV FSW). To minimise

damage to the eggs, a large ended turkey baster was used to collect them. Those animals that failed to produce enough gametes for the experiments within 20 minutes were discarded as ageing effects could have led to erroneous results.

Gametes were inspected microscopically prior to use. If sperm appeared sufficiently motile, and eggs appeared (1) mature and healthy, with central nuclear material, and (2) smooth rather than lumpy, then they were considered adequate for use in subsequent experiments. The quality of the gametes is important for development success. In all experiments, eggs-only controls were set up and checked for viability and contamination after 24 hours. In addition, if <70% fertilization success was achieved this was considered indicative of poor gamete fitness and these data were discarded in order that quality control be maintained. A control fertilization was conducted before each experiment to check gamete fitness.

2.2.2.3 Experimental Design

A number of preliminary studies were performed in order to determine the experimental procedures that would be most effective. Trade-offs were addressed between the number of (1) different treatments, (2) replicates and (3) individual crosses, which were logistically feasible. Ideally, experiments were conducted upon multiple crosses between single males and females, with 3 replicates for each cross. I attempted to adhere to this strategy wherever possible. Previous workers have used eggs pooled from many females (e.g. Leighton and Lewis, 1982; Williams, 1999).

2.2.2.4 Hypotheses tested

In order to assess fertilization success a thorough analysis of the effects of many variables is required. The following hypotheses cover the major aspects:

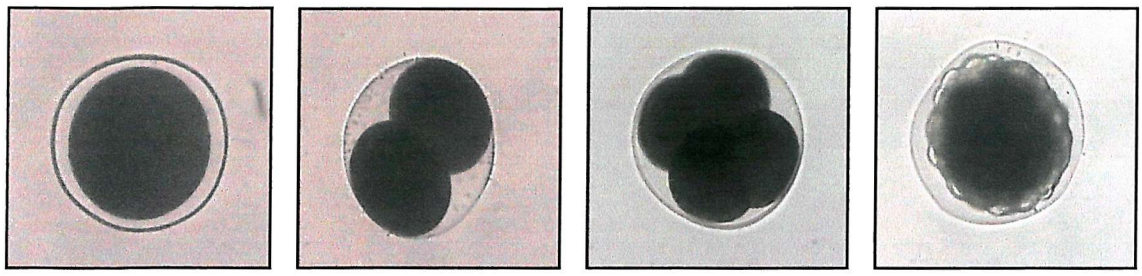
1. There is no variation in fertilization success with variable sperm-egg contact time.
2. There is no variation in fertilization success with different sperm concentrations.
3. There is no variation in fertilization success with variable sperm-egg ratios.
4. There is no variation in fertilization success with gamete age.
5. There is no variation in fertilization success with culture volume.

6. There is no variation in fertilization success with respect to temperature.
7. There is no variation in larval development with respect to temperature.

2.2.2.5 Protocols

2.2.2.5.1 General

In all experiments, sterile glassware was used for egg-sperm incubations. Polystyrene and polycarbonate vials were not used because they increase the proportion of abnormal embryos (Dinnel et al., 1987; Oliver and Babcock, 1992). Sperm concentrations were determined by examining sub-samples with a haemocytometer. The counts were made after the experiments because time was limited. Microlitre aliquots of sperm solutions were diluted with warm water to kill sperm and enable counting. Back calculations were made to derive the original concentrations. Means were calculated from 6 replicate counts. As this process was rather time consuming, it was necessary to estimate concentrations by eye in the first instance. However, for the sperm-egg ratio experiments, this 'by eye' method proved too variable. Therefore, the sperm-egg ratio experiments were repeated in July 2000 and sperm counts were conducted prior to fertilization to ensure correct ratios. The sperm concentrations used in the experiments were determined by conducting preliminary experiments. Egg density was estimated using a Sedgewick Rafter cell. For all experimental treatments, at least three replicates were conducted wherever possible. Each replicate used a different male and female. Egg-only controls were set up in each experiment to test for contamination or parthenogenesis. If >1% of these control eggs were fertilized, the experiment was abandoned. Each experimental animal was wet weighed using a Mettler PM1200 balance to an accuracy of 0.1g and the shell length recorded using callipers to an accuracy of 0.01mm. The percentage of eggs fertilized at each treatment was assessed using a compound microscope at 40x magnification by scoring uncleaved, normal or abnormal cleavage in 100 undamaged eggs, which were randomly sampled from each treatment at least 4 hours after initial fertilization to ensure fertilized eggs had undergone several cleavages and most had reached the morula stage of development (Figure 2.8). Both normal and abnormally developing eggs were counted as successfully fertilized, in line with other studies of fertilization success. Abnormal cleavage was quite distinct. Preliminary studies monitored abnormally developing embryos over time and rapid degeneration was seen.



unfertilized egg *1st division* *2nd division* *blastula*

Figure 2.8: Embryo development stages in Haliotis tuberculata

2.2.2.5.2 Sperm-egg contact time experiment

Sixty-six x 30ml sterile glass tubes were rinsed and filled with 8ml of UV FSW. Nitex mesh strainers constructed from plastic syringes and 48µm Nitex mesh were placed into these tubes for easy removal of eggs from sperm solution. A further 66 tubes were set up in parallel and filled with 10ml of UV FSW for the developing embryos. Between 100 to 200 eggs ml^{-1} were used in each treatment. Sperm were diluted to approximately 10^7 sperm ml^{-1} (standard concentration) and then diluted to provide lower sperm concentrations. Thirty of the tubes had 1ml of the standard sperm concentration added and 30 tubes had 1ml of a more dilute concentration added. The remaining 6 tubes were used as egg only controls. One ml of egg solution was then added to the tubes for varying time periods (3 replicates for each): 5 seconds, 30 seconds, 1 minute, 2 minutes, 5 minutes, 10 minutes, 30 minutes, 1 hour, 2 hours and 4 hours. After each experiment, the Nitex chamber was removed from the sperm solution and rinsed thoroughly by gentle raising and lowering of the chambers in a series of 4 x 6 litre tanks containing fresh UV FSW in order to ensure complete removal of excess sperm. Regular checks were made for efficiency. After rinsing, eggs were placed into the embryo development tubes. This experiment was conducted first to see if Nitex mesh chambers were required for subsequent experiments. Ideally, the influence of sperm-egg contact time should be examined at four different sperm concentrations but this proved not to be logistically possible with only one investigator conducting the experiments. Results of this experiment are on pages 45 and 46.

2.2.2.5.3 Sperm dilution experiment

To test the effect of sperm dilution upon subsequent fertilization success, 33 x 30ml sterile glass tubes were rinsed and then filled with 8ml of UV FSW. 100 to 200

eggs in 1ml seawater were added to each tube. A sample of the most concentrated sperm solution attainable was taken and 10 diminishing dilutions were made. 1ml of each sperm dilution was added to 3 replicate tubes with eggs and left to develop. Three tubes were used as egg-only controls. Results of this experiment are on pages 45 and 47.

2.2.2.5.4 Sperm-egg ratio experiment

The effect of 3 different sperm-egg ratios on fertilization success was determined using 4 different sperm concentrations. Twelve 30ml glass tubes, 9 x 50ml glass beakers and 18 x 600ml glass beakers were rinsed with UV FSW. Four ml aliquots of 1.25×10^5 sperm ml^{-1} were added to 9 x 30ml glass tubes. Fifty, 500 and 5000 eggs were added to each of 3 of these tubes. 50ml of 10^4 sperm ml^{-1} were added to the 50ml beakers. Fifty, 500 and 5000 eggs were added to each of 3 of these beakers. 500ml of 10^3 sperm ml^{-1} were added to 9 x 600ml beakers. Fifty, 500 and 5000 eggs were added to each of 3 of these beakers. 500ml of 10^2 sperm ml^{-1} were added to 9 x 600 ml beakers. Fifty, 500 and 5000 eggs were added to each of 3 of these beakers. Three x 30ml tubes were used as egg-only controls. Different volume containers were used in order to count a minimum of 50 eggs per experiment. Egg densities of 50, 500 and 5000 eggs ml^{-1} were added in order to achieve sperm-egg ratios of 10,000:1, 1000:1 and 100:1 (1000:1, 100:1 and 10:1 for 10^2 sperm ml^{-1} concentrations). The same culture volumes were used for all ratios at a particular concentration. Cultures were allowed to develop for ~ 4 hours before examination for fertilization.

As high levels of fertilization success were seen at low sperm densities in the 500ml volumes, the sperm dilution experiment described in section *2.2.2.5.3* was repeated using larger culture volumes of 500ml but with the same sperm dilutions and number of eggs in order to further investigate sperm-egg ratio effects upon fertilization success. Results of these experiments are on pages 49 and 50.

2.2.2.5.5 Culture volume experiment

In order to test whether the volume of the culture vessels used in these laboratory-based experiments were having any effect upon fertilization success, the following protocol was designed. 9 x 30ml glass tubes, 9 x 50ml glass beakers and 18 x 600ml glass beakers were prepared. An optimum sperm dilution was used and egg

density was varied (50, 500, 5000 eggs ml^{-1}). 5ml of the sperm solution was added to the 30ml tubes, 50ml to the 50ml glass beakers and 500ml to the 600ml glass beakers. Three replicates of each egg density were added to each volume. The cultures were left for 4 hours to develop. Results of this experiment are on page 52.

2.2.2.5.6 Gamete age experiment

The fertilizing capacity of sperm and eggs was assessed over time. 90 x 30ml glass tubes were each filled with 8ml each of UV FSW. Four decreasing sperm concentrations were used in these experiments (~10^6, 10^5, 10^4 and 10^3 sperm ml^{-1}) and both gamete types were aged at the same rate. New fertilizations were performed every 30 minutes by introducing 1ml of 100 to 200 eggs per ml, and 1ml of sperm at each concentration, with 3 replicates of each treatment until no fertilization occurred. In addition, experiments were conducted where only one gamete was aged at a time to see whether sperm or eggs lasted longer. Fresh sperm were added to ageing eggs when sperm were available from a fresh spawning. Results of this experiment are on pages 50 and 51.

2.2.2.5.7 Temperature experiment

A thermal block was set up with a temperature gradient ranging from 10 to 20°C. 21 glass tubes containing 8ml UV FSW were placed within the block. The water in these vials took around 2 hours to reach their target temperatures. 1ml of sperm concentration was added to 6 different temperatures with 3 replicates of each treatment. For controls, 3 tubes had eggs only. Sperm were left in the vials for 20 minutes to acclimate to the ambient temperatures. Following this, 1ml of egg-seawater solution was added to each vial - approximately 200 eggs per tube. These cultures were left to undergo fertilization in the thermal block for 25 minutes. Cultures were then removed from the block and placed in a constant temperature room at 20°C to develop further. An additional experiment used temperatures ranging from 6 to 33°C and 12 different temperatures were tested for fertilization success. It should be noted that with the addition of 1ml of egg-seawater solution at 20°C, the extreme lower and upper temperatures are affected in the first minute or so by the addition of the solution and therefore the actual temperature at fertilization is approximately 0.5°C higher or lower at this time. Results of this experiment are on page 53.

2.2.2.5.8 Larval development with respect to temperature experiment

A thermal block was set up with a temperature gradient ranging from 6 to 33°C. Embryos were cultured at temperatures of 6, 8.5, 11, 13.5, 16, 18, 20.5, 23, 25,5, 28 30 and 33°C. Each hour, for a total of 15 hours, development stages were scored for 20 embryos for each of 3 replicates at each temperature. Development stages are depicted by diagrams in Appendix A. Results of this experiment are on pages 54 to 55.

2.2.2.5.9 Gamete release

Total spawning duration was noted for 8 animals (6 male and 2 female). The volume of gametes released over a 1 hour period was determined for 10 males and 7 females. As each animal started to spawn, they were transferred to separate 3 litre tanks filled with either 1000 or 1500ml of UV FSW. After spawning for 1 hour, samples were taken in order to estimate egg or sperm density. The size and weight of each animal was also determined.

In one male, gamete release was monitored over a much finer time scale. Contraction periodicity was noted and sperm release rate was estimated by taking sperm samples at various intervals after the first contraction. These data were plotted to show sperm release rate in this single male. Results are on page 56 and 57.

2.2.3 Results

2.2.3.1 Experimental animal weights and lengths

Animal weights (including shell) and lengths were noted for each animal used in the experiments. See Table in Appendix B. The relationship between individual lengths and weights are presented graphically in Figure 2.9 showing the regression relationship between the two variables. A similar weight vs. length relationship was reported for 69 *H. tuberculata* collected from Cobo Bay in Guernsey (Forster, 1967).

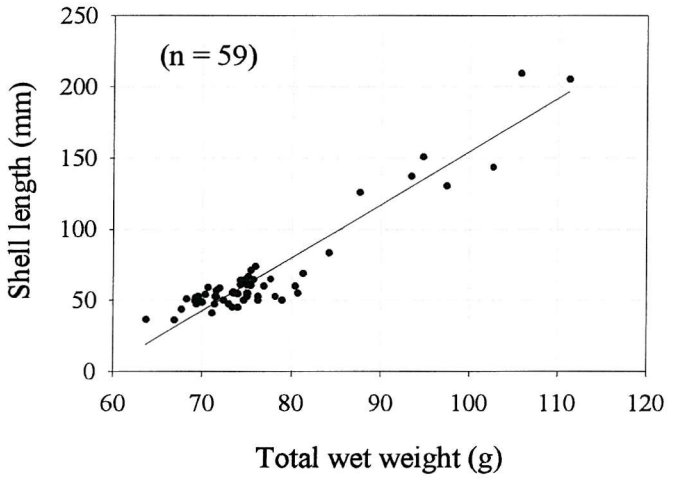


Figure 2.9: Relationship between H. tuberculata *length and weight.* $R^2 = 0.9$

2.2.3.2 Spawning Induction

The time taken for animals to commence spawning ranged from 3h 5min to 17h 40min, with an average of 8h 9min (n=83; s.d. 3h 5min). There appeared to be a correlation between spawning and the onset of the evening, even though animals were kept in darkened tanks during conditioning. Black plastic bin liners were used to shelter the animals from sunlight. However, these covers were lifted at least every 30 minutes in order to check progress. This subjected the animals to the ambient light levels in the aquarium and hence may have served to disturb their conditioning process. Regardless of what time animals were set to condition (from 04:30 to 12:30), 72% of animals spawned after 18:00 with a median spawn time of 19:30 (n=83). Removing the animals from the large tanks and placing them in individual small tanks at the onset of spawning did not appear to affect the spawning process.

Babcock and Keesing (1999) induced spawning in *H. laevigata* and found that they produced gametes between 5 and 12 hours after collection. They also set up an experiment to assess the effect of aggregation on spawning behaviours. Only those animals held together in aggregations spawned, in contrast to isolated individuals. Their findings may infer a chemical stimulus in spawning of surrounding individuals.

In 1998 and 1999, the ormer appeared to be riper earlier in the year than in 2000. The delay in spawning activity in 2000 may have been related to temperature, but mean air temperatures for the months leading up to spawning seasons (April, May and June) showed little variation, 12.4, 12.5 and 12.2°C for 1998, 1999 and 2000 respectively. However, the mean hours of sunlight for this same 3-month period were far lower in 2000 (5.9 h) than in 1998 (7 h) and 1999 (7.5 h). This may have influenced gametogenesis and have caused the observed delay in spawning.

2.2.3.3 Sperm-egg contact time experiment

Fertilization success was significantly affected by variation in sperm-egg contact time (Figure 2.10). Even after 5 seconds there was between 10 and 35% fertilization success in replicates 1 and 2. In replicate 3 fertilization success up to 65% is seen after 5 seconds. Maximum fertilization success occurred after 30 minutes contact time. One treatment in replicate 3 gave high fertilization success at 5 seconds but decreased significantly at 30 seconds. This may have been caused by experimental error in the form of contamination. At sperm-egg contact times of >5 minutes all treatments showed similar fertilization success.

2.2.3.4 Sperm dilution experiment

The concentration of sperm had a highly significant effect on fertilization success of *H. tuberculata*. Virtually no fertilization occurred at 10^2 sperm ml^{-1}. Fertilization success was ~20% at 10^3 sperm ml^{-1}. The greatest rise in fertilization success took place between 10^3 and 10^4 sperm ml^{-1}. At higher sperm concentrations fertilization success increased rapidly to reach an optimum (~100%) within a range of 5×10^4 to 5×10^5 sperm ml^{-1} (Figure 2.11a). At sperm concentrations $>10^6$ sperm ml^{-1} polyspermy occurred in some replicates. Hundreds of sperm were seen to be attached to the membrane of such eggs. These embryos were allowed to develop and resulted in a substantial increase in abnormal larvae (Figure 2.11b).

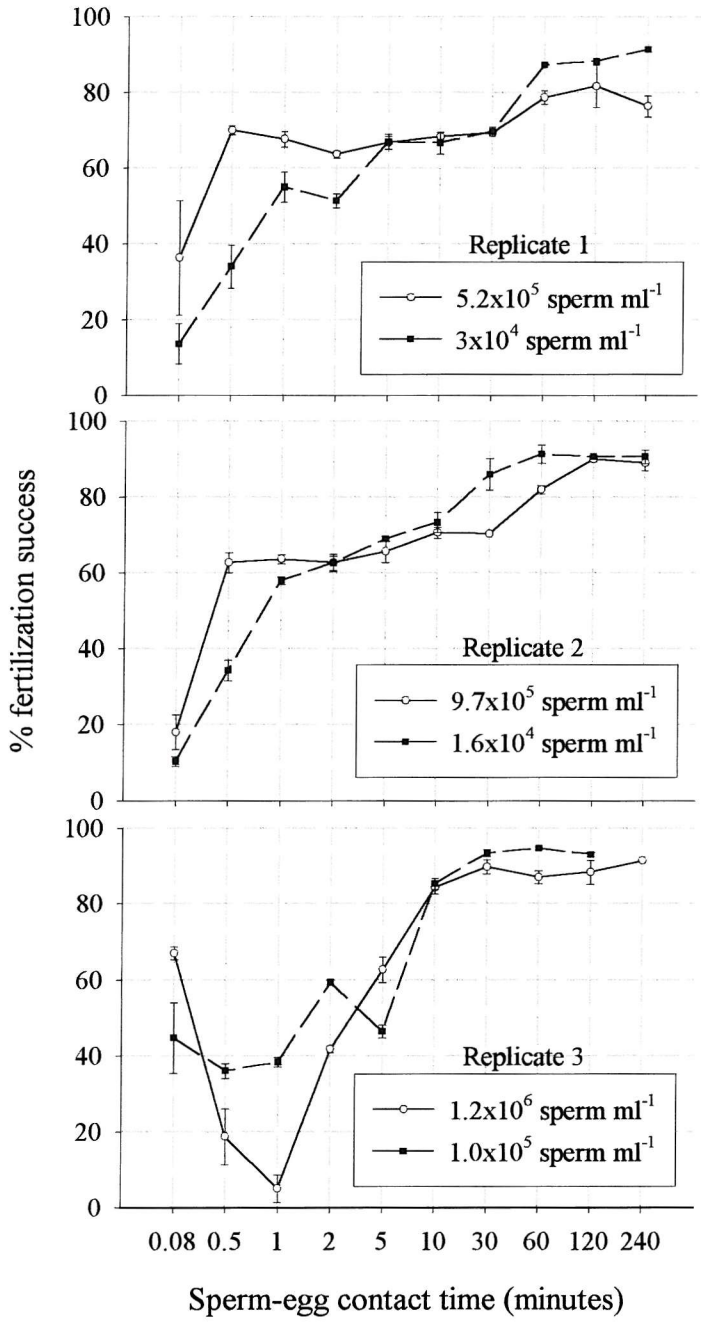


Figure 2.10: The effect of sperm-egg contact time upon fertilization success in H. tuberculata. *Bars show the SE between counts from 3 replicate tubes.*

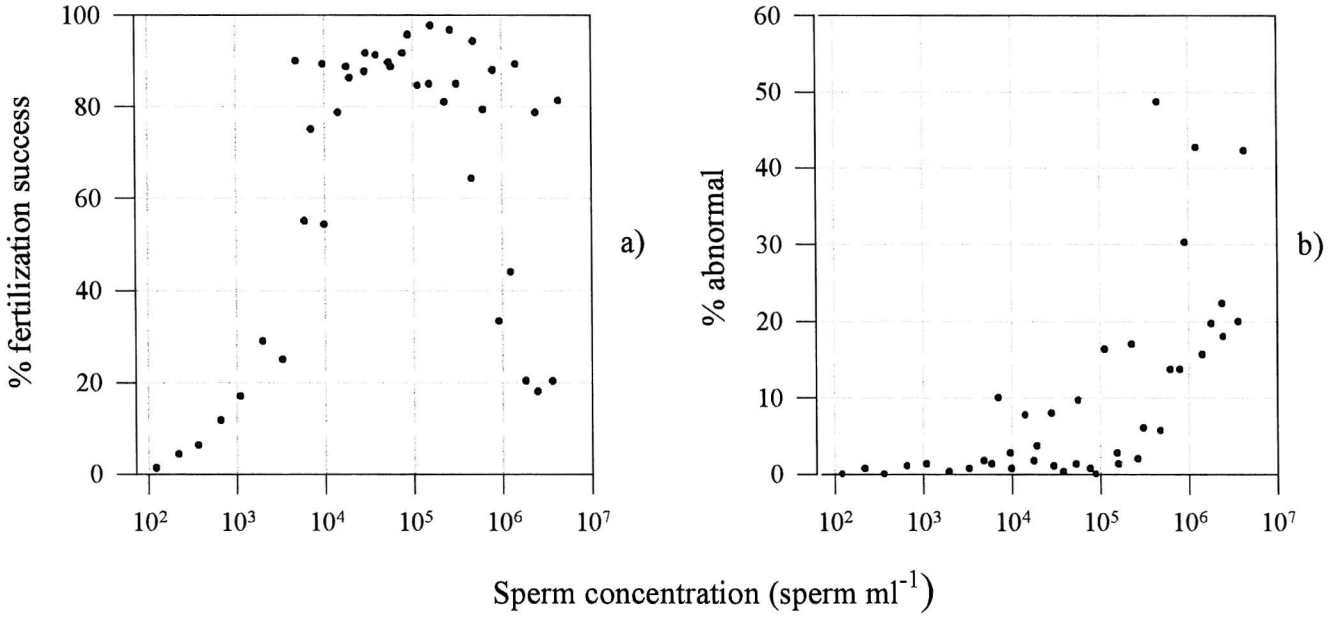


Figure 2.11: (a) The effect of sperm concentration on fertilization success H. tuberculata, *(b) Zygote abnormalities*

2.2.3.5 Sperm-egg ratio experiment

The combined effects of variations in sperm concentration and sperm-egg ratio upon fertilization success in *H. tuberculata* were investigated. Fertilization success remained generally high at each sperm-egg ratio and at each different sperm concentration, with all treatments resulting in >58% fertilization success (Figure 2.12).

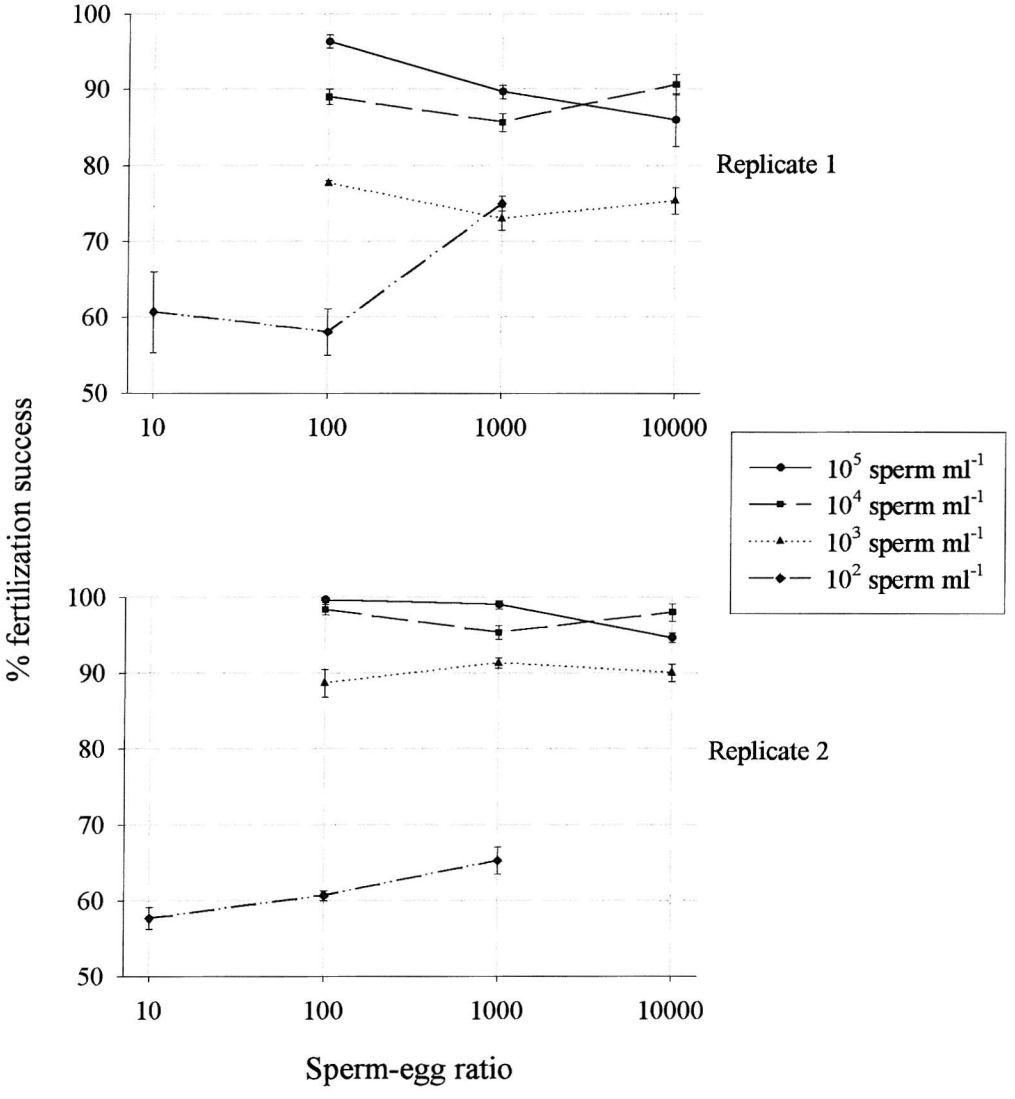


Figure 2.12: The effect of sperm-egg ratio on fertilization success in H. tuberculata. *Bars show SE between counts from 3 replicate tubes.*

These data suggest a limited deleterious effect of sperm-egg ratio upon fertilization success, but a clear underlying effect of sperm concentration is apparent. Fertilization success was greatest at high sperm concentrations, decreasing to 58% at the lowest

sperm concentrations of 10^2 sperm ml^{-1} and the lowest sperm-egg ratio of 10:1. Results from the 500ml culture volume sperm dilution experiment showed that the effect of sperm concentration in these larger volumes had little effect on fertilization success (Figure 2.13). Only at the highest sperm concentrations was any detrimental effect seen in the form of polyspermy. At 10^3 sperm ml^{-1} the sperm-egg ratio was 1000:1 in the larger culture volumes compared to only 45:1 in the 10ml culture volume.

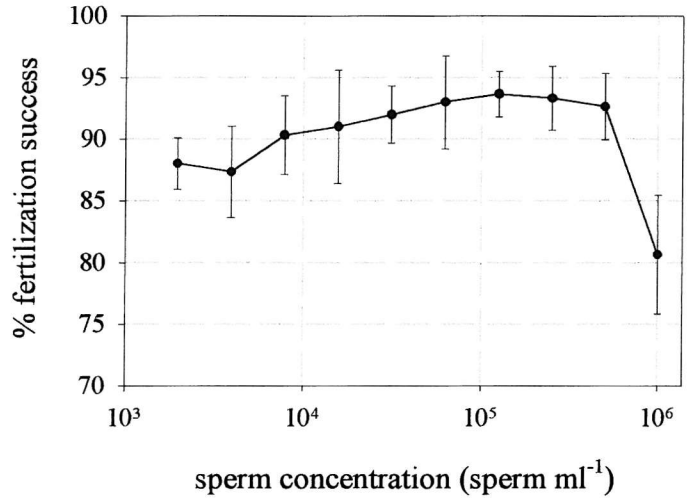


Figure 2.13: The effect of sperm dilution on fertilization success in Haliotis tuberculata *(500ml culture volume) Bars show SE between counts from 3 replicate experiments*

2.2.3.6 Gamete age experiment

Both sperm and eggs were aged simultaneously in these experiments, as would happen in the field assuming synchronous spawning among individuals within close proximity. The more concentrated the sperm solution, the longer lasting its capacity for fertilization (Figure 2.14). Maximum fertilization success occurred with newly released gametes. Fertilization success generally decreased rapidly within 30 minutes of gamete release. In all but one treatment, fertilization success was <20% when the gametes were more than 60 minutes old. In all replicates the higher sperm concentrations had sperm with a greater longevity. Some gametes remained capable of successful fertilization 120 minutes after release but after 150 minutes there was successful fertilization only at the highest sperm concentration. At the lowest sperm concentrations, sperm and eggs remained capable of fertilization for a maximum of 60 minutes.

In an uncontrolled experiment, fresh sperm were added to eggs of 120 and 150 minutes age. Fertilization success was <10% suggesting that eggs as well as sperm were ageing.

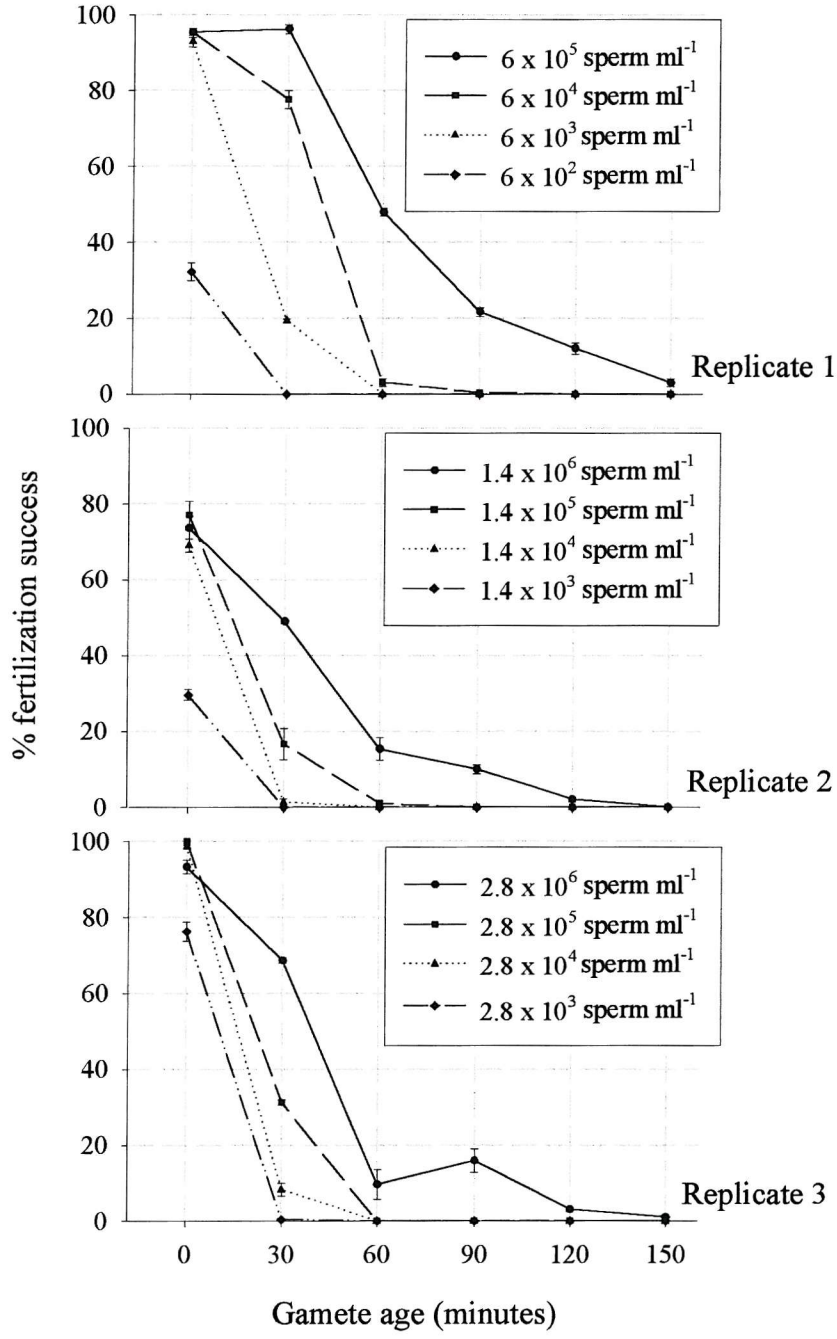


Figure 2.14: The effect of gamete age on fertilization success in H. tuberculata
Bars show SE between counts from 3 replicate tubes

2.2.3.7 Culture volume experiment

There was little effect of culture volume on fertilization success (Figure 2.15). The mean fertilization success exceeded 68% at each volume and each egg density.

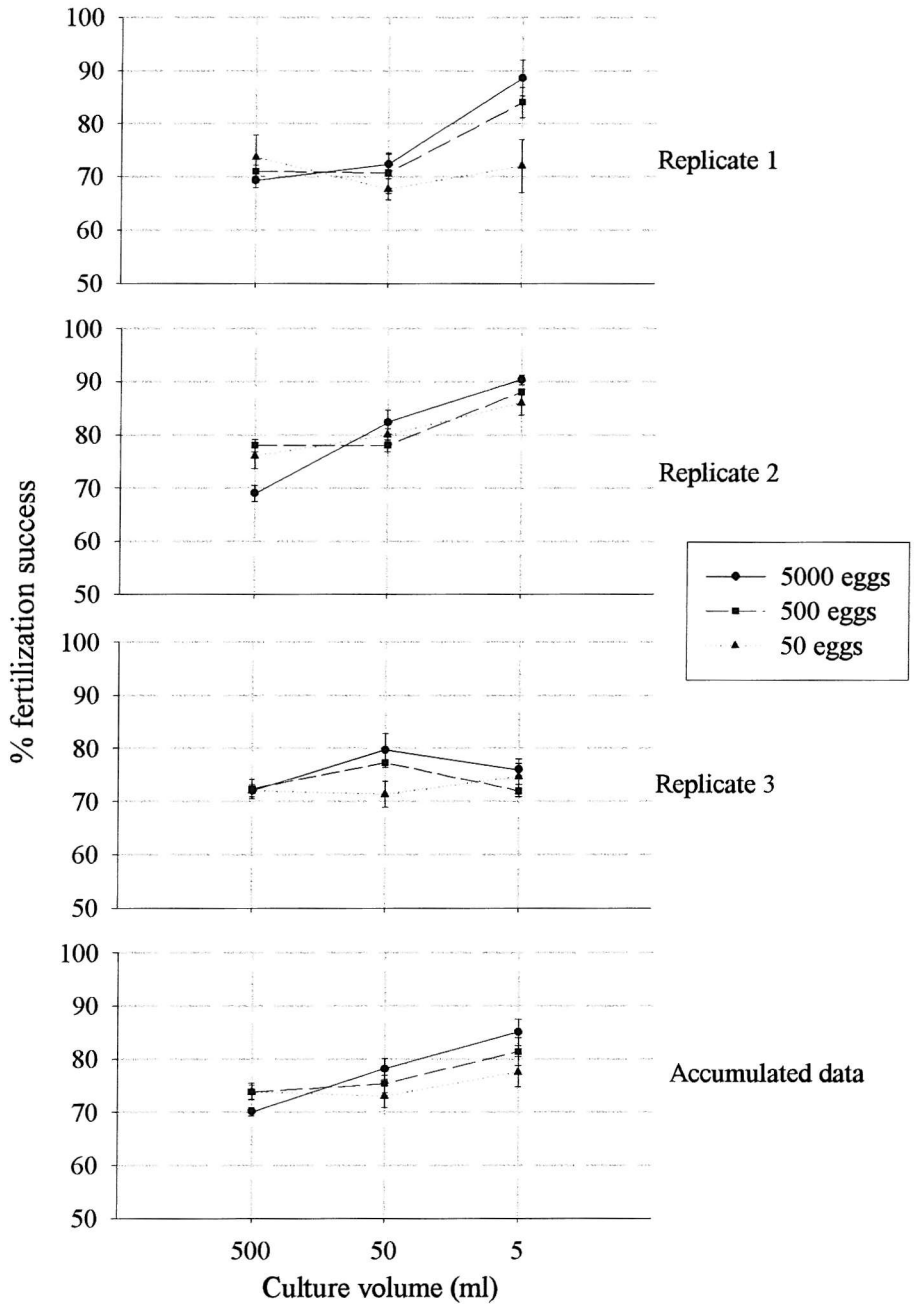


Figure 2.15: The effect of culture volume on fertilization success of H. tuberculata
Bars show SE between counts from 3 replicate tubes

2.2.3.8 Temperature experiment

Temperature had a significant effect on the fertilization success of *H. tuberculata.* Fertilization success was >90% for all treatments where gametes were held at temperatures between 13.5 to 21.5°C (Figure 2.16). No fertilization was evident in treatments <8.5°C or >30.5°C. A rapid increase in success was seen between 8.5 and 13.5°C and a rapid decrease occurred between 21.5 and 30.5°C.

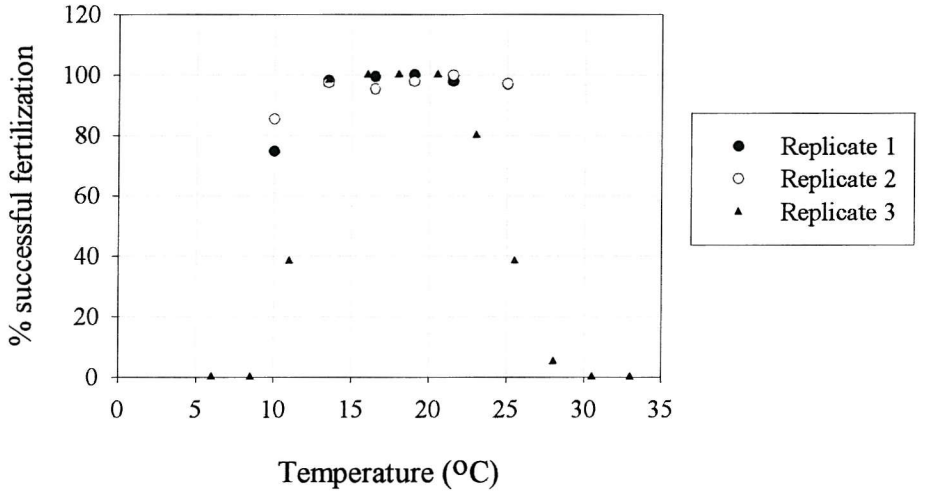


Figure 2.16: The effect of temperature on fertilization success in H. tuberculata

2.2.3.9 Larval development with respect to temperature experiment

The development rate of *Haliotis tuberculata* larvae was governed by temperature. At 18°C, 96.5% of eggs were fertilized 1 hour after initial gamete contact and the embryos were at the two-cell stage. At temperatures of 16°C and below, and 28°C and above, all eggs were uncleaved after 1 hour. The four-cell stage was reached in over 50% of eggs at 20.5°C and 20% of embryos had reached four-cells at 23°C. Developmental competence was recorded each hour over the next 15 hours up to the trochophore stage. The overall results show that the greatest frequency of embryos achieving the fastest development times occurred between temperatures of 20 and 30°C. However, cultures at temperatures of >23°C showed increased numbers of abnormally developing embryos over time. The optimum temperature therefore for both fertilization success and speedy development is between 18 and 20.5°C. The first trochophore larvae were seen after only 9 hours at temperatures between 20.5 and 23°C. The slowest development was recorded at 11°C. In this case embryos reached the two cell stage after 9 hours. These embryos, however, failed to develop normally after the first two cleavages (Figure 2.17)

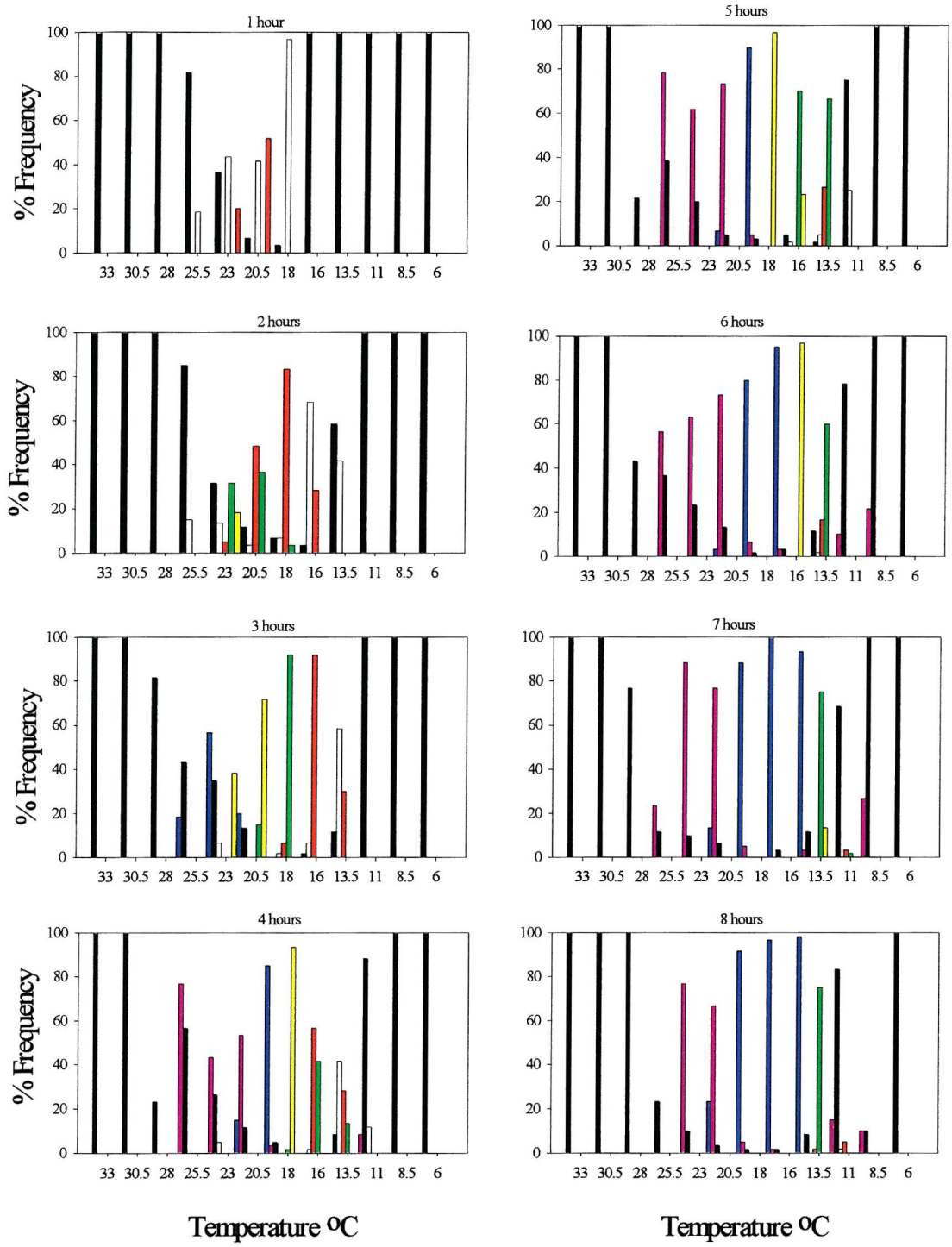


Figure 2.17: The effect of temperature on larval development in **H. tuberculata.** *Continued over page.*

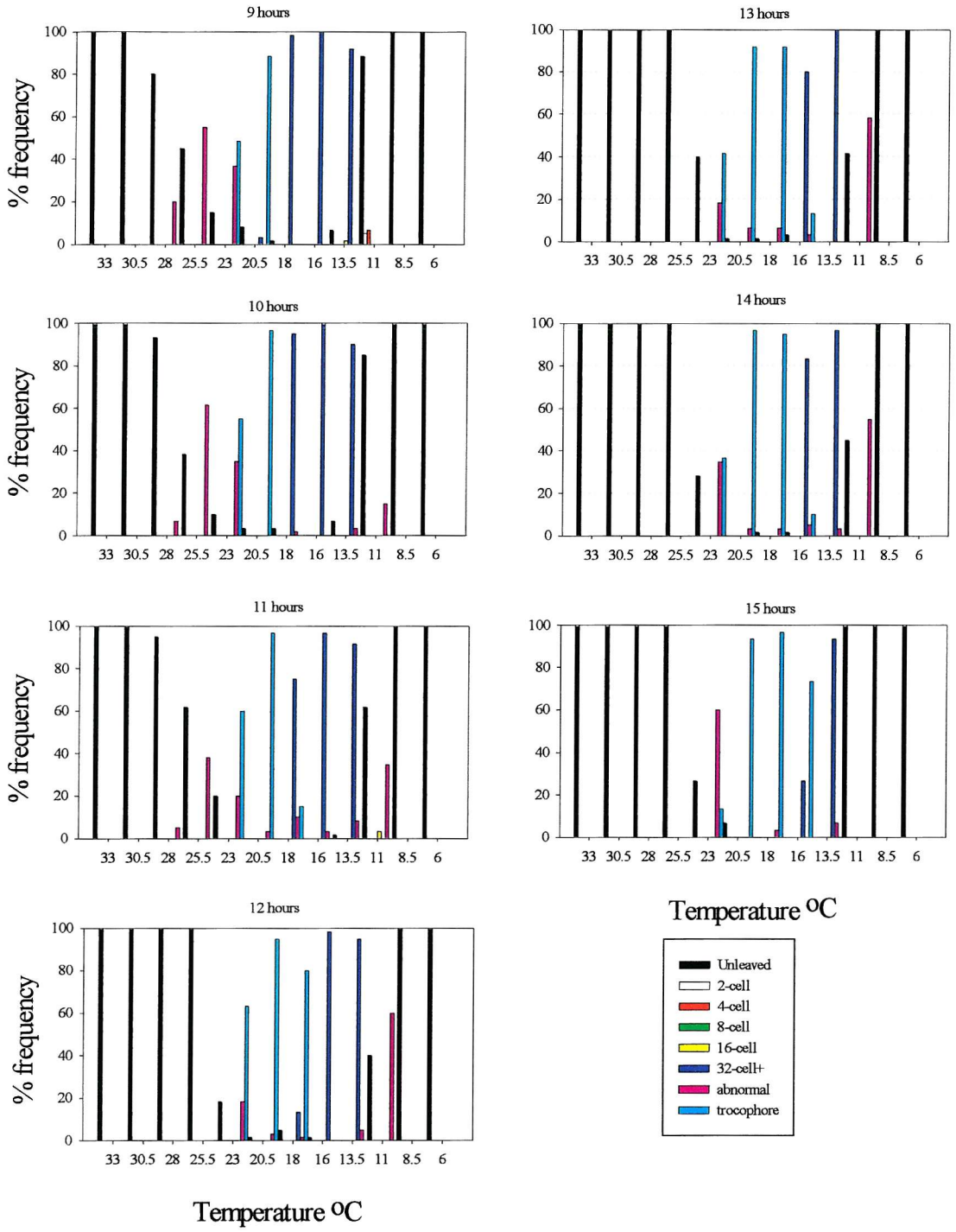


Figure 2.17: (Continued) The effect of temperature on larval development in H. tuberculata.

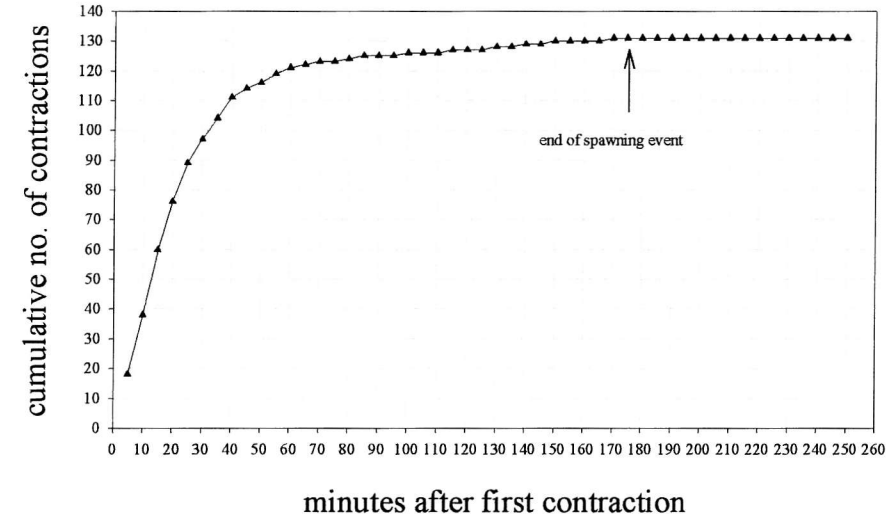
2.2.3.10 Gamete release

In *Haliotis tuberculata* spawning is characterised by a series of contractions of short time intervals for the first 30 minutes then extended intervals up to and beyond 3 hours. Total spawning duration was noted for 8 animals (6 male and 2 female). These ranged from 40 minutes to 5 hours with a mean of 2 hours 23 minutes. Figure 2.18a shows the cumulative number of contractions during a single spawning event of one male. This shows an exponential decrease in activity with time. Figure 2.18b presents the same data by displaying sperm release rate by counting the number of sperm released every few minutes. In July 1999, the numbers of gametes released per hour were calculated (Table 2.1). The mean sperm release rate calculated from these data is 1.7 x 10^7 sperm sec^{-1}.

Table 2.1

Gamete Release Rate

Sex	*Length (mm)*	*Weight (g)*	*Gametes released per hour*
Male	70.7	58.7	4.5×10^{10}
Male	75.2	66.7	3.9×10^{10}
Male	69.2	49.2	1.9×10^{10}
Male	69.6	52.5	9.1×10^{9}
Male	72.4	50.0	5.7×10^{10}
Male	71.5	52.5	2.9×10^{10}
Male	73.4	45.0	1.3×10^{11}
Male	75.1	55.0	1.5×10^{11}
Male	80.4	60.0	8.2×10^{10}
Male	74.0	45.0	5.0×10^{10}
Male	75.0	52.5	9.1×10^{10}
Female	76.9	60.0	1.4×10^{6}
Female	80.7	55.0	7.9×10^{4}
Female	76.3	52.5	2.0×10^{6}
Female	74.7	50.0	8.9×10^{4}
Female	71.1	41.0	4.2×10^{5}
Female	78.2	52.5	9.9×10^{5}
Female	79.0	50.0	5.3×10^{5}
Female	66.9	35.8	2.3×10^{5}
Female	63.7	36.3	3.6×10^{5}



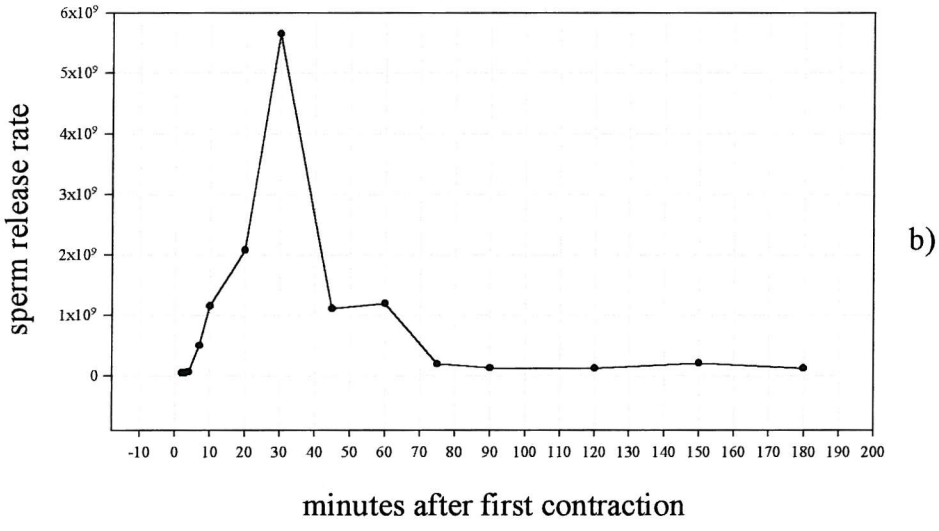


Figure 2.18: Sperm release data from 1 adult H. tuberculata. *(a) contraction rate during sperm expulsion event, (b) sperm release rate*

2.2.4 Discussion

First, it should be noted that contrary to Hayashi (1980a) who reported spawning of *H. tuberculata* in Rocquaine Bay in August and September in 1975-6, Lopez (pers. comm.), Tostevin (pers. comm.) and Baker (this thesis) have noted over the past few years that in the same Bay, the highest percent of ripe individuals occur in and around the month of July.

The laboratory experiments show that fertilization success in *Haliotis tuberculata* depends upon several variables. Sperm concentration has a considerable effect upon fertilization success in culture volumes of 10ml (as also shown by Clavier, 1992 who used culture volumes of 20ml). However, when these experiments are repeated in culture volumes an order of magnitude greater (500ml), it would appear that rather than sperm concentration *per se*, which has little effect, it is the increased probability of sperm-egg encounters resulting from higher sperm-egg ratios which leads to an increase in fertilization success at concentrations of ~10^3 sperm ml^{-1}. This is also highlighted in the sperm-egg ratio experiments where sperm concentrations of 10^2 and 10^3 sperm ml^{-1} in 500ml culture volumes result in relatively high fertilization success (between 58 and 92%) compared to a maximum 18% in the 10ml volume experiments. This would suggest that the critical factor in these cases is not sperm concentration but sperm-egg contacts. Sperm-egg ratio appears to have less effect judging by the fact that at low ratios of only 10:1, 60% fertilization success is still achieved.

Clavier (1992) used 6 male-female pairs of *H. tuberculata* from Brittany in his sperm dilution experiments and noted very wide variations among replicates. His data showed no fertilization below 10^3 sperm ml^{-1} whereas my results for similar culture volumes show up to 18% fertilization success at these low concentrations. As can be seen in both data sets, much variability exists amongst individuals and this could well account for the difference seen between the two data sets. Nevertheless, this study highlights the caution that must be shown using different culture volumes in sperm dilution experiment studying fertilization success. Smaller volume experiments may lead to misinterpretation and hence misleading conclusions. A comment by Rampersad et al. (1994) was made that gamete numbers based on sperm-egg ratios alone should not be used as they are unable to define gamete concentrations or describe proper fertilization conditions for eggs and sperm. For example, if a ratio of 30:1 was used in a large

volume, the chances that the sperm would find the eggs are far less than in a small volume. Hence, ratios should only be used when culture volume is also considered.

For the particular sperm-egg ratios tested in these experiments, this factor had little influence over fertilization success in *Haliotis tuberculata.* The only large drop in fertilization success occurred with sperm concentrations of 10^2 sperm ml^{-1} at sperm-egg ratios of 10:1 and 100:1. However, treatments examining sperm-egg ratios below 100:1 at higher sperm concentrations may have shown significant effects upon fertilization success. Certainly where sperm-egg ratios of 50:1 existed in the 10ml sperm dilution experiments at sperm concentrations of 10^3 sperm ml^{-1}, fertilization success was < 20%. Conversely, in the 500ml volumes of 10^3 sperm ml^{-1}, with sperm-egg ratios of 2500:1, fertilization success was > 85%. When sperm-egg ratios were reduced to 10:1, the lowest fertilization was seen in the sperm-egg ratio experiments. Fertilization success was still relatively high at 58% at 10^2 sperm ml^{-1} suggesting that although sperm concentration *per se* was having some effect, sperm-egg ratio at 10:1 was giving higher fertilization success than sperm-egg ratios at 5:1 in the sperm dilution, 10ml culture volume experiments. Further detailed experiments are needed to safely draw conclusions as to whether sperm dilution or sperm-egg ratio effects are more important at low sperm concentrations and low sperm-egg ratios. For example, ratios of <10:1 should be tested in 500ml culture volumes. It could be that there is competition between eggs for sperm or that multiple sperm are required in this species in order to increase the chance of sperm penetration of a specific binding site. Alternatively, the release of enzymes by more than one sperm may be necessary for initiation of chemical lysis in the egg envelope. Maybe only a fraction of all sperm are able to fertilize the egg. Brown and Knouse (1973) suggest, for the horseshoe crab *Limulus polyphemus,* that a selection mechanism determined by the egg envelope for special genetic spermatozoa may exist and so multiple sperm are required for each egg.

Lillie (1915) showed that egg concentration had a much less significant effect than sperm concentration on fertilization success in the sea urchin *Arbacia punctulata.* Levitan et al. (1991) also studied effects of sperm-egg ratio on the sea urchin *Strongylocentrotus franciscanus* and found little effect except at the lowest sperm concentrations/highest egg concentrations. At sperm-egg ratios of 12.5:1, however, they found little decrease in fertilization success when sperm concentration was at ~10^5 sperm ml^{-1}. They speculated that egg concentration is unlikely to be significant in the

field because the dilution of eggs is so rapid that only immediately following release would egg concentration be high enough to affect fertilization success. Benzie and Dixon (1994) also reached similar conclusions to Levitan et al. (1991) for the seastar *Acanthaster planci*. The sperm-egg ratio was only important in the first few seconds after egg release. Clotteau and Dube (1993) investigated the effects of sperm-egg ratio upon fertilization success in the surf clam *Spisula solidissima* and found that ratios >500:1 induced increased polyspermic fertilizations while at ratios <30:1 oocytes remained unfertilized. Good percentages of monospermic fertilizations (80%) were nevertheless obtained at sperm-egg ratios between 30:1 to 500:1. Brown and Knouse (1973) observed, in *Limulus polyphemus,* that 10^6 sperm per egg resulted in 68% fertilization success whereas 100 sperm per egg gave only 2% fertilization success.

These studies highlight the importance of incorporating the sperm-egg ratio factor into fertilization success experiments at low sperm concentrations because sperm-egg ratios required to achieve the optimum level of fertilization success differs between species. Authors, such as Babcock and Keesing (1999), who ignore this factor are leaving themselves open to misinterpretation of results - there is no discussion of sperm-egg ratio in their paper. Had they conducted their sperm dilution experiments in volumes larger than 10ml, with the same sperm concentrations but greater numbers of sperm per egg, their results could be very different in terms of this sperm dilution experiment. Williams (1999) found similar results to those found in this thesis in that higher levels of fertilization success were gained at low sperm concentrations during the sperm-egg ratio experiments when larger culture volumes were used. He suggested that sperm-egg ratio and the increase of sperm-egg interactions in a larger volume of dilute sperm acts to increase fertilization success at low sperm concentrations. Stephano (1992) reported sperm-egg ratios of between 1000:1 to 66000:1 at sperm concentrations of 3.4×10^4 to 8.3×10^7 sperm ml^{-1}, which showed no increase in polyspermy in *H. rufescens*. Kikuchi and Uki (1974) reported a minimum sperm-egg ratio of 1000:1 up to 50,000:1 at sperm concentrations between 1×10^5 and 1.9×10^6 sperm ml^{-1} was necessary for a high percent fertilization success in *H. discuss hanai*. Mill and McCormick (1989) reported a high percentage of normal cleavage with 5000:1 to 30,000:1 sperm-egg in *H. rufescens* and *H. corrugata* and 1500:1 to 30,000:1 in *H. fulgens*. Sperm concentrations were between 1.5×10^4 and 3×10^5 sperm ml^{-1}. From

this, it may be concluded that abalone have a high tolerance to ranges of sperm-egg ratios and appear to be fairly resistant to polyspermy.

The quality of the eggs produced at spawning is important to subsequent development success. Paulet et al. (1992) found variable oocyte quality with respect to environmental conditions during maturation, especially temperature. In my experiments I tried to eliminate this variability to some degree by only using data from individuals that produced >75% fertilization success at the optimum sperm concentration. Results for the upper range of sperm concentrations beyond 10^6 sperm ml^{-1} qualify Clavier's findings of vitelline layer lysis and ova destruction or abnormal development at these high sperm concentrations. Clavier did not score abnormal development, however. The abnormally developed larvae scored in this study were most likely a function of polyspermy because incidents of abnormalities increased with increasing sperm concentrations, although ploidy levels were not directly assessed in these eggs. When several spermatozoa enter an egg, either cell death or abnormalities result during the subsequent embryonic development. Blocks to polyspermy are common in many taxa (reviewed by Jaffe and Gould, 1985), but it remains unresolved whether such anti-polyspermy mechanisms exist in *H. tuberculata*. Stephano (1992) reported on the susceptibility of *H. rufescens* oocytes to polyspermy. He found oocytes were highly resistant to polyspermy in this species, even at sperm-egg ratios of 66,000:1, in contrast to findings in this thesis of polyspermic eggs at 5,000:1 in *H. tuberculata*. He suggests that these oocytes may be subjected to high sperm densities during natural spawning and hence have developed a fast electrical block to polyspermy. He identified fertilization potentials in the oocytes which are also indicative suggestive of fast blocking mechanisms.

It is also unknown whether gametic chemotaxis occurs in the abalone. In some animals, chemotaxis is thought to increase sperm-egg collision (Miller, 1989). By both (1) repelling excess sperm by using blocks to polyspermy and (2) attracting rare sperm using chemotactic mechanisms, individuals can ensure that a maximum number of eggs are fertilized and developed normally under a range of sperm concentrations. Oliver and Babcock (1992) suggest that the pronounced decrease in fertilization success seen with high sperm concentrations may be a function of the combined influences of decreased oxygen, increased CO_2 and lower pH. Inactivity of echinoid and ascidian sperm under similar conditions is well documented (Chia and Bickell, 1983). Oliver and Babcock

(1992) suggest that coral sperm are inactive when they are highly concentrated, which leads to the low fertilization success reported.

Other studies of abalone fertilization success with respect to sperm concentration have been reported in the literature. Kikuchi and Uki (1974) determined a range of suitable sperm concentrations that would achieve 100% fertilization success in *Haliotis discus hannai* during hatchery procedures. They proposed concentrations of 100,000 – 1,730,000 sperm ml^{-1}, with 200,000 sperm ml^{-1} being the optimum for hatchery procedures (to offset any measuring errors). This is a similar range to that of *H. tuberculata.* However, it should be noted that all their experiments were carried out using 50ml culture volume. The utilisation of such small volumes would be unlikely during hatchery operations and their results, therefore, should be used with caution. They also found sperm concentrations of 1.9×10^6sperm ml^{-1} caused egg membranes to dissolve and at 4.8×10^6 sperm ml^{-1} development was abnormal. Leighton and Lewis (1982) found that the concentration of sperms that yielded maximum fertilization with least abnormality in subsequent development to be ~10^6 sperm ml^{-1} in *H. rufescens, H. corrugata, H. fulgens and H. sorenseni,* all species from the California coast. Leighton and Lewis (1982) also produced hybrid zygotes by combining these 4 species and found that 10^7 sperm ml^{-1} was the optimum sperm concentration for fertilization in the hybrids. They also found that at sperm concentrations >10^8 sperm ml^{-1}, lysis of the vitelline layer occurred. In their paper they do not reveal culture volumes used in their methodology.

With very high sperm concentrations, supernumerary sperm will release sufficient acrosomal lysin to dissolve the oocyte vitelline layers (Kikuchi and Uki, 1974; Leighton and Lewis, 1982) and without this protective coat, eggs and embryos are quite fragile and prone to lysis. Chen (1989) reported that with the addition of sperm concentrations greater than 7.2×10^5 sperm ml^{-1}, eggs of *H.diversicolor* supertaxa became polyspermic with a decrease in hatchout and an increase in abnormal larvae. The most recent study of fertilization success in abalone is that of Babcock and Keesing (1999) who examined the effects of sperm concentration on fertilization success in *H. laevigata.* They found the highest fertilization rates were obtained for sperm concentrations in the range of 1×10^4 to 1×10^6 sperm ml^{-1}. Again, they conducted their experiments in 10ml culture volume and failed to address in their analysis the important question of sperm-egg ratio. Table 2.2 shows optimum sperm concentrations for 10 different species of *Haliotis* tested using either small or undefined culture

volumes. The relationship between sperm concentration and the likelihood an egg will be fertilized has been demonstrated for a number of different species (see Chapter 1 for details). All of these workers found similar relationships of positive correlation between increasing sperm concentration and fertilization success, but did not repeat their experiments in larger culture volumes. Oocyte dispersal in a larger volume increases the number of potential sperm-egg interactions that may occur prior to the eggs sinking to the bottom of the culture vessel compared to oocyte dispersal in a small, say 10ml, volume. Encena et al. (1998) found an optimal sperm concentration for fertilization and development lower than other authors.

Table 2.2

Optimum Sperm Concentrations for Fertilization Success

Species	*Optimum sperm concentration for ~100% fertilization success*	*Author*
H. tuberculata	10^5 sperm ml^{-1} ; 10^5 - 10^6 sperm ml^{-1}	Baker & Tyler (2001); Clavier (1992)
H. discus hannai	2 x 10^5 sperm ml^{-1} ; 10^5 - 10^6 sperm ml^{-1}	Kikuchi & Uki (1974); Gao et al. (1990)
H. rufescens	10^6 sperm ml^{-1} ; 10^5 sperm ml^{-1}	Leighton & Lewis (1982); Mill & McCormick (1989)
H. corrugata	10^6 sperm ml^{-1} ; 10^5 sperm ml^{-1}	Leighton & Lewis (1982); Mill & McCormick (1989)
H. fulgens	10^6 sperm ml^{-1} ; 10^5 sperm ml^{-1}	Leighton & Lewis (1982); Mill & McCormick (1989)
H. sorenseni	10^6 sperm ml^{-1}	Leighton & Lewis (1982)
H. diversicolor	2 x 10^4 sperm ml^{-1}	Chen (1989)
H. laevigata	2 x 10^5 sperm ml^{-1}; 10^4 - 10^6 sperm ml^{-1}	Fallu (1991); Babcock & Keesing (1999)
H. asinina	5 x 10^3 - 1 x 10^5 sperm ml^{-1}	Encena et al (1998)
H. iris	2.5 x 10^4 sperm ml^{-1}; 10^5 sperm ml^{-1}	Hahn (1989); Tong & Moss (1992)
H. rubra	2 x 10^5 sperm ml^{-1}	Fallu (1991)

H. asinina produced high levels of fertilization success at 5 x 10^3 to 1 x 10^5 sperm ml^{-1}. However, their experiments were conducted in 200ml culture volume. As discussed earlier, this is likely to be influencing their results.

Ambient sperm concentration in nature is a function of the distribution and abundance of males releasing sperm and the rate of dilution resulting from turbulence. Increases in population density, population size, level of aggregation, body size and spawning synchrony leads to an increase in sperm concentration. Increases in ambient current velocity, turbulence and distance between spawning individuals leads to a decrease in sperm concentration. It is important to attain a reliable measure of how sperm concentration affects the likelihood of fertilization of eggs in order to construct adequate models of fertilization success in natural populations. Also, for this research, there is a need to determine optimum sperm concentration in fertilization success experiments to ensure that effects of further experimental manipulations are not masked by an excess of sperm.

The length of time eggs are allowed to remain in contact with a sperm suspension influences fertilization success of *H. tuberculata*. The most significant changes in fertilization success occurred in the first few minutes after initial fertilization. The results must be considered with caution, however. Even though only relatively high sperm concentrations were used in these experiments, in an attempt to eliminate the affect of short-lived sperm at low concentrations, the chances are that even at sperm concentrations of 10^4 sperm ml^{-1} some gamete ageing effects are still evident. For example, the results from the gamete age experiments show a sharp fall in fertilization success within the first 30 minutes post-spawning for sperm at concentrations of $\sim 10^4$ sperm ml^{-1}. This must have some effect upon the sperm-egg contact time because ageing effects come into play prior to the sperm-egg contact time reaching a maximum fertilization success plateau at 30-60 minutes. Nevertheless, results indicate that eggs need to be in contact with sperm for several minutes in order to achieve high fertilization success. Ebert and Houk (1984) found that excess sperm was not detrimental to fertilization success in *H. rufescens* provided the excess was removed within 15-30 minutes post-fertilization. Babcock and Keesing (1999) found that contact time was not significant in the abalone *H. laevigata* and predicted that attachment of sperm to an egg must be rapid. The results presented for *H. tuberculata* in this thesis were useful in developing methods for the other laboratory-based manipulations to study fertilization success and were also taken into account when implementing the Vogel (1982) model of fertilization rates (see section *2.3* for details).

Lillie (1915) was one of the first workers to recognise the limited life span of gametes. Since then, quantification of gamete age in free-spawning marine invertebrates has spanned a number of different invertebrate groups including echinoids (Pennington, 1985; Levitan et al., 1991), asteroids (Benzie and Dixon, 1994), bivalves (Andre and Lindegarth, 1993), hydroids (Yund, 1990), ascidians (Havenhand, 1991; Bolton and Havenhand, 1996; Bishop, 1998) and chelicerates (Brown and Knouse, 1973). The data presented here are among the first for the Gastropoda. Sperm longevity in *H. tuberculata* is comparable to that of other species. Table 1.2 (Chapter 1) compares similar sperm concentrations with respect to ageing effects. Sperm age is known to vary considerably with concentration. This is a function of the 'respiratory dilution effect' reviewed by Chia and Bickell (1983). The longevity of sea urchin sperm is extended when sperm are stored at high concentrations. In the natural environment, high

concentrations of sperm are likely to be quickly diluted and hence the life span of the sperm would be drastically shortened. As can be seen from the table, results gained in this study are similar to those gained in studying gamete age in the tropical abalone, *H.asinina* (Encena, 1998) and *H. laevigata* (Babcock and Keesing, 1999). It would have been useful to determine sperm ageing effects at lower sperm concentrations, particularly at time intervals less than 30 minutes because fertilization success fell rapidly to zero during this period. More detailed experiments should be conducted in future.

Generally, oocytes of free-spawners last longer than the sperm (Pennington, 1985, Levitan, 1995). Oocyte longevity in *H. tuberculata* appears to be shorter than reported for other species. A few uncontrolled experiments, which examined the longevity of oocytes of *H. tuberculata*, indicated that they aged at approximately the same rate as sperm. Unfortunately it proved logistically impossible to conduct separate sperm and egg ageing experiments owing to the unpredictable timing in spawning. Babcock and Keesing (1999) refrigerated sperm of *H. laevigata* at 5°C and found that it did not age so were able to conduct egg age experiments. I found no such increase in sperm longevity of *H. tuberculata* with refrigeration during preliminary tests. Stephano (1992) studied oocyte ageing in *H. rufescens* and found that eggs of 5 to 6.5 hours old would allow sperm to penetrate, but they failed to activate and cleave. It would appear from his results that one component of oocyte ageing is the loss of activation response to fusing sperm. Brown and Knouse (1973) found that storing sperm at 4-5°C increased fertilization success in *Limulus polyphemus* eggs from 43% at 0-1 h to 83.5% at 72 hours. It was suggested that storage instigated selective death of abnormal spermatozoa, thereby increasing the chance of successful fertilization.

Male *H. tuberculata* are generally thought to release sperm into the water column slightly ahead of the females releasing their eggs. Certainly this is seen in laboratory induced spawning (pers. obs.). Possibly the delay of spawning by females is an adaptation to ensure that sperm are already in the surrounding water during egg release. Maybe there is little need for increased longevity in eggs as the chances are that sperm will already be around when eggs are spawned. Gamete age is not generally considered to be important in wild populations of free-spawners as the dilution of gametes below fertilizable concentrations would normally occur a long time before the viable life of gametes has expired. In some situations, however, gamete age could be a factor. For example, if animals were to spawn in a tidal pool or some other slack water

environment, the dilution effect would be lessened and gamete age may come into play. The rather short life-span of *H. tuberculata* sperm may suggest a lack of chemotactic response in gametes of this genus. If chemotaxis were to play a significant role in increasing fertilization success, it would be of great benefit for the gametes to remain viable for longer. For the purposes of this study, information concerning gamete age is particularly useful in the design of laboratory and field experiments but is not considered to be important to the overall fertilization ecology of *H. tuberculata* populations in Rocquaine Bay, Guernsey where tidal and wave action can be substantial. For hatchery purposes, extending the longevity of gametes may be of economic interest. Preliminary experiments were conducted to examine the possibility of increasing the life-span of eggs and sperm by reducing their holding temperature. Storing gametes at 12°C appeared to have no effect upon their longevity, although holding animals at lower temperatures has yet to be tested as these facilities were unavailable in Guernsey.

Culture volume was also considered to ensure it was not contributing to variations in the fertilization success of *H. tuberculata*. No significant difference was seen when using different culture volumes under optimum sperm concentrations. Sperm-egg ratio experiments that required different sized culture vessels could be conducted without the worry of this potential discrepancy.

The physiological ability to cope with environmental temperatures plays a major role in determining the distributional limits of marine organisms. The majority of marine animals are poikilothermic species with a varying body temperature that approximately follows the ambient water temperature. Temperature is known to have a major effect upon many biological processes and fertilization success is no exception. For broadcast-spawning animals, information regarding temperature effects upon gametes and embryos is of particular importance as these cells are more likely to be exposed to thermal anomalies in the ambient waters. They are solely dependent upon their own resistance mechanisms. *H. tuberculata* gametes appear to have a high thermal tolerance (eurythermic) with the ability to withstand temperatures spanning 8°C without any detrimental affects to their subsequent development to trochophore. However, this is not to say that sustained low or high temperatures would not have an effect on the later embryonic development. This was not examined. Nevertheless, there is evidence to suggest that temperature is a limiting factor primarily during the earliest period of ontogenesis: at the stages of ova, zygote and cleavage (Lonning, 1959; Moore, 1959;

Calabrese, 1969; Andronikov, 1975). Other molluscan species that have been examined for gamete thermal tolerance show similar results to those presented here. For example, temperature limits for normal embryonic development in *Mytilus edulis* span 10°C from 8 - 18°C (Kinne, 1970). In the clam *Mercenaria mercenaria*, embryos develop normally between 18 and 31°C (Loosanoff, 1937; Davis and Calabrese, 1964). In the cockles, *Cardium edule* and *C. glaucum*, fertilization does not take place at temperatures as low as 5°C, but at 15 and 20°C fertilization and early development appears normal. No development was observed in ova inseminated at 25°C (Kingston, 1974). Andronikov (1975) studied the temperature effects upon gametes and fertilization in 16 species of sea urchins and molluscs and proposed that the upper thermal limit for the normal development of embryos is only 1 to 3°C higher than those temperatures encountered under normal conditions. *H. tuberculata* may now be added to this list.

Although an overall idea of what happens to fertilization success with temperature has been gained, some important questions remain to be addressed. Namely, does temperature directly affect the sperm motility and survival? It would be possible to assess this by using video imaging methods as used by Fujita (1929) for a study on *Crassostrea gigas* development. A further question to be addressed is how temperature affects egg viability. It may be that the temperature shock itself of higher and lower temperatures affects the viability of ova or spermatozoa. If so, then the determination of fertilizability itself may not have been tested here and hence interpretation of these results for *H. tuberculata* may be questionable. A future investigation of this possible scenario is needed by subjecting fresh eggs and sperm to high and low temperatures and then returning them to ambient temperature prior to fertilization to see if they are still viable. Also, from an ecological standpoint, it would be interesting to repeat experiments on the effect of temperature on *H. tuberculata* collected from further south – perhaps Portugal – to see if larvae of the same species but living under a different temperature regime, respond in the same way.

Although larval development is widely reported for many different species no work has been published on the larval development rates of *H. tuberculata* with respect to temperature. Kioke (1978) described larval development in *H. tuberculata* for temperatures of between 20 and 21°C. Ebert and Houk (1984) reported on the development of *H. rufescens* at temperatures of 15°C. The only other abalone study which assesses veliger larvae development and growth across a range of temperatures

(from 10 to 20°C) was for *H. sorenseni*. The optimum temperature range for development of the larvae lay between 14 and 18°C (Leighton, 1972). Beaudry (1983) found an inverse relationship between temperature and survival over a temperature range of 14 to 21°C for *H. kamtschatkana*. The ambient sea water temperature in Rocquaine Bay, Guernsey, was particularly warm during the summer of 1999. This would have resulted in marginally faster development times for the larvae. Hayashi (1980a) and Forster et al. (1982) suggested that larval *H. tuberculata* had decreased survival in below-average temperatures resulting in poor recruitment in exceptionally cold years.

The rate at which gametes are released has a significant bearing upon subsequent fertilization success. In this study the sperm release rate was estimated for *H. tuberculata* from one individual. In addition, the amount of sperm and eggs extruded over the period of one hour was noted for a number of animals (Table 2.1). Hayashi (1980a) reported the total numbers of gametes released from 5 male and 7 female *H. tuberculata* with very similar results to those reported here. Clavier (1992) also induced spawning in *H. tuberculata* in order to determine fecundity and found total numbers of sperm or eggs extruded over the entire spawning period. The number of eggs shed ranged from 2×10^5 to 1.6×10^6 and the number of sperm from 1.5×10^{10} to 19×10^{10}. His results are similar to those found in this study where the number of eggs shed in one hour ranged from 7.9×10^4 to 2×10^6 and sperm from 9.1×10^9 to 1.5×10^{11}. Clavier reported total spawning time ranged from 40 to 80 minutes in contrast to my findings of between 40 minutes and 5 hours. Clavier also noted the frequency of sperm pulses, and showed that sperm were released every 30 to 45 seconds in initial spawning activity, with decreasing regularity to the end of spawning, completed after 30 to 70 pulses. Babcock and Keesing (1999) found that the number of sperm released by 24 male *H. laevigata* over a 3 hour period ranged from 7.1×10^{10} to 1.9×10^{12}, averaging 5.7×10^{11} +/- 5.4×10^{11} SD. Data from 16 females indicated that the number of eggs released ranged from 3.4×10^5 to 8.2×10^6 averaging 3.0×10^6 +/- 2.2×10^6 SD. They found that length vs. gamete output was not significant. Sperm release rate over the 3 hour period was 5.3×10^7 +/- 4.9×10^7 s^{-1}. The information gained for *H. tuberculata* in terms of sperm release rate was required in the estimation of fertilization kinetics in the field.

One factor that was not considered during the experimental procedures was that of time in breeding season. Benzie and Dixon (1994) found a reduction in gamete quality towards the end of the breeding season in the Crown-of-Thorns starfish *Acanthaster planci*. Anecdotal evidence from experiments conducted on *H. tuberculata* during June through to July 1999 showed an increase in gamete production and quality from the end of June through to the end of July. This time period most likely represented early to mid/late breeding season. Perhaps it is possible to infer from this that the most viable gametes would be expelled in the middle of the spawning season. A further consideration is the viability of an individuals gametes in terms of time during a spawning event. It may be that gametes released by an individual at the start or end of the spawning period may be of inferior quality. In this study, gametes were never collected at the end of the individuals spawning but it is not known whether the cells expelled at the start of spawning were of similar quality to those expelled a few minutes later.

There are a vast number of marine species that have adopted the mode of broadcast spawning, which affirms the effectiveness of this reproductive method and may be of particular importance to sessile organisms. *H. tuberculata* are slow growers and therefore it is all too easy to over-harvest this resource (Clavier and Chardy, 1989). Clavier produced a fecundity/size curve which shows clearly how fecundity increases with individual body size. Potential fecundity ranged from 4×10^5 for 20g wet weight to 8×10^6 for 160g wet weight of *H. tuberculata.* Similar conclusions were also drawn during work by Hayashi (1980). This variation in fecundity with size could have a real effect upon the fertilization potential of a population depending upon the size frequency of that population. Abalone do not readily disperse from reef to reef and individual reefs are mainly stocked by reproduction of abalones already on them. There is little hard evidence but most workers favour the position that there is poor geographic dispersion of larvae and that is the reason it takes so long to re-colonise an over-fished reef.

The principal conclusions implied from these results are that relatively high sperm concentrations (although not so high as to induce polyspermy) are important for successful fertilization. This would suggest that the most effective strategy to be adopted would be to have large numbers of animals living in close proximity with high levels of synchronisation and aggregation during spawning events. Where this is not possible because of fishing mortality, sperm limitation will ensue.

There are many limitations associated with laboratory experimentation. For example, Epel (1991) demonstrates a substantial decrease in fertilization success with the introduction of turbulence to a fertilization assay. For this reason, field experimentation was attempted in July 2000 (see section *2.5*).

2.3 The Vogel-Czihak-Chang-Wolf (VCCW) Model

2.3.1 Implementation

In order to estimate fertilization success in field situations, the probability of sperm-egg collisions and fertilization success must be considered. The VCCW (Vogel-Czihak-Chang-Wolf) model 'Don Ottavio' (Vogel et al.,1982) described previously in Chapter 1 (section *1.3*), was used to obtain estimates for *H. tuberculata* fertilization rate constants based on information gained from (1) gamete concentrations and (2) sperm age effects on fertilization determined in the laboratory studies. This model makes the assumption that a sperm will adhere to the first egg it encounters, regardless of whether or not fertilization occurs. The model predicts the proportion of eggs fertilized (φ) as:

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

where (S_0, sperm ml^{-1}) is the concentration of virgin sperm, (E_0, eggs ml^{-1}) is the concentration of virgin eggs, (τ, sec) is sperm half life, β_0 and β are two rate constants of fertilization. The first rate constant β_0 ($mm^3\ s^{-1}$) is that of sperm and egg collision and may be estimated from the sperm swimming velocity (v), and the *total* cross-sectional area of the egg (σ_0), where known ($\beta_0 = v \times \sigma_0$). The second, β, is the rate constant of fertilization. This is the product of the mean spermatozoan speed (v) and the *fertilization* cross-sectional area of the egg (σ) ($\beta = v \times \sigma$). This has yet to be determined experimentally for any marine invertebrate. The ratio of β/β_0 is the average proportion of sperm contacts necessary for fertilization to occur. Both of these constants may be estimated from Vogel's model. Laboratory experiments show that for maximum fertilization, sperm and eggs must be in contact with each other for approximately 30 minutes. In order to determine sperm half life for inclusion in the model, the results of the sperm age experiment were fitted to an exponential regression equation. It was not possible to include the lower dilutions in the prediction. Gamete

age at low sperm concentrations has been inferred from the exponential regression analysis performed. As no experiments were conducted for time spans of less than 30 minutes, it is not possible to gauge the accuracy of the data at these lower concentrations. The half life was calculated from the predicted time when fertilization success was 50% of the initial value at t = 0. These half life values were log transformed and then plotted as a function of log-transformed sperm concentration and a linear regression was performed in order to predict the required values for τ at each sperm concentration (Figure 2.19).

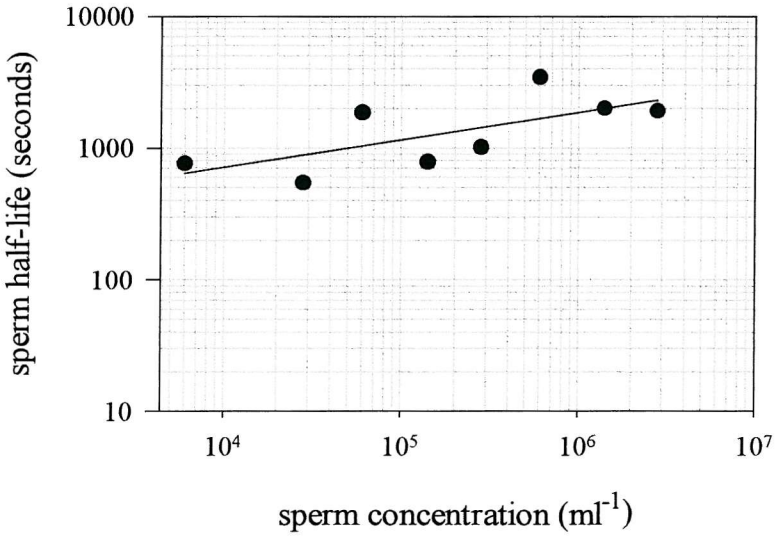


Figure 2.19: Sperm half life (τ,S) as a function of sperm concentration (ml.). Log τ = (log S x 0.2028) +2.10577, R^2 = 0.465

The slope of the regression line is directly related to sperm swimming velocity. The intercept on the y axis is inversely related to sperm swimming velocity. The higher swimming velocities are associated with lower stamina.

A Levenberg-Marquardt iterative method of non-linear regression (Jandel SigmaPlot) was used to determine the best fit values of the two rate constants for the calculated values of sperm half life and sperm concentration. The data converged and tolerance was satisfied with 15000 iterations. Increasing the number of iterations had no effect on the results. Figure 2.20 shows the predicted fertilization kinetics model line fitted to a subset of the empirically derived fertilization success data.

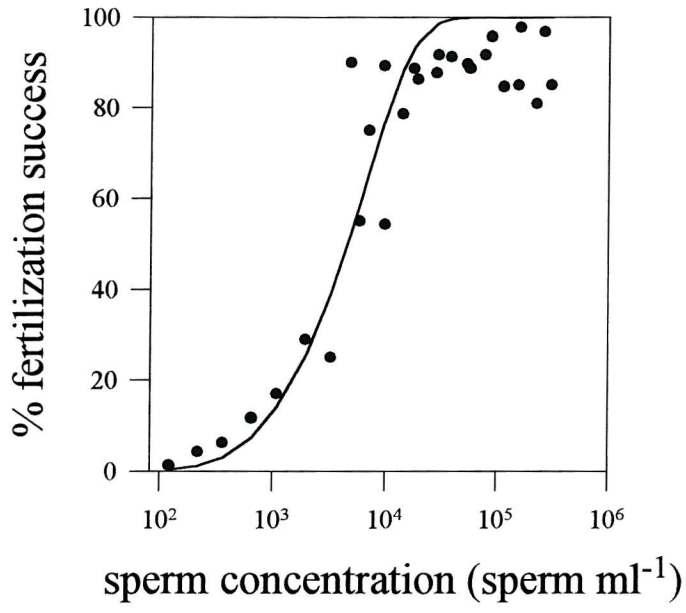


Figure 2.20: Best fit line of model to empirical data. See text for details.

The best fit for this model gave a β value of 1.6×10^{-6} (calculated from β_0), and a β_0 value of 5.5×10^{-4}. β_0 values did not vary over the range of initial parameters tested. The ratio of β/β_0 was 3.0×10^{-3}. This suggests that only around 0.3% of the egg surface is fertilizable or that only about 0.3% of the spermatozoa are able to fertilize. The empirical data were regressed against the predicted data from the model with an R^2 value of 0.898. This fitted regression line explained almost 90% of the variation in fertilization and the model is considered to be robust.

2.3.2 Discussion of Vogel Model

The predicted ratio of β/β_0 that describes the average proportion of sperm contacts necessary for fertilization to occur was 3.0×10^{-3}, an order of magnitude smaller for *H. tuberculata* than for *H. laevigata,* which had a ratio of 1.1×10^{-2} (Babcock and Keesing, 1999). The value of β increased with egg diameter in the two *Haliotis* species. Given that the total cross sectional area of the egg of *H. tuberculata* is approximately 25450μm^2, the mean sperm swimming velocity is predicted to be 0.02 mm s^{-1}, calculated from the given value of β_0 from implementation of Vogel's model. If the same calculation is done from the data of Babcock and Keesing (1999), an estimated egg diameter of 225μm gives a sperm swimming velocity of 0.07 mm s^{-1}. These values of sperm velocity are of a similar order to those that found for sea urchins (Levitan et

al., 1991; Vogel et al., 1982). It would be useful to measure sperm swimming velocity empirically to validate these predicted values. Levitan et al. (1991) did just that for species of sea urchins and compared model predictions of β_0 to empirical data. They found that the predicted and observed values were not significantly different from each other. This suggests that the Vogel model is accurate in its predictions and that egg size and sperm velocity are very important in influencing fertilization success in nature and in the laboratory.

If a comparison is made between the two *Haliotis* species in terms of fertilization success with respect to sperm concentration against egg size and incorporating the $\beta/\beta o$ parameter, one may expect to see higher success of *H. laevigata* at low sperm concentrations owing to the larger egg size and the higher fertilizability of the egg predicted by Vogel's model. This line of thinking follows that of Levitan (1993) who found a good correlation between these factors in 3 species of *Strongylocentrotus*. However, this theory does not follow for these two abalone species.

The model of Vogel et al. (1982) has a couple of shortcomings that should be considered. First, it should be noted also that this model does not account for sperm chemotaxis, which may act to increase the effective egg size. Gametic chemotaxis has, however, not been demonstrated in the abalone. Second, the model used here assumes a perfect polyspermy block occurs following initial fertilization of an egg by one sperm. For this reason, only a subset of my data was used to determine the fertilization constant values. It was not possible to fit data at higher sperm concentrations above 5×10^5 sperm ml^{-1} where polyspermy was evident in the results. Styan (1998) produced a polyspermy-adjusted model of fertilization and describes sperm-egg interaction in *Strongylocentrotus franciscanus* using different polyspermy time factors and includes eggs with no ability to block polyspermy. Vogel's model has been used by some workers who have found very good fits to the model with their data (Vogel et al., 1982; Levitan et al., 1991; Levitan, 1993, Levitan and Young, 1995, Babcock and Keesing, 1999). Styan (1998) discusses that some of these authors may have overestimated zygote production as some of the eggs scored as successfully fertilized at the raised fertilization membrane stage may not have developed normally owing to polyspermy even though they were scored as successfully fertilized in the first instance. Other workers who have scored cleaved eggs in a variety of free-spawning marine invertebrates have found decreases in fertilization success at higher sperm

concentrations - a function of polyspermy (e.g. Sprung and Bayne, 1984; Clavier, 1992; Keesing and Babcock, 1996; Desrosiers et al., 1996; Babcock and Keesing, 1999; Baker and Tyler, 2001). These data lend themselves better to the Styan (1998) polyspermy-adjusted model. Assuming estimates of parameters using the VCCW model, implementation of Styan's (1998) model may be used to estimate polyspermy block activation time. I used the model of Vogel in this study as it has been tried and tested and I was able to draw comparisons with previous studies.

2.4 Modelling of Sperm Diffusion and Fertilization

2.4.1 Introduction

The probability of successful fertilization in natural populations of free-spawning shellfish may be estimated by the development and implementation of predictive theoretical models. Despite sperm having a certain swimming capacity in the order of ~ 0.1mm s^{-1}, dispersal and dilution of gametes in the water column are largely governed by turbulent mixing processes. Aside from the biological considerations, the environment in which free-spawning organisms live will mediate fertilization success in terms of current flow (advective velocity and turbulence) topographical complexity, water depth and water quality (temperature, salinity and pH). The distance across which two individuals can successfully fertilize is in part a function of gamete dispersal and dilution in the water column. An understanding of gamete dispersal patterns is therefore critical to an evaluation of the reproductive success of free-spawning populations.

The environment in which *H. tuberculata* lives is highly complex in terms of flow patterns. The effect of advection (mean direction and velocity) on the spawned gamete cloud and its rate of spreading (diffusion) must be considered. Advection influences gamete contact time and concentration, two factors which we already know have a profound influence on fertilization success in *H. tuberculata* (Baker and Tyler, 2001). Diffusion disperses and mixes gametes, which also has a bearing on these two factors. In nature, turbulent as opposed to molecular diffusion prevails, causing particles to diffuse at higher and more variable rates. Turbulence is produced at the seabed when the mainstream flow moves past the stationary bottom. This motion causes a velocity gradient to be set up in a layer of fluid (the benthic boundary layer) adjacent to the substratum. The faster the flow, the steeper the gradient. When the gradient exceeds a certain threshold (see Schlichting, 1979 for critical values), the flow becomes unstable

and turbulent eddies are formed. Eddies are discrete water masses that flatten out, fold on themselves, and then dissipate into smaller eddies, eventually resulting in Kolmogorov eddies. These minimum length eddies can range from 0.1cm in highly turbulent water to 3.5cm in calm seas (Mitchell et al., 1985; Mann and Lazier, 1991). Turbulence may also be caused as water moves around solid objects protruding from the sea bed such as rocks, animals or kelp. The wake of each object is turbulent and these many wakes add significantly to the already turbulent environment. Turbulent eddies produce an unpredictable and patchy mosaic of ephemeral water masses (Okubo, 1988; Vincent and Meneguzzi, 1991). Because of this, rates of mixing and fertilization may only be specified in terms of statistical means. When applying predictive models, it is always necessary to recognise their limitations in terms of untested assumptions made. Models of this nature only predict mean values and are not an estimate of variance.

Csanady (1973) developed equations in an attempt to understand how turbulent water movement mixes and disperses gametes in terms of diffusion. Denny (1988) made modifications to these equations for predicting gamete concentration as a function of distance or time from a release point. Babcock et al. (1994) made adjustments to incorporate sperm reflecting off both the bottom and the seawater's surface, important in shallower waters.

By using empirical estimates of sperm release rate and the effects of gamete age, gamete concentration and gamete contact time upon fertilization success it is possible to model fertilization success *in-situ*. Such models have been used to predict fertilization success in species of echinoderm (Levitan et al., 1991; Levitan and Young, 1995; Babcock et al., 1994), bivalve (Andre and Lindegarth, 1995) and gastropod (Babcock and Keesing, 1999).

2.4.2 The Model (Denny, 1988)

Denny's (1988) equations predict gamete concentration downstream of a spawning male. This model is intended to be 'educational' in the sense that it attempts to model reality in the simplest fashion possible and serves as a guide for experimentation (Denny and Shibata, 1989). The model assumes unidirectional turbulent flow. The sperm concentration (c sperm m^3) downstream form a sperm source is a function of the sperm release rate (Q sperm s^{-1}), the velocity of advection ($\bar{u}$, m s^{-1}),

two dispersion coefficients α_y and α_z which describe the shape of the plume, the downstream position along the x axes (m) and the frictional velocity (u_*):

$$c(x) = (Q\bar{u}/2\pi\alpha_y\alpha_z u_*^2 x^2)$$

As the size of eddies can be constrained by the presence of the seabed, the vertical diffusivity coefficient α_z is typically much smaller than the horizontal diffusivity α_y in boundary layer flows. Denny (1988) used values of 2.2 and 1.25 for α_y and α_z respectively. Denny and Shibata (1989) suggested values for α_z of between 0.2 and 0.05 and they varied the α_y/α_z ratio from 1 to 3. These values were gained from data of Panofsky (1967) for diffusivity in terrestrial boundary layers, which are dynamically similar to those in water. Babcock and Keesing (1999) used values of 7.79 and 0.77 for α_y and α_z following *in-situ* dye measurements for calculation dispersion at 5m. Andre and Lindegarth (1995) used Denny's (1988) values in their model predictions for fertilization success in the cockle. It should be noted that vertical diffusivity increases with distance from the substratum. It should also be noted that turbulent diffusivity is typically an increasing function of time. In the initial stages of particle dispersal, only the smallest eddies are acting upon the particles but as these particles become further dispersed, larger and larger eddies can participate in their further dispersal and hence the rate as dispersal increases. These diffusivity values may have a profound effect on the model's predictions.

The fertilization model of Denny and Shibata (1989) has been used to explore sperm limitation in populations of *Haliotis tuberculata* inhabiting Rocquaine Bay, Guernsey. Laboratory-derived results for the effect of sperm concentration upon fertilization success and sperm release rates, and a range of parameter values (as shown in Table 2.3), have been used to construct plots showing predicted fertilization success under varying current regimes (Figures 2.21a-e). For simplicity, the sperm concentrations were evaluated at y=0 and z=0, thereby only incorporating the x directional factor.

Table 2.3

Parameter Values

Parameter Description	Symbol	Values and Units
Vertical diffusivity coefficient	α_y	2.2; 0.1984
Horizontal diffusivity coefficient	α_z	1.25; 0.124
Male-female separation distance	X	0 - 400cm
Sperm release rate	Q	1.7×10^7 ; 5.1×10^7 sperm s^{-1}
Mean flow velocity	$\bar{U}$	5 ;10 cm s^{-1}
Frictional velocity	u_*	5%; 10%; 15% $\bar{u}$

Sperm release rate was estimated in laboratory manipulations (section *2.2.3.10*). In the surf zone, the frictional velocity (u_*) is typically 5 to 15% of the maximum wave-induced velocity (Svendsen, 1987). Frictional velocity is calculated from:

$$u_* = \kappa ((\bar{u}_2 - \bar{u}_1) / (\ln Z_2 - \ln Z_1)$$

where κ is von Karman's constant (0.4) and $\bar{u}_2$ and $\bar{u}_1$ are current velocities measured at two heights Z_2 and Z_1 above the seabed. This equation provides approximate values for u_* in the log section of a well-defined boundary layer (Wimbush and Munk, 1970; Wimbush, 1976; Young et al., 1992). This value was coarsely calculated in Rocquaine Bay in water depths of 1m over a smooth sandy substrate where *in-situ* experiments were subsequently executed. Three replicate measurements were made using fluorecein dye, a tape measure and stop watch. Dye movement was timed at depths of 10 cm and 5 cm (Z_2 and Z_1)above the seabed. Results gave a mean velocity ($\bar{u}_2$ and $\bar{u}_1$) of 5.3 cm s^{-1} and 4 cm s^{-1} respectively. When input into the above equation, u_* was 0.75 cm s^{-1}. This value is 14.15% of the current speed at 10cm above the seabed. Young et al. (1992) calculated frictional velocity to be ~8% of the current speeds measured.

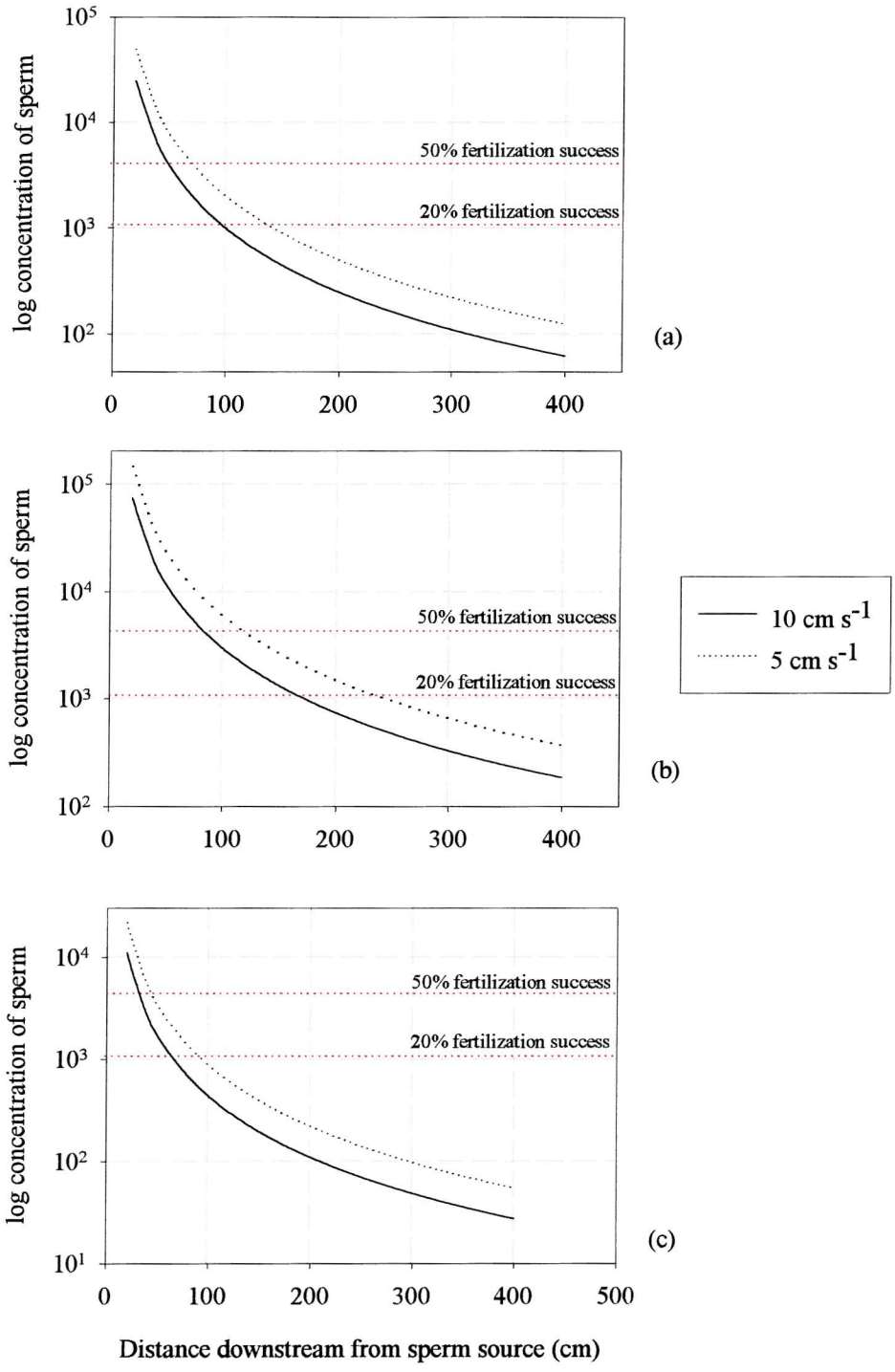


Figure 2.21 (continued on next page): Predicted sperm concentration and fertilization success under a range of different current regimes for H. tuberculata. *(a)* $\alpha_y = 1.25$; $\alpha_z = 2.2$; $u_* = 10\%$; *1 male, (b)* $\alpha_y = 1.25$; $\alpha_z = 2.2$; $u_* = 10\%$; *3 males, (c)* $\alpha_y = 1.25$; $\alpha_z = 2.2$; $u_* = 15\%$; *1 male*

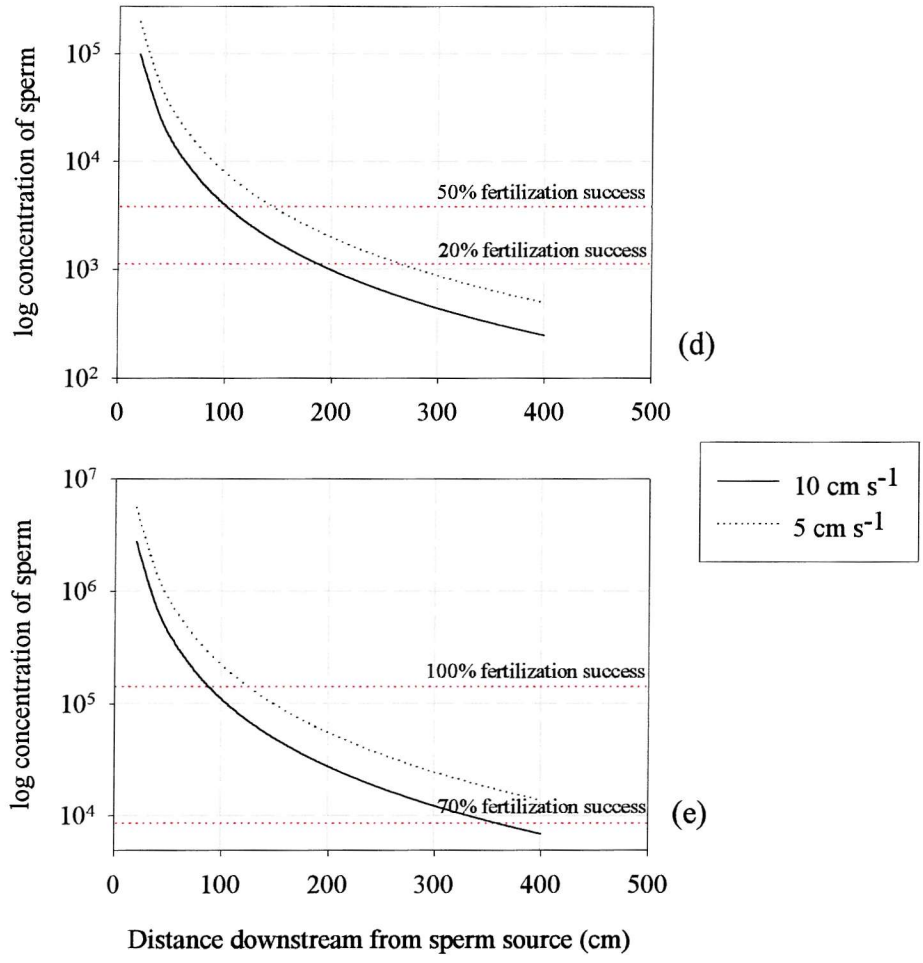


Figure 2.21 (continued): (d) $\alpha_y = 1.25$; $\alpha_z = 2.2$; $u_* = 5\%$; 1 male, (e) $\alpha_y = 0.198$; $\alpha_z = 0.124$; $u_* = 10\%$; 1 male

2.4.3 Discussion

Five different simulations are presented in terms of current speed, the number of spawning males, different diffusivity coefficients and variations in frictional velocity at varying distances upstream of egg release. Predictions of this model for *H. tuberculata* using the particular parameters chosen implies that field fertilization is highly dependent upon all of these variables.

Firstly, in terms of current speed, a decrease in fertilization success is seen with the higher current speed of 10 cm s^{-1} (solid line on each graph) compared with 5 cm s^{-1} (dotted lines). As suggested previously in this chapter, spawning at slack water appears to be beneficial in terms of fertilization success in this species. However, as can be seen in Figure 2.21e, fertilization success may be compromised as a function of polyspermy at slower current speeds when male and female animals spawn synchronously within a few

cm of each other and when diffusivity coefficients are set to 0.198 and 0.124. With increasing values of u_*, lower levels of sperm concentration and consequently fertilization success are seen, as shown in Figures 2.21 a, c and d.

In four of the simulations (2.21a-d) where diffusivity coefficients were set to 1.25 and 2.2 (Denny, 1988), fertilization success failed to reach 100%, even at a few cm from the sperm source. A very different scenario was seen when using 0.198 and 0.124 (Denny and Shibata, 1989). Here, fertilization success is predicted to be 100% at 1 m downstream, even at the higher current speed of 10 cm s^{-1}. This shows the high sensitivity of these parameters to the model predictions and hence any arbitrary choice of these factors will have a profound effect on conclusions drawn. Indeed, in some published work reasons for choice are not specified (e.g. Andre and Lindegarth, 1995).

By increasing the number of males spawning (assuming that the males are spawning synchronously and in the same position) an increase in fertilization success by ~30% is seen at 100 cm downstream. In reality, the model is likely to over-estimate fertilization success as it is much more common that the males are next to each other rather than in the same position. This would increase the chance of the sperm plume deviating from the x-axis, which leads to increased dispersion and hence decreased fertilization success. Nevertheless, it is probable that any increase in the number of males spawning synchronously within a localised patch would result in an exponentially increasing number of fertilized eggs.

This model assumes that gametes in the turbulent zone of the benthic boundary layer are transported primarily as passive particles, moving arbitrarily with the turbulent water in which they are suspended. Experiments using larvae in a subtidal habitat support this theory (Hannan, 1984; Butman, 1987). As sperm swimming speeds are generally slower than that of larvae, it is assumed that the passive particle theory holds true for gametes. Only in cases of non-turbulent flow would sperm swimming action be an issue. Although the slow sperm swimming speeds render it unlikely that sperm can actively control their position in a turbulent water column, their directed swimming action may have some effect when spermatozoa are close enough to an egg.

One issue which has been omitted in the model is that of shear dispersion. Okubo (1968, 1973) and Csanady (1973) have shown that in turbulent boundary layers, shear dispersion can substantially increase the rate at which particles are dispersed. The results from this model may therefore overestimate sperm concentrations that occur in

the field. The other aspect that is not accounted for here is the shear that may act upon the fertilization process itself This may also act to reduce fertilization success as shown by other preliminary studies (Sweezey, Denny and West, unpub.).

Another potential overestimation of sperm concentration is that the model is set up to calculate gamete advection down the x-axis only. Most plumes move away from the axis at points and here, concentrations will be lower, especially further downstream.

I have only modelled sperm plumes passing over spawning females. Drifting eggs would increase the chances of fertilization by passing over spawning males (Levitan and Young, 1995).

Thomas (1994a,b) noted that simple models of gamete mixing can be complicated by spawn consistency, which varies across species and habitats. My personal observation of abalone spawning suggests that the viscosity is similar enough to that of fluorescein dye to discount this factor.

An attempt was made to validate these model predictions with *in-situ* measures of dye diffusion and fertilization success of *H. tuberculata* in order to calculate the level of fertilization success as a function of distance away from a spawning organism. Spawning in nature is rarely witnessed. This forces the need for experimental studies *in-situ*, the design of which needs careful consideration. This model was useful in designing field experiments in terms of scale.

2.5 In-situ Experimentation

2.5.1 Animal Density Estimates

It is apparent from the above model that unless spawning animals are relatively close together, fertilization success will be severely compromised. In order to assess the current condition of ormer density in Rocquaine Bay, a survey was conducted.

2.5.1.1 Methods

A 20 m tape transect was placed, by diver, along an area of suitable substratum likely to hold ormers. Three separate sampling sites were chosen between depths of ~14 to 24 meters (see Appendix C for precise locations). The chosen sites were characterised by granite bedrock, boulders and smaller rocks. Prior to animal collection, kelp stalks were cleared away from the sites to ease sampling. Animals were collected within a 4 m^2 area (1m either side and 1m behind and ahead of the location centre). The

number of ormer within each $4m^2$ area were recorded and these animals were collected, measured and, where possible, sexed. Unfortunately the survey was conducted when many of the animals had already spawned and this made sex identification impossible in some cases.

2.5.1.2 Results

Only two of the three sample sites yielded significant ormer numbers. Sample location 1 had 1.025 ind. m^{-2}, and sample location 2 had 1.43 ind. m^{-2}. Only one individual was found at sample location 3. In locations 1 and 2, the vast majority of animals were found to be aggregated in groups of two or more on the underside of algae-covered rocks. Nine individuals were found under one boulder. Figure 2.22 are images captured from a video tape, which was made during the sampling programme by Richard Keen. Similar ormer densities to those presented here were found in Rocquaine Bay by Forster (1962). *H. tuberculata* is a sedentary gastropod and Clavier and Richard (1983) reported that movement does not increase during the spawning season. This contrasts with spawning aggregation behaviour reported for *H. laevigata* (Shepherd, 1986) and *H. kamtschatkana* (Breen and Atkins, 1980). However, *H. tuberculata* appear to aggregate year round (Tostevin, pers. comm., pers. obs.).

2.5.2 Current Velocity Estimates

A 5m long weighted pole with distance markings was placed on the seabed, parallel to the ambient water flow at a depth of ~1 meter. Approximate flow velocity was measured by releasing 20ml of fluorecein dye at release point '0' and approximately 5cm height from the seabed. A stop watch was used to gauge the time taken for the head of the dye cloud to travel a pre-determined distance along the pole in the x

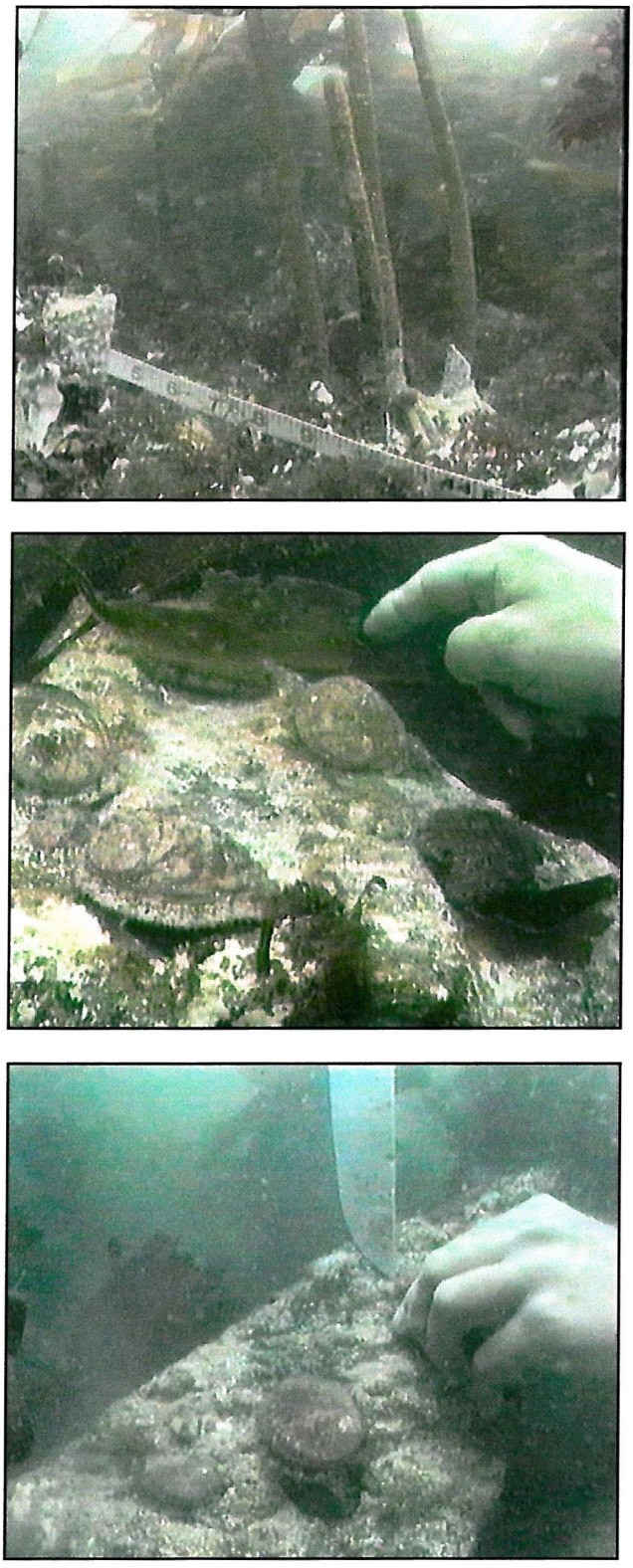


Figure 2.22: Sampling H. tuberculata *for density studies.*

direction (along current). These current velocity estimates were made just seconds before the experiment in order to ensure, as far as possible, that the current was flowing in a consistent direction for the duration of the experiment.

2.5.3 In-situ Measures of Fertilization Success

2.5.3.1 Methods

Animals were collected and gametes were obtained as described in section *2.2.2.1* and *2.2.2.2.* The spawning process took many hours, and it was often too dark to carry out experiments by the time they spawned. Setting the animals to condition early in the morning (04:00) on two occasions made no difference to the time of day they spawned.

A site was chosen in Rocquaine Bay for these *in-situ* experiments, away from other animals to try to avoid contamination. Eggs were placed in Nitex mesh chambers and carefully carried to the beach. These egg-filled chambers were then attached to the pole, laid on the seabed at ~1m depth, at sampling points at 5cm, 10cm, 25cm, 50cm, 1m, 2m, 3m, 4m and 5m. A second pole was laid perpendicular to the first at 0cm and further chambers were attached at 25cm and 50cm in each direction. Floats were attached to the tops of the chambers in order to hold them in position at 5cm above the seabed and elastic bands were used to secure the chambers. The container surfaces were oriented perpendicular to the direction of flow. One chamber was also placed 10m away (upstream of current flow) to act as a sperm-free control. Ten ml of concentrated sperm (exact concentration determined post-experimentation) was released from an upward facing syringe at 5cm above the seabed at a rate of 1ml s^{-1} for 10 seconds, which approximately amounted to 10ml of 1.7×10^7 sperm ml s^{-1} (pre-determined mean sperm release rate). Eggs were collected after 10 minutes. They were carefully taken back to the lab and washed into 30ml glass tubes in order to continue development. The percentage of eggs fertilized were assessed by examining 100 eggs after approximately 4 hours incubation.

2.5.3.2 Results

Less than 6% fertilization success was achieved in any of the treatments in any one of four replicates. All gametes used in these experiments were checked for viability in the laboratory and all showed fertilization success in excess of 90%.

2.5.3.3 Discussion

The most likely explanation for the almost complete absence of fertilization success encountered during the course of this experiment is the sperm-egg contact time

factor. As laboratory results showed (section *2.2.3.3*), sperm and eggs need to be held together for some minutes in order to achieve fertilization. The design of this experiment did not allow for this to occur. Mundy et al. (1994) designed a discrete-sampling submersible suction pump that was used by Babcock and Keesing (1999) in their *in-situ* experiments of fertilization rates. This pump contains sample cartridges in which eggs are placed and sperm are sucked into the cartridges at a series of points at varying distances downstream from sperm release. This enables sperm and eggs to experience longer contact times. This type of equipment should be employed in any further study of *H. tuberculata* fertilization *in-situ*.

Although the predictions from the model were not validated in the field, they are in line with that of Babcock and Keesing (1999) who showed a good fit between model predictions and empirical field data gathered using their pump system.

2.6 General Discussion

Reproductive output been measured typically as a function of gonad size or fecundity. However, when fertilization success falls below 100%, a common occurrence in many free-spawning marine invertebrates including *H. tuberculata*, these methods will overestimate offspring production. Thus, information regarding fertilization success in populations of *H. tuberculata* is of great interest.

The main purpose of the research contained within this chapter was to gain an insight into the possibility of enhancing the future management of the natural *H. tuberculata* fishery of Guernsey. In the absence of any *in-situ* fertilization success data, minimum stocking densities of the ormer may only be guessed at in terms of laboratory-derived results and the predictive model. Having estimated the ambient current flow in a typical ormer habitat in Rocquaine Bay, it is clear that if mean nearest neighbour distances fall below 3m, and diffusion coefficients are similar to those proposed by Denny (1988), fertilization success and hence recruitment of new juveniles would be jeopardised severely. Such recruitment failures have been reported for populations of *H. laevigata* in Australia in populations with (1) densities below ~0.3 ind. m^{-2} or (2) mean nearest neighbour >1 and 2 meters (Shepherd and Brown, 1993). To estimate the minimum stocking densities to establish harvest refugia in Rocquaine Bay, one would need to measure not only animal densities (found in the survey to be ~1 to 1.5 ind. m^{-2}) but also the nearest neighbour distances *per se*. Personal observations and reports from

local Guernsey divers show that the ormer tend to aggregate naturally on the underside of rocks, with only a few centimetres between individuals. This behaviour will lend itself to an increased chance of fertilization.

Some authors (e.g. Prince et al., 1987) suggest that abalone exist in small, localised stocks and hence management approaches and stock assessments must address exploitation on a very fine scale in contrast to other fishery types. Abalone fisheries also seem prone to unpredictable collapse. A good example of this is the Channel Islands abalone fishery (review in Breen, 1986).

The establishment of harvest refugia as a management tool used in conjunction with more traditional size limits, licences etc. are likely to be effective for the local ormer fishery in Rocquaine Bay. In addition to fishing closure, recovery of abalone stocks would be aided by the introduction of a broodstock to enhance the local population. A further consideration in the setting up of marine reserves is that of population size structure because fecundity is greater in larger individuals. Ormers are sexually mature at around 2-3 years of age, 40-49mm shell length (Hayashi, 1980a). A lower number of older individuals may produce a similar reproductive output to a larger number of relatively young specimens. Optimal stocking densities in harvest refugia should also consider trade-offs in reproductive success such as intraspecific competition for food which may result in size stunting and a reduction in gamete production. Capinpin et al. (1999) showed that the growth of individual abalone decreased as stocking density increased suggesting density-dependent competition for space and food. This only occurred at very high densities in the order of ~400 ind. m^2. The primary aim of a harvest refugia is to ensure that a portion of the spawning stock are protected from exploitation. In a refuge, density, average size and total egg production can be increased over what it would be if the area were fished. Larvae produced in reserves are then spread by oceanic currents to both exploited and protected areas, providing insurance against stock collapse.

Abalone biologists have used a variety of stock assessment tools: yield- and egg-per-recruit techniques are the easiest to apply, requiring only easily obtained data. Such tools have been used in determining the minimum legal size of animals fished in order to maintain yield efficiency and egg production. Nevertheless, problems are often encountered in terms of abalone stock assessment in understanding settlement and recruitment. Populations often show highly variable and unpredictable recruitment. If

patterns in variability are noticed and recorded, these could be incorporated into stock assessment techniques. The most important factor in successful fishery management is to elucidate the relationship between present stock and future recruitment. Bannister and Addison (1986) modelled lobster stock assessment in terms of stock-recruitment relationships. Such modelling is essential to the development of management strategies with variable stock-recruitment relations. Also stock structure needs to be assessed in terms of whether it is localised or dispersed.

In addition to the assessment of the abalone stock itself, a multi-species approach may be very important in some areas. For example, knowledge of the volume of algae available as a dietary source, and the effects of the abundance of other animals who also use the same food source, may be of great consequence. Any changes in the surrounding environment as a function of anthropogenic disturbance may also be an important consideration in some areas.

While the relationship between stock and recruitment is dependent upon knowledge of mortality for all phases of life history: larval, juvenile and adult, fertilization success is also important and is often ignored. An understanding of the scale and extent of any variability in mortality and its causes (such as density-dependent mortality - Peck and Culley, 1990) also need consideration in any long-term management plan. A good review of abalone mortality is given by Shepherd and Breen (1992).

Tegner (1989) achieved success in broodstock transplants in enhancing stocks of *H. fulgens* in California to areas of high larval retention. This generated good results in terms of recovering depleted and isolated stocks. High levels of reproduction and recruitment resulted from these transplants. However, the potential genetic consequences of such practices are unknown. Outplanting strategies are also costly. In addition, efforts to assess the success of outplanting operations have frequently been ambiguous (Burton and Tegner, 2000). Such programmes have regularly been unreliable and certainly not cost effective e.g. *H. iris* in N. Zealand (Schiel, 1993). During the course of my research in 1999 and 2000, Richard Tostevin set up a programme to introduce *H. tuberculata* larvae around the coasts of Guernsey. Local people collected large plastic containers full of larvae at pre-settling stage and discharged them around their particular area of coast. Unfortunately no success assessment programmes have been administered.

A recent survey of the severely depleted white abalone, *Haliotis sorenseni*, has shown that they are at very low numbers throughout a large portion of their range. The survey indicates that 80% of the remaining individuals observed were isolated and thus unlikely to contribute reproductively to the rebuilding of the population (Hobday and Tegner, unpub.). There has been a >99% decline in both the abundance and density of white abalone in California since the 1970s. No field studies of fertilization success in the white abalone have been conducted to date. This abalone is currently a candidate species for listing as an endangered species in the United States.

CHAPTER THREE – *FERTILIZATION SUCCESS IN OTHER COMMERCIALLY VALUABLE MOLLUSCS*

3.1 Introduction

From March to August 1998, laboratory-based fertilization experiments were conducted upon the Pacific oyster, *Crassostrea gigas* and the clam, *Tapes decussatus*. These experiments were carried out at the CEFAS laboratory in Conway, N.Wales, which held spawning stock of each of these animals. This initial period of experimental design and fine-tuning of experimental methods was invaluable to all the subsequent laboratory-based research in this thesis. In August 1998, the first useful data emerged from these experiments. These data comprised fertilization success with respect to sperm dilution for *Crassostrea gigas* and sperm dilution and temperature effects of fertilization success and gamete release rates for *Tapes decussatus*.

In addition to *C. gigas* and *T. decussatus*, *Patella vulgata* was included in fertilization success studies. The breeding habits of molluscs have been classified into 3 broad categories by Boolootian et al. (1962), namely, year-round breeders, winter breeders and summer breeders. Eighty-five percent of the species were summer breeders. *Patella vulgata* is among the few marine molluscs that spawns in the winter season.

3.2 Crassostrea gigas

3.2.1 The Fishery

Oysters are commercially valuable bivalve molluscs. The common European oyster, *Ostrea edulis*, has been fished around UK waters for centuries but the fishery has declined severely. Less than 100 years ago, the oyster was a very popular food, eaten in large quantities by a wide cross-section of the community. In 1864, almost 500 million oysters were reportedly sold at Billingsgate market in London. Official MAFF statistics

give an average annual landing of 28.4 million oysters between 1903 - 1914, but only 8.9 million during 1975 - 1985. This decline has been attributed to over-fishing, failure of spatfalls, disease and severely cold winters. Their natural habitat has also been degraded by anthropogenic disturbance in the form of recreation and construction of commercial (ports) and residential areas. A rise in suspended particulate material in the water disrupted the life cycle of these filter-feeding organisms.

During the past 100 years, non-native species of oyster have been introduced to the UK oyster fishery in an attempt to compensate for this loss. Species introduced included *Crassostrea virginica* from the USA and *C. angulata* from Portugal. Importation of *C. virginica* ceased in 1939 and *C. angulata* importation was banned after 1970 following an outbreak of viral gill disease.

The Pacific oyster, *Crassostrea gigas* is native to the Pacific Ocean and is found in the Sea of Okhotsk, in Japan, in Korea and along the Pacific coast of America from Alaska and south to California. It is the most common species of oyster in the world. *C. gigas* was introduced to the UK in 1965 and 1972 from Canada and in 1978 from the USA to the Fisheries Laboratory at Conwy, under strict quarantine guidelines. This species grows faster than the native *O. edulis* and reaches market size in only 3-4 years compared with 5-6 years for the flat oyster. The Pacific oyster is cultivated in many estuaries and sheltered coastal waters of the British Isles. Production was estimated to be ~300 tonnes per year from 36 farms in England and Wales in 1992 (Spencer et al., 1994). Many of these locally produced oysters are exported. *C. gigas* is also farmed widely on the Atlantic and Mediterranean French coasts following its introduction in the 1970s. 150,000 tons are produced annually. Only ~10% of this figure is produced from hatcheries. The majority of juveniles are collected from the wild (Ramirez et al., 1999). *C. gigas* is now the most important oyster species grown in Europe. It is an estuarine species that attaches to rocks, debris and other oyster shells and also may be found on mud or mud-sand substrata.

3.2.2 Reproductive Biology

Crassostrea gigas are broadcast spawners that mature first as males and then either change sex to female or remain as males. The females reach sexual maturity earlier in the season than males (Fujita, 1929). The ripe gonad of *C. gigas* is located near the surface of the body within a layer of connective tissue, between the digestive

gland and surface epithelium. Many follicles merge along the dorsal side of the body to form one continuous structure encompassing the visceral mass and extending ventrally to the tip of the pyloric process (Galtsoff, 1964). The fully developed gonad comprises the largest organ in the oyster. Female *C. gigas* of around 70mm shell length can be expected to release ~50 million eggs per individual (Spencer, 1990). Mature oocytes are approximately 55µm in diameter (Fujita, 1929; pers. obs.). They are colourless, transparent and finely granulated with a large nucleus. The negatively buoyant eggs are discharged into the seawater following contraction of the adductor muscle. They pass over the gills and are dispersed several centimetres away from the oyster. Spawning may last from a few minutes to nearly an hour. Sperm are expelled slowly via ciliary action. The spermatozoa move along the genital ducts before respiratory currents then sweep them away (Galtsoff, 1964). Fertilized eggs of *C. gigas* develop into free-swimming planktotrophic larvae that remain in the water column for several weeks prior to settlement.

The timing and duration of gametogenesis in *C. gigas* varies worldwide (Bardach et al., 1972; Ventilla, 1984; Dinamani, 1987; Sphigel, 1989; Ruiz et al., 1992; Paniagua-Chaves and Acosta-Ruiz, 1995; LangoReynoso et al., 1999; Steele and Mulcahy, 1999). The reproductive cycle of *C. gigas* can be influenced by a number of environmental conditions such as temperature, salinity (Mann, 1979; Muranka and Lannan, 1984; Lubet and Mann, 1987; Sphigel, 1989) and levels of nutrition (Deslous-Paoli et al., 1982). It is also influenced by exposure to pollutants (Maung Myint and Tyler, 1982) and parasite loading (Thain and Waldock, 1986). There have been a number of incidents where introduced *C. gigas* have failed to spawn e.g. Cork Harbour, Ireland. This case was linked to TBT contamination (Steele and Mulcahy, 1999). Oocyte quality is variable in wild *C. gigas* broodstock (Ramirez et al., 1999).

3.2.3 Materials and Methods

The animals used in these experiments were collected from the Menai Straits and Tinmouth and then conditioned in tanks at CEFAS. Although the natural spawning period is between July and August, experimental animals can be conditioned to spawn outside their natural spawning period by careful regulation of their food, temperature and light regime.

The effect of subjecting fresh *C. gigas* eggs to a range of sperm dilutions was examined in terms of fertilization success. Although these animals may be forced to spawn via thermal cycling (Spencer, 1990), a faster method is to strip spawn the specimens by removing the flat valve and puncturing the gonad using a Pasteur pipette (Helm and Millican, 1977). By this method, the gametes may be carefully withdrawn from the animal. Following gamete collection, the same methods were used as for *H. tuberculata* in Chapter 2, section *2.2.2.5.3*. A range of sperm dilutions were mixed with ~200 eggs to determine the optimum sperm concentration for successful fertilization. Cultures were incubated at 25°C. Counts were made after ~2 hours incubation time when embryos were at the 12 to 16 cell stage. Three replicates were conducted using 3 males and 3 females.

3.2.4 Results

Sperm concentration had a highly significant effect on fertilization success of *C. gigas* (Figure 3.1). Fertilization success reached a maximum at sperm concentrations of 10^5 to 10^6 sperm ml^{-1}. A steep decline in success occurred as sperm concentration fell from 10^5 to 5 x 10^3. Only a very slight decrease in fertilization success was seen when sperm concentrations exceeded 10^6 sperm ml^{-1}.

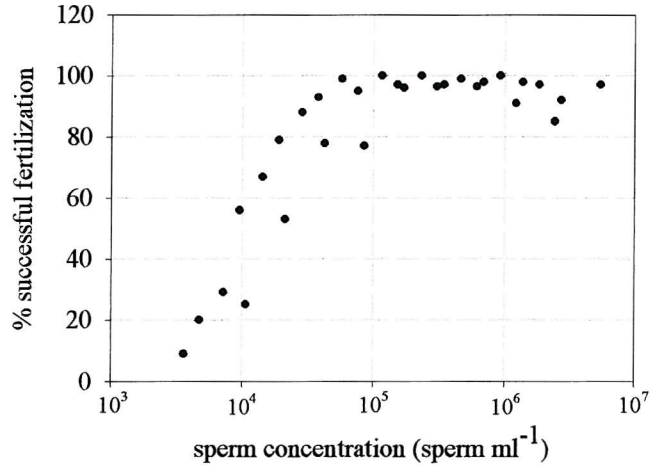


Figure 3.1: Effect of sperm concentration on fertilization success in C. gigas

3.2.5 Discussion

The concentration of sperm required to fertilize eggs of *C. gigas* reflects the general pattern seen for other free-spawning marine invertebrates using similar experimental methods, with 10 – 20ml culture volumes. However, only a slight decline in fertilization success was seen at sperm concentrations of 10^6 sperm ml^{-1} and above. This was somewhat surprising, as freshly obtained ovarian oocytes (as opposed to spawned) are highly susceptible to polyspermy even at sperm-egg ratios as low as 30:1 and only acquire resistance if they are incubated for 1hr in seawater before insemination (Stephano and Gould, 1988). Perhaps the incidence of polyspermic fertilizations would increase exponentially with slightly higher sperm concentrations than tested here.

Staeger and Horton (1976) conducted a similar study to quantify the gamete concentrations required to maximise larval production. They used smaller and variable culture volumes during their experimentation. Nevertheless the results presented here were very similar to those of Staeger and Horton (1976), with an optimal sperm concentration of 2.4 to 7.3 x 10^5 sperm ml^{-1} producing the best larvae compared to the present study that found an optimal range of 10^5-10^6 sperm ml^{-1} to achieve optimum fertilization success. Unlike the present study, Staeger and Horton (1976) did, however, find an increase in abnormal larvae at higher sperm concentrations, most likely as a function of polyspermy.

The effects of sperm density on hybridization between *C. gigas* and *C. virginica* were studied (Lyu and Allen, 1999). The results showed that the affinity between heterologous gametes was weaker compared to homologous gametes and a higher concentration of sperm was required for successful fertilization and survival for hybrids.

As discussed in chapter two, care should be taken in the interpretation of the data given here describing sperm concentration only, as effects of sperm-egg ratios have not been tested for specifically to see if they have a bearing on fertilization success. The range of sperm-egg ratios in the sperm dilution experiment varied between ~115:1 and 630,000:1. It could be that sperm-egg ratios at the lower end of the scale reduce fertilization success rather than sperm concentration *per se*, However, this is unlikely as fertilization success at sperm-egg ratios at 5 x 10^3 sperm ml^{-1} are still relatively high. Further experiments would need to be conducted to qualify this point.

Sperm of the zebra mussel (*Dreissena polymorpha*) showed that those sperm that had been removed manually from the animal exhibited significantly lower motion

characteristics than those spawned naturally (Mojares et al., 1995). Similar results were also found for sperm from the scallop, *Pecten maximus*. Stripped sperm were of poorer quality than those spawned using thermal shock methods (Faure et al., 1994). Perhaps if the experiments on *C. gigas* were repeated with spawned rather than stripped sperm, the fertilization success curve may shift over to the right as more sperm may be fit enough to fertilize more eggs at lower sperm concentrations, given that the sperm concentrations reported here may be less than they suggest, as they may incorrectly assume 100% fit sperm. In addition to this, polyspermy may have occurred within the range of sperm dilutions examined here, with an increase in the number of fit sperm.

Increases in the fertilization success of *C. gigas* were found when freshly stripped eggs were immersed in seawater for 60 minutes prior to fertilization. The addition of vitamine solutions to the egg water was also found to improve fertilization success (Wang et al., 1997).

The effects of gamete concentration on fertilization success was examined in the oyster *C. rhizophorae* (Rampersad et al., 1994). They found an optimum fertilization efficiency was achieved with sperm densities of 5×10^4 sperm ml^{-1} and egg concentrations of 30 eggs ml^{-1}. When egg concentrations were increased, a decrease in normal larvae was seen. At 200 eggs ml^{-1}, only 10% normal larvae occurred. This contrasts with the results presented here on *C. gigas* in which fertilization success reached 100% with 200 eggs ml^{-1}, albeit at 10^5 sperm ml^{-1}. Rampersad et al. (1994) suggest that the decrease in normal larvae with an increase in egg density may be a function of some deleterious water-soluble substance being emitted from the fertilized eggs. This phenomenon is found in sea urchin eggs where hydrogen peroxide is thought to be emitted from fertilized eggs as a defence against polyspermy (Boldt et al., 1981).

C. gigas is already an important species in commercial terms and any information which may lead to a more thorough understanding of its reproductive behaviour will be of great importance to both aquaculture and natural fisheries management. Investigations concerning the reproductive health of *C. gigas* (Li et al., 1997; Park et al.,1999) and triploidy induction (Quillet and Panelay, 1986; Guo and Allen, 1994; Guo-fan et al., 1998; Leitao et al., 2001) have recently been conducted and further studies are needed to assess the most economically feasible methods of harvesting this highly sort after shellfish. The data presented here add to the increasing body of knowledge concerning the potential fertilization success of *C. gigas* under laboratory conditions.

Although *C. gigas* is generally considered to be a broadcast spawner, Stephano and Gould (1988) suggest that sperm actually enter the gills of females. Fertilization success occurs as oocytes are discharged through the gills. This gamete behaviour may increase the chances of fertilization success in *C. gigas* under natural conditions. Gamete longevity was assessed by Fujita (1929). Eggs and sperm are both able to live longer than 15 hours at 29°C. In comparison to other free-spawning molluscs the sperm live longer (see Table 2, Chapter 1) and have a relatively slow swimming speed (at 0.05mm s^{-1}). It probably makes sense for *C. gigas* to invest in longer-lived sperm if they are to be retained on the gills of the female.

One final consideration is that the experimental animals used in this study were conditioned for some time in the laboratory. Conditioning may cause physiological changes in the animal and may effect fertilization success as gametes may be either more or less fit, depending upon diet, temperature and illumination. The chances are, however, that an even greater degree of variability in terms of gamete fitness will exist in those animals spawning naturally in the field.

Although the eggs of *C. gigas* are negatively buoyant, forcible ejection into the water column is likely to increase their chance of fertilization. Eggs are only ejected when sperm is present in the surrounding waters (Galtsoff, 1964). Oysters are also thought to spawn more than once during each breeding season. Figure 3.2 is a rare opportunity to see spawning activity in nature. Gamete clouds of *C. gigas* are being carried by the tide. With this amount of spawning synchrony, high levels of fertilization success are likely.

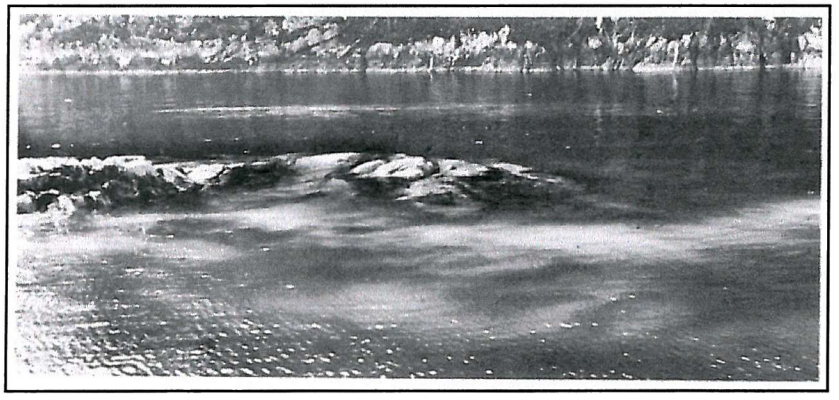


Figure 3.2: C. gigas *gamete cloud near Vancouver, British Columbia. After Galstoff (1964)*

3.3 *Tapes decussatus*

3.3.1 The Fishery

The clam, *Tapes decussatus*, is commonly known as the carpet shell or palourde clam. The culture of this high-priced bivalve mollusc is a relatively recent activity and research into preferred rearing conditions has only occurred over the last few years (Alpbaz et al., 1981; Mercer, 1981; Breber, 1985; Gutierrez, 1991; Perez Gragera et al., 1991; Rossi and Paesanti, 1992; Puigcerver, 1996; Laing and Child, 1996; Lamela et al., 1996). *T. decussatus* is native to Europe and occurs along Atlantic coasts from the British Isles, South as far as Morocco and Senegal and also into the Mediterranean Sea. It lives on the lower shore and shallow sublittoral in sand, muddy gravel or clay (Hayward and Ryland, 1995). Natural populations of *T. decussatus* also occur on the west and north-west coasts of Ireland and these have been subjected to harvesting. However, these populations are very susceptible to overfishing and recovery is slow. This has stimulated research into clam culture (Rice, 1993).

3.3.2 Reproductive Biology

Tapes decussatus is a protandric hermaphrodite. Populations of 2 and 3 year olds have a 1:1 sex ratio. Individual females of 35-45mm shell length spawn an average of 5-8 million eggs. Viable larvae cannot be obtained from artificially stripped gametes because eggs need to undergo a maturation process during passage down the oviducts. The ultrastructure of the mature oocytes of *Tapes decussatus* has been described (Bozzo et al. 1996) and mean oocyte diameter is 42.6μm (Xie et al., 1994). Rodriguez et al.

(1993) were able to condition *T. decussatus* individuals to reach gamete maturation outside of the natural reproductive period. During spawning, eggs and sperm are expelled through the exhalent siphon. Ripe gametes were found to be present and continually released between April and August 1997. Major spawning occurred in August/September in a population of *T. decussatus* in northwest Spain (Rodriguez-Moscoso and Arnaiz, 1998). Further studies of reproductive timing and environmental influence on this timing are given by Beninger and Lucas (1984), Navarro and Iglesias (1995) and Xie et al. (1994). A population from southern Ireland also showed ripe specimens in June with a major spawning commencing in August in 1991, a distinctly unimodal gametogenic cycle. *T. decussatus* have planktotrophic larvae that remain in the watercolumn for around 10-20 days before settling.

3.3.3 Materials and Methods

3.3.3.1 Sperm dilution experiments

Animals used in these experiments were collected from Tal-y-foel, Anglesey, North Wales. Sperm dilution experiments were carried out by inducing animals to spawn using thermal shock. Twenty animals were placed, evenly spaced, in a 2 metre-long fibreglass tank. Seawater of 20°C was added to the tank, enough to cover the animals, and left for 20 minutes. This water was then drained from the tank and replaced with seawater of 30°C for a further 20 minutes. This process of thermal shock cycling was repeated until spawning was seen. This usually took in the region of 3-6 hours. At this stage, spawning animals were quickly removed from the tank and placed into separate glass beakers containing filtered seawater. Batches of eggs that did not round-off after ~10 minutes in seawater were discarded. For the remainder of the method see Chapter 2, section *2.2.2.5.3.* Three replicates were made using a total of 3 males and 3 females.

3.3.3.2 Temperature experiments

Fertilization success was also assessed with respect to temperature for *Tapes decussatus* using the methodology set out in Chapter 2, section *2.2.2.5.7.* Twelve experimental temperatures were used ranging from 5 to 31°C. Three replicates were made using 3 males and 3 females. In addition to fertilization success, larval development stages were also determined for each temperature treatment.

3.3.3.3 Gamete Release Rates

Rates of gamete release were assessed for *T. decussatus* by allowing animals to spawn in a known quantity of filtered seawater for 1 hour. Eggs were counted using a Sedgewick Rafter cell and sperm concentration was determined using a haemocytometer.

3.3.4 Results

3.3.4.1 Sperm dilution experiments

Sperm dilution had a highly significant affect upon fertilization success. In *T. decussatus*, wide ranging sperm concentrations of 5 x 10^3 to 5 x 10^6 sperm ml^{-1} achieve the optimum fertilization success of >80% (Figure 3.3). Fertilization success declines rapidly at sperm concentrations in excess of 5 x 10^6 sperm ml^{-1} and to <20% at 10^2 sperm ml^{-1}.

3.3.4.2 Temperature experiments

Effects of temperature within the range tested were only significant at the lower end of the spectrum (Figure 3.4). Fertilization success declined from ~100% in the 14 to 31°C treatments to ~70% in one replicate at 11.5°C. Percentage fertilization success fell to zero at temperatures of 9°C and below. Larvae reached the most advanced stages of development within 4 hours at temperatures >23.5°C but with a slight increase in abnormalities occurring at 31°C in all three replicates (Figure 3.5).

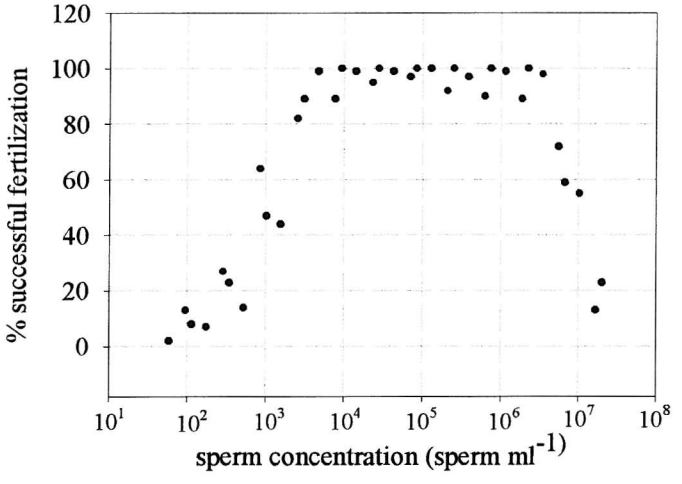


Figure 3.3: The effect of sperm concentration on fertilization success in T. decussatus.

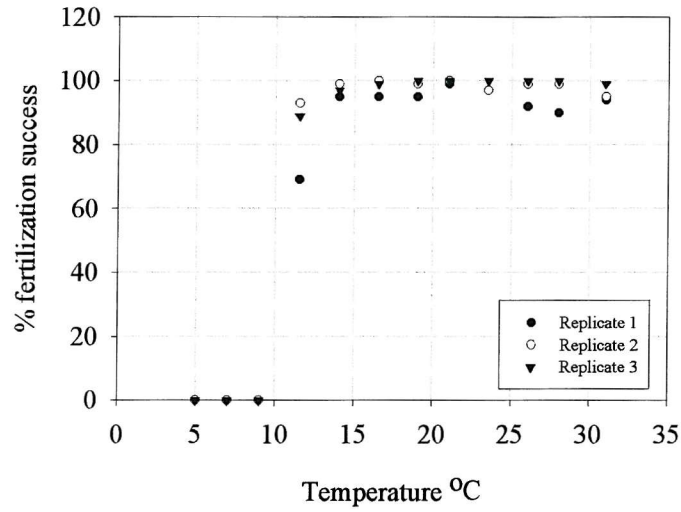


Figure 3.4: The effect of temperature on fertilization success in T. decussatus.

3.3.4.3 Gamete Release Rates

Gamete release rates are listed in table 3.1.

Table 3.1

Gamete release rates

Animal Reference	*Sex*	*Shell length (mm)*	*Wet weight (g)*	*Gametes released per hour*
1	M	31.0	7.72	7.6×10^9
2	M	32.4	7.61	7.8×10^9
3	M	31.8	7.76	4.8×10^9
4	M	30.5	6.36	3.1×10^9
5	M	31.1	6.90	6.9×10^8
6	M	30.5	6.65	5.8×10^8
7	M	32.5	7.46	2.0×10^9
8	M	30.7	6.27	1.6×10^9
9	F	31.6	6.96	5.5×10^5
10	F	30.8	6.68	1.1×10^6
11	F	31.6	6.65	1.0×10^6
12	F	31.5	6.90	1.9×10^6
13	F	32.3	7.34	1.3×10^6

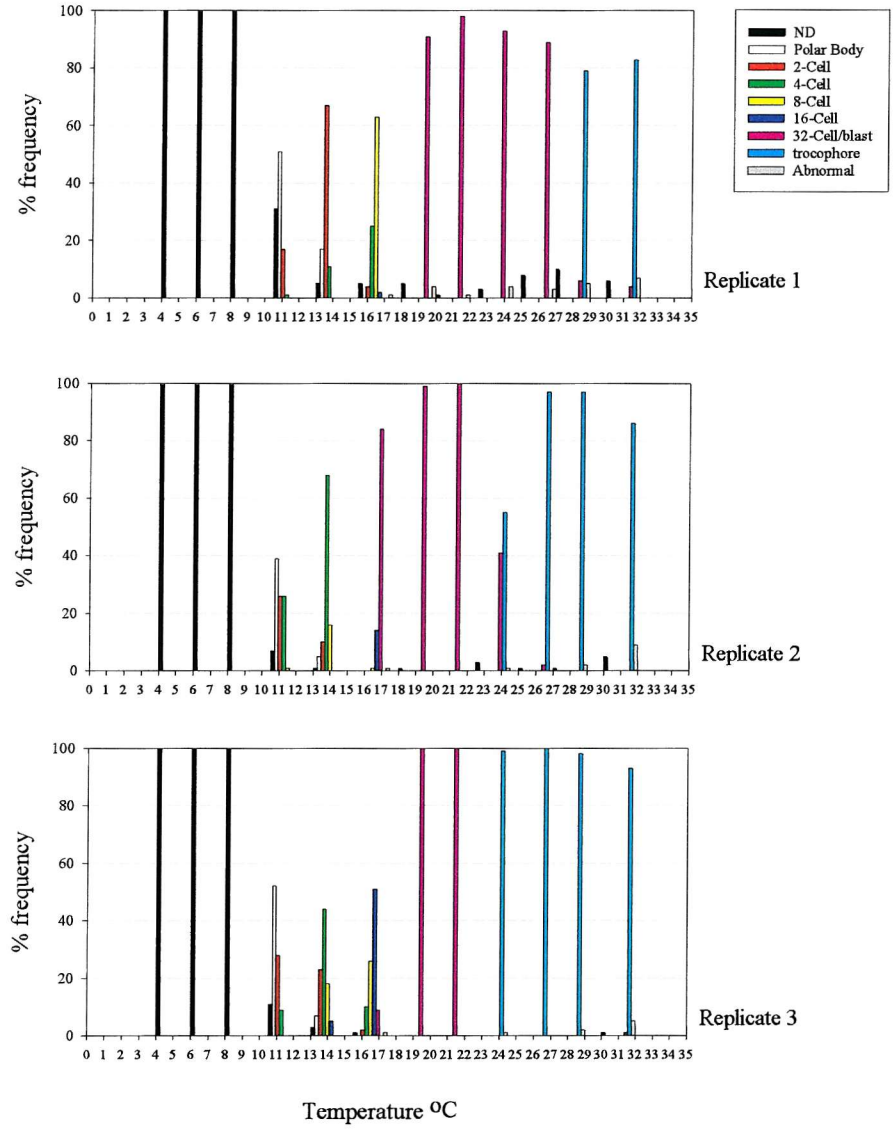


Figure 3.5: The effect of temperature on larval development in T. decussatus

3.3.5 Discussion

The successful fertilization of eggs of *T. decussatus* is rather less dependent upon sperm concentration than for other species. *T. decussatus* attains optimum fertilization success over a far wider range of sperm concentrations that span 3 orders of magnitude. This has implications for the introduction of propagules to a population, especially in more turbulent environments were sperm may be dispersed quickly. Maybe *T. decussatus* has adapted to blocking polyspermy and attracting rare sperm using chemotactic mechanisms. *T. decussatus* also displays a high tolerance to temperature in terms of fertilization success. It would appear that this animal is able to thrive in rather unstable environments.

The temperature experiments conducted here show that at 9°C and below, no successful fertilization occurs. However, Laing and Child (1996) conducted low temperature experiments on *T. decussatus* to ascertain the tolerance of juveniles, both fed and unfed. Their experimental temperatures ranged from 3 to 9°C over a period of 11 weeks. At 9°C good juvenile survival was seen. The findings in the present study suggest that low sea temperatures may limit fertilization success and subsequent recruitment of *T. decussatus*. Knowledge of optimum temperatures for larval development are useful for hatchery purposes but these elevated temperatures that produce viable larvae in the shortest time are obviously far higher than those that occur under natural conditions.

Another species of commercially harvested clam, *Mercenaria mercenaria*, used to thrive in Southampton Water. This animal is a non-native species but established a self-sustaining population. A reasonable fishery existed but over the period of a few years, populations were decimated, leaving only a few individuals to reproduce. In 1998, I joined local fishermen and spent a week dredging the area to collect all the remaining clams. We then replaced them on one chosen site in order to enhance reproductive potential with a view to replenishing stocks for future generations of fishermen. Unfortunately this programme failed as the animals were placed on an unstable area of sediment, and it would appear from diving surveys that they were smothered by slumping mud (Jensen, pers. comm.).

3.3.6 Denny Model (1988)

By using the sperm release data and sperm dilution data predictions of fertilization success in the natural environment were made for *T. decussatus* under different current regimes using the Denny (1988) model. A detailed description of this model is given in Chapter 2, section *2.4*. The average sperm release rate of 1.96 x 10^6 sperm sec^{-1} was obtained from data on 8 males and was used in these calculations. The results of the model are depicted in Figure 3.6.

3.3.6.1 Discussion

The gamete release rate in *T. decussatus* was relatively low compared with that of *Haliotis tuberculata* and *Psammechinus miliaris* (pers.obs.). Consequently, this factor had a profound effect upon the predicted values of fertilization success at current speeds of 5 and 10 cm s^{-1}. In Figure 3.6 (a) to (d), fertilization success is reduced to 50% at distances only 80cm or less away from the sperm source. Prediction (e), which uses different values for the dispersion coefficients, shows improvements in fertilization success with 100% success 1m away from the sperm source in currents of 5 cm s^{-1}. Nevertheless, this model predicts that sperm limitation in populations of *T. decussatus* with nearest neighbour distances in excess of 1m is far more likely than in populations of *H. tuberculata* or *P. miliaris* (see Chapter 4). This has important implications in the consideration of re-stocking programmes as abundance of *T. decussatus* would be required to be relatively high in order to attain high reproductive success and hence increase chances of recruitment to the population.

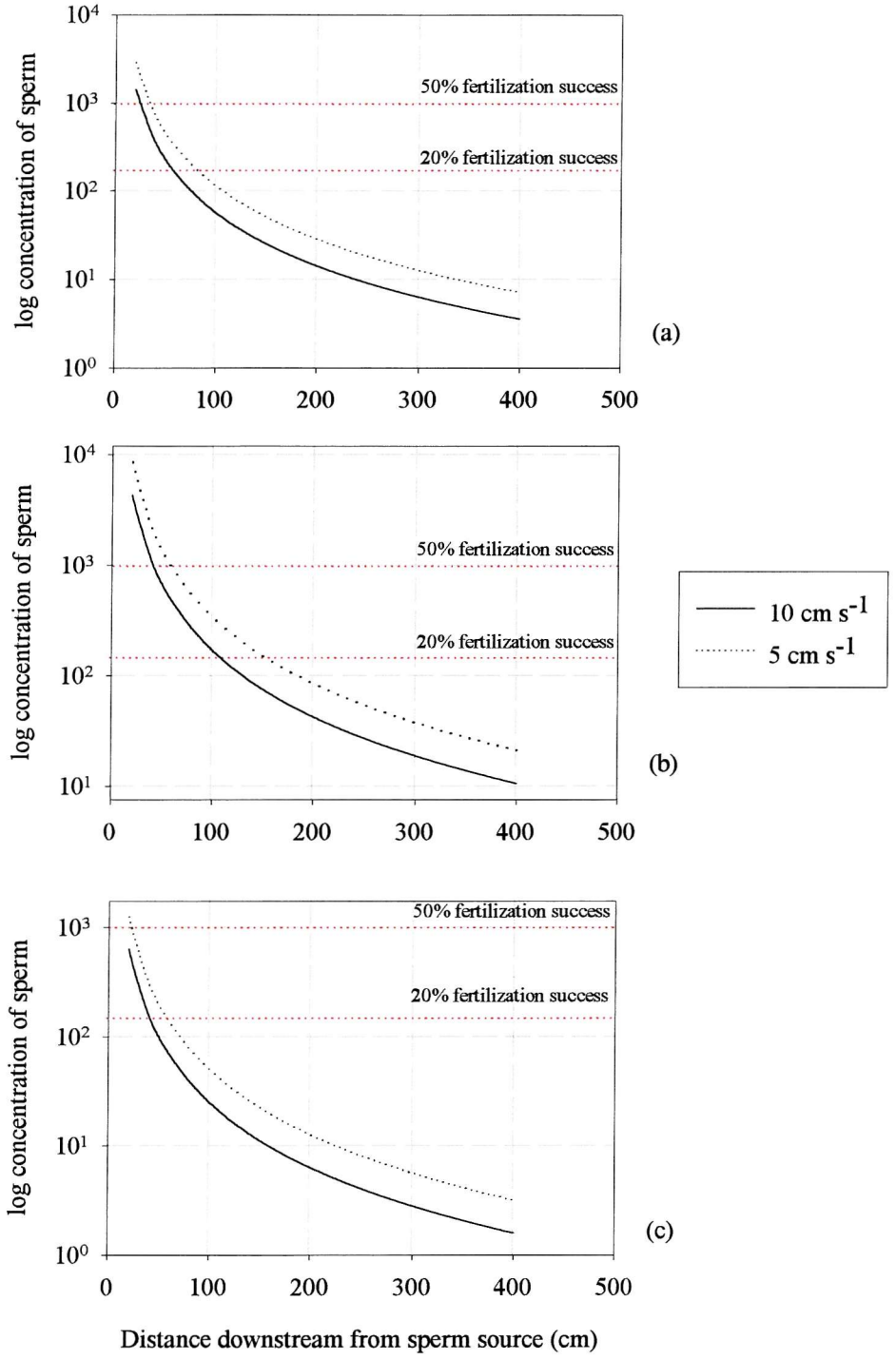


Figure 3.6 (continued on next page): Predicted sperm concentration and fertilization success under a range of different current regimes for T. decussatus. *(a)* $\alpha_y = 1.25$; $\alpha_z = 2.2$; $u_* = 10\%$; *1 male, (b)* $\alpha_y = 1.25$; $\alpha_z = 2.2$; $u_* = 10\%$; *3 males, (c)* $\alpha_y = 1.25$; $\alpha_z = 2.2$; $u_* = 15\%$; *1 male.*

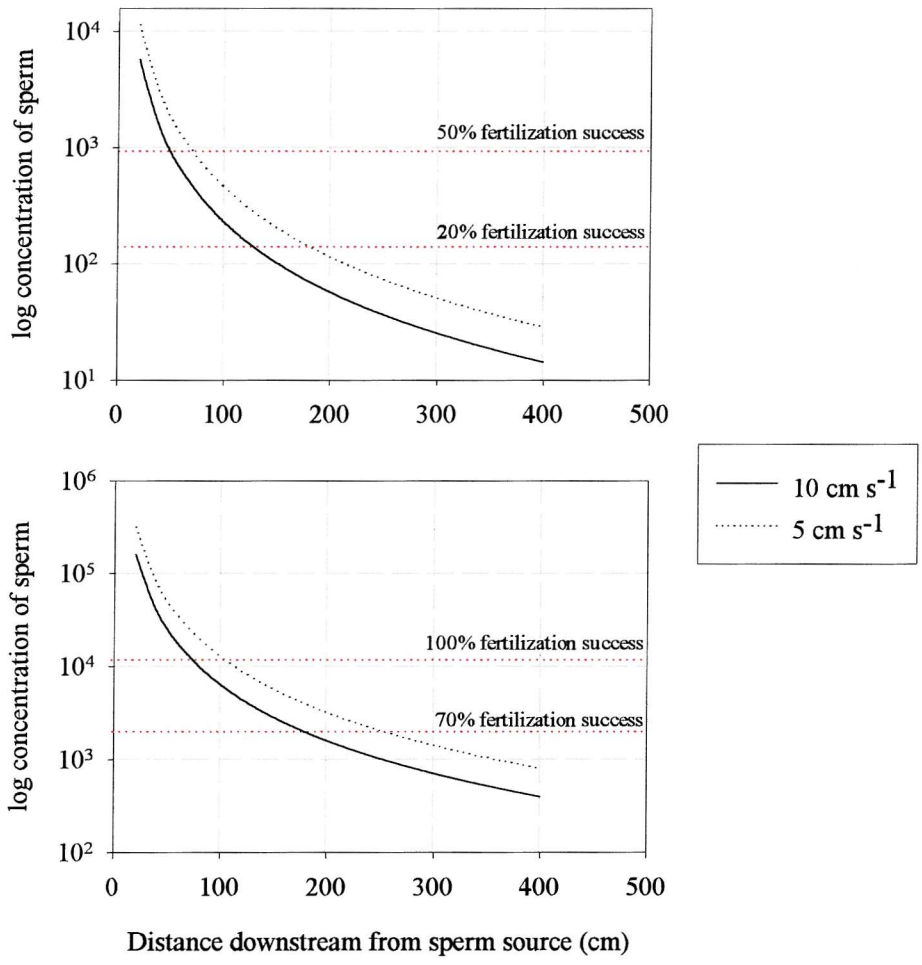


Figure 3.6 (continued): (d) $\alpha_y = 1.25$; $\alpha_z = 2.2$; $u_* = 5\%$; 1 male, (e) $\alpha_y = 0.198$; $\alpha_z = 0.124$; $u_* = 10\%$; 1 male.

3.4 *Patella vulgata* Linnaeus 1758

3.4.1 Introduction

The common limpet, *Patella vulgata*, is a gastropod mollusc found in cold waters in the Northern Hemisphere along the Atlantic coasts of Western Europe, from Northern Norway to Southern Portugal. By far the most frequently found species of limpet on rocky shores, *P. vulgata* grazes on young algae. Its shell is light coloured both outside and inside, while the foot is green-grey, yellow or orange. The average size of *P. vulgata* is ~40mm long. This species generally lives in colonies on rocky shores as deep as ~10m. Although this animal is sold for commercial purposes, the fishery is on a local scale and hence no information pertaining to the fishery was found in the literature.

3.4.2 Reproductive Biology

The reproductive biology of *P. vulgata* is well known (e.g. Orton, 1920, 1928; Smith, 1935; Orton et al., 1956; Blackmore, 1969; Wright & Hartnoll, 1981; Baxter, 1983; Garwood, 1986). It is a dioecious, protandrous hermaphrodite that develops male gametes in the first 2-3 years of life, after which some animals undergo an irreversible sex change. This transitional stage is thought to be very brief as only 30 hermaphroditic specimens were found among 64,576 animals (Dodd, 1957). The large gonad underlies the gut and visceral mass and swells in size during maturation and eventually consists of up to half the body tissue weight. Because of the location of the gonad, sexes may not be distinguished externally, only by dissection or by inserting a hypodermic needle into the gonad and retrieving gametes. It is possible to distinguish sexes by gonad coloration and/or microscopic examination. Gametes are released into the water column via the right kidney to the right renal papilla (Davis & Fleure, 1903; Fretter & Graham, 1962). Dodd (1957) showed that only eggs characterised by the rounding of the egg after

removal from the ovary are capable of being fertilized and developing normally. Eggs are ~150μm in diameter (pers. obs.). An individual female *P. vulgata* of ~50mm shell length has a fecundity of approximately 500,000 eggs (Ballantine, 1961). Gonad development shows a distinct annual cycle with the peak spawning occurring between October and December in Britain (Orton et al., 1956) although Baxter (1983) reported spawning between December and February in populations from the Orkney Islands. Variation in the pattern of spawning activity both between sites and between years is well documented. Ballantine (1961) showed that 80% of a *P. vulgata* population may spawn over a two day period. Bowman (1985) has suggested that early partial spawnings may occur in populations inhabiting exposed shores, whilst more sheltered populations tend to accumulate gametes to be released at a late major spawning. Orton et al. (1956) linked spawning with rough seas and storms and subsequent workers have tended to support this hypothesis (Ballantine, 1961; Bowman & Lewis, 1977; Thompson, 1980). Bowman (1985) suggested the spawning may be stimulated by a temperature drop to below 12°C although this remains to be demonstrated in laboratory studies. Spawning is not obviously related to tides or to moon phases. Morton (1958) states that close proximity leads to spawning. In the Antarctic patelid limpet, *Nacella concinna,* spawning appears to be cued by the spring phytoplankton bloom (Stanwell-Smith & Clarke, 1998). No worker has managed to induce *P. vulgata* to spawn under laboratory conditions. *P. vulgata* have free swimming trochophore and veliger stages in their larvae and are planktotrophic (Dodd, 1957).

3.4.3 Materials and Methods

3.4.3.1 Sperm dilution experiments

In November 1998, the limpet *Patella vulgata* were collected from the sea wall at Netley (50°51'7N; 1°20'8W). Many weeks were spent in an attempt to induce spawning in these animals but with no success. Thermal shock, osmotic shock, hydrogen peroxide, mechanical stimulation and the addition of gametes to surrounding seawater were all attempted. For the sperm dilution experiments gametes were strip spawned. A small scalpel was inserted into the left side of the foot. The knife was gently traced around the rim of the shell and great care was taken not to puncture the gonadal sac. The gonad was then pierced using a glass pipette. Gametes were removed and collected in filtered seawater. Eggs were rinsed prior to experimentation. For the

remainder of the sperm dilution experiment methodology see Chapter 2, section *2.2.2.5.3.* Only one experiment using one male and one female was achieved. In December 2000, additional attempts were made to fertilize stripped eggs of *P. vulgata* for more replicates of this sperm dilution experiment. Unfortunately, none of the eggs were viable. They appeared rather irregular and deformed, with only about 5% appearing round. The sperm appeared to be motile and healthy-looking.

3.4.3.2 Pairing behaviour

During animal collection, pairing behaviour was noted among the limpets. In order to investigate the possibility of pairing for reproductive purposes, 60 pairs were sampled and sex was determined by dissection.

3.4.4 Results

3.4.4.1 Sperm dilution experiments

Fertilization success is affected by sperm concentration in *Patella vulgata* but the results are rather less clear in this species than for other species examined (Figure 3.7). Only one experiment was conducted and the range of sperm concentrations was not broad enough to determine the lower fertilization success percentages. The highest mean fertilization success achieved in this single study was only 78% and this when sperm concentration was at 4.4 x 10^6 sperm ml^{-1}. Fertilization success was still >60% at sperm concentrations of >10^8 sperm ml^{-1} and was only seen to drop to ~35% in treatments of 2.84 x 10^8 sperm ml^{-1}.

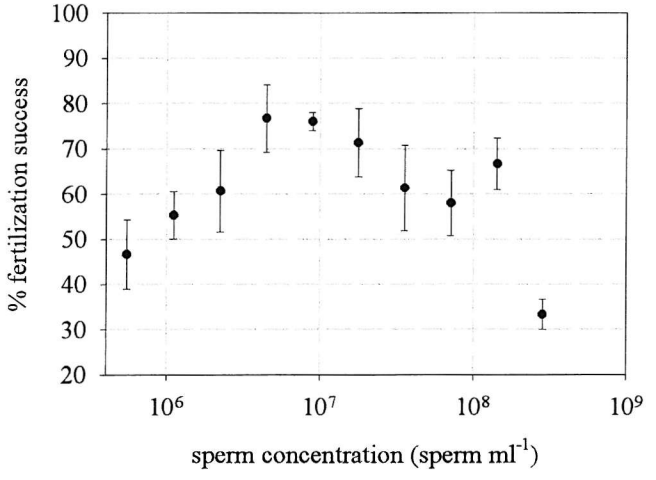


Figure 3.7: The effect of sperm concentration on fertilization success in P. vulgata
Bars show SE between counts from 3 replicate tubes

3.4.4.2 Pairing behaviour

Figure 3.8 shows a typical pair of limpets. Results showed 99% confidence that male-female pairs are more common than would be expected by chance (Chi-squared test, χ^2 $P = 6.66$). One would expect a ratio of 1:1 male-female pairings, whereas 67% were found.

Figure 3.8: a) P. vulgata *pairing,, b) dissected* P. vulgata *showing female (green) and male (yellow) gonads*

3.4.5 Discussion

At Netley in the Solent on the south coast of England, *P. vulgata* were ripe throughout October, November and December, with a mass spawning occurring on December 25-26th in 1998 (pers. obs.). The spawning trigger at Netley was thought to be the storm conditions that were experienced over the Christmas period. This follows Orton's theory suggesting that mechanical wave action triggers spawning in this species. During the course of this study, many attempts were made in the laboratory to induce spawning in *P. vulgata* via mechanical action. A wave machine was set up with limpets placed in the tank along a gradient of wave action but no spawning occurred. During storm conditions, atmospheric pressure changes occur. Spawning may be achieved by subjecting ripe animals to such pressure changes. All attempts to spawn *P. vulgata* in the laboratory failed and hence gamete release rates could not be determined in this species.

More replicates are needed for the sperm dilution experiments with an increase in sperm concentration range in order to determine the limits of fertilization success. Pennington (1985) had similar high sperm concentrations for optimum fertilization success in the sea urchin *Strongylocentrotus droebachiensis*. He found that sperm concentrations between 10^7 to in excess of 10^{10} produced maximum fertilization success. The Antarctic limpet, *Nacella concinna* also displays a similar pattern of fertilization

success with respect to sperm dilution (Powell et al., in press) although there was a switch from normal to abnormal development when sperm concentration exceeded 10^6 - 10^7 sperm ml^{-1}.

The evidence of pairing behaviour in *Patella vulgata* was rather unexpected. Not only was it noted in the field but when animals were held in aquaria, pairing behaviour occurred in almost all cases (n=30). Other patellacean limpets, *Helcion pellucidus* and *Patella caerulae* (Ankel, 1936) and *P. lusitanica* (von Medem, 1945) also pair during spawning. Another limpet, *Nacella concinna*, is unique in that it forms stacks of two to six animals during spawning (Picken, 1980). Pairing behaviour during reproduction has also been noted for some deep-sea echinoderm species (Young et al., 1992; Tyler et al., 1992). It is thought that this aggregative behaviour during the spawning season may act to increase the chances of fertilization success in broadcast spawners. Certainly in the deep sea, the chances of meeting a con-specific are rare and aggregative behaviour would have obvious advantages in terms of reproduction. *P. vulgata* appear to spawn in rough weather conditions, thereby increasing the probability of sperm dilution which decreases fertilization success. Pair formations would help to alleviate this problem.

CHAPTER FOUR – *FERTILIZATION SUCCESS IN* PSAMMECHINUS MILIARIS

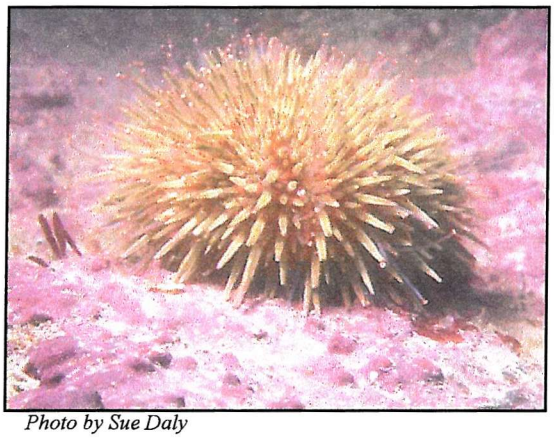


Photo by Sue Daly

4.1 Introduction

4.1.1 Sea Urchin Fisheries

Sea urchins are an exotic food that have been fished extensively and consumed in Japan more than any other country. Statistics produced by the Food and Agricultural Organisation (FAO) show an exponential increase in sea urchin harvests throughout the world since the 1940s. Total world production was estimated at around 117,000 tonnes in 1998 with a market value of around £500 million (Keesing and Hall, 1998). France, Chile and Asia are also substantial consumers of sea urchin roe. There has been a widespread collapse in wild stocks of sea urchin resulting from over fishing. The collapse began in Japan and, as a consequence, Japan increased imports from the USA and Chile. This, in turn, triggered a collapse of the USA fisheries. Europe urchin fisheries were also subject to collapse. The French over-harvested in the 1970s and the Irish in the 1980s. The extensive fishing efforts were attempting to supply France, Spain and Italy with their sea urchin roe requirements (Allain, 1975; Regis et al., 1986). Although new fishing grounds are still being discovered, these too are over exploited. To meet demands, therefore, the rise of sea urchin cultivation is inevitable. As a result, research is increasing in sea urchin aquaculture.

The Japanese have started to examine methods of wild sea urchin transplantation (Agatsuma, 1998) and re-stocking with hatchery reared juveniles (Hagen, 1996). Studies on the development of artificial diets for sea urchins in land-based cultivation systems have recently been undertaken in the USA and France (Fernandez and Pergent, 1998; Walker and Lesser, 1998; Klinger et al., 1998; Lawrence et al., 1997; Barker et al., 1998; McBride et al., 1998; Watts et al., 1998; Grosjean et al., 1998).

In the U.K., there are two endemic species in Scotland that are harvested on a small scale and exported from the Shetland Isles (Penfold, 1995). These are *Echinus esculentus* Linnaeus and *Psammechinus miliaris* (Gmelin) (previously *Echinus miliaris*). Their roe quality is generally relatively poor and *P. miliaris* is rather small. Nevertheless, studies to enhance roe production in *P. miliaris* have shown positive results, when the animals are held in salmon cages stocked with the Atlantic salmon, *Salmo solar*, compared with monocultures or wild stocks (Kelly et al., 1998). *P. miliaris* also appears to develop well when naturally settled upon commercial scallop lines in Loch Fyne (Cook 1999). The roe of *P. miliaris* is sold for a high price in the Persian market place. It is currently fished in Brittany and also exported from Southern Ireland (Cook, 1999).

As the market demand increases for this rather exotic food, the fishery will become increasingly attractive economically. Recent studies examining the economic feasibility of sea urchin cultivation have been carried out (Burke, 1998; Cook, 1999). In a recent Ph.D. study, Cook (1999) examined the potential for commercial cultivation of *P. miliaris* from waters near Oban. Cook found that in mature animals, somatic growth, gonad quantity and quality could be influenced by feeding the animals with a high quality diet of salmon feed. The urchins were also shown to have the ability to exploit a diverse diet of both animal and plant origin, to tolerate high densities and to survive well during prolonged periods of aerial exposure. These combined factors make *P. miliaris* a suitable species for aquaculture.

4.1.2 Distribution

P. miliaris is commonly found all round the British coasts. Its wider distribution is from the Trondhjem Fjord, along the Scandinavian coasts to the Baltic and North Sea, and from Iceland, Rockall and Dogger Bank to the Atlantic coast of Morocco and west to the Azores. It is not found in the Mediterranean where it is replaced by the smaller *P. microtuberculatus*. *P. miliaris* does not occur in Greenland or on the Atlantic coasts of America (Mortensen, 1943). A detailed description of *P. miliaris* (common name, Green Sea Urchin) is given by Mortensen (1927). The urchin is essentially green in colour with purple-tipped spines. It is a littoral form, living among sea-grass, algae and often found under stones and on rocks within the tide limits but may also be found in the sublittoral. It has omnivorous feeding habits with a large part of the diet made up of

macroalgae and encrusting organisms. It may be found down to a maximum depth of 100m.

4.1.3 Natural Abundance

P. miliaris can be found in aggregations of 15 to 120 ind. m^{-2} (Cook, 1999). This range is similar to that reported for *Strongylocentrotus droebachiensis* (Miller and Colodey, 1983) but higher than for *Echinus esculentus* (Comley and Ansell, 1988). Studies on wild populations of *S. droebachiensis* showed that test and gonadal growth were reduced at an average density of 37 ind. m^{-2} (Lang and Mann, 1976). Orton (1914) states that *P. miliaris* tends to associate in pairs, most of which consist of opposite sexes, at spawning time.

4.1.4 Reproductive Biology

Individuals of *P. miliaris* found in the wild generally have a maximum test diameter (excluding spines) of 30mm, although larger specimens up to 48mm diameter have been found. The largest animals were from the North coast of Brittany (Allain, 1978). The adult varies in colour from light to dark green with purple-tipped spines (Hayward et al., 1996). *P. miliaris* is an iteroparous species with a lifespan of >10 years (Allain, 1978). They are dioecious and display sexual dimorphism, which is very rare in echinoids. The gonopores of *P. miliaris* are mounted on short papillae in males but not in the females. Minimum size at sexual maturity is 8mm (Jensen, 1969). The reproductive system of regular echinoids, such as *P. miliaris*, consists of five gonads, sometimes more or less fused, suspended from the aboral side of the test via mesenterial strands (Hyman, 1955). The gonad may constitute >20 % of the total weight of soft parts just prior to the onset of spawning. Eggs of *P. miliaris* have a maximum diameter of 97 - 115μm (Shearer et al., 1913) and when ripe they are encompassed by a transparent gelatinous coat. A vitelline membrane is observed beneath the gelatinous coat. Eggs are negatively buoyant and larvae are planktotrophic. *P. miliaris* are free-spawners and shed their gametes via gonopores. Spawning in echinoderms may generally occur a few times during a breeding season (Kanatani and Nagahama, 1983), although in many species, most of the eggs are released in the first spawning. In sea urchins, fully mature eggs resulting from the two maturation divisions are shed. Fecundity has not been studied in this species.

Generally, echinoderms go through an annual reproductive cycle and *P. miliaris* follows this pattern. The spawning season is in the early summer months (see Plymouth Marine Fauna) but ripe specimens have been found from February to November (Orton, 1923; Mortesen, 1943). A large variation in spawning has been noted between populations from different geographical localities. For example in the Clyde Estuary, *P. miliaris* breeds from June to August (Elmhirst, 1922) whilst near Bergen, Norway from June to October (Runnstrom, 1925). According to Bruce et al. (1963), *P. miliaris* plutei were found from July to September at Fleshwick, Scotland and adults taken in late August had spawned.

The population sampled for these experiments was from Torquay and these animals were found to be ripe in April (pers. obs.). Temperature is known to be a significant factor in the timing of spawning (Byrne, 1990), although the factors affecting spawning in *P. miliaris* are currently under investigation (Cook, 1999).

Immediately following activation by the sperm, the vitelline membrane detaches itself from the egg to form the fertilization membrane. Polar body formation in sea urchins occurs prior to fertilization. The first cell division occurs after ~1 hour. Cleavage is holoblastic and equal up to the 8-cell stage. Following this, the 4 vegetal cells each give off a small micromere at the vegetal pole and this unequal division leaves the vegetal cells slightly larger than those of the animal half or mesomeres, which continue to divide equally. The cells of the blastula develop flagella and the blastula begins rotating within the fertilization membrane that then ruptures and releases the embryo. After a period of approximately 12 hours, the free-swimming blastula stage is reached (Hyman, 1955). The gastrula stage is completed within 1 to 2 days. The gastrula alters into a characteristic larval type - the echinopleuteus. This transformation consists of the elongation of the gastrula into a conical shape (the prism). The gut and larval body cavity develop and the larvae develops arms. The 4-arm stage is generally reached within a few days. At this stage, the larvae requires food in order to survive and develop further. For a full description of development see Hyman (1955). Full development time is 58 days at 10-16°C (Lonning and Wennerberg, 1963).

4.2 Fertilization Kinetics - Laboratory Experimentation

4.2.1 Introduction

As discussed earlier, *Psammechinus miliaris* has been shown to be a suitable species for sea or land based cultivation (Cook, 1999). Therefore, as this species of sea urchin has good commercial potential, it is important to understand its fertilization kinetics. Sea urchins are a particularly well studied class of marine free-spawning animals in terms of fertilization because they are relatively easy to sample and manipulate.

Spawning induction in sea urchins can be achieved easily by injecting potassium chloride or acetylcholine or by applying an electric stimulus. The gonads respond immediately to these stimuli, which causes contraction of the gonad and the discharge of a conspicuous amount of gametes (Iwata, 1950). In sea urchins, oocytes within ripe ovaries are already mature and do not have firm follicles, thereby enabling the ovarian wall to contract immediately upon application of the spawning stimulus.

The procedure for fertilization in sea urchins in the laboratory is well established (Fenaux et al., 1988; Leighton, 1995; Grosjean et al., 1998).

4.2.2 Materials and Methods

4.2.2.1 Collection of animals

Psammechinus miliaris were collected by hand from littoral pools and from beneath rocks below high water level at Corbyn Head, Torquay, England (50°6N; 3°32W) on 30th March 1999 (Figure 4.1).

Figure 4.1: Opposite views of Corbyn Head, Torquay

They were covered with damp seaweed and transported in an insulated box to Southampton Oceanography Centre. On arrival, they were transferred to a re-circulating seawater aquarium of salinity 33.6 at 17.5°C . All animals were used for experimental manipulations within 2 weeks of capture.

4.2.2.2 Collection of gametes

One ml of 0.55M KCl was injected through the peristomal membrane into the coelomic cavity of *P. miliaris* in order to induce spawning. Fresh needles were used for each animal in order to avoid spurious fertilizations. Animals were inverted and balanced over glass beakers. Within a few minutes, spawning commenced and gametes were collected in the bottom of the glass beaker. Animals were left to spawn for a few minutes until enough gametes had been extruded for use in experiments. Sperm were collected 'dry' in an attempt to prolong their lifespan whereas eggs were spawned directly into small amounts of filtered seawater.

4.2.2.3 Hypothesis tested

1). There is no variation in fertilization success with variable sperm-egg contact time
2). There is no variation in fertilization success with different sperm concentrations
3). There is no variation in fertilization success with gamete age

4.2.2.4 Protocols

4.2.2.4.1 General

The general methodology is described in section *2.2.2.5.1* as the same protocol was used for *P. miliaris* as was used for *H. tuberculata* with following exceptions. The test diameter (excluding spines) of each experimental animal was recorded using callipers to an accuracy of 0.01mm.

4.2.2.4.2 Sperm-egg contact time experiment

See section *2.2.2.5.2* for methodology. Sperm were diluted to approximately 10^7 sperm ml^{-1} giving 10^6 sperm ml^{-1} in experiments and only this one pre-determined sperm dilution was used.

4.2.2.4.3 Sperm dilution experiment

See section *2.2.2.5.3* for methodology.

4.2.2.4.4 Gamete age experiment

The longevity of sperm and eggs of *P. miliaris* were determined. 240 x 30ml glass tubes were filled with 8ml each of UV FSW. Four decreasing sperm concentrations were used in these experiments from 5 x 10^5 sperm ml^{-1} to 7 x 10^3 sperm ml^{-1} in order to account for respiratory dilution effect (Chia and Bickell, 1983). Eggs and sperm were aged simultaneously. New fertilizations were performed at hourly intervals by introducing 1ml of 100-200 eggs ml^{-1} and 1ml of sperm at each concentration with 3 replicates of each treatment until zero fertilization was evident. The volume of gametes collected from the first male-female pair was too low to continue with the experiment beyond 10 hours. In order to determine the effects of ageing on gametes >10 hours old, a further male-female pair were spawned and gametes were aged for 10 hours before recording data for the following 12 hour period. Fresh sperm were added to the 10 hour-old eggs, and fresh eggs added to the 10 hour-old sperm in order to assess individual gamete age. A total of six animals were used for this single experiment. Only one gamete age experiment was conducted for *Psammechinus miliaris* as all animals in the holding tank spawned rendering them unsuitable for further experiments.

4.2.2.4.5 Gamete Release

Gamete release rates were estimated for 9 male and 4 female urchins. Animals were induced to spawn using the KCl injection method described previously. Gametes were collected in a known volume of seawater over the period of 15 minutes. The number of eggs and sperm released by each animal during this set time period were determined by examination of 3 replicate sub-samples. A Sedgewick Rafter cell was used to count the eggs and a haemocytometer was used to estimate the number of sperm. The gamete release rate was then calculated for each individual. This release rate was compared to individual test diameters to identify any correlation.

4.2.3 Results

4.2.3.1 Experimental animal weights and test diameters

Animals weights (including test) and test diameters were noted for each experimental animal and are displayed in Table 4.1. A linear regression was fitted to these data in order to determine the relationship between the two variables, with an R^2 value of 0.84 (Figure 4.2).

TABLE 4.1

Experimental Animal Weights and Test Diameters

Animal Reference	*Experiment*	*Sex*	*Test diameter (mm)*	*Wet weight (g)*
1	sperm dilution	M	33.0	12.14
2	sperm dilution	F	32.1	11.41
3	sperm dilution	M	48.5	47.64
4	sperm dilution	F	27.5	7.33
5	sperm dilution	M	24.5	6.51
6	sperm dilution	F	21.5	4.34
7	sperm dilution	M	24.5	4.19
8	sperm dilution	F	20.0	3.01
9	contact time	F	40.8	19.1
10	contact time	M	34.0	16.8
11	contact time	F	42.5	36.6
12	contact time	M	32.5	14.9
13	contact time	F	26.6	7.50
14	contact time	M	41.0	30.0
15	gamete age	F	30.5	13.2
16	gamete age	M	30.5	13.0
17	gamete age	F	36.2	16.5
18	gamete age	M	29.5	8.2
19	gamete age	F	32.5	15.0
20	gamete age	M	40.5	19.3

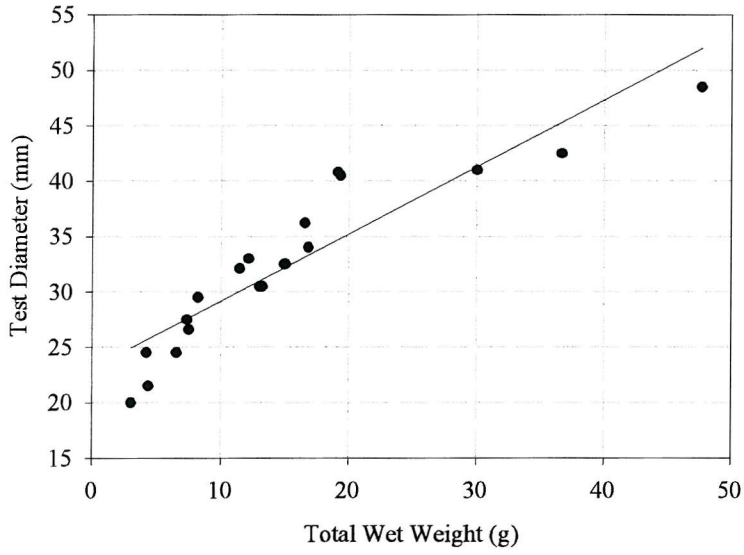


Figure 4.2: Relationship between individual weight and size in P. miliaris. $R^2=0.84$

4.2.3.2 Sperm-egg contact time

Fertilization success was significantly effected by variation in sperm-egg contact time (Figure 4.3). At just 5 seconds contact time ~20% fertilization success was achieved. A steady increase in fertilization success with time was seen for each of the 3 replicates. After 5 minutes, fertilization success had increased to ~60%. Maximal fertilization success occurred at 30 minutes contact time. All treatments showed very similar fertilization success.

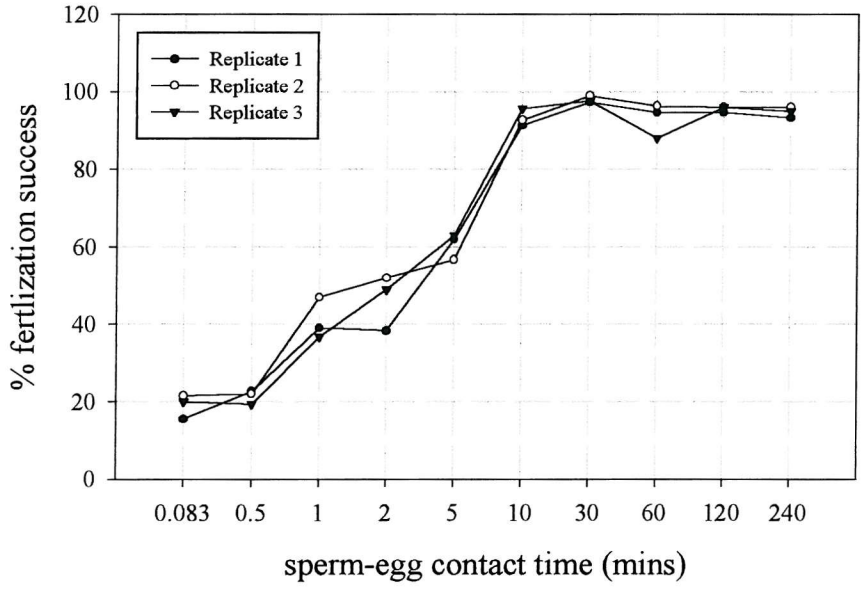


Figure 4.3: The effect of sperm-egg contact time on fertilization success in P. miliaris.

4.2.3.3 Sperm dilution experiment

Sperm concentration had a highly significant effect on fertilization success of *P. miliaris* (Figure 4.4a). A substantial rise in fertilization success took place between 10^3 and 10^5 sperm ml^{-1}. At sperm concentrations of around 5 x 10^4 to 1 x 10^6, fertilization success reached an optimum (between 85 - 100%). A decline in fertilization success is seen with sperm concentrations >10^6 sperm ml^{-1}. At these high concentrations polyspermy occurred. Abnormally developing embryos were also noted (Figure 4.4b).

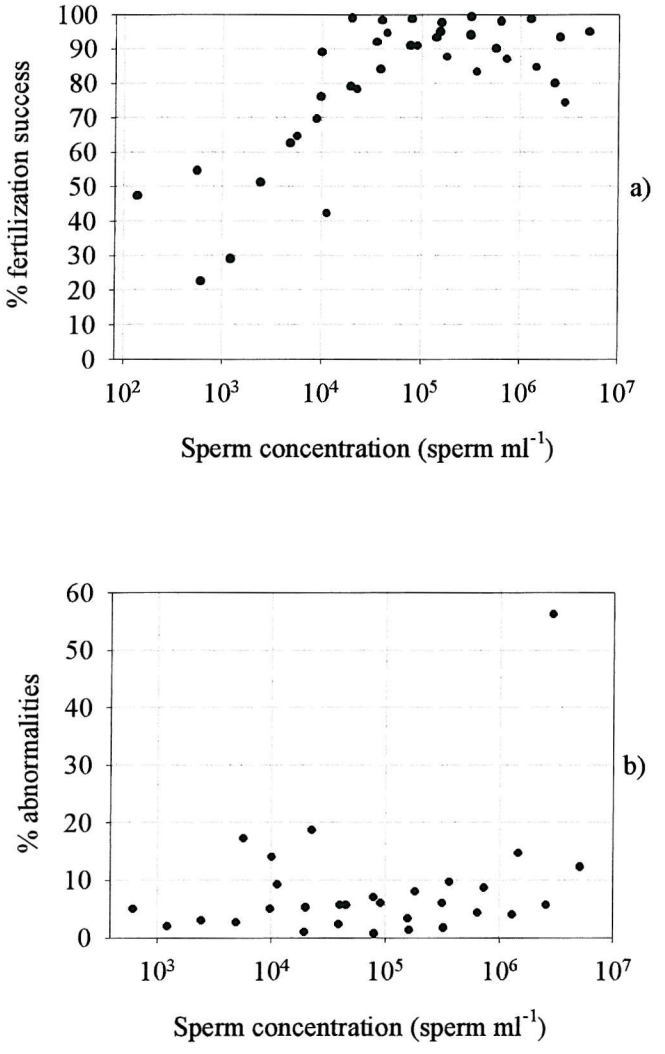


Figure 4.4: The effect of sperm concentration on a) fertilization success and b) abnormal development in P. miliaris

4.2.3.4 Gamete age

Experiments using the first male-female pair, where eggs and sperm were aged simultaneously, showed significant effects of gamete age upon fertilization success. Significant effects of sperm concentration were also clearly seen in these experiments (Figure 4.5a). At one hour old, treatments at each different sperm concentration showed > 80% fertilization success. This success rate declined rapidly in the lowest sperm concentration to <20% after only 2 hours. It took 7 hours to reach zero fertilization at this lowest concentration. Conversely, in the highest sperm

concentration, fertilization success still exceeded 60% even after 10 hours. In all replicates the higher sperm concentrations had sperm with a greater longevity.

The second part of this gamete age experiment showed that some gametes remained capable of successful fertilization up to 20 hours after release but by 22 hours there was no successful fertilization (Figure 4.5b). Results indicate that eggs age faster than sperm in *P. miliaris*. At the highest sperm concentration, in excess of 90% fertilization success still existed even when sperm was 16 hours old. Eggs of the same age when added to fresh sperm at 10^6 sperm ml^{-1} only achieved 60% fertilization success. In those treatments with lower sperm concentrations, fertilization success approached zero after 14 hours with a steep decline in success from 10 hours old.

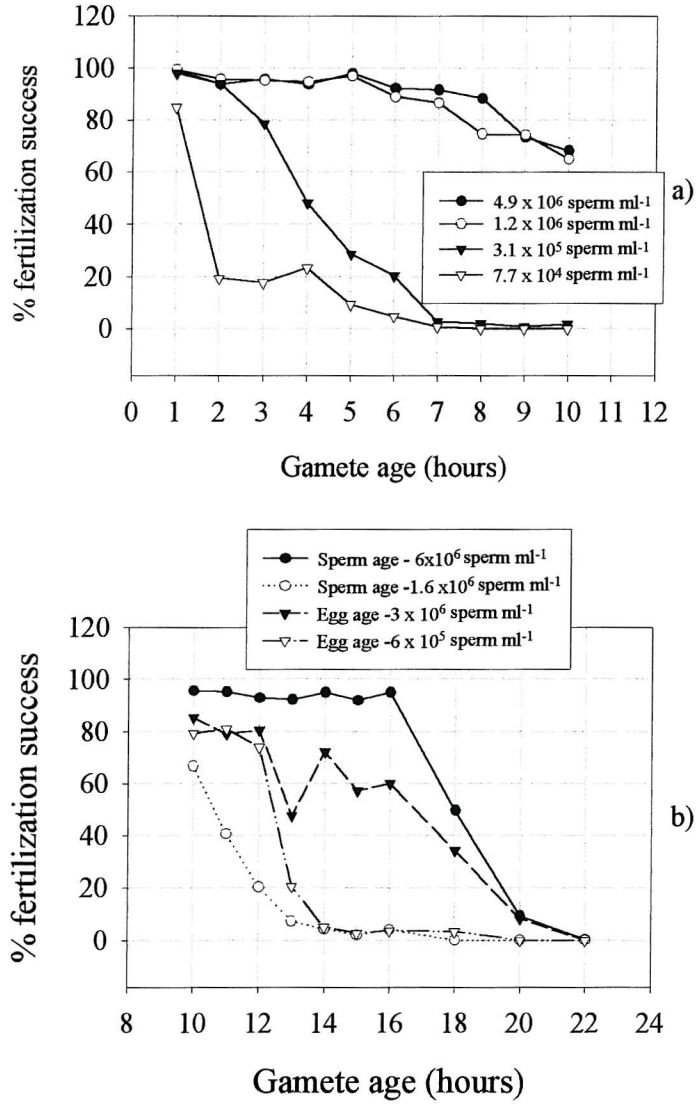


Figure 4.5: The effect of gamete age on fertilization success in P. miliaris *a) from time zero, b) fresh gametes after10 hrs*

4.2.3.5 Gamete Release

Gamete release rates have been calculated for male and female *P. miliaris* and are displayed in Table 4.2 .

TABLE 4.2

Gamete Release Rates

Sex	*Test Diameter (mm)*	*Mean no. gametes released in 15 minutes*	*Gamete release rate (gametes per second)*
F	32.0	1.78×10^5	197.7
F	25.5	2.95×10^4	32.7
F	24.0	4.16×10^4	46.2
F	30.0	1.18×10^5	131.1
M	40.0	1.59×10^{10}	1.76×10^7
M	32.0	3.83×10^9	4.26×10^6
M	33.0	8.33×10^8	9.26×10^5
M	30.0	7.83×10^8	8.7×10^5
M	29.5	1.18×10^{10}	1.31×10^7
M	22.5	4.09×10^9	4.5×10^6
M	30.0	6.25×10^9	6.95×10^6
M	24.0	1.43×10^8	1.59×10^5
M	23.5	1.46×10^9	1.62×10^6

The mean sperm release rate was calculated from these data as 5.55×10^6 sperm s^{-1} (n=9). A very weak relationship was seen between male size and gamete output (R^2 = 0.38, n = 9).

4.2.4 Discussion

Each of the three variables tested were shown to have a significant effect upon the successful fertilization of eggs by sperm in *P. miliaris*. Firstly, the length of time that sperm and eggs are in contact with each other has a marked effect on fertilization success. However, the experiment only examined the effects at one particular sperm concentration and different sperm-egg ratios and sperm concentrations may have a compounded effect upon the results of the sperm-egg contact time. Nevertheless, the three replicate experiments show very similar results and it is clear that gametes need to be in contact for 10 minutes in order to achieve the optimum fertilization success at this particular sperm concentration. These results are similar to those found for *H. tuberculata* (see Chapter 2), which also showed increased success after 10 minutes contact time. Similar results were also found for the echinoid *Strongylocentrotus*

purpuratus (Levitan et al., 1991) and for the polychaete annelid *Nereis virens*, the asteroid *Asterias rubens* and the echinoid *Echinus esculentus* (Williams, 1999). These results imply that unless sperm and eggs are spawned synchronously at slack water, fertilization success may be reduced severely. Rothschild and Swann (1951) investigated the effects of sperm-egg contact time upon fertilization success in *P. miliaris*. After 5 seconds, they found approximately 20% of eggs fertilized in a sperm concentration of 3.67×10^5 sperm ml^{-1}. Their results showed a rapid increase from this point, with fertilization success reaching 90% in only 1 minute. Instead of removing the eggs from the sperm suspension following the particular contact time, they added hypotonic seawater (45% seawater in distilled water) to kill the spermatozoa and prevent any further fertilization. The sperm concentrations used in the experiments were different between the two investigations (10^7 sperm ml^{-1} in this study compared to 10^5 sperm ml^{-1} used by Rothschild and Swann). However, Rothschild and Swann (1951) showed a vast decrease in fertilization success with increased sperm concentrations with the probability of fertilization (as calculated by modelling spermatozoa moving as random gas molecules) decreasing by an order of magnitude from 0.115 at 3.67×10^5 sperm ml^{-1} to 0.011 at 9.62×10^6 sperm ml^{-1} after 1 minute contact time. Had the experiments in the present study used lower sperm concentrations, the results may have been similar to those found by Rothschild and Swann (1951). An increase in sperm-sperm contact at higher sperm concentrations could increase the time needed by sperm to find and fertilize eggs. With this in mind, it could be that with situations of dilute sperm, more likely to be encountered during natural spawning events, sperm-egg contact time necessary for successful fertilization may be reduced.

The second set of experiments showed the influence of sperm concentration upon fertilization success in *P. miliaris*. A typical pattern as reported for other echinoids was seen. An increase in sperm concentration up to 10^5 sperm ml^{-1} led to optimum fertilization success with a slight downward trend in success with increased sperm concentrations beyond this level. One of the factors associated with higher sperm concentrations is an increase in polyspermic fertilizations leading to abnormal development or death. Perhaps this condition would have been more evident at higher sperm concentrations than those used in this experiment. Abnormally developing embryos were also noted during the counts. However, these results should be treated with caution because the counts were made during the first few cleavages. At this stage

it can be difficult to tell if apparently normally developing embryos may become abnormal or arrest development at a later stage. Certainly when conducting sperm dilution experiments and scoring abnormalities in *H. tuberculata* (Chapter 2) it was often necessary to leave the embryos to develop to trochophore in order to be sure of abnormal larvae counts. One may have expected to see a larger increase in the incidents of abnormal development at the higher sperm concentrations as a function of polyspermy. Rothschild and Swann (1951) investigated the time of the block to polyspermy in *P. miliaris* and found it to be in the order of seconds rather than fraction of a second. Should these animals aggregate and spawn synchronously in slack water environments, such as tidal pools, incidents of polyspermy may be expected to be high in nature. A further finding of Rothschild and Swann (1951) was that no gametic chemotaxis was observed in this species in contrast to many other echinoid species that are known to have chemo-attractants as a mechanism for increasing the chance of sperm-egg collision. As discussed in Chapter 2, the results of the sperm dilution study should be interpreted with caution as all experiments were conducted in 10ml culture volumes. Very different results may have been obtained by increasing culture volumes, thereby altering the sperm-egg ratios. Specific effects of sperm-egg ratio were not tested for this animal.

Results shown by the gamete age experiments are based on one replicate only and are therefore subject to verification by additional replicates. Nevertheless they give an idea of gamete response to ageing and the compounded effect of sperm concentration in this species. One of the most interesting points to note here is that eggs appeared to age faster than sperm - an unexpected finding in light of previous studies of gamete age. There have been relatively few studies of unfertilized gamete longevity. However, those studies that have been conducted, over a variety of free-spawning invertebrate groups, have reported that sperm are generally shorter lived than eggs (see Table 2, Chapter 1 for references). However, in many cases, egg viability has not been directly measured and it has been assumed that eggs live longer than sperm. One study that did determine egg age, independent of sperm age, was by Andre and Lindegarth (1993) who reported a similar lifespan in both gamete types. Studies on *H. tuberculata,* (Chapter 2) showed a similar pattern. Should the mode of reproduction in terms of synchronous spawning of male and female individuals prevail in these species, similar gamete longevity would seem an ecologically sound option. This, in addition to the likely dispersal of gametes in

the water prior to their death, would obviate the need of expending energy during gametogenesis to prolong gamete lifespan. Longer-lived sperm and eggs are found in *Arenicola marina* (Williams, 1999) where the mode of reproduction is somewhat different. Here, eggs may be spawned earlier than sperm and are capable of successful fertilization up to 5 days after spawning. Sperm of this animal is spawned in puddles, which are then washed down into the female burrow. This process may take many hours and hence the possession of longer-lived gametes in this species is an ecological necessity. In *P. miliaris*, it could be that gametes are only spawned in slack water conditions and sperm are usually extruded before eggs. This may explain the slightly shorter life of eggs compared to sperm. Orton (1914) noted pairing behaviour in spawning *P. miliaris*. He found that the pairs were usually made up of animals of the opposite sex. This natural aggregation behaviour could act to reduce the risk of sperm limitation and alleviate the need for long-lived gametes. Seasonal breeding aggregation has also been noted for deep-sea dwelling echinoids, *Stylocidaris lineata* (Young et al., 1992).

4.3 The Vogel-Czihak-Chang-Wolf (VCCW) Model

4.3.1 Implementation

In the same way that the VCCW model was used to analyse the likelihood of fertilization in *H. tuberculata* in Chapter 2, it was also used for *P. miliaris* by incorporating data from laboratory experiments on gamete behaviour. One additional variable is known for this species. Gray (1955) estimated that mean sperm velocity in *P. miliaris* was 0.19 mm s^{-1}. This factor together with the calculated cross-sectional area of the mature egg was incorporated into the model. A full description of the model implementation is given in Chapter 2, section *2.3.1.* Laboratory experiments have shown that for fertilization success to reach a maximum in *P. miliaris*, sperm and eggs must be in contact with each other for approximately 10 minutes.

A regression analysis was conducted to estimate sperm half life in *P. miliaris* from laboratory experimental data (Figure 4.6). However, few data were generated only at the higher sperm concentrations. Sperm half-life from the lower end of the sperm concentration range can be inferred only from the higher concentrations and are likely to be subject to error. The regression line was set to pass through the origin in order to avoid a minus figure on the y-axis intercept that would have been unrealistic.

Using the regression equation, estimates of sperm half life were incorporated into the Vogel model. Initial parameters were then estimated and tested in order to determine the best fit of the for the empirical data. Figure 4.7 shows the predicted fertilization kinetics model line fitted to a subset of the data.

The same iterative method of Levenberg-Marquardt for non-linear regression (Jandel SigmaPlot) was used to determine the best fit values of βo and β/βo for the empirically derived data points relating to sperm concentration and fertilization success.

The best fit values gave a β value of 3.7×10^{-4}, a βo value of 6.5×10^{-3} and a β/βo ratio of 5.7×10^{-2}. This suggests that ~5.7% of the egg surface is fertilizable or that only ~5.7% of the spermatozoa are able to fertilize the egg. The empirical data were regressed against the predicted data from the model with an R^2 value of 0.78. This fitted regression line explained almost 80% of the variation in fertilization and the model is hence considered to be robust.

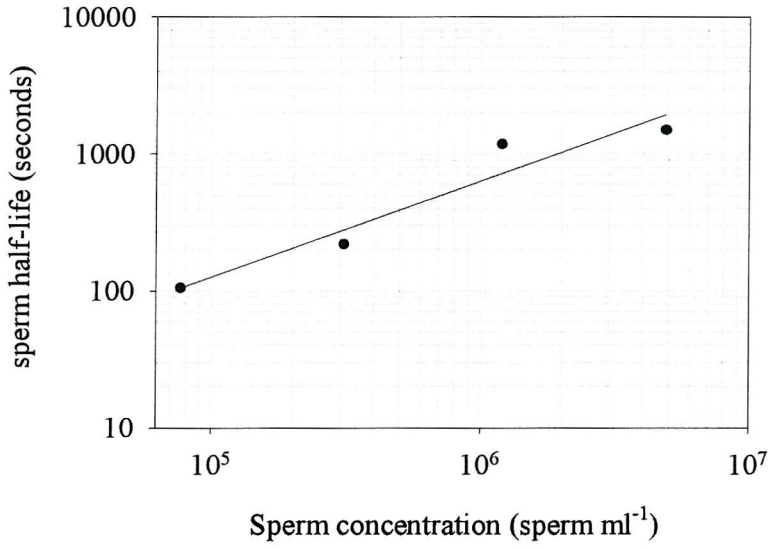


Figure 4.6: Sperm half-life (τ,S) as a function of sperm concentration (ml.). Log τ = (log S x 0) +0.4011

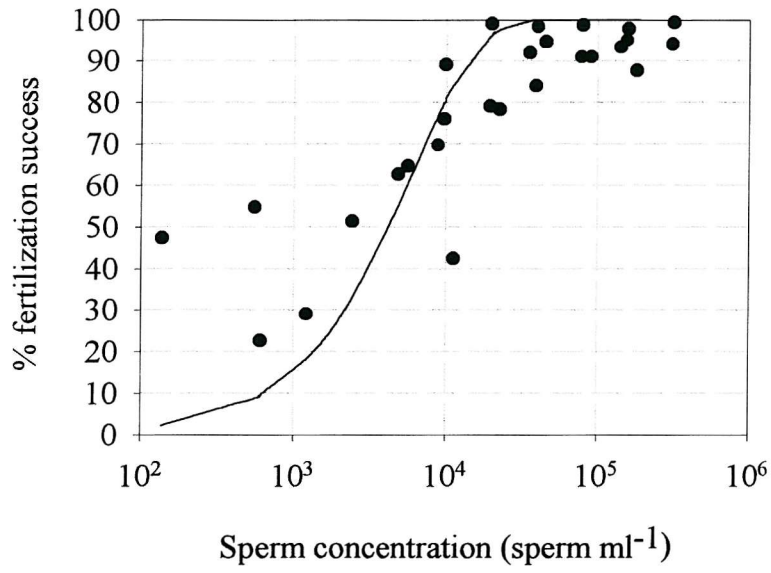


Figure 4.7: Best fit line of model to empirical data. See text for details.

In addition, determination of the two rate constants by use of the model, one of the rate constants, βo, was already known and an independent estimate of this value was made. Sperm velocity was taken to be 0.19mm s^{-1} (Gray, 1955) and the diameter of mature *P. miliaris* eggs fell in the range 97 - 115μm. βo was calculated from these empirical data with the following equation:

$$\beta o = \nu\sigma$$

where ν is the mean sperm velocity (mm s^{-1}) and σ is the cross-sectional area of the egg (mm^2).

$$\beta o = 0.19 \times (\pi \times (0.106/2)^2) = 1.7 \times 10^{-3}$$

When this is compared with the estimate of βo from the VCCW model, the results were in the same order of magnitude, but nevertheless were 3.8 times lower. This could have led to over estimation of sperm velocity if this factor was not already known.

4.3.2 Discussion of the Vogel Model

The results found for the two rate constants β and βo were compared to results gained from implementation of the VCCW model on other sea urchin species (Table 4.3).

Table 4.3

VCCW Model Values For Three Urchin Species

Species	*Authors*	β	βo	$\beta/\beta o$
Psammechinus miliaris	This thesis	3.7×10^{-4}	6.5×10^{-3}	5.7×10^{-2}
Strongylocentrotus franciscanus	Levitan et al. (1991)	6.0×10^{-5}	1.76×10^{-3}	3.4×10^{-2}
Paracentrotus lividus	Vogel et al. (1982)	3.8×10^{-6}	3.3×10^{-4}	1.1×10^{-2}

The ratio of $\beta/\beta o$ describes either the area of egg surface able to be fertilized or the percentage of sperm that can successfully fertilize eggs. All the ratios are in the same order of magnitude, but that of *P. miliaris* is greatest and is over five times greater than the ratio estimated for *Paracentrotus lividus* (Vogel et al., 1982). The latter estimation of 1% was used by Denny and Shibata (1989) to predict fertilization in the field. The use of the increased figure here would increase Denny and Shibata's predictions of *in-situ* fertilization by a factor of five from 1% to almost 6% in highly turbulent water. This would have a relatively large impact upon the potential recruitment prediction.

If the model-derived value for βo was used in an attempt to determine mean sperm swimming speed, given an egg diameter of 0.106mm as a modal value between 0.097 and 0.115mm, the result would overestimate the actual speed of 0.19mm s^{-1} and give a value of 0.74mm s^{-1}. Although there is bound to be some individual variability in terms of sperm swimming speed, this estimate is rather high. Levitan et al (1991) noted variable sperm swimming speeds within and among individuals of *Strongylocentrotus franciscanus* ranging from 0.05 to 0.3 mm s^{-1}, a difference of 0.25 mm s^{-1}, half the difference of 0.55 mm s^{-1} for *Psammechinus miliaris.* If the egg diameter was set to the upper limit for mature *P. miliaris* eggs, at 115µm, this would result in a predicted sperm velocity of 0.62mm s^{-1}, reducing the difference slightly to 0.43mm s^{-1}. One would still conclude, however, that this figure is excessive and therefore these model predictions should be treated with caution. Perhaps with an increased number of empirically derived data points for both sperm concentration and gamete age with respect to fertilization success, more accurate predictions could be obtained.

As stated above, the ratio of sperm-egg collisions to actual fertilizations ($\beta/\beta o$) varies among echinoid species. This is likely to be a function of sperm vigour and age/health of the egg or to the number and distribution of sperm receptor sites on the egg surface.

4.4 The Denny Model

This model, previously described in Chapter 2, was used to predict fertilization success of *P. miliaris* in nature given approximations of sperm release rates and flow conditions. Table 4.4 shows the initial parameters used .

Table 4.4

Parameter Values

Parameter Description	*Symbol*	*Values and Units*
Vertical diffusivity coefficient	α_y	2.2; 0.198
Horizontal diffusivity coefficient	α_z	1.25; 0.124
Male-female separation distance	X	0 - 400cm
Sperm release rate	Q	5.55×10^6 ; 1.6×10^7 sperm s^{-1}
Mean flow velocity	$\bar{U}$	5 ;10 cm s^{-1}
Frictional velocity	u_*	5%; 10%; 15% $\bar{u}$

Model predictions are displayed graphically in Figure 4.8 a-e.

4.4.1 Discussion

Five different simulations are presented following those given in Chapter 2 for *H. tuberculata*. The differences between the models were a function of the difference in sperm release rate determined experimentally for each species. The model simulations indicate three *P. miliaris* males have almost the same effect on fertilization success as one *H. tuberculata* male in terms of sperm dispersal, although fertilization success is ~50% at 10^3 sperm ml^{-1} in *P. miliaris* compared to only 20% at the same sperm concentration for *H. tuberculata*. This suggests that in nature, and given the same stocking density of these two free-spawning invertebrates, *P. miliaris* is generally more efficient at achieving high levels of fertilization success than is *H. tuberculata*, despite the lower amount of sperm released. For example, if one male *H. tuberculata* spawned 1 metre upstream of a spawning female in current speeds of 10cm s^{-1}, the model would predict around 20% of these eggs would be fertilized compared to ~40% of *P. miliaris* eggs. Slight differences in the spawning environment between the two species are not accounted for in the implementation of Denny's model. For example, the model

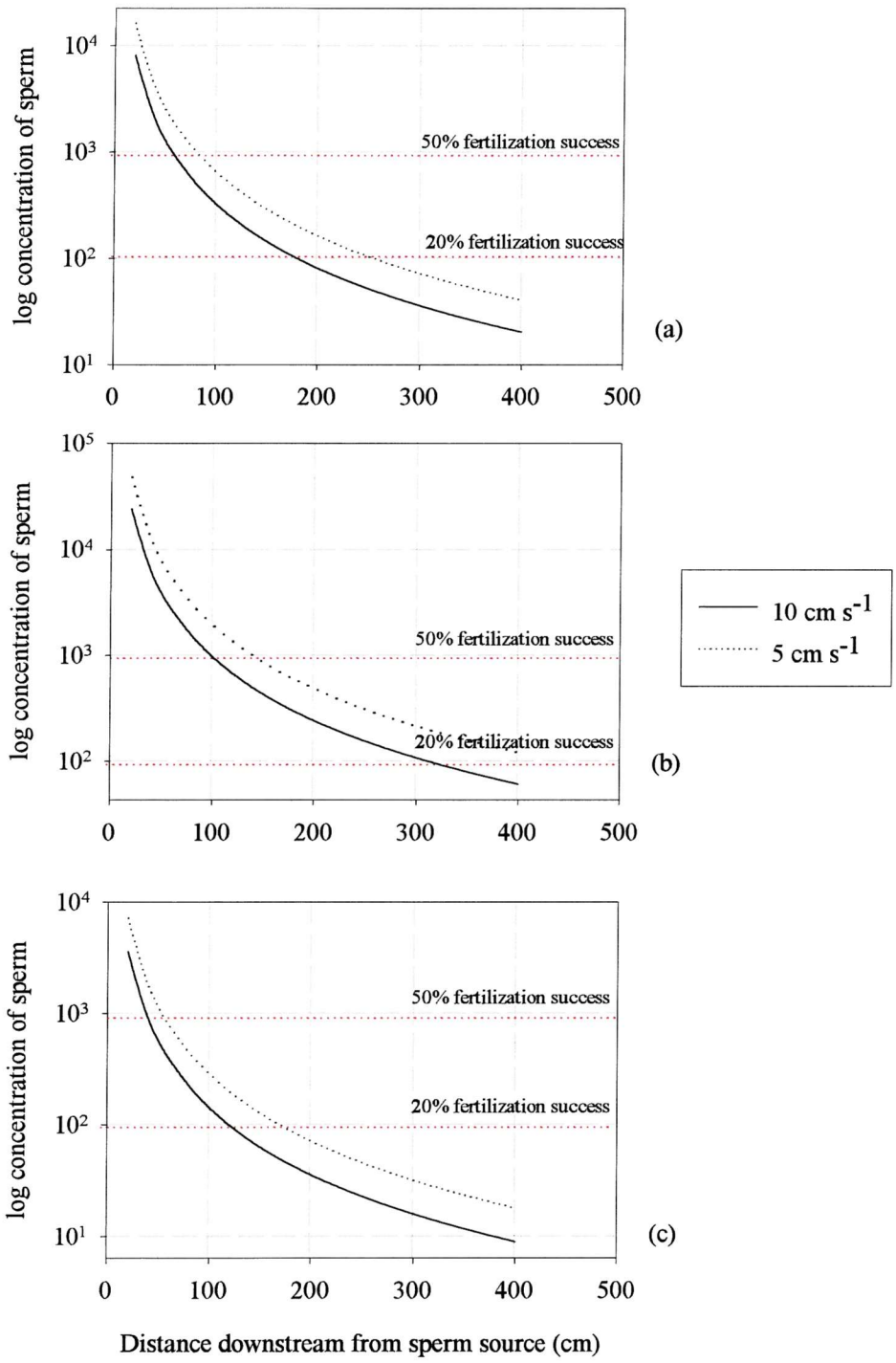


Figure 4.8 (continued on next page): Predicted sperm concentration and fertilization success under a range of different current regimes for P. miliaris. *(a)* $\alpha_y = 1.25$; $\alpha_z = 2.2$; $u_* = 10\%$; *1 male, (b)* $\alpha_y = 1.25$; $\alpha_z = 2.2$; $u_* = 10\%$; *3 males, (c)* $\alpha_y = 1.25$; $\alpha_z = 2.2$; $u_* = 15\%$; *1 male.*

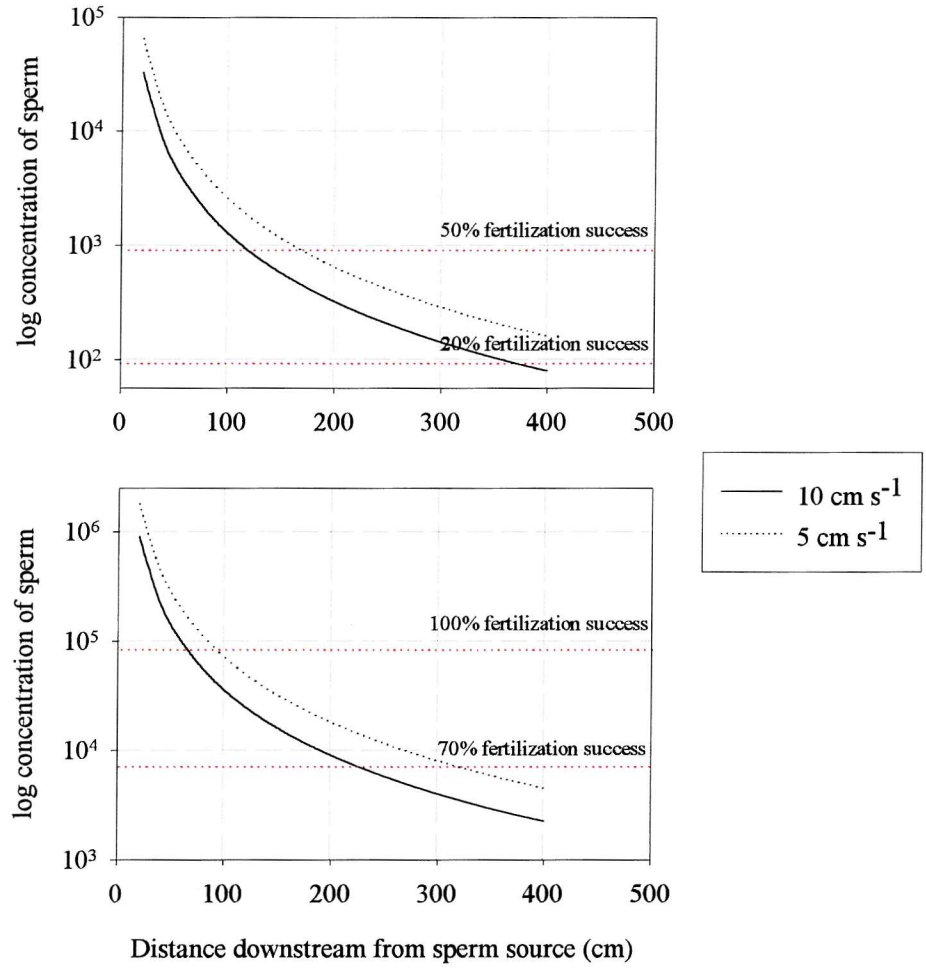


Figure 4.8(continued): (d) $\alpha_y = 1.25$; $\alpha_z = 2.2$; $u_* = 5\%$; 1 male, (e) $\alpha_y = 0.198$; $\alpha_z = 0.124$; $u_* = 10\%$; 1 male.

fails to account for the effect of bottom shear on the sperm plume. Also, the accuracy of model predictions could be improved by conducting more detailed surveys of the behaviour of a dye cloud shape in order to asses directional diffusivity. Incorporation of knowledge of gamete viscosity may also be important in some species. The proportion of gametes released during both optimal and sub-optimal spawning times could also have a large effect on fertilization success and should be considered in the estimation of lifetime reproductive success. Nevertheless, the use of this model in its current form gives a good guide to natural gamete interaction as a function of dispersal.

4.5 Fertilization Kinetics - In-Situ Experimentation

4.5.1 Introduction

In order to validate predictive models of sperm dilution in nature, *in-situ* experiments were performed, similar to those outlined in Chapter 2 for *H. tuberculata*.

4.5.2 Materials and Methods

4.5.2.1 Collection of animals

Individuals of *Psammechinus miliaris* were collected during their natural spawning season from tidal pools at Corbyns Head, Torquay, England (50°6N; 3°32W) as the tide was receding. They were usually found in groups (from 2 upwards) attached to the underside of rocks with particle-laden spines. Ten animals were collected in the first instance and placed in a bucket of aerated seawater at ambient temperature.

4.5.2.2 Collection of gametes

It was not practical to use the actual animals in these experiments as they have rather unpredictable spawning. Therefore gametes were collected prior to experimentation as outlined in section 4.2.2.2.

4.5.2.3 In-situ experiments

Experiments were conducted between Friday 7th April and Sunday 9th April 2000. Freshly spawned eggs from one to three females were diluted in ~2 litres of filtered seawater (0.02μm) in a 3 litre plastic tank to an approximate concentration of 10 eggs ml^{-1}. Pre-filtered seawater in a 5 litre plastic container was kept at ambient temperature by immersion in seawater. Nitex mesh chambers with labelled plastic floats were immersed in the egg water. Chambers were then sealed, capturing approximately 200 eggs per chamber. Eggs from more than one female were used when required. The volume of the chambers was approximately 20 ml. Egg-filled chambers were transferred to water-filled plastic bags and carried to the experiment site a few meters away.

A suitable site was chosen away from other sea urchins in order to avoid contamination from their sperm. Experiments were carried out as the tide was actively ebbing or flowing in an attempt to achieve current flow in a consistent direction. One 5 m long weighted pole with distance markings and hooks was placed on the seabed parallel to the ambient water flow. A second pole of 2 m, was positioned perpendicular to the first. Figure 4.9 shows this equipment being carried to the experimental site. Flow velocity was estimated by releasing fluorescein dye at approximately 1 ml s^{-1} at point "0" ~10cm above the seabed and timing its movement in the x direction along a predetermined distance. The dye was injected upwards from the seabed mimicking sperm release.

Figure 4.9: Transport of poles to work site at Corbyn Head

Nitex chambers containing eggs were hooked onto the pole at predetermined sampling points at 5 cm, 10 cm, 25 cm, 50 cm, 1 m, 2 m, 3 m, 4 m and 5 m away from '0'. Chambers were also placed on the perpendicular pole at 25 cm, 50 cm and 1 m in each direction. On sperm-free control sample was placed ~10 m away from the point of sperm release. Each chamber was held upwards in the water column ~ 10 cm above the sea bed by use of floats. Chamber surfaces were naturally oriented perpendicular to the direction of flow. Although these chambers may introduce experimental error by inhibiting flow, Levitan (1991) found this technique produced similar relative values across treatments when compared to unconfined eggs.

Freshly spawned sperm (less than 30 minutes old, spawned dry and activated with seawater minutes before the experiment) was injected at point '0' from an upward facing syringe at ~10 cm above the seabed at a release rate of approximately 1 ml per second. A total of 20 ml were released in each experiment. A sub-sample was preserved for determination of sperm concentration. Samples of the eggs and sperm used in the *in-situ* experiment were mixed together as a viability control. Eggs-only controls were also conducted to check for contamination. Eggs were collected after 10 minutes, rinsed in filtered seawater to remove excess sperm, and transferred from the mesh chambers into 30 ml glass tubes to continue development in filtered seawater of ambient temperature (~13°C). One hundred randomly sampled eggs from each distance downstream of sperm release were later scored for fertilization success.

4.5.3Results

4.5.3.1 In-situ experiments

Preliminary experiments were attempted on the first day and problems with equipment and logistics encountered and rectified. The female spawned on day one had non-viable eggs. It was noted, during the spawning of animals, that there appeared to be a higher proportion of females to males. Out of 29 animals spawned for the first three experiments, only 6 were male. Details of each replicate are outlined in Table 4.5. The amount of sperm released for each replicate was estimated from four replicate counts via haemocytometer.

Table 4.5

Replicate Details

Replicate No.	*Date/Time*	*Current Speed (cm sec^{-1})*	*Male test diameter (mm)*	*Amount of sperm released x 10ml*	*Female test diameter (mm)*
1	08-04-00/ 14:19	3.91	41; 38; 31	5.9 x 10^{8} sperm ml^{-1}	38; 27; 32
2	08-04-00/ 16:53	2.73	25; 24	4.2 x 10^{7} sperm ml^{-1}	27
3	09-04-00/ 15:10	2.33	37; 19	4.1 x 10^{7} sperm ml^{-1}	24
4	09-04-00/15:57	1.42	37	8.2 x 10^{7} sperm ml^{-1}	30; 25

The results from the field experiments are presented in Figure 4.10. In all replicates, fertilization success decreases substantially within the first meter downstream from the sperm source.

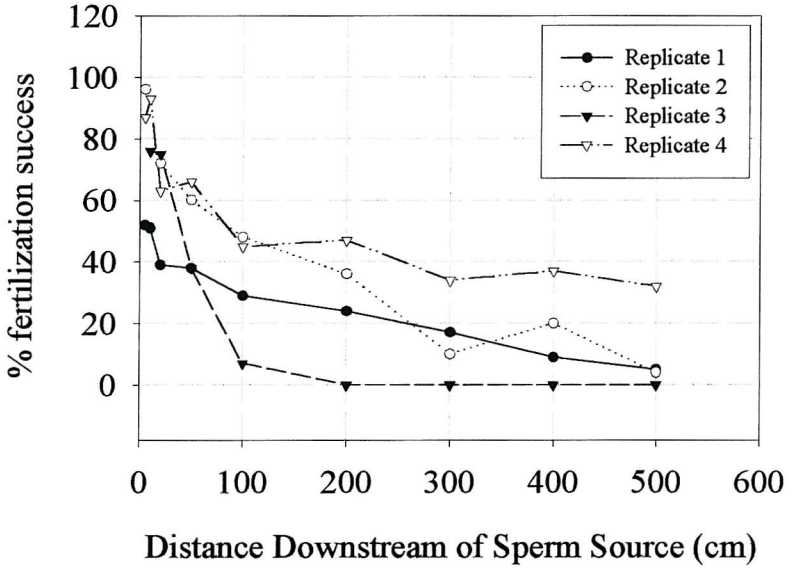


Figure 4.10: Fertilization success of P. miliaris *with respect to distance downstream of sperm source*

4.5.4 Discussion

In comparing model and experimental data, it was apparent that more sperm were released in the experiments than in nature. In addition, the model uses estimates of fertilization success based upon current speeds of 5 and 10cm s^{-1} whereas the field current speeds were lower than this. If more realistic data were used in the model, for example, current speeds of 2.5 cm s^{-1} and sperm release rate of 4.2 x 10^7 sperm ml^{-1}, as for replicate two, the model predicted very similar results to the empirical data, confirming the robustness of the model. Figure 4.11 shows the predicted results calculated via the model and the corresponding empirical values from experimentation. The model data were estimated from the laboratory-derived results of % fertilization success vs. sperm dilution (Figure 4.4a) and extrapolating for the given sperm concentrations calculated in the model.

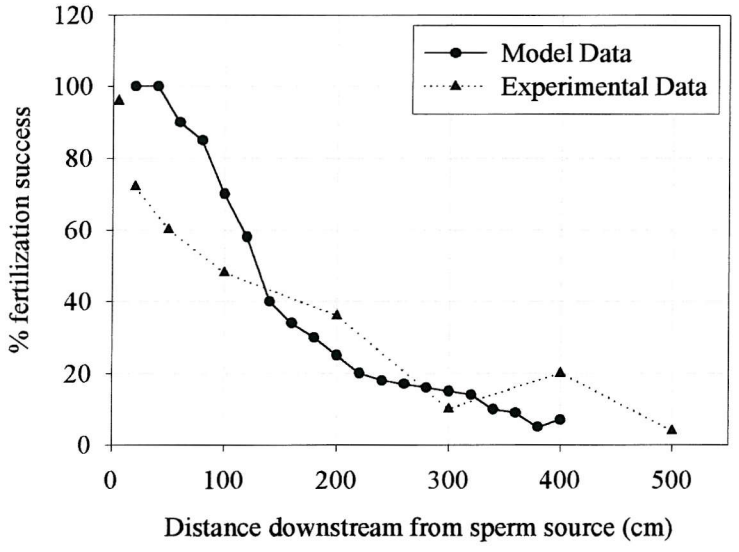


Figure 4.11: Field vs. model data for fertilization success of P. miliaris *eggs* in-situ

The estimated fertilization success was then plotted vs. downstream distance and compared with experimental data. A Mann-Whitney Rank Sum test was performed on the two data sets and no significant difference was found between the two treatments (P=0.666) suggesting a good fit of the model to the field situation.

In the field data, a clear pattern is seen in replicates 1, 2 and 4. Fertilization success corresponded well with an increase in current speed. At lower current speeds, in areas where mainstream velocities fell below 0.13m s^{-1}, urchin gametes may form

large clumps or strings resulting from inter-gamete stickiness and/or viscosity of their surrounding material (Thomas, 1994a). In such circumstances, sea urchin gametes are found to be increasingly negatively buoyant and have lower dispersion rates than freely diffusing fluorescein dye that is regularly used in determining dispersion rates for use in the models. Therefore, the use of this model to determine fertilization success in more slack water conditions is questionable. Furthermore, during conditions of increased turbulence, fertilization rates of eggs released by females may be substantially decreased at only a few centimetres from the sperm source. An increase in population density may be less effective at combating low fertilization success in highly turbulent environments (Denny, 1988; Denny and Shibata, 1989).

In replicate 1, a rather higher concentration of sperm was used in the experiment. Fertilization success close to the sperm source was rather lower than for the other three replicates. This may have been a function of the higher sperm concentration causing polyspermy and therefore retarded development in a percentage of the eggs examined. Alternatively, or additionally, the current speed in replicate 1 was somewhat higher than in the other three replicates. In this case the sperm may have dispersed more rapidly, even within the first few centimetres, leading to reduced fertilization success.

It is curious that the field experiments with *P. miliaris* worked better than with *H. tuberculata*. Several reasons may account for this. First, the sperm-egg contact time might have been important. The main reason for the low fertilization success for *H. tuberculata* was that the eggs and sperm in this species need to be in contact with one another for a few minutes. The experimental set up did not allow the sperm and eggs to drift simultaneously and may have resulted in close to zero fertilization. However, laboratory-derived results for sperm-egg contact time for *P. miliaris* also showed the need for gametes to be in contact for a few minutes. At lower sperm concentrations, similar to those encountered in nature, sperm-egg contact times of only a few seconds to minutes may be necessary. This was not so for *H. tuberculata* (see section *2.2.2.5.2*).

Levitan and Young (1995) attempted to infer the reproductive success of a species over a larger scale from small-scale model predictions. They used the Denny model to simulate large-scale spawning events in a natural population of the sea biscuit *Clypeaster rosaceus*. Their results suggested that both population size and density are important to fertilization over a very large range (2 to >250,000 individuals). They also reported that in small populations, population density in terms of clumping was a very

important factor. However in large populations, the density factor was negligible. They suggested that this might explain the lack of aggregation of some species during spawning.

4.6 General Discussion

The work presented in this chapter highlights the importance of factors affecting pre-larval stage processes in *P. miliaris,* processes that have not been considered in any detail until now. In light of the recent commercial interest in this species, such information could aid in the development of fisheries and aquaculture projects such as those proposed by Cook (1999), more specifically polyculturing techniques. Although *P. miliaris* harvesting has, to date, only been conducted on small scale, as the market demand increases, this fishery is bound to increase its economic viability. Economic feasibility studies have already shown that there are considerable financial returns to be made and that there is room for expansion in the global market for sea urchin roe (Burke, 1998).

Knowledge of the optimum sperm density required for fertilization, gamete longevity and sperm-egg contact time effects on fertilization success all have an important place in the successful development of spat cultivation for stocking new fishing grounds or polyculture programmes. Another future possibility, with the increase in sea urchin roe, is that of managing wild stocks of *P. miliaris* and the establishment of harvest refugia. By using information from predictive models of fertilization probability (Vogel et al., 1982; Denny, 1988) along with the existing information on stocking densities with respect to growth and food availability, optimum stocking density estimates may be made. It has been shown that these animals can tolerate high stocking densities (Cook, 1999). If a marine reserve was established and high numbers of adult spawning stock introduced, the chances of sperm limitation during the fertilization process would be reduced. If animals were separated by less than 1m, then reasonable fertilization success would be guaranteed, given synchronous spawning in relatively slack waters. A marine reserve site must be chosen carefully in terms of hydrodynamics in that it should supply the surrounding areas with larvae.

Quinn et al. (1993) suggest that maintaining harvesting refuges may be a better alternative to overall size or weight limits for urchin fisheries. Source populations need to be chosen with a good knowledge of recruitment patterns in the chosen population in

terms of distribution, abundance and size structure of individuals and also local environmental conditions which could influence fertilization on a local scale or the larval transport on a larger scale which may contribute more offspring than others. Denny and Shibata (1989) pointed out that certain locations along exposed shorelines may be suitable for settlement, recruitment and growth but would not be conducive to fertilization. These populations, although appearing to thrive, may contribute rarely as a larval source.

CHAPTER FIVE – SUMMARY AND GENERAL DISCUSSION

5.1 Introduction

The complexity of the fertilization process in free-spawning marine invertebrates cannot be over-stressed. In order to investigate this critical life-history process a considerable number of variables must be taken into account. This thesis explores a subset of these factors and emphasises the importance of some of the ecological processes involved in fertilization success in five commercially exploited marine species. Fertilization success is a key component in the determination of propagule supply to and from a population. By studying this important aspect, a more thorough understanding of life-history processes can be attained. Ultimately this may have a direct impact on shellfisheries management programmes. It is hoped and expected that a re-evaluation of existing programmes may arise from such fertilization kinetics experiments.

Indeed, fertilization success is already being incorporated into some plans. A rather elaborate study is currently being undertaken in the Indian River Lagoon, Florida to test alternative strategies for population enhancement of hard clams, *Mercenaria* spp. (Arnold et al., 1999). The existing commercial fishery has been subject to unpredictable availability of clams. Tests to find the right combination of management tools in an attempt to stabilise the fishery are being implemented, including spawner transplants, seeding and larval injection. The monitoring of these three strategies is currently underway and results from this study will be very important in terms of fisheries management, not only for this region, but for extrapolation to other over exploited shellfish populations. Another important study has been conducted on the sea scallop, *Placopecten magellanicus* (Dumais et al., 1999). The effects of adjusting spawning density and distribution upon resultant fertilization success were modelled and were combined with laboratory studies of fertilization success and the Denny model.

At low population densities, the polychaete *Arenicola marina* has reduced fertilization success (Williams, 1999) increasing the likelihood of recruitment failure. Protected areas have already been established to prevent over exploitation of populations and prevent habitat damage. Immigration of adults to over-exploited populations of lugworms may aid their recovery (Olive, 1993).

5.2 Synopsis of Achievements

The experimental animals used in this thesis were all chosen for their actual or potential commercial value and the need for improvements in management strategies of natural fisheries and/or aquaculture. The results herewith contribute to this aim.

Haliotis tuberculata:

- A significant relationship was found between animal length and wet weight with an R^2 value of 0.9 (n=59).
- The time taken for *H. tuberculata* to commence spawning in the laboratory via osmotic and thermal shock and UV-light irradiated water ranged between 3h 5min and 17h 40min.
- Sperm-egg contact time was found to have an effect on fertilization success, with maximum fertilization success occurring after 30 minutes.
- Fertilization success was shown to be a function of sperm dilution in 10ml culture volume experiments with a steep incline in success seen between 10^3 and 10^4 sperm ml^{-1}, reaching an optimum level (~100%) within a range of 5 x 10^4 to 5 x 10^5 sperm ml^{-1}. Polyspermic fertilizations were apparent at sperm concentrations >10^6 sperm ml^{-1}.
- In culture volumes of 500ml, sperm concentration ranging from 10^3 sperm ml^{-1} to 10^6 sperm ml^{-1} had little effect on fertilization success.
- Sperm-egg ratio was thought to have little bearing on fertilization success.
- The longevity of the sperm of *H. tuberculata* showed a clear response to sperm dilution with dilute sperm ageing faster than more concentrated sperm. Both eggs and sperm were shown to age relatively quickly, with a decline in fertilization success observed over a period of 2 hours. Only the most concentrated sperm showed any signs of retaining the ability fertilize eggs at 2 hours.
- The optimum range of temperatures for fertilization of *H. tuberculata* eggs was found to lie between 13.5 and 21.5°C. Fertilization was non-existent at temperatures <8.5°C or >30.5°C.
- The development rate of *H. tuberculata* larvae was found to be governed by temperature. The optimum temperature for the least time taken for normal development to trochophore was between 18 and 20.5°C.

- The mean sperm release rate observed for *H. tuberculata* was 1.7×10^{7} sperm sec^{-1} (n=11). Mean egg release rate was 188 eggs sec^{-1} (n=9).
- Implementation of the Vogel model (1982) gave best fit values for the two rate constants of $\beta=1.6 \times 10^{-6}$ and $\beta_0=5.5 \times 10^{-4}$. The ratio of $\beta/\beta_0=3.0 \times 10^{-3}$. This finding suggests that only around 0.3% of the egg surface is fertilizable or that only about 0.3% of the spermatozoa have the ability to fertilize the eggs.
- Predictions were made using the Denny (1988) model concerning fertilization success in the field under particular conditions of current flow, number of spawning males and distance downstream from sperm source. Field fertilization is highly dependent upon each of these variables.
- Animal density in Rocquaine Bay, Guernsey was assessed by diver and found to be in the order of 1 to 1.5 animals per m^{-2}.
- Fertilization success was <6% in field trials even at distances of 5cm from sperm source. This may be a reflection of the need for substantial sperm-egg contact time.

Crassostrea gigas:

- Fertilization success in the region of 100% was attained with sperm concentrations ranging from 10^5 to 10^6 sperm ml^{-1} in culture volumes of 10ml. A severe decline in fertilization success occurred as sperm concentration fell from 10^5 to 5×10^3 sperm ml^{-1}. Evidence of polyspermy was negligible at the sperm concentrations tested.

Tapes decussatus:

- Sperm concentration ranging from 5×10^3 to 5×10^6 sperm ml^{-1} resulted in fertilization success in excess of 80%. A reduction in success was observed with sperm concentrations outside this range, with evidence of polyspermy at the higher concentrations.
- Fertilization success remained relatively constant when temperature was varied from 11.5 to 31°C but fell to zero at temperatures of 9°C and below.

- Larvae of *T. decussatus* reached the most advanced stages of development within 4 hours at temperatures of >23.5°C. A slight increase in abnormalities occurred at 31°C.
- Mean sperm release rate was 9.8 x 10^5 sperm sec^{-1} (n=8). Mean egg release rate was 325 eggs sec^{-1} (n=5).
- The Denny (1988) model predicts sperm limitation in populations of *T. decussatus* with nearest neighbour distances in excess of 1m is far more likely than in populations of *Haliotis tuberculata* or *Psammechinus miliaris.*

Patella vulgata:

- Fertilization success is affected by sperm concentration. The highest levels of success are evident when sperm concentration is around 10^7 sperm ml^{-1}, with gradual declines extending either side of this optimum.
- Pairing behaviour was noted during the spawning season with male-female pairs significantly more prolific than single sex pairs.

Psammechinus miliaris:

- A significant relationship was found between test diameter and wet weight with an R^2 value of 0.84 (n=20).
- Gametes of *P. miliaris* required 10 minutes contact time in order to achieve fertilization success in excess of 90%. After 5 seconds contact only ~20% fertilization success was achieved.
- Optimum fertilization success (>80%) was achieved with sperm concentrations of 5 x 10^4 to 1 x 10^6 sperm ml^{-1} in 10ml culture volumes.
- Significant effects of gamete age were seen upon fertilization success. Sperm age was also a function of sperm concentration. With the most concentrated sperm of ~6 x 10^6 sperm ml^{-1}, fertilization success remained >90% up to 16 hours, after which a steep decline was seen until zero fertilization occurred at 22 hours. Conversely, fertilization success fell to zero after only 7 hours at sperm concentrations of 7.7 x 10^4 sperm ml^{-1}. Eggs of *P. miliaris* were also seen to age within a similar time scale with fertilization success reaching zero between 20-22 hours.

- Mean sperm release rate was 5.55 x 10^6 sperm sec^{-1} (n=9). Mean egg release rate was 101.9 eggs sec^{-1}.
- Implementation of the Vogel model (1982) gave best fit values for the two rate constants as β=3.7 x 10^{-4} and β_0=6.5 x 10^{-3}. The ratio of β/β_0=5.7 x 10^{-2}. This finding suggests that ~5.7% of the egg surface is fertilizable or that ~5.7% of the spermatozoa have the ability to fertilize the eggs.
- The Denny (1988) model showed that *P. miliaris* is generally more efficient than *H. tuberculata* at achieving high levels of fertilization success, despite the lower amount of sperm released.
- *In-situ* fertilization success experiments showed that in current speeds of 2.33 cm sec^{-1} and above, fertilization success is reduced to <20% at 3 meters downstream from the sperm source. The fastest current speed recorded during these experiments was 3.9 cm sec^{-1} and at these speeds fertilization success was reduced to <60% just 5cm from the sperm source.
- A comparison of predicted and empirical data suggests that the Denny (1988) model predictions are robust.

5.3 Limitations

For laboratory studies of fertilization success, the first objective is to obtain ripe gametes from the experimental animals. Echinoids feature predominantly in studies of fertilization success because of the ease of artificial spawning of these animals that has led to their wide use. Conversely, molluscs, are not so easily manipulated in terms of gamete extraction. One of the most difficult aspects of experimentation during the studies involving *H. tuberculata* and *T. decussatus* was acquiring male and female gametes simultaneously, in order that gamete age would not confound results.

As gametes deteriorated with age all experimental manipulations were conducted speedily. For this reason, sperm concentrations were determined after the sperm had been used in experiments. With experience, however, sperm counting was determined quickly prior to experimentation in some cases, using a via haemocytometer. This proved far better in terms of more precise replication.

A significant amount of time and effort was spent trying to induce spawning in the limpet, *Patella vulgata*. Finally, these attempts were abandoned and animals were strip-spawned. Many extremely ripe animals were dissected but very few viable eggs

resulted. In some cases sperm motility also appeared rather sluggish. It is thought that fertilization experiments would be far more reliable with naturally extruded gametes.

As with many biological processes, a large degree of variation exists between the physiology and behaviour of individuals. Even at the cellular level, diversity in gamete quality undoubtedly has an affect on fertilization success. Fertilization success varies considerably between individuals of the same population as shown by experimental results.

Field experiments of fertilization success posed a further set of problems. Again, timing was imperative. Freshly spawned gametes needed to be prepared for experimentation and transported from the laboratory to the shore as quickly as possible with minimal disturbance. Meanwhile, current flow and direction needed to be determined immediately prior to setting up the experiment. This required two scientists for the current determination and one or two for gamete preparation. Following the experiment, the fertilized eggs needed to be very carefully transported back to the laboratory for subsequent counting. In the case of *P. miliaris* this consisted of a 45 minute car journey to reach the Plymouth Marine Laboratory. Transportation of oyster, clam and scallop embryos from N.Wales to Southampton, a 5 hour journey, resulted in embryo lysis in all cases. However, controls showed near to 100% fertilization success in *P. miliaris* so it was assumed that no negative effects on fertilization were incorporated by this shorter journey.

In order to assess and compare problems associated with holding eggs in Nitex containers and collection in plankton nets, Levitan (1991) conducted experiments using both methods for an estimation of fertilization success of the sea urchin *Diadema antillarum*. As Levitan explains, there are recognised artefacts associated with both methods. The mesh of Nitex bags tends to impede flow through the containers and holding eggs stationary increases the amount of water and sperm sampled by the eggs. With the plankton net method, the injection of gametes via syringes may lead to increased estimates of fertilization success as a result of the high extrusion rates of sperm (Denny and Shibata, 1989). Also, the eggs are released and captured in the centre of the passing sperm plume. In an attempt to overcome such sampling problems, Mundy et al. (1994) developed a diver-operated plankton pump able to filter eggs from the water column. If used with care, this device is able to provide a relatively artefact-free estimate of fertilization. Styan (1997) developed an inexpensive and portable sampler for *in-situ* egg collection but did not determine experimental error. He

suggested that it would be low because previous experiments showed that most sperm-egg collisions occurred within the first few seconds of exposure, suggesting the bias would be slight. This piece of kit would have been very useful in the *H. tuberculata* field experiments.

Genetic markers have been used in some instances (Grosberg, 1991) to look at sperm mediated gene flow. This methodology avoids artefacts introduced by the above sperm-tracking methods. Yund and McCartney (1994) and Atkinson and Yund (1996) also used genetic markers in testing the hypothesis that male reproductive success is reduced in the presence of other males in a colonial ascidian and a bryozoan. They determined fertilization success and the proportion of offspring sired by each male.

The field experiments conducted in this thesis give rough estimates of field fertilization success. It is necessary in such work to make many assumptions. For example, this method assumes spawning synchrony between 1 male and 1 female. It is clear from conducting laboratory-based experiments that not all eggs and sperm are extruded in one spawning event by an individual. Experiments are based upon a spawning event, not the total reproductive potential of each animal. McShane and Naylor (1995) noted inter-annual variation in the magnitude and timing of spawning of *Haliotis iris*, indicating that the number of gametes available for fertilization could vary as the proportion of eggs released in spawning varies.

There will always be inevitable artefacts introduced into any field or laboratory study on the fertilization success of free-spawning organisms and in the construction of models based on field observations. By combining methodologies, increased predictive accuracy may be achieved.

5.4 Future Work

As with the vast majority of research programmes, many of the results gained through experimentation serve to raise further pertinent questions. This thesis is no exception. The first few months of Ph.D. study were spent conducting fertilization experiments on the scallop *Pecten maximus*. This was the preferred experimental animal owing to it's high commercial value being the largest British bivalve fishery with 17,417 tonnes being harvested in the UK in 1991 (FAO statistics). It is also possible to condition this species to spawn throughout the year, which would have enabled an extensive suite of experiments to be conducted. Unfortunately, working with the gametes of *P. maximus* proved very difficult as they are highly sensitive to

temperature and handling in general, as previous workers have noted (Manahan, pers. comm.). This fact made assessment of fertilization success rather problematic and hence experiments with *P. maximus* were abandoned. Now that techniques have been thoroughly practised with other species, fertilization kinetics of *P. maximus* should be attempted as this work would be of great value to fisheries management.

Many other factors that were not tested in this study may have an important effect upon fertilization success in natural populations of shellfish. For example, consideration of the effects of pollutants, increased turbidity of organic and inorganic particles in terms of sperm-sediment interactions or sediment induced egg lacerations, variations in salinity and the effect of turbulence-induced bubbles and their potential interference with gametes could all have some influence of unknown magnitude. Differing levels of turbulence upon fertilization success could be assessed by use of a flume tank. *Haliotis tuberculata* tends to mass-spawn in storm conditions (Tostevin, pers. comm.) and it would be interesting to see if increased levels of turbulence may somehow act to enhance fertilization success. Pennington (1985) found that when sea urchins spawned in still water, little fertilization occurred. Fertilization success increased to a point in faster moving currents but then decreased above a certain threshold. Another important consideration in terms of management strategies is the depth at which spawning stock may be planted. In their recent paper, Babcock et al. (2000) have investigated depth effects on fertilization success in the sea star *Coscinasterias muricata*. They found that although there was higher fertilization success in shallow water (<1m) than deep water (>5m), this effect was only seen when eggs were released directly downstream from a sperm source and was not seen in the lateral diffusion of sperm.

Nearest neighbour distances and abundance (to address density and population size) of both spawning and non-spawning animals could be estimated by use of time-lapse video cameras. It would be useful to duplicate the density and population measurements out of the spawning season in order to assess potential aggregation for spawning. It would also be useful to determine whether the gamete release rate and amount vary on a temporal scale throughout the spawning weeks and to assess the degree of spawning synchrony in the population.

Another important aspect of fertilization would be to investigate the percentage of egg surface area which may be fertilized as this has implications for fertilization success. Assessment of sperm receptor distribution could be made independent of the

Vogel Model. The distribution of receptors on the egg surface could be identified with a fluorescent antibody or synthesised sperm bindin-type protein (Yund, pers. comm.). The results could be compared to those predicted by the Vogel et al. (1982) model used in this thesis for *H. tuberculata* and *P. miliaris*.

In addition to determination of receptor sites, the proportion of live and dead sperm in a sample could be determined by use of a sperm viability kit. This kit uses fluorescent stains that are excited in the visible wavelength range. Sperm viability is finally assessed either by flow cytometry or fluorescence microscopy. It would also be useful to determine any anti-polyspermy mechanisms and gametic chemotaxis in these species.

This list of possible future work is not exhaustive but gives just a few ideas for potential future research concerning the nature of fertilization success in free-spawning marine invertebrates.

5.5 Final Comments

The idea that marine resources are inexhaustible is slowly being discarded and hence marine fishery management has become increasingly important. Some fisheries have been exploited to their limits and are now in need of drastic management action. As is often the case, it is not just the fished species itself that is affected by over-exploitation, but also those organisms that are associated with it. For example, devastation of a kelp community would result in a loss of habitat for several other commercially important community members such as sea urchins and lobsters. The ramifications in such a scenario are therefore compounded.

Economics are the principal factor to be considered when assessing the potential impact of implementing specific management strategies. The livelihoods of local communities or even entire nations (e.g. Iceland) that are dominated by fishing activities are of fundamental importance. It is not just fish catch *per se* that generates significant income. Jobs in ship building and vessel maintenance, in fish processing and even the electronics industry in terms of navigation equipment and sonar and radar all play their part. Hence, substantial resistance to restrictions imposed upon fisheries often needs to be addressed.

This highlights the need for the best possible management strategies to ensure sustainable and profitable fisheries. In order to achieve this, all fundamental aspects of the exploited animal's biology need to be addressed and incorporated into plans.

Regulations are the most common protection method for fisheries. However, harvest refugia have been established in some areas to serve as sanctuaries for commercially important species. Studies have shown increased diversity and average size among populations in refugia and significant replenishment of natural stocks in both the protected habitat and adjacent fisheries (Dugan and Davis, 1993). With an increase in the average size, fecundity will likely increase leading to enhanced recruitment. An optimum stocking density based upon knowledge of fertilization kinetics of a species should be implemented to augment this increased fecundity to achieve the highest possible recruitment to the population. Fishery managers need the best information possible, from both the fishermen themselves and from scientists, using both experimental work and models, to manage properly and forecast future resources. Effective management can allow the fisheries to continue to be profitable and sustainable and also environmentally friendly.

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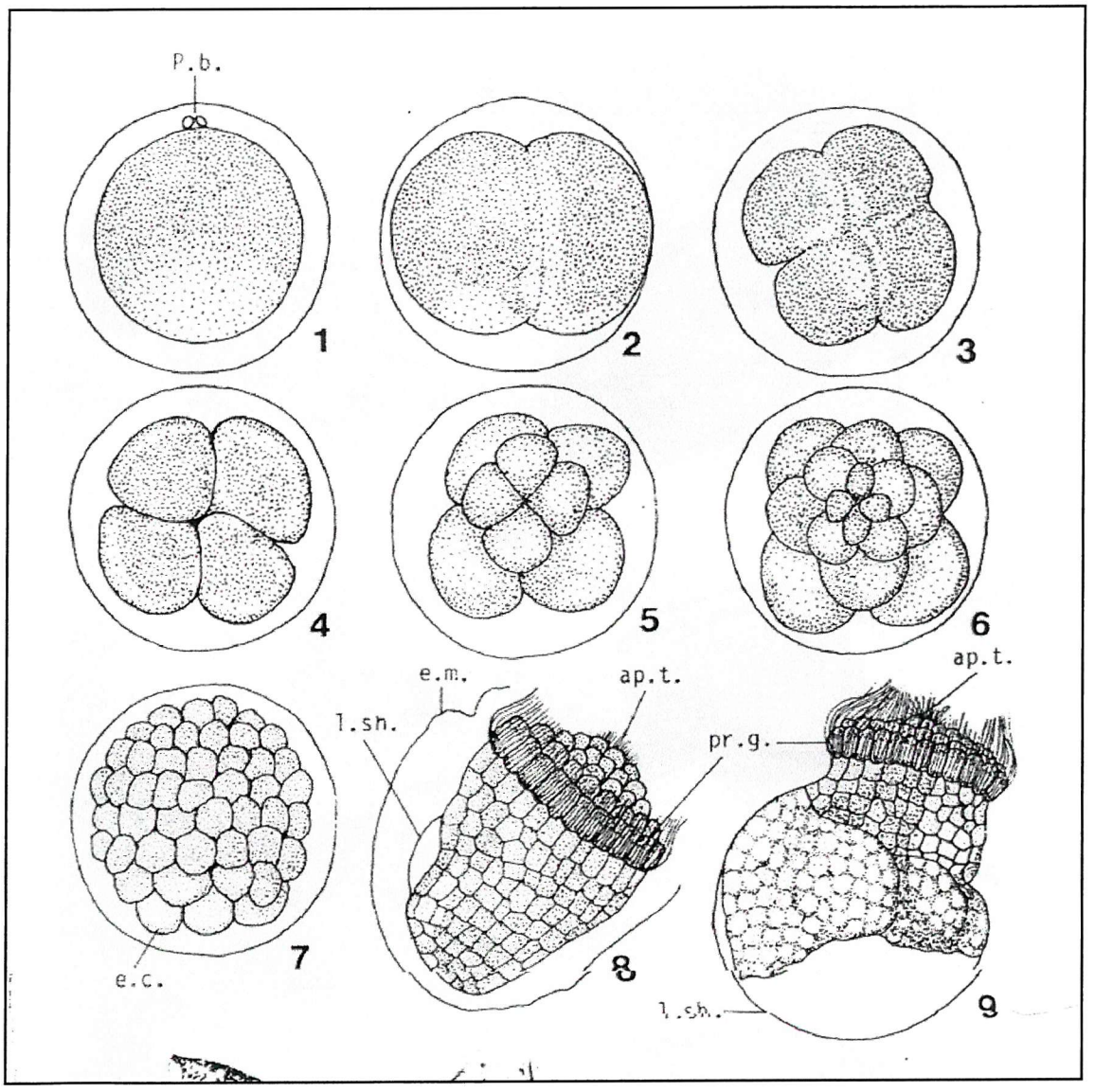
Appendix A

Development stages of embryos of H. tuberculata.
(After Koike, 1978)



1: Fertilized egg with polar bodies. 0.21 mm in diameter including egg membrane
2: Egg in 2 cell stage
3. Egg at the beginning of second cleavage
4. Egg in 4 cell stage
5. Egg in 8 cell stage (animal pole view)
6. Egg in 16 cell stage (animal pole view)
7. Embryo in morula stage
8. Embryo in trochophore stage
9. Larvae in early veliger stage

Abbreviations: ap.t. apical tuft; e.c. endodermal cell; e.m. egg membrane; l.sh. larval shell; pr.g. prototrochal girdle.

Appendix B

Shell Lenghts and Weights of Experimental Animals (*Haliotis tuberculata*)

Animal Reference	*Experiment type*	*Sex*	*Shell length (mm)*	*Wet weight (g)*
1	Temperature	M	111.30	205.26
2	Temperature	M	87.60	125.56
3	Temperature	F	81.30	68.89
4	Temperature	F	93.4	137.03
5	Temperature	M	94.7	150.56
6	larval development	F	93.4	137.03
7	larval development	M	94.7	150.56
8	contact time	M	105.8	209.37
9	contact time	F	97.4	129.96
10	ratio	M	84.2	83.4
11	ratio	F	102.7	143.10
12	ratio	M	74.3	64.12
13	ratio	F	75.5	71.24
14	sperm dilution	M	70.7	58.7
15	sperm dilution	F	66.9	35.76
16	sperm dilution	M	75.2	66.72
17	sperm dilution	F	63.7	36.29
18	sperm dilution	M	75.8	64.4
19	sperm dilution	F	75	60.5
20	sperm dilution	M	74.3	60.9
21	sperm dilution	F	70	48.52
22	gamete age	M	69.4	47.2
23	gamete age	F	67.7	43.7
24	gamete age	M	73.4	55.2
25	gamete age	F	76.3	49.87
26	gamete age	M	68.3	50.8
27	gamete age	F	73.5	56.0
28	contact time	M	69.3	51.9
29	contact time	F	71.4	47.1
30	ratio	M	74.8	64.5
31	ratio	F	73	47.3
32	contact time	M	74	54.3
33	contact time	F	75.5	60.3
34	gamete age	M	72	58.5
35	gamete age	F	77.7	65
36	gamete age	M	76	74
37	gamete age	F	71.6	52.1
38	culture volume	M	71.6	56.8
39	culture volume	F	75	55
40	culture volume	M	75.3	61
41	culture volume	F	70.4	54
42	culture volume	M	71.5	52.5
43	culture volume	F	73.6	55
44	gamete release	M	69.2	49.2
45	gamete release	M	69.6	52.5
46	gamete release	M	72.4	50
47	gamete release	M	71.5	52.5
48	gamete release	M	73.4	45
49	gamete release	M	75.1	55
50	gamete release	M	80.4	60
51	gamete release	M	74	45
52	gamete release	M	75	52.5
53	gamete release	F	76.9	60
54	gamete release	F	80.7	55
55	gamete release	F	76.3	52.5
56	gamete release	F	74.7	50
57	gamete release	F	71.1	41
58	gamete release	F	78.2	52.5
59	gamete release	F	79	50
60	ratio	F	81.5	not weighed
61	ratio	F	82.4	not weighed
62	ratio	M	83.0	not weighed
63	ratio	M	106	not weighed
64	sperm dilution 500ml	F	97.6	not weighed
65	sperm dilution 500ml	F	77.1	not weighed
66	sperm dilution 500ml	F	98.7	not weighed
67	sperm dilution 500ml	M	95.9	not weighed
68	sperm dilution 500ml	M	91.2	not weighed
69	sperm dilution 500ml	M	103	not weighed

Appendix C

Map of Rocquaine Bay, Guernsey, Channel Islands showing sample site positions.