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Aspects of Fertilization Ecology in
*Evechinus chloroticus*
and
*Coscinasterias muricata*

Elke Sabine Franke

Photo (by Elke S. Franke) shows fertilized 1-cell stage egg of *E. chloroticus*
Egg diameter approx. 80 micrometers
Abstract

Broadcast spawning marine invertebrates have been widely used as model organisms to study processes of evolution. One of these is the study of various life history stages associated with reproduction. Fertilization ecology in broadcast spawning marine organisms, i.e. the process by which sperm and egg fusion occurs once released into the ocean, has been the subject of intensive study for roughly the last 20 years, and represents thus a recent field in ecological sciences. This growth in interest was sparked by studies that showed spawning events may take place predominantly under sperm limiting conditions. More recent findings however, suggest that the occurrence of sperm competition, and the risk of polyspermy (multiple sperm entry in to the egg, which is lethal in echinoderms) can also occur during reproductive events in marine invertebrates. This has also been predicted on theoretical grounds. Even though polyspermy has been observed previously, particularly during aquaculture studies, evidence to assess the occurrence of polyspermy in situ, and the conditions under which it occurs is lacking. Simulated field studies in Evechinus chloroticus as well as laboratory studies in E. chloroticus and Coscinasterias muricata found high levels of polyspermy, even under the sperm limiting conditions that are naturally found in the field in other marine broadcast spawners. Furthermore, laboratory results in both Evechinus and Coscinasterias showed that polyspermy is most likely to increase when sperm concentrations are increased. In addition, even though increasing sperm concentrations increases monospermy to a certain extent, an increase in gamete contact times generates similar levels of monospermy. Results from these studies confirmed the mathematical model predicting some of these events.
The timing of gamete release to achieve maximum fertilization success (monospermy) in a broadcast spawner is thought to be under strong selective pressures, because reproductive success is directly related to fitness. The spawning patterns in the sea urchin *E. chloroticus* observed in this study demonstrated that *E. chloroticus* spawns under varying environmental and ecological conditions. Interestingly, it appeared that highly synchronous and widespread spawning was found to occur when highly turbulent conditions existed. This may reduce the potential for polyspermy that may exist during mass spawnings under shallow and calm conditions.

The demonstration of polyspermy and the confirmation of the mathematical model, suggest that polyspermy is a common and frequent occurrence during broadcast spawning events. Thus polyspermy represents an evolutionary force that may shape the evolution of reproductive phenomena at levels ranging from the gamete to the population.
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CHAPTER 1

General Introduction
FERTILIZATION ECOLOGY

Its Importance to Evolutionary and Ecological Studies

The processes that have given rise to the biodiversity we see today have been the subject of scientific scrutiny and speculation for a very long time (e.g. Lamarck 1809, Hutchinson 1959, Darwin 1979, Wilson 1988, Dawkins 1989, Gould 1989). Sexual reproduction and reproductive processes such as gametogenesis, are thought to be a key component to evolutionary change, in part because these facilitate variation, which is fundamental for evolutionary processes (Mayr 2001). Furthermore, the study of sexual selection, i.e. the selection that drives processes and patterns associated with reproduction (but see Arnold 1994 for further definitions of sexual selection), is central to the study of evolutionary processes and patterns.

Life is thought to have evolved in the sea (e.g. Rhodes 1962) and the commonly believed ancestral mode of sexual reproduction is one where gametes are released into the external environment and fertilization takes place (broadcast spawning). This is also the most commonly found mode of sexual reproduction among marine invertebrates (Thorson 1950). Thus many studies investigating ways in which reproduction shapes evolution, and evolution changes reproductive traits have used broadcast spawners to elucidate these issues.

From this, a synthesis is beginning to emerge of the ways in which reproductive success and reproductive behaviour may interact with environmental and phylogenetic constraints to influence traits as diverse as egg size (e.g. Levitan 1993, Podolski and Strathmann
1996, Levitan 2000), developmental mode (Strathmann 1985), adult size (e.g. Menge 1975) and population density (Levitan 2002). The role of fertilization and reproductive success in influencing population dynamics on ecological time scales is also beginning to be better appreciated (Levitan 1991, Tyler and Young 1992, Quinn et al. 1993, Claerebout 1999, Meidel and Scheibling 2001).

Over 60 years ago, it was suggested that despite the hundreds of thousand to millions of eggs released by broadcast spawning marine invertebrates, variable or low recruitment levels observed may be due to rare fertilization events in the field (Mortensen 1938). Since that time however, research has tended to focus on the importance of larval life history stages rather than fertilization as a cause of variation in recruitment success. Studies elucidating the former include research on settlement cues (e.g. Maki et al. 1988, Satuito et al. 1997), metamorphosis (e.g. Pechenik 1986), predation (e.g. Cowden et al. 1983, Gaines and Roughgarden 1987, Young and Chia 1987, Ishii et al. 2001), starvation (e.g. Hoegh-Guldberg and Pearse 1995, Klinzing and Pechenik 2000) and oceanic transportation of larvae (e.g. Lee and Williams 1999, Zheng and Kruse 2000, Ishii et al. 2001, Warlen et al. 2002). Furthermore, the study of sources and sinks (e.g. Coffroth and Mulawka 1995, Mladenov et al. 1997, DiBacco and Levin 2000), as well as mathematical modelling of recruitment events (e.g. Alexander and Roughgarden 1996, Conolly and Roughgarden 1999) present important fields of research of larval ecology. As a result, the biological and physical factors influencing the ecology of larvae have often been viewed as the major contributors to low recruitment (e.g. Thorson 1950, Underwood and Fairweather 1989, Rumrill 1990, Leviton 1995, Arnold et al. 1998). However, within the
last two decades, the earlier postulation of Mortenson (1938) has received increased attention, and studies have started to focus on fertilization and reproductive success in explaining variable and low recruitment.

Fertilization, i.e. the fusion of one egg with one sperm, which represents the beginning of an organism’s life, has been studied for a long time and expression of interest dates as far back as Aristotle (reviewed in Birkhead and Møller 1998 and Gilbert 2000). Experiments on fertilization in echinoderms were conducted as early as the last quarter of the 19th century, and Oskar Hertwig and Herman Fol are recognized to be the founders of the study of sea urchin fertilization and development (reviewed by Tyler and Tyler 1966, Ernst 1997 and Gilbert 2000). Following these studies were many that investigated the morphology of gametes and their interaction in the laboratory (e.g. Lillie 1914, 1915, Hultin and Hagström 1956, Tyler and Tyler 1966, Presley and Barker 1970). Much initial work on fertilization was conducted in vitro only for possibly at least two reasons. First, SCUBA diving was not available until the early 1940’s and second, interest in fertilization in vitro was more predominant amongst researchers studying this phenomenon. Since then, increased interest in fertilization and its ecology has become important in at least two situations. Firstly, when decreases in abundances below sustainable population densities of commercially and ecologically important species lead to failure of successful reproduction (Allee effect) (e.g. Quinn et al. (1993), Babcock and Keesing (1999), Levitan and McGovern, in press). Secondly, when animals need to be artificially cultured in tanks (e.g. Bardach et al. 1972, Clotteau and Dubé 1993, Desrosiers et al. 1996, Gribben in prep., Williams in prep.).
Since Pennington’s (1985) important field demonstration of the exponential decline of fertilization with increasing distance from the sperm source, increased efforts have been made to assess the influence and relative importance of abiotic and biotic factors affecting fertilization in situ (e.g. Levitan 1991, Levitan and Petersen 1995, Brawley 1992, Serrão et al. 1996, Levitan 2002). A large amount of factors including gamete, individual, population and even environmental aspects have been shown to have profound influences on fertilization success (summarized in Levitan 1995). Some of the factors positively correlated with fertilization success include increase in body size (e.g. Levitan 1991, Babcock et al. 1994) and population density (e.g. Levitan 1991, Levitan et al. 1992, Levitan and Young 1995, Coma and Lasker 1997a), as well as a decrease in water depth (Levitan 1998) and water motion (e.g. Serrão et al. 1996, Brawley et al. 1999). Early studies and mathematical modelling of fertilization suggested that fertilization success was variable and usually low and that sperm limitation prevailed during spawning events (e.g. Denny and Shibata 1989, Levitan 1991, Babcock et al. 1992, Lasker et al. 1996, Mead 1996). As a result many reproductive phenomena, such as egg size (Levitan 1993, Levitan 1996a), sperm chemotaxis (e.g. Levitan 1995), anisogamy and gender (Levitan 1996b) are thought to have evolved in response to lack of sperm during spawning events (Levitan and Petersen 1995). However, increased available data to date on natural fertilization levels suggest that both low and high fertilization success are likely to be common in nature (e.g. Babcock et al. 1992, Brawley 1992, Sewell and Levitan 1992, Coma and Lasker 1997b, Grant et al. 1998). Particularly, high levels of fertilization are thought to occur when organisms spawn synchronously.
(Pearse et al. 1988, Smith 1989, Coma and Lasker 1997a, Sewell and Levitan 1992, Hay 1997), because this would cause sperm concentrations to be high enough for sperm competition (Yund and McCartney 1994, Yund 2000) and polyspermy (Brawley 1992) to be present during these events.

POLYSPERMY

Its possible Occurrence under Conditions of Sperm Competition and its Role in Sexual Conflict and Speciation

Sperm competition, recently redefined by Parker (1998) as “the competition between the sperm from two or more males for the fertilization of a given set of ova”, has been demonstrated in many taxa and plays a central component of sexual selection (reviewed in Birkhead and Møller 1998). In addition, Parker (1998) includes the possibility for sperm from the same male to compete for fertilization, but the evolutionary importance of this competition would depend on the influence of the sperm’s rather than the adult male’s genotype in mediating this competition. Traditionally, external features such as increased ejaculate/test size, nuptial gifts from the male to the female and elaborate characters, such as large horns, or colourful plumage are often cited as evidence for sexual selection and in turn sperm competition (e.g. Møller 1998). For marine invertebrates that release gametes into the watercolumn, the occurrence of polyspermy, which is the multiple entry of sperm into the egg may be a consequence of the competition or of the high sperm density which gives rise to competition (e.g. Rice 1998, Frank 2000, Gavrilets 2000). When multiple entry of sperm into the egg occurs the resulting embryo usually dies (but see exceptions below) and thus, loss in fitness of the
adult is experienced. This has been postulated as a driving force for eggs to delay sperm entry (Palumbi 1998). In addition, theories of sexual conflict suggest that it is in the interest of eggs to delay sperm entry, because it may allow the egg to choose a suitable sperm (Palumbi 1998) or reduce parasite entry into the egg during fertilization (Rice 1998). Fertilization processes viewed from the perspective of the sperm propose that sperm are selected for increased swimming speed and size (e.g. Snook and Karr 1998), particularly under sperm competitive conditions (e.g. Ball and Parker 1996); thus sperm are selected for increased rate of sperm entry into the egg. This is also relevant when sperm are limiting (Palumbi 1998).

The observed rapid evolution of gamete recognition proteins (bindin in urchins and lysine in abalone, e.g. Vacquier and Lee 1993, Metz and Palumbi 1996, Vacquier et al. 1997, Swanson and Vacquier 1998, Palumbi 1999) has been linked to this conflict of gametes. The resulting evolutionary results (e.g. high polymorphism in gamete recognition proteins) in these systems has been linked to the occurrence of polyspermy which is thought to lock the evolution of recognition proteins into co-evolutionary arms races, similar to those observed in parasite-host systems (Rice 1998). Consequently, polyspermy has been implicated (Parker and Partridge 1998, Rice 1998) and mathematically modeled (Gavrilets 2000, Frank 2000) to facilitate speciation, because high levels of variation may facilitate rapid evolution of mating guilds within a population, which would subsequently become reproductively isolated (Wu 1985, Palumbi 1998).
Polyspermy Blocks

Polyspermy is lethal in most instances because the chromosome pairs fail to align themselves on the central plane of eggs during mitotic division (Jaffe and Gould 1985, Gilbert 2000). Mechanisms employed by eggs to prevent polyspermy (polyspermy blocks) have been observed in many taxa and are thought to have evolved and be maintained in response to the frequent occurrence of polyspermy (Brawley 1992). Polyspermy blocks have been well studied in organisms such as humans (e.g. Wolf et al. 1997), pigs (e.g. Xia et al. 2001), mice (e.g. Wassarman 1990) amphibians (e.g. Iwao 2000), birds (e.g. Stepinska and Olszanska 2000, 2001) fish (e.g. Ginzburg 1968), ascidians (e.g. Lambert et al. 1997), various marine invertebrates (e.g. Jaffe and Gould, 1985, Stephano and Gould 1988, Misamore et al. 1996) and marine algae (Brawley 1991). The mechanisms involved vary among these taxa, but all appear to involve two or sometimes more processes. The first is usually a change in egg membrane potential from negative to positive charge, which occurs upon entry of the first sperm and temporarily halts secondary sperm entry (Jaffe and Gould 1985, Wassarman 1990, Brawley 1991, Kobayashi and Yamamoto 1994, Lambert et al. 1997, Togo et al. 1995). Secondly, cortical granule exocytosis and the release of ovoperoxidases into the previtalline space of the egg take place upon the entry of sperm. This release causes a formation of a protective layer around the egg (fertilization envelope), which prevents further sperm from entering (Ginzburg 1968, Jaffe and Gould 1985, Wassarman 1990, LaFleur et al. 1998, Xia et al. 2001).
Other mechanisms to avoid multiple entry of sperm into the egg include the presence of a micropyle, which is a narrow channel through the egg jelly coat allowing sperm to enter. Teleost fish (e.g. Amanze and Iyengar 1990) as well as some invertebrates and plants possess micropyles, but despite the restricted area of sperm entry, polyspermy is still possible under excess sperm conditions (Ginzburg 1968, Jaffe and Gould 1985).

Interestingly, in birds, some amphibians and reptiles, insects, possibly pigs and a few marine invertebrates, polyspermy is not necessarily lethal, but “physiological” (Ginzburg 1968, Jaffe and Gould 1985, Stepinska and Olszanska 2001, Xia et al. 2001, Luttikhuizen and Pijnacker 2002). This means that many sperm are allowed to enter the egg, but only one sperm fuses with the egg nucleus (Ginzburg 1968, Jaffe and Gould 1985, Iwao 2000). Cellular mechanisms exist within the egg to break down supernumerary sperm and involve complex enzymatic activity (e.g. Stepinska and Olszanska 2000, Xia et al. 2001).

This thesis is concerned for the most part with the occurrence of polyspermy in echinoderms and the polyspermy blocks of the Orders of the two study animals used in this thesis are described in more detail next.

**Polyspermy Blocks in Echinoids and Asteroids**

In echinoderms, polyspermy is lethal (e.g., Rothshield and Swann 1952, Ginzburg 1968, Jaffe and Gould 1985, Ernst 1997) and various blocks are thought to counteract multiple sperm entry. Firstly, the egg-jelly coat, which is present around the surface of asteroid and echinoid eggs, has been postulated to act as a physical barrier against multiple sperm entry (e.g. Rothshield and Swann 1952, Hagström 1956, Farley and Levitan 2001). In
addition, a two-step polyspermy block is also thought to exist. Firstly, a fast block, whereby the membrane potential of the egg changes from negative to positive upon contact of a sperm. This is thought to halt further sperm entry for up to 3 seconds (Jaffe and Gould 1985, Schuel 1984). Despite earlier debate on the nature and the presence of fast blocks in these groups (e.g. Byrd and Collins 1975), it is now established that this rapid block exists, although it may be overcome by excess amounts of sperm (Nuccitelli and Grey 1984, Jaffe and Gould 1985). The efficiency of this fast block may be dependent on the maturation stage of eggs (e.g. Fujimori and Setsuro 1979). The second step is associated with the previously mentioned release of ovoperoxidases from cortical granules, of which approximately 15–18 x 10³ are distributed beneath the egg plasma membrane in urchins and starfish (Kay and Shapiro 1985, Gilbert 2000). The cortical granules fuse upon sperm entry with the egg plasma membrane, and their contents are released into the previtelline space. The previtelline space lies between the egg plasma membrane and the vitelline layer. In the two Orders considered here, the contents of the cortical granules not only include ovoperoxidases, but also proteases, glycoproteins and mucopolysaccarides (Kay and Shapiro 1985, Gilbert 2000). Following the release of these compounds, a fertilization envelope starts to form at the point of sperm entry, which elevates around the egg in all directions (Kay and Shapiro 1985, see Schuel (1985) for schematic representation of this process). Complete elevation can take up to 2 minutes (Jaffe and Gould 1985, pers. obs.) and is thought to act as the second and major block to polyspermy. However, sperm can enter the egg until envelope elevation is complete (Schuel 1985).
Despite various mechanisms that are thought to have evolved to prevent the multiple entry of sperm, polyspermy has frequently been detected in laboratory studies of some species where the fertilization was investigated via staining of sperm within eggs (e.g. Rothschild and Swann 1952, Stephano and Gould 1988, Clotteau and Dubé 1993, Desroisier et al. 1996). Polyspermy has also often been inferred from developmental arrest and uneven cell division of fertilized eggs (e.g. Levitan et al. 1991, Benzie and Dixon 1994, Styan and Butler 2000). This however is not necessarily always a reliable indicator of this phenomenon, due to factors that will be discussed later in this thesis.

Polyspermy in situ

Although extensive documentation exists on the occurrence of polyspermy under laboratory conditions, primarily from the aquaculture literature (e.g. Sprunge and Bayne 1984, Mill and McCormick 1991), little attention has been paid to the occurrence of polyspermy when eggs and sperm are spawned into the field during natural spawning events (Frank 2000) and no data today exist on polyspermy under natural conditions for broadcast spawning marine invertebrates. Some information is available from algae (e.g. Brawley 1992, Pearson and Brawley 1996, Serrão et al. 1999), however, in these organisms the occurrence of polyspermy has been linked, partly, to ionic differences in the surrounding waters compared to normal seawater, which affect the fast block to polyspermy (Brawley 1992, Serrão et al. 1999). The lack of information on polyspermy in situ for marine broadcast spawning invertebrates is to some extent due to the rarity of sightings of natural spawning events.
To measure natural levels polyspermy or sperm limitation in the field, it is essential to understand spawning patterns, because this may allow reliable collection of gametes during these events. Also, to assess the relative importance of polyspermy and sperm limitation, knowledge of environmental conditions and individual behaviours during spawning is fundamental. This is because, as discussed above, both environmental and behavioral factors are thought to increase fertilization success (e.g. Serrão et al. 1996, Olive 1992, but see Pearse 1990, Kiflafi et al. 1998).

**SPAWNING PATTERNS, CUES AND BEHAVIORS IN MARINE INVERTEBRATES**

Various spawning patterns can be observed among marine invertebrates. These include multi-specific spawning (mass spawning) or spawning of a single species (epidemic spawning) defined as such by Watson et al. (2000). Either one can be predictable (Babcock et al. 1986, Babcock et al. 1992) or unpredictable (e.g. Pennington 1985, McEuen 1988, Pearse et al. 1988, Babcock et al. 1992, Sewell and Levitan 1992, Watson et al. 2000). Animals either release eggs and sperm into the water (broadcast spawning) or release sperm only, while eggs are retained within the adult (free-spawning) (Levitan 1998). Synchronous spawning appears vital to ensure sufficient fertilization, however varying degrees of synchrony exist (e.g. Dix 1970a, Babcock et al. 1986, Pearse et al. 1988, Smith 1989, Lamare and Stewart 1998).

Individual behaviors observed during spawning include climbing (e.g. Stekoll and Shirley 1993), rearing (Babcock et al. 1992), arm waving (Fishelson 1968, McEuan 1988),
stacking (Picken and Allan 1983), pseudocopulation (Hamel and Mercier 1995, Young 1994, pers. obs.), arching (e.g. Babcock et al. 1986, Minchin 1987, pers. obs.) and aggregation (e.g. Smith 1989, Young et al. 1992, Hamel and Mercier 1995). Evidence from the majority of reports on spawning so far suggests that female gametes are often released after male gametes (e.g. Thorson 1950, Clifton 1997).

The spawning of non-conspecifics (e.g. Babcock et al. 1992) as well as conspecifics (e.g. Slattery and Bosch 1993) has been indicated as spawning inducers (e.g. Hamel and Mercier 1995). Other cues are thought to include phytoplankton blooms and their chemicals (e.g. Himmelmann 1975, Starr et al. 1992), moon phase (e.g. Korringa 1947, Pearse 1975), tidal cycles (Pearse 1975), rapid changes in temperature (e.g. Watson et al. 2000), storms (e.g. Keuskamp 1997) pressure changes (Watson et al. 2000) and spawning pheromones (Miller 1989). Cues that are thought to entrain the gametogenic cycle of the organism leading to spawning over the period of a whole year to include increasing or decreasing photoperiod and/or water temperature (e.g. Hardege et al. 1998, Watson et al. 1998, Lawrence 1996, Giese and Kanatani 1987). A good understanding of how cues immediately preceding spawning (phytoplankton blooms, moon phase, rapid temperature decrease/increase, storms, pressure changes) act as “zeitgebers”, i.e. “timers”, to induce gamete release is still lacking, however it has been postulated these may act in a cascading fashion or synergistically lead to spawning (e.g. Babcock et al. 1992, Wahle pers. com.).
Unfortunately, natural spawning of Echinoids and Asteroids is far from predictable, however gametes from these groups are easily obtained by induced spawning (Giese and Kanatani 1987). Members of the two Orders have therefore been widely used as model organisms for the study of many aspects in biology and fertilization in particular (e.g. Branham 1972, Ernst 1997). In this respect, the two animals introduced next represent perfect candidates to investigate various aspects of fertilization ecology.
EVECHINUS CHLOROTICUS
(Valenciennes 1864)

KINA

Photos by Chris Denny
EVECHINUS CHLOROTICUS (KINA)

*Evechinus chloroticus* (see previous page) is a member of the Class Echinoidea, Family Echinometridae, Order Camarodonta. *Evechinus chloroticus* is endemic to New Zealand, and occurs throughout its coastal waters within 0-12 m depths (Dix 1970b). It is also found around the Kermadecs, Snares and Chatham Islands (Dix 1970b), and generally occupies shallow areas of algal abundance, urchin barrens and kelp forests. Populations around New Zealand have been found to be genetically uniform, with the exception of one population in Fiordland, South Island (Perrin and Roy 2000). The distribution and abundance of *E. chloroticus* in various habitats and areas has been well documented (e.g. Dix 1970b, Kerrigan 1987, Choat and Shiel 1982, Shiel *et al.* 1995), and its influence and interaction within the subtidal community has been studied extensively (e.g. Ayling 1981, Andrew and Choat 1982, Choat and Andrew 1986, Andrew and MacDiarmid 1991). As a result of this work, *E. chloroticus* is recognized to be one of the dominant grazers, if not the most dominant grazer, of subtidal reef communities within New Zealand (Ayling 1981, Andrew and Choat 1982, Choat and Andrew 1986, Babcock *et al.* 1999). It feeds on algae and other encrusting organisms (Ayling 1978, Cole and Haggitt 2001) thereby creating urchin barrens (Ayling 1981, Babcock *et al.* 1999), similar to those reported in the Atlantic Ocean (e.g. Scheibling 1994, Scheibling *et al.* 1994). Furthermore, *E. chloroticus* is an important food source for predators such as Snapper (*Pagrus auratus*) and Lobster (*Jasus edwardsii*) (Babcock *et al.* 1999). *Evechinus chloroticus* has also been commercially harvested and has been considered as a candidate for aquaculture (Barker *et al.* 1998).
This species is dioecious. Males and females exhibit increased gonad size during the months of November until March with highest values being attained during December, January, and February (Dix 1970a, Walker 1982, McShane et al. 1996, Keuskamp 1997, Brewin et al. 2000). Eggs and sperm are released into the water column where external fertilization takes place; however, aggregation is not thought to be prerequisite for spawning (Dix 1969). Its planktotrophic larvae can take between 30 and 63 days to develop and metamorphose (Dix 1970a, Walker 1984).
COSCINASTERIAS MURICATA
(Verrill 1867)

THE ELEVEN-ARMED STARFISH

Photo by Dr. Russ Babcock
Male *C. muricata* spawning after injection with 1-Me-Ade
COSCINASTERIAS MURICATA (ELEVEN ARMED STARFISH)

*Coscinasterias muricata* (see picture of spawning male on previous page) is a member of the Order Forcipulatidae within the Class Asteroidea, of the Phylum Echinodermata. Within the genus *Coscinasterias*, three species are known, but *C. muricata* is the only one widely distributed within the Indo Pacific region (Crump and Barker 1985). It occurs in depths ranging from 0-140 m (Rowe and Gates 1995) and is found in the waters surrounding Chatham Island, Kermadec Islands, the South African coast (Crump and Barker 1985), from Norfolk Island to Wanganella Bank in the northeastern Tasman sea (Rowe 1989, Rowe and Gates 1995) the coasts of Australia (Rowe and Gates 1995) Taiwan (Chao and Chang 1989) and it is the most ubiquitous forcipulate starfish along the east and west coasts of New Zealand (Crump and Baker 1985).

Arm number in adult *C. muricata* can vary from 7 to 13 (Crump 1969), and adults can attain sizes of over 350 mm in diameter (Babcock *et al.* 2000). They can vary in color, with larger individuals appearing predominantly creamy yellow and smaller ones blue (Crump 1969). Like many asteroids, it has the ability to regenerate limbs, which are lost as a result of anti-predatory or escape responses. *Coscinasterias muricata* is purely carnivorous and preferably feeds on bivalves, but consumes gastropods and crabs when the former are not available (Crump 1969). Due to its voracious activity as a predator, it has been postulated to be a keystone species in some ecosystems (www.melbourneaquarium.com.au/education/ffelevenstar.htm).
Coscinasterias muricata reproduces sexually and asexually. Asexual reproduction involves fissiparity, a process by which the adult splits into two or more parts (Crump and Barker 1985). This species is dioecious and similar to E. chloroticus, in that sexual dimorphism is absent. Sexual reproduction involves the release of sperm and eggs into the water column and gametes can be obtained through hormonal treatment (1-Methyl-Adenine) of gonads from June until February (Barker 1977). Its planktotrophic larvae develops for approximately 27 days through the bipinnaria stage to settlement in the laboratory (Barker 1978). The late brachiolaria larval stage attaches to a range of substrata, prior to metamorphosis, but requires a surface film (Barker 1977).
THESIS OUTLINE

Various aspects of the fertilization ecology of *Evechinus chloroticus* and *Coscinasterias muricata* will be the focus of this thesis. The success of fertilization during fertilization events in the laboratory and in the field will be subject to investigation. In addition, because conditions of spawning events determine fertilization success, this thesis will also assess the occurrence of natural spawning events in the field. The following section outlines specifically, the aspects of fertilization ecology for *E. chloroticus* and *C. muricata* that will be addressed in each chapter.

- Chapter 2 will quantify the occurrence of polyspermy and sperm limitation during experimental spawnings of *Evechinus chloroticus* from rockpools along the shores of northeastern New Zealand.

- Chapter 3 will assess polyspermy and sperm limitation under controlled laboratory conditions in *E. chloroticus*.

- Chapter 4 will investigate the occurrence of *in situ* spawning events in *E. chloroticus* over the course of three consecutive spawning seasons and assess fertilization success during these events.

- Chapter 5 will further investigate the occurrence of polyspermy and sperm limitation under controlled laboratory conditions for *Coscinasterias muricata*. 
Data on fertilization and polyspermy within each of the chapters 2, 3, and 5 will be used to test the mathematical model proposed by Styan (1998). This model is an amended fertilization kinetics model originally developed by Vogel et al. (1982), but additionally incorporates polyspermy. The importance of the results established throughout this thesis, and the application of the mathematical model used, will be addressed in the general discussion.
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CHAPTER 2

This chapter was published in 2002 in The American Naturalist 160: 485-496 titled Sexual conflict and Polyspermy under Sperm-limited Conditions: In situ Evidence from Field simulations with the free-spawning marine echinoid Evechinus chloroticus

Picture shows test of *E. chloroticus*

Amended from http://www.nhm.ac.uk/paleontology/echinoids/GENERA/ECHINOID/EVECH4.HTM
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CHAPTER 3

This chapter is in preparation for submission to Marine Biology titled Fertilization Kinetics and the Importance of Polyspermy in Fertilization Ecology of the sea urchin *Evechinus chloroticus*

Picture shows test of *E. chloroticus*

Amended from http://www.nhm.ac.uk/palaeontology/echinoids/GENEROECHINOID/EVECH4.HTM
Abstract

Fertilization success in broadcast-spawning marine organisms is determined by numerous biological interactions of sperm and eggs as well as the physical environment. Recognizing the parameters controlling early life history events of marine invertebrates has wide-ranging implications for our understanding of evolutionary, ecological and economic importance of reproductive processes.

The potential occurrence of polyspermy (multiple entry of sperm into the egg which is lethal) has largely been ignored in marine invertebrates, although the phenomenon itself is well known in algae. Here we demonstrate the influence of sperm concentration, sperm-egg contact time and gamete age on total fertilization, polyspermy and ultimately the proportion of monospermic fertilizations that potentially develop into healthy larvae. Polyspermy reached levels as high as 40%, halving apparent maximal fertilization of 80%, for fresh gametes in contact for only 2 minutes. Furthermore, polyspermy was also shown to occur under sperm-limiting conditions. *Evechinus*’ fertilization kinetics, including polyspermy, was in strong agreement with a probabilistic model of sperm-egg interaction that predicted both these outcomes. This was the first empirical test of the model using controlled gamete concentrations as well as nuclear staining to reliably estimate polyspermy. Alternative methods used to assess polyspermy, such as cell cleavage or larval development, were found to produce polyspermy estimates that could differ markedly from estimates derived from sperm staining.

Our observations, and their agreement with theoretical predictions, indicate that the occurrence of polyspermy during fertilization events is potentially an important
driving force for the evolution of reproductive characteristics in broadcast-spawning marine invertebrates.
Introduction

Lack of sperm, or sperm-limitation, is thought to be a major driving force in the evolution of sperm chemotaxis, anisogamy, gender and egg size (Levitan 1995, 1996, 1998). Egg size is thought to have implications for the evolution of larval development times (Levitan 1996), which in turn are correlated to larval life histories (Havenhand 1993). However, recent reports reviewing the likelihood of sperm-limitation in nature (e.g. Yund 2000) have pointed out the potential importance of conditions under which excess sperm are present, such as when sperm competition exists. When sperm is present in excess, adaptations to reduce the effects of sperm-limitation may lead to polyspermy (multiple entry of sperm) and should be selected against, because polyspermy is lethal (Jaffe and Gould 1985). Polyspermy is known to impose serious costs in algal populations inhabiting brackish environments (Brawley 1992, Pearson and Brawley 1996, Serrão et al. 1999). However, the potential occurrence of polyspermy among marine invertebrates has been largely ignored until recently (Franke et al. 2002). The widespread occurrence of polyspermy in invertebrates is indicated by the ubiquitous presence of polyspermy blocks (Jaffe and Gould 1985), which are thought to have evolved in response to the need to avoid multiple fertilizations.

A widely used mathematical fertilization kinetics model (Vogel et al. 1982), recently extended to integrate the occurrence of polyspermy as well as sperm-limitation (Styan 1998), predicts that polyspermy should occur under sperm-limiting conditions. This is an important prediction because it means that polyspermy can provide a simultaneous selective force balancing many of the selective pressures resulting from sperm-limitation. One example of this is the evolution of egg size; although the evolution of
egg size has long been argued to underlie energetic constraints (e.g. Vance 1973, Podolsky and Strathmann 1996), more recent findings state that prezygotic processes and in turn sperm-limited conditions during fertilization events should select for larger and larger eggs (Levitan 1996a, Levitan 2000). However, bigger eggs also have a higher probability of polyspermy (e.g. Ginzburgh 1972, Styan 1998), placing a constraint on maximum egg size. It has been suggested that the sexual conflict resulting from the competing demands imposed by sperm-limitation and polyspermy is a factor in the co-evolution of gamete recognition proteins, since they may act as a selective partial polyspermy block (e.g. Vaquier et al. 1997, Rice 1998, Palumbi 1999). The tension between sperm-limitation and polyspermy may influence aspects of spawning behavior such as level of aggregation and synchrony. In each of these scenarios, polyspermy is predicted to be an important driving force. Since this co-evolution produces rapid changes in recognition proteins, polyspermy may play a key role in the processes of reproductive isolation and speciation (Gavrilets 2000, Rice 1998).

Many studies have examined the fertilization kinetics of broadcast-spawning marine invertebrates (e.g. Rothschild and Swann 1951, Vogel et al. 1982, Pennington 1985, Levitan et al. 1991, Oliver and Babcock 1992, Benzie and Dixon 1994, Williams et al. 1997). However, only few of these studies have examined the occurrence of polyspermy reliably, via sperm staining, and under a range of gamete parameters that might occur during natural spawning events (Clotteau and Dubé 1993, Desrosier et al. 1996). Moreover, these studies have focussed upon the issue from an applied perspective, in which aquaculturists have sought to reduce polyspermy and maximise the number of eggs that grow into healthy larvae. A recent field study has shown the
occurrence of polyspermy for the urchin *Evechinus chloroticus* under simulated spawnings in rockpools (Franke *et al.* 2002); however, quantification of polyspermy under controlled conditions is required in order to more fully test the predictions of Styan’s (1998) model under a wider range of controlled conditions. While the model has been tested before on scallops (Styan and Butler 2000), egg cleavage was used to assess fertilization. Many studies have attributed abnormal cleavage/larval death to polyspermy (e.g. Encena *et al.* 1998, Benzie and Dixon 1994, Williams *et al.* 1997, Buchanan 1998, Styan and Butler 2000). However, bacterial contamination, oxygen deprivation of developing embryos and fungal growth have also been shown to cause abnormal division, development and death (e.g. Bardach *et al.* 1972); hence to link such losses to polyspermy may give an inaccurate estimate of this phenomenon. Here sperm within the egg have been stained to unambiguously assess total fertilization (= polyspermy + monospermy), polyspermy and monospermy at a range of sperm concentrations, gamete ages and contact times. The reliability of the more rapid method of inferring polyspermy (i.e. equating polyspermy with irregular cleavage or abnormal/arrested development of larvae) is also tested in this study.

**Materials and Methods**

**Collection of Animals and Gametes**

*Evechinus chloroticus* is endemic to the shores of New Zealand, from the intertidal to the sub-tidal zone (>20 m). Upon collection, animals were placed in tanks, fed and provided with fresh running seawater. Laboratory based experiments were conducted from the 29th of January until the 17th of March 1998, which falls within *Evechinus*’ spawning season. For each experiment, animals were used within one week of collection. Gametes were obtained through injection of approx. 5 ml of 0.5 M KCl
through the perianal membrane. Between injections, syringes were rinsed in fresh water to kill any sperm present from an injection and to prevent subsequent contamination of eggs when injecting a potential female. For each experiment, eggs of different females were pooled to reduce any individual variability in gamete viability/fertilizability and to obtain the necessary volumes of eggs. Similarly, sperm from more than one male was used to produce concentrated sperm stock solutions. Prior to setting up sperm solutions, two replicate haemocytometer counts determined the concentration of the stock sperm solution. The mean was calculated and the sperm stock solution adjusted shortly prior to the experiment to give 1x10^{10} or 10^{11} sperm ml^{-1}.

**Experimental Protocol**

Stock sperm suspensions were serially diluted ten-fold to produce sperm concentrations of 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 sperm ml^{-1}. A petri dish containing seawater served as a sperm free control. The series of sperm suspensions were placed in a row of seven 9 cm petri dishes (100 ml). Alongside those petri dishes were placed two corresponding rows. The first comprised 200 ml beakers of 0.01 g/L^{-1} Sodium Lauryl Sulphate (hereafter SLS) and the second 5 L buckets containing 1 µm filtered seawater (Fig. 1). Sperm solutions were prepared from sperm stock solution within five minutes of use, to minimise any respiratory dilution effect. Between 4-5x10^4 eggs were placed in each of 14 plastic tubes (2 cm diameter). The tubes were sealed at one end with 60 µm nitex mesh and held in buckets of filtered seawater (1 µm). Two tubes were held by a clothes peg, seven of which were attached to a length of rod (hereafter egg-dipper), which could be suspended horizontally across all seven treatments. Raising and lowering the rod allowed treatments to be carried out.
simultaneously at all concentrations for any given contact time. Contact times chosen were 10 sec, 30 sec, 1 min, and 2 min. Each contact time experiment was repeated three times, giving a total n=6.

Upon withdrawal of eggs from the sperm solutions, eggs were submerged in SLS for 10 seconds, causing sperm to stop swimming for five seconds (99% effective, Presley and Barker 1970) or more (pers. obs) once submerged, and thus allowed accurate exposure of gametes to particular contact times, by stopping the sperm from swimming until rinsed off, and allowing the zygotes to develop normally. While fertilized eggs were submerged in SLS, a subsample of eggs from the mesh vials were removed and fixed in 10% formaldehyde. The immediate fixing of eggs was necessary to allow sperm to be visible when stained, as sperm is visible only within 3 min. after fertilization when stained in *Evechinus*’ eggs (Franke, unpub. data). Subsequently, the remaining eggs in the vials were rinsed thoroughly to remove unattached sperm by lifting the egg-dipper in and out of the buckets 80 times.

The effect of gamete age on female and male gametes was assessed simultaneously using the same sperm and egg suspensions as in the first experiment (T=0). Gamete ages were T=15 min, T=30 min and T=60 min after the initial sperm concentrations had been prepared, and the process described above was repeated at these times. *Evechinus chloroticus* has been observed to spawn synchronously, thus gametes would age simultaneously *in situ* (Levitan *et al*. 1991). Hence, even though sperm has been shown to be more sensitive to ageing effects than eggs, the approach of aging gametes simultaneously has been taken.
For contact times 1 min and 2 min at gamete age T=0 two further subsamples were taken. One subsample was fixed 2-3 hours after fertilization (hereafter “cleavage” sample) and the other was fixed 24 hours after fertilization (hereafter “embryo” sample). The time lag between fixation of the first sample and cleavage/embryo samples could potentially lead to higher fertilization values in the latter, however, submersion of all eggs in SLS as well as thorough rinsing of cleavage and embryo eggs was designed to counteract this problem.

**Identification of Fertilized and Polyspermic Eggs**

For the first samples fixed, fertilization was initially assessed as presence of a raised fertilization envelope (hereafter "envelope" data). A minimum of 100 eggs per sample was counted. Following this, unless envelope data showed zero fertilization, all samples which experienced high sperm concentrations (10^6-10^8 sperm ml^{-1} for contact times 10 sec-1min, and 10^5-10^8 sperm ml^{-1} for contact time 2 min) and/or where envelope fertilization levels were ≥10% were stained using the DNA specific stain Ethidium Homodimer-1 (Molecular Probes®, hereafter “staining” data). This included all samples that were exposed to one order of magnitude higher sperm concentration regardless of whether polyspermy was detected. Of the remaining samples (45 in total), 38 exhibited fertilization levels ranging from 0 to 5%. 3 out of those 45 samples were stained to test for presence of polyspermy, but none was detected.

To stain sperm in eggs, a subsample of eggs was placed into a micro titre well and eggs were rinsed free of fixative in phosphate buffer saline (Oxoid®, standard solution, hereafter PBS). Subsequently, gamete membranes were permeabлизed by
three minutes exposure to 98% methanol. This allowed the nuclear stain to penetrate the gametes and adhere to nuclear structures. Excess methanol was rinsed off with PBS and eggs were then exposed to the nuclear stain at concentration of 0.001818 mg mL\(^{-1}\) for five minutes, after which excess stain was rinsed off with PBS. Eggs were mounted in glycerol (Citifluor Alltech®) and the number of sperm that had entered eggs (up to a maximum of ten) was determined using a fluorescence microscope, at 40x magnification, focusing through the egg. Representative samples (>200 eggs in total) of sperm entry were verified using Confocal Laser Microscopy (40 x oil, Fig. 2). At least 50 eggs per sample were counted.

Only sperm present within the egg were scored as a fertilizing sperm, whereas sperm attached to the outside of the egg periphery were not. This conservative criterion could have led to an underestimation of fertilization, particularly for short contact times (10 and 30 sec), because the time taken for urchin sperm to enter and the fertilization envelope to fully develop is approximately one minute (Schatten 1981, Eisen et al. 1984, Tyler and Tyler 1966, Schuel 1984).

For cleavage samples, embryos with an even number of blastomeres (two, four, eight etc) or eggs with a fertilization envelope (1-cell stage) were scored as fertilized. Some of the latter egg type may have been polyspermic, however, this egg type was also present in samples which showed very low fertilization levels (0-10%), and where polyspermy was not detected. Since it was impossible to distinguish between polyspermic and monospermic 1-cell stage eggs, we had to assume that all one-cell stage eggs were monospermic. Embryos with irregular division, such as three, five, seven or nine segments, were scored as polyspermic. Monospermy was inferred by
subtracting the amount of polyspermy from the total fertilization. A minimum of 100 eggs per sample were counted.

For scoring the embryo samples a developed embryo (late prism/early pluteus stage) was equated with monospermy. 1-cell stage eggs and eggs showing other signs of fertilization (e.g. segmented cell bundles) were included in total fertilization data. At least 100 eggs per sample were counted. Polyspermy was inferred by subtracting the proportion of healthy larvae from the total amount of fertilization. Eggs exposed to seawater only (control) were examined to provide a baseline appearance of unfertilised eggs.

For envelope, staining, cleavage and embryo samples, percentages were calculated from the number of eggs counted. This method unfortunately did not allow to determine the total number of eggs that may have been lost in the process of development or subsequent analysis. A summary of all samples analysed and the time of their egg fixation after fertilization is presented in Table 1.

**Statistical Analysis of Envelope and Staining Data**

Neither the envelope data assessing fertilization nor the stained data assessing fertilization, polyspermy or monospermy conformed to the assumption of normality required for ANOVA, even after transformation. Data were therefore analysed by Logistic Regression for the following reasons:

1. All fertilization data are binary and more likely to conform to a binomial or logistic distribution than a normal distribution.
2. The logistic regression assumes a non-linear relationship between dependent variables (fertilization, polyspermy, monospermy) and the independent variables (sperm concentration, contact time, gamete age) (Tabachnick and Fidell 2001). All previous studies of fertilization kinetics indicate highly non-linear relationships between the variables (e.g. Pennington 1985, Levitan et al. 1991, Benzie and Dixon 1994, André and Lindegarth 1995, Encena II et al. 1996, Williams et al. 1997). Furthermore, the logistic regression produces odds ratios, which indicate the likelihood of an increase (if odds are > 1) or decrease (if odds are <1) of the dependent variable occurring with each unit change of the particular independent variable.

No significant interactions between the independent variables were found in the analysis of fertilization in the envelope data nor in the analysis of fertilization and polyspermy in the staining samples. In these cases the analysis was repeated omitting insignificant interactions. Statistically significant (p=0.01) interaction between the three independent variables was found for monospermic fertilizations. This interaction was subsequently removed from the model, because the odds ratios of the interaction indicated a small effect on the outcome of the dependent variable (monospermy). The estimated odds ratio was 1.01, and hence the interaction would only contribute a 1% change in monospermy.

In addition, the percentage fertilization of raw means of stained data for all gamete contact times and all gamete ages was plotted against the percentage monospermy to determine at what level of fertilization monospermy would cease to increase.
Comparison of Methods

Raw means of staining data were compared with envelope data as well as data from cleavage and embryo samples (n=6 per contact time). Data were pooled between all contact times (10 sec, 30 sec, 1 min and 2 min) and fertilization rates were compared for each gamete age using regression analysis (e.g. envelope fert. vs. stained fert.). For gamete ages T=0 and T=15 min, no difference in trends of subsequent regression analysis were found (i.e. the relationship remained positive), regardless of whether the data were analysed for each contact time individually, or if data were pooled. However, for gamete ages T=30 and 60 min, data for one particular contact time were too rare to be able to perform a regression analysis, thus data were pooled to give a large enough sample size to allow subsequent regression analysis.

For comparison between staining and both cleavage and embryo samples, mean raw data were analysed for the two contact times. When data were observed for each contact time individually (1 min and 2 min), no difference in trends of regression analysis (i.e. the relationship remained positive) were found compared to when data of 1 min and 2 min were pooled; hence to increase likelihood of detecting a significant regression, the two data sets of the two contact times were pooled. In the embryo samples, one set of replicates per contact time was lost due to insufficient supply of eggs, hence n=4 per contact time.

For all comparisons, simple regression analysis was performed using Excel®. To test if regressions differed from the expected relationship of y=x, slopes (b) were tested to see whether they were equal to 1, using t tests (Zar 1999). If the slope was not different from 1, the intercept was tested to determine if it was significantly different.
from zero using Excel®. The null hypothesis that the fertilization rates were equal (y=x) was rejected if either the slope was not equal to 1 or the intercept was not equal to zero.

**Modelling Fertilization**

Styan’s (1998) fertilization kinetics model was fitted to raw data from envelope and staining samples for contact time 2 min at gamete age T=0. The number of successful fertilisations was a function of the concentrations of eggs (approx. 1000 eggs ml⁻¹) and sperm (0-10⁵ sperm ml⁻¹), egg diameter (0.087 mm, Franke et al. 2002), sperm swimming speed (0.112 mm sec⁻¹, Franke et al. 2002), and the length of time sperm and eggs can potentially interact (contact time). The model predicts fertilisation efficiency (hereafter $F_e$, which is an estimation of the fertilizability of the egg surface) and the time taken to develop a block to polyspermy ($t_b$). For stained data a least squares fit using a quasi-Newton algorithm was applied that minimised the pooled differences between the measured and the predicted proportion of unfertilised eggs (Eq. 6, Styan 1998), and polyspermy (Eq. 15, Styan 1998). For envelope data the same equations were used, however the pooled differences between the measured and predicted proportion of unfertilised eggs only were minimised.

**Results**

**Stained Data**

The percentage fertilization, polyspermy and monospermy increased significantly with increasing sperm concentration and contact time (p<0.001). Logistic regression analysis produced the following equations, where $y$ is the dependent variable, $u$ the
linear regression equation, SC=sperm concentration (log_{10}), CT=contact time (min), GA=gamete age (min) and errors are ±1 S.E.

Fertilization/Polyspermy/Monospermy  

\[
y = \frac{e^u}{1+e^u}
\]

Fertilization:  
\[u_f = 1.1076 \pm 0.0552 \text{ (SC)} + 0.8218 \pm 0.0781 \text{ (CT)} - 0.0509 \pm 0.00356 \text{ (GA)} - 9.8391\]

Polyspermy:  
\[u_p = 1.7756 \pm 0.1086 \text{ (SC)} + 0.5963 \pm 0.0821 \text{ (CT)} - 0.0605 \pm 0.00453 \text{ (GA)} - 16.1515\]

Monospermy:  
\[u_m = 0.8729 \pm 0.0447 \text{ (SC)} + 0.7518 \pm 0.0713 \text{ (CT)} - 0.041 \pm 0.00313 \text{ (GA)} - 8.624\]

However, with increasing gamete age, percentage fertilization, polyspermy and monospermy decreased significantly (Fig.3; p<0.001). Sperm concentration was the most important predictor for fertilization, polyspermy and monospermy, because it generated the highest odds ratios (Table 2). Polyspermy was most affected by a 10-fold increase in sperm concentration within the range of 1x10^5 to 10^8 sperm ml^{-1} compared to fertilization and monospermy, because the likelihood of polyspermy increased 490% for a 10-fold increase in sperm concentration (Table 2). The same change in sperm concentration would increase the likelihood of total fertilization by 202.7% and monospermic fertilizations by only 139.4%. Interestingly, for each minute increase in contact time the likelihood of an increase in monospermy (112.1%) was similar to the likelihood predicted by each 10-fold change in sperm concentration. Hence, sperm concentration and contact time are of almost equal
importance in terms of their influence on successful monospermic fertilization (Table 2).

Mean maximum fertilization from stained samples was 85% at 1x10^8 sperm ml^-1, gamete age T=0, contact time 2 min (Fig. 3 and Fig. 4). The highest levels of polyspermy (40%) were also recorded under these conditions (Fig. 3). Therefore, polyspermy occurred under sperm limiting conditions, i.e. when fewer than 100% of eggs are fertilized. Maximum levels of monospermic fertilization (~40%) were also observed for these parameters at sperm concentrations of 10^7 and 10^8 sperm ml^-1 (Fig. 3) and ranged between 0 to 20% for gamete ages T=15, 30 and 60 min across all contact times (Fig. 3). Monospermy ceased to increase beyond 40-45% once levels of fertilizations were above 50% (Fig. 4).

**Comparison of Methods**

Staining vs. Envelope samples assessing Fertilization

There was a highly significant relationship between envelope and stained samples assessing fertilization for gamete ages T=0 and T=15 min (p<0.001), but this relationship was not significant for gamete ages T=30 and T=60 min (Fig. 5). The slope of the regression generated was significantly different from b=1 for gamete age T=0 (p<0.01) and compared to staining data, envelope data tended to be higher at low levels of fertilization and lower at high levels. The slope was not significantly different from b=1 for gamete age T=15 min. and the intercept was not significantly different from zero, hence the regression generated was equal to the expected relationship of y=x. Low sample size and large variability in the data were probably
the main reasons for the failure to detect a significant regression for gamete ages T=30 and 60 min.

**Staining vs. Cleavage samples assessing Fertilization, Polyspermy and Monospermy**

There was a significant relationship between staining and cleavage samples assessing fertilization, polyspermy and monospermy (p<0.01) (Fig. 6). Slopes were not significantly different from b=1 for fertilization and monospermy at alpha 0.05, but the slope was different from b=1 for polyspermy (p<0.05). The intercept was significantly different from zero for fertilization, but not for monospermy. Hence, the regression generated for fertilization is parallel to the expected relationship of y=x. Estimates of fertilization based on cleavage will therefore always generate higher values than if fertilization is assessed via staining. However, estimates of monospermy will be the same regardless of whether cleavage or staining method is used, because the regression for monospermy is like the expected relationship.

**Staining vs. Embryo samples assessing Fertilization, Polyspermy and Monospermy**

A significant relationship was found for fertilization and monospermy (p<0.001), but not for polyspermy (Fig. 7). The slope was not significantly different from b=1 for fertilization and monospermy. This test could not be performed for polyspermy, because the regression was not significant. The intercept was significantly different from zero for fertilization, but not for monospermy. Thus, similar to comparison of staining and cleavage samples, the regression for fertilization is parallel, and like the expected relationship of y=x for monospermy. Therefore, estimates based on embryo
development will generate higher values for fertilization than staining, but give the same amount of monospermy.

**Modelling Fertilization**

The proportion of unfertilised eggs (1 - fertilization) for envelope data showed a close fit between data and predictions generated by the model ($r^2=0.73$). However, using data from stained samples for unfertilised eggs, polyspermy resulted in an even closer fit with the fertilization kinetics model ($r^2 = 0.93$). Least squares fitting generated a polyspermy block latency ($t_b$) of 8.07 seconds and a fertilization efficiency ($F_e$) of 0.001 for both envelope and stained data (Fig. 8).

**Discussion**

**Fertilization, Polyspermy and Monospermy observed via nuclear staining**

Styan’s (1998) prediction that polyspermy should occur under sperm-limited conditions (i.e. less than 100% fertilization) has been confirmed by our results. Furthermore, results from logistic regression analysis confirm previously established results of fertilization kinetics studies, in that sperm concentration is the most important predictor for fertilization compared to contact time and gamete age (e.g. Levitan *et al.* 1991). However, of all three fertilization categories (fertilization, polyspermy and monospermy) investigated, it is important to note that polyspermy was most influenced by change in sperm concentration. This is important, because it means that even if organisms increased environmental sperm concentration by 10-fold, the chance of increasing monospermy, would be less than the chance of increased polyspermy. This highlights that increasing sperm concentration *per se*, and
in turn fertilization, may not be the preferred solution to obtaining maximum levels of monospermy (see Fig. 4), but that increasing other factors, such as contact time, may be more important if higher fertilization success is to be achieved.

Furthermore, we have demonstrated that in the laboratory polyspermy can halve the apparent maximal fertilization of 80% to 40% at sperm concentrations of $1 \times 10^8$ sperm ml$^{-1}$, for contact time 2 min, gamete age $T=0$. These results are similar to previously measured polyspermy levels for marine invertebrates in the laboratory using sperm staining methods. For example, maximum polyspermy levels at $1 \times 10^6$ sperm ml$^{-1}$ were approximately 37% for the giant scallop *Placopecten magellanicus* (Desroisers *et al.* 1996) and 30% to 70% at $1 \times 10^7$ sperm ml$^{-1}$ depending on egg concentration, for the surf clam *Spisula solidissima* (Clotteau and Dubé 1993). Franke *et al.* (2002) measured polyspermy levels *in situ* of up to 76.5% at $6 \times 10^6$ sperm ml$^{-1}$. However, for these studies, contact times may have been longer than in our laboratory experiments (no data for contact time given in Desroisers *et al.* (1996), 10 min in Clotteau and Dubé (1993) and 2 - 10 min for Franke *et al.* (2002)).

Polyspermy was recorded at concentrations from $10^6$ to $10^8$ sperm ml$^{-1}$ and at contact times as short as 10 seconds. Polyspermy was also measured at longer contact times (30 sec, 1 min and 2 min). The parameters chosen were likely to be within the range experienced in nature, because $1 \times 10^6$ sperm ml$^{-1}$ and above have been measured in the field for marine invertebrates (e.g. Hamel and Mercier 1996, Williams *et al.* 1997). Populations of a variety of organisms (e.g. Babcock *et al.* 1986) including *Evechinus* (Lamare and Stewart 1998) have been observed to spawn over the course of hours, indicating that such concentrations may persist for substantial periods of
time; hence the potential for polyspermy to occur during natural spawning events clearly exists. This is even more likely since we have confirmed that polyspermy occurred even under sperm-limiting conditions (i.e. when not all eggs were fertilized), as has previously been demonstrated by Franke et al. (2002) for in situ fertilizations of Evechinus.

**Comparison of Methods**

**Staining vs. Envelope samples assessing Fertilization**

Comparison of fertilization levels as assessed by envelope and staining methods produced different results for gamete age T=0. There are two possible reasons for this. The first reason is the conservative criterion used in assessment of fertilization for stained eggs, i.e. sperm sitting on the periphery of the egg were not counted as fertilizing sperm. In fact, some of the sperm may have been fertilizing, but had not yet fully penetrated the egg. Secondly, eggs experiencing severe polyspermy may fail to raise a fertilization envelope and could have been classified as an unfertilised egg. A similar phenomenon has been observed in other urchin species (Levitan pers. com.) and thus represents a reasonable explanation. In this study, this can be observed where stained fertilization levels are >60% and corresponding envelope data fall below the expected line. Thus both factors may explain why envelope data produced higher levels of fertilization relative to staining data, when fertilizations were low, but lower levels when fertilization was high.

The regression generated for gamete age T=15 min was not significantly different from the expected relationship of y=x, hence when gametes are older, scoring fertilization levels using either method produce similar results. No significant
relationship was found for gamete ages T=30 and 60 min. possibly due to high variability and low sample size.

**Staining vs. Cleavage samples assessing**

**Fertilization, Polyspermy and Monospermy**

Fertilization as assessed by cleavage gave consistently higher fertilization values than did staining data for the same sample. The most likely explanation for this is again that eggs from stained samples were fixed very rapidly after fertilization and sperm attached to the periphery were not scored as a fertilizing sperm. In addition, some sperm still adhering to eggs, even after initial immobilization and thorough rinsing may have fertilized more eggs over course of two to three hours. This is particularly true for higher sperm concentrations, i.e. $10^7$ and $10^8$ sperm ml$^{-1}$.

When high levels of polyspermy were present, polyspermy as assessed by cell cleavages was far lower than for staining data (see Fig. 6). There are two likely reasons for this. Firstly, when stained polyspermy levels are high, eggs that failed to raise their membrane due to severe polyspermy were scored as unfertilized. Secondly, 1-cell stage eggs that may have been polyspermic could not be distinguished from monospermic eggs, and were thus scored as monospermic, as discussed previously.

For monospermy, a regression line not different from the expected was generated; hence, the same estimates of monospermy are obtained using either method.
Staining vs. Embryo samples assessing

Fertilization, Polyspermy and Monospermy

Fertilization as assessed by embryo development gave consistently higher values than did staining. Again, the conservative estimate of sperm entry when stained may explain this, and/or remaining sperm present in samples could have subsequently fertilized eggs over the course of 24 hours. In addition, unfertilised eggs often disintegrate over the course of 24 hrs (pers. obs.), hence increasing the apparent fertilization levels. Similar to comparison of staining and cleavage samples, the regression slope for monospermy was not significantly different from 1 and the intercept was not significantly different from 0. Thus both methods estimate the same amount of monospermy. No significant relationship was found for polyspermy estimates, most likely due to the high variability in the data. However, it is apparent that the majority of polyspermy estimates in embryo samples were higher compared to staining samples (see Fig. 7). Again, this may be due to unfertilised eggs disintegrating over the course of 24 hours, leaving disproportionately higher numbers of abnormally developed embryos.

Both methods, i.e. staining or cleavage/larval development resulted in similar estimates of the level of monospermy. However, this was not true for estimates of polyspermy, where cleavage data appeared to underestimate polyspermy and embryo data bore no significant relationship to polyspermy as previously assessed by staining. Staining is particularly important if low levels of polyspermy are to be detected, because even low levels of polyspermy may be evolutionarily important (Gavrilets 2000). Furthermore, staining is also important to detect high levels of polyspermy, because of failure of eggs to raise their envelope when penetrated by many sperm.
Overall, fertilizations appear to be systematically higher when cleavage or embryo development was assessed. This was probably due in part to residual sperm effects which may result in longer effective contact times and thus bias fertilization estimates when assessed by this method.

Despite the possibility that staining data underestimate sperm entry relative to envelope data, the magnitude of this effect is far smaller than those seen when cleavage or embryo data are used to assess fertilization. In addition, envelope data cannot be used to assess polyspermy. Assessment of monospermy and polyspermy by staining is clearly the most accurate method to use in this application.

**Modelling Fertilization**

Predicted levels of unfertilised eggs, polyspermy and monospermy for stained data based on Styan’s (1998) model showed a close fit with data of contact time 2 min gamete age \( T=0 \). Similarly, for the same parameters a close fit was also achieved between the predicted levels of unfertilised eggs for envelope data. The polyspermy block latency \( (t_b) \) of 8 sec generated by the model was half of what was predicted for *in situ* data in this species, which was 16 sec (Franke *et al.* 2002), but was of the same order of magnitude as the fast block to polyspermy in other urchin eggs (3 sec, Jaffe and Gould 1985).

The \( F_e \) value of 0.001 for both stained and envelope data sets was more than two orders of magnitude lower than the one generated for the *in situ* spawnings in rockpools in this species, which was 0.782 (Franke *et al.* 2002). Furthermore, the \( F_e \) value reported here is one order of magnitude below predicted \( F_e \) values in other
species of urchins and broadcast-spawning marine invertebrates. For example, Levitan et al. (1991) calculated the $F_e$ value at 0.03, and Buchanan (1998) at 0.05. The calculation of $F_e$ values is based on sperm-egg collisions, and one reason for different measures of $F_e$ values may be related to differing sperm swimming speeds at different sperm concentrations. Such respiratory dilution effects (Chia and Bickell 1983) are not accounted for in the model, which assumes that sperm swimming speed/behaviour does not vary with sperm concentration (Rothshild and Swann 1951, Styan and Butler 2000). However, this is an unreasonable assumption, because when sperm concentrations and/or sperm swimming speeds are high, more sperm/egg collisions are likely to occur (Styan and Butler 2000). Future incorporation of possible differences in sperm swimming speeds as well as possibly different sperm behaviours at different concentrations may give more consistent estimates of $F_e$ values. The inconsistencies of generated $F_e$ values could however also result from mathematical artefacts of model fitting, a possibility that has been suggested elsewhere (Farley 2002) to explain counter-intuitive results on model generated $F_e$ values.

The importance of polyspermy in fertilization ecology of broadcast-spawning marine invertebrates

The occurrence of polyspermy under sperm-limited conditions and the close fit of the model to a subset of the data demonstrated here suggest that polyspermy is not rare but a frequent event during natural spawning events, particularly if conditions of sperm competition are common (e.g. Yund 2000). The fact that polyspermy occurs under sperm-limited conditions is of particular importance, because polyspermy and sperm-limitation are balancing selective forces that act simultaneously in opposite
directions. The observed variability of egg sizes among taxa and optimal egg size within taxa (Levitan 1996b) may be stabilized by sperm-limitation (selecting for larger eggs thus increasing sperm target size) and polyspermy (selecting for smaller eggs thus reducing chance of polyspermy).

If broadcast-spawning invertebrates lose approximately 20% of their reproductive output due to polyspermy, as suggested by our laboratory data as well as by results from the field (Franke et al. 2002), then models of larval recruitment based on 100% fertilization need major revision. Hence, not only does sperm-limitation lead to potentially large egg losses but polyspermy could further contribute to substantial egg losses during spawning events. Thus polyspermy avoidance may explain various apparently contradictory phenomena in reproductive biology of broadcast-spawning invertebrates. For example, spawning under turbulent conditions (e.g. Grange 1976, Creese and Ballantine 1983, Franke and Babcock, unpub. data), would seem maladaptive if fertilization is sperm-limited, yet it may be advantageous if it reduces the costs of polyspermy. Another apparent contradiction can be found in the rapid co-evolution of sperm-egg receptors in urchins (Palumbi 1999, Vaquier et al. 1997) and abalone (Swanson and Vacquier 1998). Gamete recognition systems are thought to be driven by an evolutionary arms race between sperm and eggs, similar to parasite/host co-evolutionary events in which sperm receptor proteins rapidly change making it more difficult for sperm to attach to and penetrate the egg (Rice 1998). Changes such as this make little sense in the context of sperm-limitation, yet could prove adaptive as selective partial polyspermy blocks. Broad-scale generalizations about the relative importance of polyspermy on the reproductive biology of marine organisms will
require fertilization kinetics and behavioral data from many more species, preferably from natural spawning and fertilization events

**Figures**

**Figure 1**

Experimental setup for fertilization kinetics study in *Evechinus chloroticus*. The egg dipper (A) holds vials, which bear nitex mesh (60 µm) at one end, into which the eggs are placed (4-5 x 10^4 per vial). Petri dishes (B) hold sperm solutions ranging from 10^3 to 10^8 sperm ml⁻¹; filtered seawater (1 µl) served as sperm free control. Adjacent to petri dishes, 250 ml glass beakers (C), held 200 ml of 0.01g Sodium lauryl sulfate (SLS), which caused sperm to temporarily cease swimming. Next to the SLS dishes were 5 L water buckets (D) holding filtered seawater (1µl) and served to rinse eggs free of excess sperm attached to apparatus and/or eggs.

**Figure 2**

A 2µm slice of an *Evechinus chloroticus* egg (contact time 2 min, 10^8 sperm ml⁻¹, gamete age T=0) stained with Ethidium Homodimer-1 as viewed under the Confocal Lazer Microscope (40 x oil). The slice is taken 9 µm into the eggs from their top end. Arrows indicate sperm that have entered the egg. Eggs shown are monospermic (one sperm, M) and polyspermic (two and more sperm, P).

**Figure 3**

Raw means of staining data assessing percentage fertilization, polyspermy and monospermy vs. sperm concentration (log₁₀) and gamete age (min) for gamete contact times 10 sec, 30 sec, 1 min, and 2 min. Error bars have been omitted for clarity. No staining for samples experiencing less than at 10^5 sperm ml⁻¹ was conducted, either
because samples showed less than 10% fertilization when envelope was assessed and/or no polyspermy was detected in corresponding samples experiencing an order of magnitude higher sperm concentration when stained. Thus data for $10^5$ to $10^8$ sperm ml$^{-1}$ only is shown. Numbers on contour graphs represent the raw means of fertilization parameters.

**Figure 4**
Staining data (●) of fertilization (%) vs. monospermy (%) for all raw means data pooled between all gamete ages and gamete contact times. Note that after 50% fertilization monospermy only ranges between 30-45%, even though total fertilization levels reach up to 85%.

**Figure 5**
Comparison of the envelope and staining method assessing percentage fertilization for each gamete age. T shows age of gametes (min). n indicates the number of samples used for regression analysis. Data points represent raw means (n=6 per contact time) at one particular sperm concentration for contact times 10 sec, 30 sec, 1 min and 2 min, and data were pooled from all four contact times for analysis. Thick lines designate significant regressions. Thin lines indicate the expected slope $y=x$. Significant $r^2$ values are shown on graphs. n.s. indicates non-significance of regression analysis. The regression generated for gamete age $T=15$ min only is like the expected relationship of $y=x$.

**Figure 6**
Comparison of staining vs. cleavage method assessing percentage fertilization (F), polyspermy (P) and monospermy (M). Data points indicate raw means (n=6 per contact time) at a particular sperm concentration. Data from contact times 10 sec, 30 sec, 1 min and 2 min were pooled for analysis. Thick lines show significant regressions with $r^2$ values indicated on corresponding graphs. Thin lines represent the expected relationship of $y=x$.

**Figure 7**

Comparison of staining vs. embryo method assessing percentage fertilization (F), polyspermy (P) and monospermy (M). Data points indicate raw means (n=4 per contact time) at a particular sperm concentration. For regression analysis data from contact times 10 sec, 30 sec, 1 min and 2 min were pooled. Thick lines indicate significant regressions and corresponding $r^2$ values are indicated on the corresponding graphs. Thin lines show the expected relationship of $y=x$. n.s. indicates a non-significant result from regression analysis.

**Figure 8**

Styan’s (1998) fertilization kinetics model fitted to staining (●, —) and envelope (○,……) data for gamete contact time 2 min and gamete age $T=0$. Symbols represent experimental data and lines indicate the model’s prediction. Estimates of sperm swimming speed $= 0.112 \text{ mm sec}^{-1}$, egg concentration of 1000 ml$^{-1}$ and egg diameter of 0.087 mm were used in the model. Polyspermy block latency was calculated as 8.07 seconds. $F_e$ (fertilizability of eggs) values were calculated as 0.001 for stained and envelope date, however $r^2$ values differed, with stained data generating a higher
value (0.93) compared to envelope data (0.73). For consistency with data presented in Fig. 3, proportions along the y-axis were converted back to percentages.
Fig. 2
Fig. 4
Fig. 5

![Graphs showing correlation between staining and envelope percentage at T=0, T=15 min, T=30 min, and T=60 min. R² values are 0.88, 0.81, n.s., and n.s. respectively.](image-url)
Fig. 6

**F**

Cleavage (%) vs. Staining (%)

$r^2 = 0.88$

**P**

Cleavage (%) vs. Staining (%)

$r^2 = 0.72$

**M**

Cleavage (%) vs. Staining (%)

$r^2 = 0.9$
Fig. 7

F: $r^2 = 0.9$

P: n.s.

M: $r^2 = 0.79$
Fig. 8

Unfertilized (%)
(100 - Fertilized)

Polyspermy (%)

Monospermy (%)

Sperm Concentration (log_{10})

100
0
4 5 6 7 8

100

Sperm Concentration (log_{10})

100

Sperm Concentration (log_{10})
Tables

Table 1
Overview of samples analysed. Time of fixation indicates the time between the completion of gamete interaction and fixation in 10% formaldehyde. Methods used to assess total fertilization, polyspermy and monospermy are also outlined.

Table 2
Odds ratios generated from logistic regression analysis of raw means (n=6 per contact time at one particular sperm concentration) for envelope and staining data assessing fertilization, polyspermy and monospermy in relation to sperm concentration, contact time and gamete age. The odds ratio is calculated by raising the coefficient (b) in the regression equation of the dependent variable to the base of the natural log (e), i.e. odds ratio=\( e^b \). Odds ratios below 1 designate a decrease, and above 1, an increase of the dependent variable (e.g. fertilization) in response to a change in the independent variable (e.g. sperm concentration). From the odds ratios, the percentage likelihood of an increase/decrease of the dependent variable in response to a unit increase/decrease of the independent variable is calculated as follows: \(((\text{odds ratio} -1) \times 100)\), and indicated in brackets.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Time of Fixation after Contact Time Experiment completed</th>
<th>Method of Assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Envelope</td>
<td>As quickly as possible (approx. 1 min)</td>
<td>Envelope elevation, eggs of samples showing ≥10% fertilization, were subsequently stained</td>
</tr>
<tr>
<td>Staining</td>
<td>As quickly as possible (approx. 1 min)</td>
<td>Sperm stained with Ethidium Homodimer-1 inside an egg</td>
</tr>
<tr>
<td>Cleavage</td>
<td>2-3 hours</td>
<td>Regular and irregular cleavage</td>
</tr>
<tr>
<td>Embryo</td>
<td>24 hours</td>
<td>Larvae and cleaved cell bundles</td>
</tr>
</tbody>
</table>
Table 2

<table>
<thead>
<tr>
<th>Independent / Dependent Variable</th>
<th>Sperm Concentration (log_{10})</th>
<th>Contact Time (min)</th>
<th>Gamete Age (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fertilization</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Envelope)</td>
<td>2.374 (137.4%)</td>
<td>1.123 (12.3%)</td>
<td>0.968 (3.2%)</td>
</tr>
<tr>
<td>(Staining)</td>
<td>3.027 (202.7%)</td>
<td>2.275 (127.5%)</td>
<td>0.95 (5%)</td>
</tr>
<tr>
<td><strong>Polyspermy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Staining)</td>
<td>5.9 (490%)</td>
<td>1.815 (81.5%)</td>
<td>0.941 (5.9%)</td>
</tr>
<tr>
<td><strong>Monospermy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Staining)</td>
<td>2.394 (139.4%)</td>
<td>2.121 (112.1%)</td>
<td>0.96 (4%)</td>
</tr>
</tbody>
</table>
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Table 3

Summary of regression analysis testing for differences between staining and envelope, cleavage and embryo samples assessing percentage fertilization, polyspermy and monospermy.* indicates significant results. n.s. indicates non-significant results and n.a. indicates if the test was non-applicable, either because the regression was non-significant or because $b \neq 1$. The numbers in brackets indicate $r^2$ values or $t$ values as indicated. Significant $p$ values are bold, and regression lines not significantly different from the expected line $y=x$ are shown in italics.
Fertilization Kinetics and the Importance of Polyspermy in Fertilization Ecology of the Sea Urchin *Evechinus chloroticus*

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CHAPTER 4

This chapter is in preparation for submission to the New Zealand Journal of Marine and Freshwater Research titled Reproductive Patterns of the New Zealand sea urchin *Evechinus chloroticus*
Abstract

Identifying spawning patterns under natural conditions is pivotal to the understanding of evolutionary and ecological processes in the fertilization ecology of marine invertebrates. Variability in these patterns may be an important component of life history patterns, but is still poorly understood. Observations of *Evechinus chloroticus* indicate that different types of spawning occur under a wide range of different conditions. To elucidate this issue further, weekly assessment of gonad indices and daily assessment of eggs exposed to seawater *in situ* (fertilization assays or bioassays) allowed observation of various spawning patterns during three consecutive spawning seasons in this species. The spawning patterns observed included sub-population spawnings that occurred up to several times throughout a spawning season. These were not always associated with a significant decrease in gonad index. In addition, a large-scale epidemic (whole population) spawning in response to high wave-energy conditions was also detected accompanied by a highly significant decrease in gonad index and fertilizations in bioassays. Furthermore, a natural sub-population spawning event was observed under calm/moderate environmental conditions. This spawning was not associated with a significant decrease in gonad index, but fertilization was detected in the bioassay. Severe sperm limitation (0-34.1% fertilization) was observed when fertilization was measured during this spawning event. Environmental parameters, such as moon phase, surge height and chlorophyll a concentrations were not significantly correlated with spawning events. Hence, natural spawnings were variable and unpredictable in *E. chloroticus*, agreeing with previous results established for *E. chloroticus* and most other echinoids.
Introduction

Marine broadcast-spawners, (i.e. organisms that release sperm and eggs into the water column where external fertilization and subsequent zygote formation takes place), face the problem that the chance of gamete fusion can be significantly reduced by physical and biological factors. For example, dilution of gametes may be so great that sperm and eggs fail to meet. This lack of sperm to fertilize all eggs, termed sperm limitation has been shown to occur frequently during natural spawning events (e.g. Oliver and Babcock 1992, Babcock et al. 1992, Lasker et al. 1996, Williams et al. 1997) as well as during experimental assessments of field fertilization events (e.g. Pennington 1985, Levitan and Young 1995).

Natural selection is thought to have given rise to means of overcoming problems of sperm limitation, such as synchronous spawning (e.g. Babcock et al. 1992, Hay 1997) preferably during calm conditions (Pearson and Brawley 1996, Serrão et al. 1996, Brawley et al. 1999, Serrão et al. 1999). Timing of gamete release under these conditions is thought to enhance fertilization success, which has been postulated to be one of the main evolutionary driving forces for synchronous spawning (Olive 1992, but see Kiflafï et al. 1998).

Many physical and biological cues, including water temperature, day length, lunar phase, hydrostatic pressure, presence of phytoplankton and chemicals have been suggested to entrain endogenous seasonal, tidal and diel cycles of reproduction thus causing synchronized spawning within populations. Often these cues are thought to act synergistically over different time scales (e.g. Babcock et al. 1986, 1992). However, direct evidence demonstrating that these cues cause spawning is ambiguous
and often circumstantial (e.g. Thorson 1950, Pearse et al. 1988, but see e.g. Starr et al. 1992). Furthermore, not much is known about the interaction and relative importance of different spawning cues. For example, spawning might take place when rough weather conditions occur, but during this time phytoplankton may also be abundant (Wahle pers. com.); or peak temperature co-occur with a phytoplankton bloom (Hamel and Mercier 1995).

Adding to the complex interactions between biological and physical factors causing and affecting synchronized gamete release and in turn fertilization success (summarized in Levitan 1995) are indications of excess sperm conditions during spawning and fertilization events (e.g. Brawley 1992, Yund and McCartney 1994, Levitan 1998, Yund 2000, Franke et al. 2002). This and other evidence brings into question the universality of sperm limitation and suggests that conditions of excess sperm concentrations are probably very common during synchronous spawning events (e.g. Levitan 1998, Petersen and Warner 1998, Yund 2000). Furthermore, some of these studies have shown that significant levels of polyspermy can occur even under sperm limited conditions (Brawley 1992, Pearson and Brawley 1996, Franke et al. 2002). Thus any adaptation to decrease the effects of sperm dilution may risk increasing the level of polyspermy.

_Evechinus chloroticus_ is a gonochoric echinoid, endemic to New Zealand, with its spawning season occurring during the Indo-Pacific mid to late summer (Walker 1982). Levels of sperm limitation and polyspermy have been measured in simulated natural spawnings in this species (Franke et al. 2002). While epidemic spawning of _Evechinus chloroticus_ has been reported during calm conditions (Lamare and Stewart
1998), the consensus of commercial urchin fishers is that major spawnings occur after storms or periods of high swell (Mead 1996). Minor spawnings of varying synchrony or intensity have also been reported for *E. chloroticus* (e.g. Keuskamp 1997, Brewin *et al.* 2000).

To determine the generality of these contrasting results, the timing and frequency of spawning in *E. chloroticus* was investigated in more detail, with particular focus on the occurrence of spawning in response to high wave-energy conditions.

**Materials and Methods**

**Time and Location**

The study was conducted during the summer months (December to February/March) of the years 1997-98 1998-1999 and 1999-2000 in and adjacent to the Leigh Marine Reserve, Northland, New Zealand (Fig.1). Matheson’s Bay, situated 5 km south of the Reserve, and Waterfall Reef (within the Reserve) served as main sampling sites (Fig. 1). A more limited set of observations of a spawning event of *E. chloroticus* was also obtained during the 1996-1997 spawning season on the shores around Waterfall Reef. Eggs exposed to seawater *in situ* (hereafter fertilization assays or bioassays) were sampled daily and gonad indices were sampled weekly over three consecutive spawning seasons (late November until end of February/March). Two natural spawning events of different magnitudes were also observed during this study, which allowed the linkage between direct evidence and indirect indicators of spawning to be established.
Determination of Egg Longevity

Observations of natural spawning events are inherently difficult, but methods exposing eggs to *in situ* seawater have proven useful in detecting natural spawning events (Meidl and Yund 2001, Wahle and Gilbert 2002). A similar approach has been used here, in which eggs were placed into a bucket, which received a constant supply of seawater from Waterfall Reef (Fig. 1). In order to monitor spawning events, it was necessary to determine how long eggs were viable. Eggs and sperm were obtained from urchins during January 1998 through injection of 2-3 ml of 0.5 M KCl through the perianal membrane. Syringes were washed in fresh water between injections to prevent sperm contamination of eggs. At this time of the year, gametes of *E. chloroticus* are easily accessible because this period falls within the peak spawning time of this species (Dix 1970, Walker 1982, Brewin *et al.* 2000). After spawning a female, her eggs were examined for the presence of germinal vesicles (x40 magnification) and if > 5% of eggs showed evidence of these, the egg batch was discarded and new eggs were obtained from a different female. Between 5-10 x 10³ eggs were held in scintillation vials (20 ml) at room temperature (~20°C) and mixed with sperm. Fifteen to 30 min. after insemination, gametes were fixed in 2 ml of 10% formaldehyde. Sperm stock solutions (~10¹⁰ sperm ml⁻¹) were held in the fridge (4°C) and were not used if older than three hours. Similar studies in echinoderms have shown that sperm held at high concentration for comparable amounts of time do not show significantly reduced sperm swimming speeds or longevity even under warmer conditions (e.g. Pennington 1985, Levitan *et al.* 1991, Benzie and Dixon 1994, Williams and Bentley 2002). Before mixing sperm and eggs, sperm motility was visually confirmed (x 40 magnification). If sperm were immobile, or swimming more slowly than one hour previously, new sperm were taken from a fresh male. Eggs were
exposed to three different sperm concentrations \((10^3, 10^4 \text{ and } 10^5 \text{ sperm ml}^{-1})\) and aged up to 15 hours. The experiment was carried out twice for egg ages 1, 3, 7, 8, 9 and 11 hours \((n=2)\). For all other egg ages, i.e. 2, 5, 6, 10, 12, 13, 14, and 15 hours only one replicate was obtained. Eggs were scored as fertilized when a fertilization envelope was present and scored as unfertilized if no envelope was apparent \((\times 40 \text{ magnification})\).

**Fertilization Assays**

Approximately 50 *E. chloroticus* were collected once every 7-14 days from habitats of high algal abundance (shallow mixed algae, 2-5 m. depth) around the shores of the Leigh Marine Laboratory. Urchins were held in tanks that contained running seawater and algae (*Ecklonia radiata*).

Eggs were obtained as described above. Upon obtaining eggs, these were examined for the presence of germinal vesicles. Only egg batches that consisted of at least 80% fertilizable eggs (i.e. no germinal vesicle present) were used. Sometimes the majority of eggs were immature for approximately one week after a spawning event in the field was detected. This took place once to three times during a spawning season. Daily sampling was continued none-the-less even under these circumstances and eggs containing germinal vesicles were not counted.

Approximately \(1\times10^4 \text{ to } 1\times10^5 \text{ eggs ml}^{-1}\) were placed twice a day into a 200 ml plastic jar, covered with a 30 μm mesh. The jar was placed into a 20 L bucket through which seawater was pumped directly from the sea at a filling rate of approximately 2-3 min per 20 L. The inlet of the pipe (80m in length, ~20 cm in diameter) transporting water
to the bucket was situated at Waterfall Reef (Fig. 1), adjacent to urchin populations at densities of approximately 2 m$^{-2}$. Thus sperm present in the seawater could interact with eggs suspended in the treatment vial through the mesh.

Because the water was carried through an 80 m pipe before reaching the bucket, sperm present in the seawater would be greatly diluted and thus possibly lead to very low fertilization rates. These would not represent fertilization rates that can be expected to occur during a spawning event, but primarily indicated presence of sperm in the seawater.

A separate vial containing the same concentration of eggs was placed into a bucket filled with aged (24 hrs) seawater. Sperm are unable to fertilize eggs after a long time when highly dilute (e.g. Chapter 3, Chia and Bickell 1983), thus sperm present in the aged seawater were unlikely to be viable after 24 hours. This served to control for sperm contamination of eggs prior to exposure of water from Waterfall Reef as well as false fertilization envelopes.

**Assessment of Fertilization Assays**

Eggs from treatment and control vials were checked for fertilization by taking two replicate samples of 1-5 x 10$^4$ eggs and randomly examining eggs at 40x magnification. Eggs were scored as “fertilized” if cleaved eggs (i.e. two, four to multi-cell embryos) were apparent and the fertilization level (%) was calculated. If fertilizations were detected in controls (aged seawater) and treatments at the same time or in controls only, the corresponding treatment samples were disregarded.
Gonad Index

Concurrent to monitoring fertilization assays, gonad indices from urchins inhabiting Matheson Bay’s shallow mixed algal habitat (Fig. 1) were determined weekly for the 1997-1998 (n=30 per sample), 1998-1999 (n=20 per sample) and 1999-2000 (n=20 per sample) spawning seasons. Gonad indices were measured within 24 hrs of collection. Two measurements of urchin test diameter were taken (± 0.5 mm) using calipers and the mean calculated to give the size of the animal. The volume of gonads was assessed as the displacement volume of dissected gonads and gonad index (GI) was calculated as follows:

\[ GI = \frac{\text{gonad volume (ml)}}{\text{body size (mm)}} \times 100 \]

While measuring gonad volume, the sex of the urchin was also determined.

If fertilizations were detected in the bioassay, additional gonad index measures were taken from Matheson’s Bay within 24 hrs to determine whether presence of fertilization coincided with a decrease in gonad index.

Because one of the main objectives of this study was to explore if spawning coincided with high wave-energy conditions, additional gonad index sampling was conducted at Waterfall Reef (n=16-20 per sample) and Matheson’s Bay (n=20 per sample) upon reports of approaching cyclones, storms or big swells.
In situ spawning of an urchin barren population at Matheson’s Bay

Upon returning from an urchin collection dive on the 1st Mar. 1999, a few male and female urchins inhabiting urchin barren habitat at Matheson’s Bay (Fig.1), were observed to start spawning at ~17:00 hrs in 6 m depth. More dive tanks were necessary to observe this spawning event in detail, thus these were brought back from the Leigh Marine Laboratory. On returning immediately to the site (~18:00 hrs) with additional SCUBA tanks a large number of urchins covering an area of approximately 75 x 75 m$^2$ were observed spawning. Using SCUBA, a 10 m transect line was laid across the area of spawning and the number of spawning and non-spawning urchins were recorded within 1 m to either side of the transect line for 7.2 m. In addition, the sex of spawning urchins was recorded. Following this, eggs were haphazardly collected by drawing in the seawater and eggs by holding the tip of a syringe (25 ml, filled with 5 ml of 10% formaldehyde) approximately 5-10 cm above spawning females. This ensured immediate fixation of eggs, which was necessary for subsequent sperm staining (Franke et al. 2002). In total, 24 syringe samples were collected. At the end of the dive (~19:30 hrs) the number and sex of spawning and non-spawning urchins within a 4 m$^2$ quadrat were noted. Two replicate measures of current speed were recorded by measuring the transit of drifting particles along the transect line.

Fertilization levels of the syringe samples were assessed in the laboratory. Eggs with an elevated fertilization envelope were scored as fertilized and at least 100 eggs were counted per sample (hereafter “envelope” samples); if less than 100 eggs were present (25% of samples) all eggs were counted. Samples that exhibited >10% fertilization were subsequently stained with Ethidium Homodimer-1 to enable histological
determination of total fertilization, polyspermy and monospermy (Franke et al. 2002). This was important if true reproductive success during this spawning event was to be elucidated, because only monospermic eggs develop into healthy larvae (e.g. Jaffe and Gould 1985, Ernst 1997). On the day following spawning, gonad indices were determined for urchins (n=20) from within the area of the spawning event.

Statistical Analysis

Fertilization Assays

The frequency of fertilizations among bioassays was analysed to determine whether fertilization levels correlated with lunar phase, surge heights, water temperature or presence of phytoplankton (chl. a). These environmental data were obtained from the Leigh Marine Laboratory, where daily surge heights (m), water temperatures (°C) and weekly chl. a (µg L⁻¹) levels are measured. Moon phases were obtained from LunarPhase®. A Pearson’s correlation analysis in Excel® (Zar 1999) was performed correlating surge heights, water temperature and chlorophyll levels separately to the levels of fertilization obtained for a particular date within a spawning season. Each spawning season was analysed separately. Moon phases were plotted for the months that fertilization assays were sampled. Because one of the main objectives of this study was to determine if spawning coincided with high wave-energy conditions, surge heights (m) in relation to data from bioassays and gonad indices were plotted for each separate spawning season to illustrate large-scale patterns between spawning events and water motion.

Data of frequencies of fertilizations detected in treatments and controls were tested to determine if the frequency of fertilizations was like the expected frequencies. Expected frequencies of fertilizations were such that all fertilizations should be
present in treatments and no fertilizations should be present in controls. A goodness-of-fit G-test (log likelihood ratio test) was used for this. Frequency data from all three spawning seasons were pooled for this analysis to achieve a meaningful sample size.

**Gonad Index**

The assumption that gonad index is independent of body size (Gonor 1972) has recently been questioned and re-examined (Bonardelli and Himmelman 1995). This assumption was first tested by regressing size (log\(_{10}\) transformed) against gonad volume (log\(_{10}\) transformed) for each sex and each spawning season. For male and female data of the 1999-2000 spawning season and male data of the 1997-1998 spawning season, the slopes ranged between 3.08 and 3.5. This indicated that the increase in gonad volume with body size was isometric and the calculation of gonad index as gonad volume/body size did standardize for size differences (Bonardelli and Himmelman 1995). However, male and female data of the 1998–1999 spawning season and female data of the 1997-1998 spawning season generated regression slopes ranging between 1 and 2.6. This indicated that the relationship between body size and gonad volume was allometric, and dividing gonad volume by size did not standardize for size differences. The relationship between gonad index and body size was further assessed using linear regression for pooled raw data between sexes and spawning seasons. If the slope of the regression line equaled 0, then this indicated that gonad index was independent of body size (Bonardelli and Himmelman 1995). However, a weak, but highly significant relationship was found between body size and gonad index (\(r^2=0.22, p<0.00001, \text{Fig.2}\)). Hence further statistical analysis of data was necessary, taking size into consideration as a covariate (Grant and Tyler 1983).
Gonad indices and urchin size data for each season could not be consistently transformed to give normality (Table 2). However, for non-normal data the residuals were examined (SAS®) and all exhibited homoscedasticity (Table 2). ANOVA, (SAS®) was subsequently used to determine whether a difference in gonad index between sexes and dates for each spawning season existed. ANOVA has been shown to be robust against violations of normality of data (Zar 1999, McGuiness 2002), and because homoscedasticity of residuals was found for non-normal data (Table 2) the parametric test was applied. Following this, ANCOVA (SAS®) tested differences in gonad indices between dates, with body size as the covariate (Grant and Tyler 1983, Bonardelli and Himmelman 1995) for each spawning season separately. In cases when ANOVA detected a significant difference between sexes (Table 3), ANCOVA (SAS®) was used to analyse data for each sex separately. When body size was found to be a significant covariate a post-hoc multiple comparison Tukeys test (SAS®) was used to assess whether differences in mean gonad index, body size and gonad volume existed between consecutive dates. This determined whether changes in gonad index were due to changes either in gonad volume or body size or both. This was important, because gonad index was not independent of body size, and an observed decrease in gonad index may be due to a decrease in mean body size within the sample and not due to a spawning event.

To determine if the variance of gonad indices observed for each spawning season was related to the variance in body sizes, ANOVA (SAS®) assessed this for each year and sex. Furthermore, raw body size data were pooled for both sexes and compared between years using ANOVA and a subsequent post-hoc multiple comparison Tukeys test (SAS®) to determine between which years body size differed. Also, the variances
of gonad indices at Matheson’s Bay and Waterfall Reef were assessed before and after major spawning events in Dec. 1998 and Jan. 1999.

Box plots were established for gonad index data obtained immediately before and after inferred spawning events. This was to assess whether a decrease in variance of gonad indices was due to loss of possible outliers, i.e. loss of very large gonads due to spawning of a few individuals thus homogenizing the data and in turn reducing the variance. Homogenization of data and reduction of variance would also occur due to synchronized spawning of a whole population.

**In situ spawning observation of a rockflat population at Matheson’s Bay**

Observations on climbing behaviour of spawning females, which elevated themselves upon rocks or higher surfaces, were made throughout this spawning event. Raw means for gonad indices (pooled for both sexes) were compared before and after the spawning observation using t-test (Zar 1999). The variances of gonad indices before and after the spawning event were observed and box plots established for reasons outlined above. From egg counts performed on syringe samples the percentage fertilization in envelope samples and the percentage fertilization, polyspermy and monospermy in staining samples was determined.

**Results**

During a pilot study conducted in March 1997 a spawning event was noted while highly turbulent conditions existed. A significant proportion (between 50 and 80%) of urchins ceased to spawn in response to 0.5 M KCl injections in rockpools adjacent to
Waterfall Reef after cyclone Gavin compared to after the event (t-test, \( p < 0.0001 \), Fig. 3). The proportion of urchins not responding to injection of KCl before the cyclone ranged between 5 and 40% (Fig. 3). Surge heights during this event were between 4-5 m, which were the highest recorded during the 1996-1997 spawning season (Table 5).

**Determination of Egg Longevity**

Eggs aged \( \leq 8 \) hrs. showed fertilization levels between 20-80 \% at \( 10^3 \), \( 10^4 \) and \( 10^5 \) sperm ml\(^{-1}\) (Fig. 4). Eggs aged 8.5 hrs. exhibited fertilization levels <10\% at all three sperm concentrations and only \( \sim \)1\% of eggs older than this were fertilized (Fig. 4). When eggs were aged for 13 hours or longer, no fertilizations were recorded in any of the treatments (Fig. 4). Fresh eggs were placed into the bucket twice a day when monitoring the fertilization assays, hence eggs were able to detect sperm reliably, i.e. exhibiting medium fertilization levels (\( \sim 15-30 \) \%) at low sperm concentrations (\( 10^3 \) and \( 10^4 \) sperm ml\(^{-1}\)), for a total of 16 hours per day. Overall, the level of fertilization decreased with decreasing sperm concentration, and mean maximum fertilization of 80\% was achieved at \( 10^5 \) sperm ml\(^{-1}\) for eggs aged one hour.

**Fertilization Assays**

During the 1997-1998 spawning season, treatments and controls were each measured 121 times. Five fertilization events with fertilization levels ranging between 0.1-0.6\% were detected in treatments (Fig. 5, Table 5). No fertilizations were ever recorded in controls only, but fertilizations were recorded in treatments and controls simultaneously three times with fertilization levels ranging between 0.2-2.4\% (not shown). Surge heights throughout this spawning season were between 0.5-2.6 m.
During the time when most fertilizations were detected in treatments, surge heights were low (0.5-1.5 m) compared to those when no spawning was detected (Fig. 5, Table 5).

For the 1998-1999 spawning season, a total of 184 treatment/control samples were measured. In treatment samples, fertilizations were recorded ten times ranging between 0.03-1.96% (Fig. 6, Table 5) and in control samples fertilization was recorded on three occasions with fertilization levels ranging between 0.01-6% (not shown). Fertilizations were recorded in controls and treatments simultaneously four times ranging between 0.01-25% (not shown). Surge heights throughout this season were between 0.3-2.8 m. The majority of surge heights were 1.5 m and above, when most fertilizations were detected in treatments (Fig. 6, Table 5).

During the 1999-2000 spawning season 233 treatment/control samples were recorded. Fertilization (0.01%) was detected only once in treatments (Fig. 7, Table 5) and twice in control samples. Percentage fertilizations in the controls were 0.4 and 2.7% (not shown). Fertilization was recorded once in treatments and controls at the same time and was ~1% (not shown). Surge heights varied between 0.3-2.5 m throughout this spawning season and the majority of surge heights attained values of ~1 m when fertilization was detected in treatments (Fig. 7, Table 5).

No significant correlation was found between fertilization levels in treatments, and the environmental parameters such as moon phase, water temperature (°C), surge height (m) or phytoplankton abundance (ug·L⁻¹) for the spawning seasons 1998-1999 and 1999-2000. For 1997-1998, a weak, but significant negative correlation was detected.
between fertilization (%) and surge height (m) \((r=-0.226, p=0.05)\); however, all other environmental parameters showed no significant correlation to the percentage fertilization detected for this spawning season.

The log likelihood ratio G-test showed that the frequency of fertilizations present in treatments and controls only were not like the expected frequency of all fertilizations present in treatments and no fertilizations present in controls \((p<0.001)\).

**Gonad Index**

For the 1997-1998 and 1998-1999 spawning seasons significant differences in gonad index among dates were found \((\text{ANOVA, } F=7.43, F=16.98, p<0.0001, \text{ Table 3})\), but no difference in gonad indices existed between sexes \((p<0.36 \text{ and } 0.088 \text{ respectively})\). However, for the 1999-2000 spawning season gonad indices differed between sexes \((\text{ANOVA, } F=17.36, p=0.0001)\) as well as among dates \((\text{ANOVA, } F=9.62, p=0.0021)\). For all three spawning seasons, urchin size was a significant covariate \((\text{ANCOVA, } F=135.5 \text{ for } 1997-1998, F=74.9 \text{ for } 1998-1999, F=66.36 \text{ (female) and } F=85.29 \text{ (male) for } 1999-2000; \ p<<0.01 \text{ in all cases, Table 3})\).

**Inferring Spawning Events from**

**Fertilization Assays and Gonad Index Analysis**

Statistical analysis of the frequency of fertilization present in treatments and controls showed that controls contained more fertilizations than expected.

In addition, analysis of gonad indices demonstrated that size was a significant covariate, which indicated that in some instances significant changes in gonad indices
may be due to changes in body size, and not changes in gonad volume only (Table 4).

For example, for the 1997-1998 spawning season, a significant decrease in gonad index occurred between the 22\textsuperscript{nd} –29\textsuperscript{th} Dec. 1997 (Fig. 5, Table 4); this decrease was associated with a significant decrease in body size (Table 4). Also, during the 1999-2000 spawning season, an increase and then a decrease in female gonad index was observed between the 4\textsuperscript{th} -17\textsuperscript{th} Jan. 2000, again, most likely due to similar changes in body size (Table 4). Thus, to infer a spawning event from a decrease in gonad size between two dates, a significant decrease in body size should not be detected. Ideally, to be able to deduce a spawning event reliably, data from gonad index analysis and fertilization assays need to indicate spawning at the same time. As a result, a spawning event was inferred the following ways. If a significant decrease in gonad index occurred, which could not be attributed to a significant decrease in body size, and/or if the variance in gonad index decreased, a spawning event was designated as such. However, this was only done, if either of the two gonad index responses coincided with fertilizations in the treatment bioassay and/or a direct spawning observation and/or both. A decrease in gonad index variance was also used to deduce a spawning event because during an observed sub-population spawning event no significant decrease in gonad index could be detected, however, the variance in gonad index did decrease (please refer to section at a later stage titled “\textit{in situ} spawning of an urchin barren population at Matheson’s Bay”).

During the 1997-1998 spawning season a significant decrease in mean gonad index occurred between the 22\textsuperscript{nd}-29\textsuperscript{th} Dec. 1997 (Tukey’s test \(p<0.0001\), Fig.5, Table 4 and 5). While this may in part be due to a mean decrease in size of urchins (Table 4),
various other indicators of spawning demonstrated that a spawning event took place. Box plot analysis of gonad index data shows that outliers of extremely large gonads were lost between the two dates and that the variance was reduced (Fig 8 A). Because three male urchins have been observed to spawn in the vicinity of the intake pipe on the 27th Dec. 1997 (Heltzel, pers. com., Fig. 5, Table 5), this could have lead to large gonads being reduced in size, homogenizing the data and in turn reducing the variance from 30 to 3 (Fig. 8 A). Also, fertilizations (0.6%) were detected in the bioassay on the 23rd Dec. 1997 (Fig. 5). This definite trickle and possibly even subpopulation spawning was detected around the full moon, and surge heights were one of the lowest for this season, ranging between 0.6-0.7 m. (Fig. 5, Table 5).

Even though other fertilizations were recorded throughout that spawning season (Fig. 5, Table 5), none of them were associated with a significant decrease in mean gonad index. However, the variance of gonad indices decreased between the 9th – 12th Jan. 1998 and between the 19th-24th Jan. 1998. Again, large outliers were lost after the inferred spawning event (Fig. 8 B and C respectively), and fertilizations were present in the bioassays between both sets of data (Fig. 5). Similar to the spawning event noted on the 27th Dec. 1997, trickle or subpopulation spawning occurred.

Three additional decreases in gonad index variance were detected during this spawning season, however, neither of them were associated with either fertilizations in the bioassay, and/or a direct spawning observation, hence no other spawning event was inferred.
Overall, during 1997-1998, the variances (expressed as standard deviations, which equal the square root of the variances) of gonad indices were highest in both sexes (Fig. 5) compared to the other two spawning seasons. While this may be due in part to the larger variance in size due to the inclusion of size classes not present in the other two spawning seasons (Table 1), the large standard deviations per se indicated continuous sub-population spawning throughout the season, i.e. some urchins were ready to spawn, while others had just spawned.

During the 1998-1999 spawning season, three significant decreases in gonad indices occurred. One took place between the 21st-28th Dec. 1998 (Fig. 6) and the variance decreased from 15 to 14 (Fig. 8 D). During this time fertilizations (0.1%) was detected on the 26th Dec. 1998 (Fig. 6, Table 5). Both indications suggest a subpopulation or even epidemic spawning event. Surge height on this day was 1.4 m and 1.5 m and the moon was around its first quarter (Table 5).

Another decrease in gonad index was detected between the 4th-5th Jan. 1999 and fertilization (1.17%) was observed on the 5th Jan. 1999 (Fig. 6, Table 5). The variance in gonad indices decreased from 17 to 13 (Fig. 8 E). Again, a subpopulation or epidemic spawning event can be inferred from these observations, which occurred three days after the full moon and surge height was 0.8 m. (Table 5).

The third significant decrease in gonad index in 1998-1999 was detected between the 17th-26th Jan. 1999 at two sites, Matheson’s Bay and Waterfall Reef (Fig. 6). Two fertilization events (0.7 and 6%) were detected on the 23rd and 24th Jan. 1999 respectively. The latter was the highest fertilization recorded in treatments throughout
all three seasons. The variances in gonad indices decreased from 7 to 4 at Matheson’s Bay and from 23 to 6 at Waterfall Reef after the spawning event (Fig. 8 F and G respectively). Significant decreases in gonad index in two locations, which are approximately 5 km apart indicated a reef (epidemic) spawning. The moon was in its first quarter and surge heights ranged between 2.3-2.4 m. on the 23rd and 24th Jan. 1999. These were among the highest recorded during this season (Table 5).

Three additional decreases in gonad index variance were detected during this spawning season, however none of them were associated with a direct spawning observation nor fertilization in the bioassay, and thus, no further spawning event could be deduced.

During the 1999-2000 spawning season no significant decreases in gonad index of both sexes simultaneously occurred (Fig. 6, Table 5), although significant decreases in male and female gonad indices separately were recorded (Table 4). Also, the variance of gonad indices decreased six times. However, neither of them was associated with fertilizations in the assay, nor a direct spawning observation, thus no spawning event was inferred from this data. A decrease in gonad index variance from 9.1 to 5.1 was detected between the 6th-14th Mar. 2000 (Fig. 8 H) and fertilization (0.1%) in the bioassays was detected on the 10th Mar. 2000 (Fig. 7). During this possible trickle or subpopulation spawning surge height attained 1.8 m and the moon was 4 days into the new cycle (Fig. 7, Table 5).
In situ spawning of an urchin barren population at Matheson’s Bay

Spawning Densities and Environmental Data

36% (32 of 89) of urchins were spawning along the transect at ~18:00 hrs. Spawning density was 2.2 m⁻² (32 urchins over 14.4 m²). Nineteen of the spawning urchins were males and 13 were females. At ~19:30 hrs. 23.5 % (12 of 51) of urchins were spawning within the 4 m² quadrat and spawning density was 3 m⁻² (12 urchins over 4m²). Ten were male and two were female. The two estimates of current speeds averaged to 5 cm sec⁻¹ only and recorded surge height was 1 m. The event occurred one day before the full moon and low tide was at 13:45 hrs.

Fertilization and Gonad Index

Fertilization levels assessed in “envelope” and “staining” samples ranged from 0-47.5% (envelope samples) and from 0-34.1% (staining samples, Table 6). Polyspermy was not detected in any of the staining samples. No significant decrease in gonad index after the event was observed compared to indices 11 days prior to the sub-population spawning (t-test p=0.19, Fig. 9). However, the variance decreased from 7.5 to 4.6, due to loss of extreme high and low gonad indices (Fig. 8 I).

Discussion

Testing assumptions of the Gonad Index

Before examining the patterns of spawning exhibited in E. chloroticus, it is important to note that methods widely used in assessing the timing and periodicity of spawning events (i.e. gonad index assessment) need careful examination before any conclusions on the occurrence of such an event can be drawn. In this study, even though some gonad index decreases can be accurately interpreted as spawnings, others would have
indicated a spawning event where the decrease was due to sub-sampling individuals of smaller sizes. For example, during the 1997-1998 spawning season, a decrease in gonad index between the 22nd-29th Dec. 1997 was possibly in part due to a decrease in urchin size, even though three male urchins were also observed spawning in the field around the intake pipe during that time. Also, during the 1999-2000 spawning season, a decrease in female gonad index between the 10th-17th Jan. 2000 was detected. Again, a significant decrease in body size also occurred, making it impossible to distinguish if the decrease is due to a drop in gonad volume (and thus spawning) or body size.

Therefore, the assumption that gonad index measures are independent of body size and that dividing gonad volumes by body size standardized gonad measures needs testing. This is particularly important if accurate sampling of individuals is inherently difficult (e.g. strong swell conditions), or if body size varies within a population where only a finite amount of individuals are available, so that individuals of a particular size class cannot be reliably obtained. Multivariate analysis followed by post-hoc multiple comparison tests have been for the most part successfully used in this study to differentiate between decreases in gonad index due to real spawning events or decreases in body size.

**Spawning patterns observed in E. chloroticus**

Fertilization levels in bioassays were often very low (i.e. ~ 1%) and this was most likely attributable to the strong dilution that sperm experienced when transported up the 80 m pipe to the bucket in which eggs were held. Hence, fertilization levels detected in the fertilization assays most likely do not reflect those from an *in situ*
spawning event. Also, frequencies of fertilizations obtained in controls and in treatments showed not to be like the expected frequencies, thus fertilizations in treatments could in some cases indicating contamination of eggs with sperm. However, the fertilizations in treatments were useful in confirming spawning events, when a significant decrease in gonad index or its variance was detected. Even though a decrease in variance of gonad indices occurred many times throughout the three spawning season, with no other indicator of spawning associated with it, it was used as a spawning indicator, because a direct spawning observation was associated with a decrease in gonad index variance only during 1998-1999. As a result, a hierarchy of reliability for inferring spawning events can be established and was used in this study. The most reliable indicator of a spawning event is actual observation of such, which occurred twice throughout this study. Next, a combination of a significant decrease in gonad index and fertilizations present in bioassay were also used to reliably infer a spawning event. This occurred five times throughout all three spawning seasons. Lastly, a combination of gonad index variance decrease and fertilizations in bioassays were used to deduce a spawning event and occurred three times throughout this study. However, the latter needs to be viewed with some caution, because, decreases in gonad index variance was detected many other times throughout the three spawning seasons, and fertilizations were present in controls more often than expected.

Despite some of the potential shortcomings of few of the methods used in this study to deduce a spawning event, several discrete spawning patterns were apparent in *E. chloroticus.*
Sub-population spawning seemed to be the most common occurrence. This was indicated by two findings. Firstly, the high levels of variation in gonad index, which prevailed throughout all three spawning seasons, but was particularly apparent during 1997-1998, indicated that some urchins were ready to spawn, while others had just spawned. Similar conclusions have been drawn for two scallop species (Styan and Butler 2003) as well as New Zealand South Island populations of *E. chloroticus* (Brewin *et al.* 2000). Secondly, confirmed indications of subpopulations spawnings in this study, i.e. a decrease in gonad index variance associated with fertilizations in the bioassay and/or direct spawning observation, were detected four times throughout the three spawning seasons. The lack of correlation between decreases in gonad index and sub-population spawning has been reported previously for *E. chloroticus* (Dix 1970). In addition, observations of sub-population spawnings have been made for *E. chloroticus* South Island populations (Brewin *et al.* 2000) as well as urchins from the North Atlantic (Wahle and Gilbert 2002).

A second pattern that could be recognized was that when high wave energy or turbulent conditions arose, epidemic spawning (i.e. whole population spawning) and/or sub-population spawning can occur. A highly significant drop in gonad index at two locations (Matheson’s Bay and Waterfall Reef) and fertilizations in the bioassays were associated with such a spawning event. Surge heights attained some of the highest values recorded for the 1998-1999 spawning season, yet fertilization in bioassay was the highest recorded during all three seasons. In addition, failure to spawn in response to KCl injections indicated a spawning event in March 1997 during the highest wave-energy conditions recorded during that particular season. However, it could not be determined whether this spawning occurred over a large scale, because
urchins were injected at one site only. Spawning in response to KCl injection has previously been correlated to ripeness of gonads (King et al. 1994), therefore a decreases in spawning response to KCl injection can reliably indicate a decrease in gonad volume and thus spawning. Release of gametes in response to storms or strong swell has previously been inferred in *E. chloroticus* (Mead 1996, Keuskamp 1997) and rough weather has been associated with spawning in other broadcast-spawning marine invertebrates (e.g. Grange 1976, Creese and Ballantine 1983, Desrosiers and Dubé 1993). An epidemic spawning under calm conditions in January 1994 has been reported for *E. chloroticus*, but this occurred in Fiordland, New Zealand, where high wave action is always absent (Lamare and Stewart 1998). Evidence from urchins in California and Guam suggests that spawning densities can also be high under calm to moderate conditions (Pennington 1985). This is important, because cyclones or extremely rough weather are a rare occurrence and were not consistently linked to spawning events in this study. For example, during the 1998-1999 spawning period, two significant decreases in gonad index and associated fertilizations in the bioassay were observed when surge heights were only 0.8 m and 1.5 m. These could have represented reef spawnings, but could not be confirmed as such, because one site was sampled only. Also, during the 1999-2000 spawning season rough weather conditions occurred, but no epidemic spawning was detected.

The few fertilizations observed in the bioassays during 1999-2000 compared to previous years may be due to the decrease in urchin abundance around Waterfall Reef over the course of this study (Shears and Babcock 2002, 2003). However, this does not explain the absence of asynchronous decreases in gonad indices in both sexes during this season.
The third spawning pattern that was recognized in this study was trickle spawning. Trickle spawning differs to subpopulation spawning, in that males only are spawning, which was observed on the 27th Dec. 1997. Associated with this direct observation of three males spawning were a decrease in gonad variance due to loss of extremely large outliers, and fertilizations in the bioassay. These were similar indicators which have been associated with an observed subpopulation spawning event in this study. The two indicators have been observed three times throughout this study (excluding the actual spawning event observed on the 1st Mar. 1999), and trickle spawnsings are well reported in the literature (e.g. Pennington 1985, Pearse et al. 1988, Babcock et al. 1992).

Overall, spawning events in *E. chloroticus* were similar to observations of urchins overseas in that they were variable and unpredictable (e.g. Pennington 1985, Pearse 1990). This was supported by the fact that spawning events did not seem to correlate to any particular environmental parameters. We propose that the variability in spawning densities and spawning behaviors observed under different environmental conditions in this study is real and not an observational artifact. Animals are likely to need to respond to the wide range of environmental variables that they are constantly subjected to. The use of a range of spawning cues that possibly interact in various ways may constitute a form of bet hedging which ensures that spawning during any particular season occurs during favorable conditions each season, even if those conditions are not optimal in terms of conditions that may be experienced over longer periods of time.
Fertilization success during different spawning events in *Evechinus chloroticus*

The fertilization parameters measured showed that monospermy, i.e. eggs entered by one sperm only, and unfertilized eggs only were observed. Polyspermy, i.e. eggs fertilized by more than one sperm which consequently die, was not detected. This is important, because any eggs experiencing polyspermy will not develop into healthy larvae, whereas this is still possible for unfertilized eggs when fertilized at a later stage. Sub-population spawning under calm to moderate environmental conditions are possibly sufficient to produce enough offspring, even though sperm appear limiting during these events. However, when comparing the fertilization levels obtained here under open ocean conditions (0-34.1%, staining) with those obtained in rockpools (0-96.1%, staining, Franke *et al.* 2002), it becomes clear that fertilization success can still be high even though sperm are limiting, because polyspermy increases exponentially once fertilization levels are >50% (Fig. 4 in Franke *et al.* 2002). Thus it may be advantageous for eggs not to be fertilized immediately during a spawning event, because this may subject them to too many sperm. This may be particularly relevant, considering that eggs of urchins as well as other invertebrates are fertilizable for a long time (e.g. 8 hours, this study; 2-3 days for *Strongylocentrotus droebachiensis*, [Meidel and Yund 2001], 3-4 days in the polychaetes *Arenicola marina* and *Nereis virens*, and 4-8 hours for the starfish *Asterias rubens*, [Williams and Bentley 2002]). Furthermore, during natural spawns eggs are held within the spines (Thomas 1994, pers. obs.), possibly ensuring that the majority of eggs do not drift off in the current instantly. As a result, fertilization success in a subpopulation spawning that lasts for at least 2-3 hours (e.g. this study, Lamare and Stewart 1998) may in fact be much higher. Thus, increased egg longevity, persistence of eggs within the close vicinity of release and long population spawning times may negate the need
for eggs to be fertilized shortly after release from the adult female and significant fertilization success may be achieved by low to medium sperm concentrations.

Reef wide or epidemic spawnings in response to strong environmental conditions must also ensure efficient fertilization and subsequent larval production. Turbulent conditions may ensure good gamete mixing (Denny et al. 1992), and possibly counteract the high frequency of polyspermy that can occur in this species when spawning under calm and shallow conditions (Franke et al. 2002). However, measures of fertilization rates under these conditions are necessary to draw definite conclusions in this regard. Collecting fertilization data during high wave energy events is inherently difficult, but possible. For example, methods used by Wahle and Gilbert (2002), where eggs are held in protective containers within the ocean among urchin populations, may prove useful to measure fertilization levels in situ under strong turbulent conditions. Furthermore, water mixing possibly produces favorable conditions for subsequent larval development, such as mixing of nutrients, oxygenation of water and large-scale larval transport.

Trickle spawning per se, particularly by males only as demonstrated in this study, is possibly not sufficient for subsequent larval production. However, arguments have been raised that sperm competition among males during free-spawning events (Yund and McCartney 1994, Yund 1998) may cause males to release sperm before the female (Levitan submitted), a phenomenon commonly observed among broadcast-spawners (e.g. Thorson 1950, Babcock et al. 1992, pers. obs.). Thus, trickle spawning may be an attempt for males to fertilize eggs before other males do, in case a
subsequent spawning event takes place. Furthermore, trickle spawnings may also indicate subsequent subpopulation or even epidemic spawning events.

Clearly, the attempt to balance sperm limitation as well as sperm competition during natural spawning events, as well as the interdependent relationship between water turbulence and spawning density demonstrated in this study, are not the only factors influencing spawning events and subsequent fertilization success however. Many other factors such as water depth (Levitan 1998), habitat structure (Wahle and Peckham 1999), and gamete characteristics (Thomas 1994, Young 1994, Levitan 1995, 2002) influence fertilization rates. Partitioning out the relative importance of each of these parameters represents the real challenge in determining the evolutionary importance of spawning behaviors for fertilization ecologists in the future. A mathematical modeling approach or Geographic Information Systems (GIS) may prove useful to determine these, because they allow explicit spatial and temporal examination of these questions.
**Figures**

**Figure 1**
Sampling sites within and outside the Leigh Marine Reserve. ● indicates sampling sites of urchins at Matheson’s Bay and Waterfall Reef. ○ designates the location of the bucket situated at Leigh Marine Laboratory (■). □ shows site of natural spawning event at Matheson’s Bay.

**Figure 2**
Pooled raw data of gonad index between all three spawning seasons and both sexes (N=1098) was regressed against test diameter (mm). A weak, but highly significant regression was found ($r^2 = 0.22$, $p < 0.00001$, $F=302$). The slope of the regression line (b) equaled to 0.388. The dotted lines indicate 95% confidence intervals.

**Figure 3**
The proportion of urchins (%) inhabiting rockpools at Waterfall Reef within the Leigh Marine Reserve spawning in response to KCl (0.5M) injection before and after the occurrence of Cyclone Gavin which in March 1997. □ are males, ■ females and ▲ represents urchins for which sex was unknown. The number associated with each bar indicates the total number of urchins injected.

**Figure 4**
Raw mean fertilization rates obtained at different sperm concentrations (—●— 10^3, – ■ – 10^4 and ▲ 10^5 sperm ml^-1) at different gamete ages (hrs.). Closed symbols are samples for which n=2. Open symbols represent samples for which n=1.
Figure 5
Mean gonad indices (total n=30), fertilization events (%) and surge height (m) of the spawning period 1997-1998. Error bars are ± 1 S.D. ● represents mean male, ■ represents mean female gonad index of urchins from Matheson’s Bay. ▼ indicate fertilizations (%, 2, 4 and/or multicell) in treatments. Note that mainly no fertilizations were obtained in treatment samples. The boxed area in black indicate periods of a significant decrease in mean gonad index and associated with a fertilization present in treatment vials only. It marks a definite spawning event designated as such by the fact that all these three factors co-occurred, and that a direct observation of spawning was obtained. The boxed areas in grey indicate periods where a decrease in gonad index variance occurred, which was associated with a fertilization in the bioassay. These were designated as potential spawning events, because decreases in gonad index variance occurred at other times throughout this spawning season, and contamination of controls occurred more times than was expected throughout all three spawning seasons.

Figure 6
Mean gonad indices (total n=20), fertilization events (%) and surge height (m) for the spawning period 1998-1999. Error bars are ± 1 S.D. Circles represent mean male and squares represents mean female gonad indices. Black symbols represent indices from Matheson’s Bay and grey symbols indicate indices from Waterfall Reef within the Leigh Marine Reserve. ▼ indicates fertilizations (%, 2, 4 and/or multicell) in treatments. The boxed area in black indicate periods of a significant decrease in mean gonad index and its variance with which a fertilization present in treatment vials only was associated. It marks a definite spawning event designated as such by the fact that
all these three factors co-occurred, and/or that a direct observation of spawning was obtained.

Figure 7
Mean gonad indices (total n=20), fertilization events (%) and surge height (m) during the spawning period 1999-2000. Error bars are ± 1 S.D. Circles represent mean male, and squares represents mean female gonad indices. Black symbols represent indices from Matheson’s Bay and grey symbols indicate indices from Waterfall Reef. ▽ indicate fertilizations (%), 2, 4 and/or multicell in treatments. A potential spawning event was inferred (boxed area in grey), because a decrease in the variance of the gonad index and fertilizations (%) were detected in treatments between indicated dates.

Figure 8 A-I
Box plot representation (SASS®) for gonad indices before and after spawning events for which dates and location are given. Numbers in brackets underneath the box plots indicate the variance of the particular data set. The results of gonad index analysis and fertilization assays in response to a spawning event are indicated. A decrease in variance occurred every time a significant decrease in gonad index was observed (A, D, E, F, G,) or when a spawning event was observed in the field (I). Thus, if no direct observation of spawning was obtained and no significant decrease in gonad index was detected, a spawning event was inferred when a decrease in gonad index variance was associated with fertilization in the bioassay (H). A decrease in gonad index variance was detected several other times throughout three the spawning seasons (please refer to text), however, none of them were associated either with a direct observation of
spawning, nor fertilizations in the bioassay, hence no other spawning event was deduced from them.

Figure 9

Gonad indices for urchins before and after the observed spawning event on the 1st March 1999. ● represents mean male, ■ mean female gonad index. Bars are ±1 S.D. Above the data, the number of urchins for which gonad index was assessed in each sample is indicated. Urchins were collected from barrens habitat.
Fig. 1
Fig. 2

Test diameter (mm)

Gonad Index
Fig. 3

Spawning Individuals (%)

Date (March 1997)

Cyclone Gavin

- 5
- 6
- 7
- 8
- 9
- 10
- 11
- 12
- 13
- 14
- 15
- 16
- 17
- 18
- 19
- 20

- 5
- 6
- 7
- 8
- 9
- 10
- 11
- 12
- 13
- 14
- 15
- 16
- 17
- 18
- 19
- 20
Sighting of 3 males spawning at Waterfall Reef

Date (1997-1998)
Fig. 6

Mean gonad indices

Date (1998-1999)

October  December  January  February  March

Natural Spawning of urchins in urchin barrens at Matheson's bay

Surge height (m)

Fertilization (%)
Fig. 7

Mean gonad index

Surge height (m)

Date (1999-2000)

Fertilization (%)
Fig. 8  

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- **Date**
- **Location**
- **Gonad Index response**
- **Results from Fertilization Assay**
- **Additional Information**
  where necessary

- 22nd-29th Dec. 1997
- Matheson's Bay
- significant decrease in GI and body size
- fertilization in bioassay
- sighting of 3 males spawning

- 9th-12th Jan. 1998
- Matheson's Bay
- no significant decrease in GI
- fertilization in bioassay

- 19th-26th Jan. 1998
- Matheson's Bay
- no significant decrease GI
- fertilization in bioassay
- Dec. 1998 - Matheson’s Bay - significant decrease in GI fertilization in bioassay

- Jan. 1999 - Matheson’s Bay - significant decrease in GI fertilization in bioassay

- Jan. 1999 - Matheson’s Bay - significant decrease in GI fertilization in bioassay

- Jan. 1999 - Waterfall Reef - significant decrease in GI fertilization in bioassay

- March 1999 - Matheson’s Bay - no significant decrease in GI fertilization in bioassay - observation of subpopulation spawning

- Mar. 2000 - Matheson’s Bay - no significant decrease in GI fertilization in bioassay
Fig. 9

Mean Gonad Index

Date (2000)

February
March

natural spawning
Tables

Table 1
Summary of size data from all urchins collected for gonad index assessment. Data given for spawning seasons 1997-2000 are from urchins collected form shallow mixed algal habitat at Matheson’s Bay and Waterfall Reef. * indicates that mean size classes (data of both sexes were pooled) differed between seasons (ANOVA F=152, p<0.0001).

Table 2
Summary of normality tests for each sex and spawning season of gonad index and test diameter. Data are from urchins inhabiting the shallow broken rock habitat at Matheson’s Bay. Table entries indicate whether data were normal or not. Bold letters represent results of arcsin squareroot transformed data. Before transformation, gonad index as well as body size data were converted to proportions (i.e. divided by 100). Furthermore, body size data were also log_{10} +1 transformed, but the transformed data still did not confine to assumptions of normality. For non-normal data, residuals were checked for homoscedasticity. H.S. indicates that residuals of data were homoscedastic.

Table 3
Results of ANOVA and ANCOVA analysis of gonad indices (dependent variable) vs. week and sex (independent variable). Body size was used as the covariate in the ANCOVA analysis. * indicates a significant effect (p<<0.01 in all cases), n.s. indicates a non significant effect.
Table 4

Significant increases or decreases detected by Tukey’s test of gonad indices, gonad volumes and test diameters between consecutive dates. Raw data were analysed for each spawning season with data pooled between sexes for the spawning season 1997-1998 and 1998-1999. ANOVA analysis showed a difference between sexes for the spawning season 1999-2000, hence analysis was performed for each sex separately. △ indicates a significant increase, ▽ a significant decrease of the particular category examined. ▼ indicates a significant decrease in gonad index independent of a decrease in body size. These were associated with fertilizations in the bioassay and thus designated as definite spawning events. ▼* indicates significant decreases in gonad index, not associated with a significant decrease in body size. These were not equated with a spawning event however, because no fertilizations in the bioassays were associated with them. ▼? indicates a significant decrease in gonad index which was associated with a significant decrease in body size. In this case, it could not be determined whether the decrease in gonad index was due to a spawning event or a decrease in body size, because a spawning event in situ had been observed and fertilizations were detected in the bioassays.

Table 5

Summary of all data collected from fertilization assays, gonad indices and spawning observations, for the spawning season 1996-1997, 1997-1998, 1998-1999 and 1999-2000. Dates of all fertilizations (%) detected in treatments as well as significant decreases and increases in gonad index and/or its variance at Matheson’s Bay and Waterfall Reef are listed. Gonad index drops not associated with a decrease in body size are listed only, unless stated otherwise. n.a. indicates categories, which were not
applicable, because no survey had been conducted for that particular season. Environmental parameters of surge heights (m) on days when fertilizations (%) were detected and the range of the surge height for the whole spawning season are designated. Furthermore, the phase of the moon at the particular dates is indicated as the number of days b.n. (before new), a.n. (after new), b.f. (before full) and a.f. (after full). Rows indicated in bold represent definite spawning events, which could be inferred as such either through direct observation of these, or significant decreases in gonad index associated with a fertilization in the bioassays. Possible spawning types deduced as such from decreases in gonad index and/or its variance associated with fertilization in the bioassay, are indicated with a question mark. Some instances of fertilizations in bioassays or decreases of gonad indices and their variances were not designated a spawning type, because only one of the indicators of spawning were present. For completion purposes they have been listed here, because they might indicate other spawning events.

Table 6
Fertilization rates (%) of envelope and staining samples obtained from the natural spawning event recorded on the 1st of March 1999 for an urchin barren population at Matheson’s Bay.
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<td>57-81</td>
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<td>61-88</td>
<td>62.5-89.5</td>
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<td>70-88</td>
<td>62.5-85.5</td>
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<td><strong>Mean (mm)</strong></td>
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<td>68</td>
<td>75</td>
<td>76</td>
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<td><strong>Standard Deviation</strong></td>
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Table 2

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<td>99-00</td>
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Table 3

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<tr>
<td>Weeks (ANOVA)</td>
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<td>Sex (ANOVA)</td>
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<td>Size (ANCOVA)</td>
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### Table 4

|-----------------------|-----------|-----------|-----------|------|--------|


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<tr>
<th>Spawning season</th>
<th>Date and fertilization (%)</th>
<th>In situ spawning observation</th>
<th>Dates of significant decreases in GI and/or its variance at Matheson’s Bay</th>
<th>Surge height (m)</th>
<th>Surge range (m)</th>
<th>Moon phase</th>
<th>Type of spawning</th>
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<td>1996-1997</td>
<td>n.a.</td>
<td>10.-13.3.97 Rockpool urchins cease to spawn in response to KCL injection</td>
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<td>4.5</td>
<td>0.4-5</td>
<td>2 a. n.</td>
<td>Subpopulation or Reef?</td>
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<td>1997-1998</td>
<td>23.12.97 (0.6)</td>
<td>22.-29.12.97 (but body size also decreased!) + decrease in GI variance</td>
<td>0.7</td>
<td>6 b.n.</td>
<td>(3rd quarter)</td>
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<td>Trickle or subpopulation?</td>
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<td>27.12.97 3 males, Waterfall Reef</td>
<td>9.-12. 1. 98 Decrease in GI variance only</td>
<td>0.6</td>
<td>2 b.n.</td>
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<td>29.12.97-5.1.98 Increase in GI and GI variance</td>
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<td>2.1.98 (0.1)</td>
<td>29.12.97-5.1.98 Increase in GI and GI variance</td>
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<td>11.1.98 (0.3)</td>
<td>9.-12. 1. 98 Decrease in GI variance only</td>
<td>0.6</td>
<td>2 b.f.</td>
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<td>22.1.98 (0.1)</td>
<td>19.-26. 1. 98 Decrease in GI variance only</td>
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<td>6 b.n.</td>
<td>(3rd quarter)</td>
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<td>1998-1999</td>
<td>11.12.98 (0.1)</td>
<td>7.-15.12.98 No decrease in GI, Increase in GI variance</td>
<td>0.8</td>
<td>7 b.n.</td>
<td>(3rd quarter)</td>
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<td>26.12.98 (0.1)</td>
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<td>1.4-1.5</td>
<td>4 a. n.</td>
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<td>29.12.98 (0.2)</td>
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References


ACKNOWLEDGEMENTS

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CHAPTER 5

This Chapter is in preparation for submission to Marine Biology titled Fertilization Kinetics and the Importance of Polyspermy in the Broadcast-spawning starfish *Coscinasterias muricata*

Photo by Dr. Russ Babcock
Males (3) and female (1) *C. muricata* spawning after injection with 1-Me-Ade
Abstract

Laboratory fertilization kinetics experiments using the forcipulate asteroid *Coscinasterias muricata* assessed the influence of sperm concentration, gamete age and sperm-egg contact time on total fertilization, polyspermy and monospermic fertilization. Mean polyspermy levels as high 76% were recorded and polyspermy was found to be more sensitive to sperm concentration than total fertilization or monospermy. In addition, polyspermy was also found to occur under sperm-limiting conditions, as predicted by a mathematical fertilization kinetics model which incorporates polyspermy as well as sperm limitation and is an extension to fertilization kinetics model used frequently in the past. Model predictions and experimental results correlated very well ($r^2$ between 0.7-0.9), providing further confirmation of the theoretical model. Methods of assessing fertilization, polyspermy and monospermy based on egg or embryo development, were less accurate than samples where sperm was stained within eggs using the DNA specific stain Ethidium-Homodimer-1. This indicated that staining is the preferred method. The significant levels of polyspermy shown in this study provide further evidence for the potential of polyspermy to be an important driving force in the evolution of many aspects in reproductive ecology, such as egg size, degree of spawning aggregation, timing of spawning and the rapid evolution of gamete recognition proteins.
Introduction

Many aspects of reproduction in marine organisms, such as degree of spawning aggregation, timing of spawning, evolution of egg size and mode of larval development have recently been suggested to be influenced by polyspermy (Franke et al. 2002, Chapter 3). Sperm limitation has also been shown to be an important factor influencing reproductive success in marine organisms and it will interact with these same reproductive processes (e.g. Levitan 1998). For example, if aggregation is too great, polyspermy due to excess sperm concentration may lead to reduced fertilization success. If individuals are too sparsely distributed, sperm will be too dilute, once again reducing fertilization success. Importantly, theoretical models predict, and results confirm that polyspermy will be important even when sperm is limiting (Styan 1998). Recent mathematical modeling (Gavrilets 2000, Frank 2000) as well as the theory of intergenomic antagonistic co-evolution (Vacquier et al. 1997, Rice and Holland 1997, Rice 1998) suggest that polyspermy may be a driving force producing rapid divergence in traits responsible for gamete recognition. This divergence may in turn facilitate speciation.

Few in situ measurements of polyspermy levels have been obtained for marine organisms, with the exception of algae (Brawley 1992, Serrão et al. 1999). However, the recent report on the frequent occurrence of polyspermy in situ for *Evechinus chloroticus* under simulated spawning conditions (Franke et al. 2002) and the controlled quantification of polyspermy in the laboratory (Chapter 3) call for further examination of this phenomenon, and assessment of its occurrence and evolutionary importance in other broadcast spawning taxa.

Natural spawning observations are inherently difficult to make, due to the often unpredictable nature of such events; hence, the opportunity to collect gametes and quantify polyspermy under natural conditions is rare. Specifically, the timing of natural spawning in *Coscinasterias*
*muricata* is not well known, although this species sometimes spawns in spring in conjunction with other species on the southern coast of Australia (Styan and Melville pers. com.). In addition, individuals of *C. muricata* have been observed to spawn (Barker pers. Com, McClary pers. Com.), however, these spawnings are unlikely to generate sufficient larval supply for subsequent population growth (but refer to Chapter 4 for suggested benefits of this type of spawning).

Consequently, this study assesses the occurrence of total fertilization, polyspermy and in turn monospermy under controlled laboratory conditions in *Coscinasterias muricata*, including a comparison of assessment methods. The comparison provides further verification of whether staining is the only method of reliably inferring polyspermy. This is important, because many studies have equated irregular division or death of larvae with polyspermy (e.g. Encena II *et al.* 1998, Benzie and Dixon 1994, Williams *et al.* 1997, Buchanan 1998). However, this may be a false assumption, because bacterial contamination of samples, oxygen deprivation, fungal growth etc. can also cause abnormal division and/or development or even death of embryos (e.g. Bardach *et al.* 1972). This study also tests the mathematical fertilization kinetics model (Styan 1998), which incorporates sperm limitation as well as polyspermy. The model is an extension of the widely used fertilization kinetics model originally proposed by Vogel *et al.* (1982) and testing provides further evaluation of this model.

**Materials and Methods**

**Collection of Animals and Gametes**

Adult *Coscinasterias muricata* occur sub-tidally in New Zealand waters and were collected using SCUBA on the coastline of northeast New Zealand. Upon collection, animals were supplied with running seawater, and fed bivalves throughout captivity. Animals were used for experiments within one week of collection. A total of 15 experiments were conducted from
the 3rd of November until 8th of December 1997. These months fall within the time where
peak gonad indices have been observed for this species in the South Island, New Zealand
(Crump 1969) and Babcock (pers. com.) has successfully induced animals from the North
Island to spawn during this time. For each experiment a separate pair of adults were used.

Gonads were dissected from an arm of a female \textit{C. muricata} and placed into a glass dish to
which approximately 20 ml of $10^{-3}$ M 1-Methyl-Adenine (hereafter 1-MeAde) was added.
After 45 min. eggs were examined to assess maturation stage, i.e. presence/absence of the
germinal vesicle. If more than 50% of eggs were still immature after one hour of being placed
into 1-MeAde the experiment was terminated, and a new female chosen. If the eggs were
suitable, gonads were removed from the glass dish and egg concentration determined.
Subsequently, male gonads were removed from an arm of \textit{Coscinasterias} and placed in a
different glass beaker to which approximately five ml of $10^{-3}$ M 1-MeAde was added. Once
approximately $10^{9-10}$ sperm ml$^{-1}$ were present, male gonads were removed from the beaker
and the precise sperm concentration of the solution (hereafter “sperm stock solution”)
determined using a haemocytometer. Two replicates of five counts each were performed to
establish the mean concentration present in the sperm stock solution.

\textbf{Experimental Protocol}

Sperm stock solutions were adjusted to give $1 \times 10^{9-10}$ and subsequently diluted ten fold to give
$1 \times 10^{3}$, $10^{4}$, $10^{5}$, $10^{6}$, $10^{7}$ and $10^{8}$ sperm ml$^{-1}$. These were experimental sperm concentrations,
and prepared within five minutes of use to minimise any sperm respiratory dilution effect. A
petri dish containing only seawater served as a control for sperm contamination. The series of
sperm solutions were placed in a row of seven 9 cm petri dishes (100 ml). Alongside two
corresponding rows comprised of 200 ml beakers of 0.01g/l Sodium Lauryl Sulphate
(hereafter SLS) and five L buckets containing filtered (1 $\mu$m) seawater were placed (please
refer to Fig. 1 in Chapter 3). Between $5-8 \times 10^3$ eggs were placed in each of 14 plastic tubes (2 cm diameter). The tubes were sealed at one end with 60µm nitrex mesh and held in buckets of filtered seawater (1µm). Two tubes were held by a clothes peg, seven of which were attached to a length of rod (hereafter egg-dipper). Lifting the egg dipper in and out of various solutions allowed experiments to be carried out simultaneously and to submerge and remove eggs from sperm solutions for any given contact time. Contact times chosen were 10 sec, 30 sec, 1 min, 2 min, 5 min and each contact time experiment was repeated three times, hence $n=6$ per contact time.

Upon withdrawal of eggs from the sperm solutions, eggs were submerged in SLS for 10 seconds, causing sperm to stop swimming for at least five seconds after submergence into the solution (99% effective, Presley and Barker 1970 and pers. obs.). Following this, eggs were rinsed thoroughly to remove excess sperm attached to gametes and the apparatus by lifting the egg-dipper in and out of the filtered seawater 80 times. Upon washing, a sub-sample of eggs was placed into vials, and fixed 15 min. after fertilization.

The effect of gamete age on female and male gametes was assessed simultaneously, using the same sperm solutions and eggs as in the first experiment (T=0). Other gamete ages were T=15 min, T=30 min and T=60 min after the initial sperm concentrations had been prepared, and the process described above was repeated at these times. *Coscinasterias muricata* has been observed to spawn synchronously (Styan and Meville pers. com.), thus it can be assumed that gametes age simultaneously under natural conditions.

For contact times 2 min and 5 min at gamete age T=0 two further subsamples were taken and fixed in Formaldehyde (10%). One subsample was fixed 2-3 hrs after fertilization (hereafter cleavage samples), i.e. when most eggs had reached their four to eight cell stage. The other
sample was fixed 24 hours after initial fertilization (hereafter embryo samples). The time lag between fixation of the first sample and cleave/embryo samples could potentially lead to higher fertilization values in the latter, however, submersion of all eggs in SLS as well as thorough rinsing of eggs was designed to counteract this problem.

**Identification of Fertilized and Polyspermic Eggs**

For samples fixed 15 min after fertilization, fertilized eggs were initially assessed as those with a raised fertilization envelope (hereafter "envelope" data). A minimum of 100 eggs in total were counted. Where samples showed “envelope” fertilization levels >10%, or where mean fertilization was >10% at a particular sperm concentration, the eggs were stained using the DNA-specific stain Ethidium Homodimer-1® (hereafter “staining” samples). Some samples showing lower fertilizations were also stained to check for presence of polyspermy, however none was detected in these samples. For example, for gamete age T=0, 64 envelope samples showed < 10% fertilization, and 49 of these samples exhibited fertilization levels between 0 and 5%. Seven of these were stained to check if polyspermy was apparent, but none was detected.

To stain sperm in eggs, a sub-sample of eggs (approx. 300-400) was placed into a micro-titre well and eggs were rinsed free of fixative in phosphate buffer saline (Oxoid®, standard solution, hereafter PBS). Subsequently, gamete membranes were permeablized by five minute exposure to 98% methanol. This made it possible for the subsequently added nuclear stain to penetrate the gametes and adhere to nuclear structures. Excess methanol was rinsed off with PBS and eggs were then exposed to the nuclear stain Ethidium Homodimer-1 (Molecular Probes®) at concentration of $1.818 \times 10^{-3} \text{ mg mL}^{-1}$ for five to ten minutes. Excess stain was subsequently rinsed off with PBS. Eggs were mounted in glycerol (Citifluor Alltech®) and the number of sperm that had entered the eggs (up to a maximum of ten) was determined.
using a fluorescent microscope (40 x) by focusing through the egg. Representative samples (at least 200-300 eggs in total) of sperm entry were verified using Confocal Laser Microscopy (x 25 oil, Fig. 2). Only sperm present within the egg were scored as fertilizing sperm, whereas sperm attached to the outside of the egg periphery was not. At least 50 eggs per sample were counted.

For cleavage samples, embryos with an even number of blastomeres (two, four, eight etc.) or eggs possessing a fertilization envelope (1-cell stage) were scored as fertilized. Some of the latter egg type may have been polyspermic, however, in corresponding samples with envelope fertilization levels <10%, 1-cell stage eggs also occurred, which were not polyspermic when stained. Hence, it was impossible to distinguish between monospermic and polyspermic 1-cell stage eggs, and we had to assume that all were monospermic. Embryos with irregular division, such as three, five, six, seven or nine segments, were scored as polyspermic eggs. Monospermy was inferred by subtracting the amount of polyspermy from total fertilization.

For embryo samples, scoring was such that monospermy was equated to a 24 hr embryo (blastula stage). 1-cell stage eggs and eggs showing signs of fertilization (e.g. irregular cell segments) were counted towards the total amount fertilized. Polyspermy was inferred by subtracting the proportion of healthy larvae from the total amount of fertilization.

For cleavage and embryo samples at least 100 embryos per sample were counted. Furthermore, eggs exposed to seawater only (control) provided a reference for the appearance of unfertilised eggs. A summary of all samples analysed and the time of their egg fixation after fertilization is presented in Table 1.
Statistical Analysis

Staining data assessing Fertilization, Polyspermy and Monospermy

Raw data for total fertilization, polyspermy and monospermy via staining did not conform to assumptions of normality even after arcsin-squareroot transformation. Hence, raw data means were subsequently analysed by Logistic Regression using SAS®, because all fertilization data are binary and more likely to conform to a binomial or logistic distribution than a normal distribution. In addition, the logistic regression assumes a non-linear relationship between dependent variables (fertilization, polyspermy, monospermy) and the independent variables (sperm concentration, contact time, gamete age) (Tabachnick and Fidell 2001) and all previous studies of fertilization kinetics indicate highly non-linear relationships between the variables (e.g. Pennington 1985, Levitan et al. 1991, Benzie and Dixon 1994, André and Lindegarth 1995, Encena et al. 1996, Williams et al. 1997, Buchanan 1998). This type of analysis produces odds ratios, which indicate the likelihood of an increase (if odds are >1) or decrease (if odds are <1) of an event with each unit change of the particular independent variable (sperm concentration (log_{10}), gamete age (min) and contact time (min)).

Significant interactions were found among all three independent variables for each of the three categories examined. However, the interactions were subsequently removed and the model was rerun again omitting the interactions for reasons outlined in Chapter 3. The odds ratios of the interaction indicated that any influence on fertilization was of very small magnitude (≤ 2 %); hence effect of the interaction on fertilization rates is very small.

Comparison of Methods

Raw means (n=6 per contact time) for data assessing total fertilization of stained and envelope data were compared at each particular sperm concentration using linear regression
analysis for each gamete age. Data from all five contact times were pooled for analysis to give sufficient sample size that allowed subsequent regression analysis.

For comparisons between staining and cleavage samples assessing total fertilization, polyspermy and monospermy the raw mean of each contact time experiment was calculated in staining samples (recall, one contact time experiment had two replicates) and compared with the corresponding raw mean in the cleavage and embryo samples at one particular sperm concentration for samples of gamete age T=0 contact time 2 min and 5 min. This allowed examination of egg batch development through time.

For all comparisons simple regression analysis was performed using Excel®. To test if significant regressions differed from the expected relationship of $y=x$, slopes were tested to see if they were equal to 1, using t-test (Zar 1984). If this was found to be the case, then the experimental regression was further tested to determine if the intercept was significantly different from zero using Excel®. Similar to data treatment in Chapter 3, only if the slope was equal to 1 and the intercept equal to zero, was it concluded that the regression generated was not significantly different from $y=x$.

**Modelling Fertilization**

Raw data from stained samples (gamete age T=0 for contact times 10 sec, 30 sec, 1 min, 2 min and 5 min) were used to fit Styan’s (1998) model. In this model the number of successful fertilizations is a function of the concentrations of eggs and sperm, egg size, sperm swimming speed and the time sperm and eggs can potentially interact (contact time). Sperm concentrations determined for the fertilization kinetics experiments, published measures of average *C. muricata* egg diameter (0.150 mm ± 0.01, Babcock *et al*. 2000) as well as sperm swimming speed (0.122 ± 0.0057 mm s$^{-1}$, Babcock *et al*. 2000) were used. Egg density was 0.1
eggs μl⁻¹. The model predicts fertilization efficiency (hereafter \(F_e\), which is an estimation of the fertilizability of the egg surface) and the time taken to develop a block to polyspermy (\(t_b\)). A least-squares fit using a quasi-Newton-algorithm was used to iteratively estimate the parameters \(F_e\) and \(t_b\). The least-squares model minimises the pooled differences between the measured and the predicted proportion of unfertilised and polyspermic eggs.

## Results

### Staining Data

#### Logistic Regression Analysis

The percentages of fertilization, polyspermy and monospermy increased significantly with increasing sperm concentration and contact time (p<0.0001, Fig. 2). However, with increasing gamete age, fertilization, polyspermy and monospermy decreased significantly (p<0.0001; Fig. 2).

Based on the general equation \(y = \frac{e^u}{1 + e^u}\) the following equations were generated from logistic regression analysis, where \(y\) is the dependent variable, \(u\) the linear regression equation, \(SC=\)sperm concentration (log\(_{10}\)), \(CT=\)contact time (min), \(GA=\)gamete age (min) and errors are ±1 S.E..

Fertilization \(u_F = 0.7831 \pm 0.01 (SC) + 0.4327 \pm 0.0001 (CT) - 0.0293 \pm 0.0007 (GA) - 5.7869\)

Polyspermy \(u_P = 1.2912 \pm 0.0276 (SC) + 0.4126 \pm 0.000168 (CT) - 0.0385 \pm 0.0012 (GA) - 11.6547\)

Monospermy \(u_M = 0.2352 \pm 0.0123 (SC) + 0.1916 \pm 0.00011 (CT) - 0.00849 \pm 0.000675 (GA) - 2.4287\)

Sperm concentration was the most important predictor for total fertilization, polyspermy and monospermy, because it generated the highest odds ratios (Table 2). Polyspermy was most
affected by any change in sperm concentration, because the odds of it increasing were predicted to be 257% with each ten-fold increase, but only 115.1% for fertilization and 25.8% for monospermy (Table 2). Furthermore, for monospermy a unit change in contact time (1 min) was of almost equal predictive importance to a unit change in sperm concentration (10 fold), because one additional minute of contact time was predicted to increase the chance of monospermy by 25%, whereas a ten-fold increase in sperm concentration was predicted to increase the likelihood of monospermy by 21% (Table 2).

At gamete age T=0 maximum fertilization was achieved at $10^7$ and $10^8$ sperm ml$^{-1}$ for contact time 5 min. However, these conditions also produced the highest level of polyspermy (up to a mean of 70%, Fig. 2). Hence for gamete age T=0, maximum levels of monospermic fertilization (80%) were generated for contact time 2 min at $10^6$ sperm ml$^{-1}$ (Fig. 2).

Interestingly, gamete age T=15 min generated even higher mean fertilization rates, reaching 95% at $10^7$ and $10^8$ sperm ml$^{-1}$ for contact times 2 min and 5 min (Fig. 2). Polyspermy levels reached a maximal mean of only 30% for contact time 2 min (Fig. 2) and 70% for contact time 5 min (Fig. 2). Hence maximum monospermy level of all stained data was 85% at $10^7$ sperm ml$^{-1}$ gamete age T=15 min for contact time 2 min (Fig. 2).

Polyspermy occurred even over short contact times (10 sec and 30 sec) at high sperm concentrations ($10^7$ and $10^8$ sperm ml$^{-1}$) but was more pronounced over longer contact times (1 min, 2 min and 5 min) at medium and high concentrations ($10^6$, $10^7$ and $10^8$ sperm ml$^{-1}$) (Fig. 3). Total fertilization never reached 100%, yet polyspermy was detected under these apparent sperm limiting conditions.
**Comparison of Methods**

**Staining vs. Envelope data assessing Fertilization**

There was a significant positive relationship between embryo and stained data assessing total fertilization for all gamete ages (Fig. 3). However, none of the regressions matched the expected relationship of $y=x$, and slopes of the generated regressions were all significantly different from $b=1$. For gamete ages $T=0$, 15 and 30 min, envelope samples gave consistently higher values than stained samples when stained fertilization levels were between 0 and 75%. However, when stained fertilization levels were above 75%, envelope data were lower than stained data (Fig. 3). At gamete age $T=60$ min, envelope data always generated higher values compared to stained data.

**Staining vs. Cleavage data assessing Fertilization, Polyspermy and Monospermy**

There was a significant positive relationship between staining and cleavage samples with total fertilization generating a high $r^2$ value (0.88), but low $r^2$ values for polyspermy and monospermy (0.1 and 0.4 respectively; Fig.4). For total fertilization, the slope was not significantly different from $b=1$ and the intercept was not significantly different from 0, hence the regression was like the expected relationship $y=x$. For polyspermy the slope was significantly different from $b=1$ and at high levels of stained polyspermy, cleavage assessment generated lower values (Fig.4). For monospermy the slope was equal to $b=1$, but the intercept was significantly different from 0, hence the generated regression is parallel to the expected line; therefore cleavage always generates higher values than staining when assessing monospermy.

**Staining vs. Embryo data assessing Fertilization, Polyspermy and Monospermy**

There was a significant positive relationship for all three fertilization parameters, with total fertilization and monospermy generating high $r^2$ values (0.79 and 0.69 respectively), but a low
value for polyspermy (0.4; Fig. 5). Again, analogous to the comparison between staining and cleavage samples, none of the three fertilization parameters generated a regression like the expected relationship. Even though the slope for monospermy is the same as the expected relationship (i.e. $b=1$), the intercept was significantly different from 0. Embryo samples gave consistently higher values when monospermy was assessed, and lower values when polyspermy was assessed (Fig. 5).

**Modelling Fertilization**

Styan’s (1998) fitted model to data of contact times 10 sec to 5 min at gamete age $T=0$ generated high $r^2$ values ranging from 0.73-0.93 (Table 3). Predicted fertilizability ($F_e$ values) increased with increasing contact time ranging from 0.00005 to 0.0156; predicted polyspermy block latency ($t_b$) was 0.21 sec for contact times 10 sec, 30 sec and 1 min, 0.3 sec for contact time 2 min, and an order of magnitude higher for contact time 5 min (2.17 sec, Table 3).

**Discussion**

**Fertilization, Polyspermy and Monospermy observed via nuclear staining**

This study reveals that polyspermy occurs under apparent sperm limited conditions, i.e. when less than 100% of eggs were fertilized, as predicted by Styan (1998) and previously demonstrated in studies of the sea urchin *Evechinus chloroticus* (Franke *et al.* 2002, Franke *et al.* submitted). Furthermore, in agreement with results obtained for *E. chloroticus* and other studies investigating the fertilization kinetics in marine invertebrates, logistic regression analysis showed that all dependent variables, but particularly polyspermy, were most sensitive to increases in sperm concentration (Levitan *et al.* 1991, Benzie and Dixon 1994, Chapter 3). In contrast to studies that have not distinguished between monospermy and polyspermy, this study shows monospermy is almost as likely to occur with a unit increase in sperm concentration as it is per unit contact time. Again, this result is analogous to those obtained...
for *E. chloroticus* (Chapter 3). This highlights the fact that increases in sperm concentration *per se* may not be the evolutionary best response to increase fertilization success.

Polyspermy reduced apparent maximal mean fertilization from an apparent maximum of approx. 100%, to 30% monospermy at $10^6$ sperm ml$^{-1}$ for contact time 5 min, gamete age $T=0$. Hence, maximum monospermy is not achieved for longest contact times and highest sperm concentrations but at $10^6$ sperm ml$^{-1}$ for contact time 2 min at gamete age $T=15$ min. Again, these results are in good agreement with the similar study on *E. chloroticus*, where maximum monospermy was also achieved for contact time 2 min at $10^6$ sperm ml$^{-1}$, but at gamete age $T=0$ (Chapter 3). The high levels of polyspermy (up to 76%) observed under medium to high sperm concentrations as well as short and long contact times for *C. muricata* were similar to previously measured polyspermy in marine invertebrates under laboratory and *in situ* conditions using sperm staining methods. For example, maximum polyspermy levels at $10^6$ sperm ml$^{-1}$ were approximately 37% for the giant scallop *Placopecten magellanicus* (Desroisers *et al.* 1996) and 30% to 70% at $10^7$ sperm ml$^{-1}$, depending on egg concentration, for the surf clam *Spisula solidissima* (Clotteau and Dubé 1993). In the oyster *Crassostrea gigas* maximum polyspermy levels reached 25-28% in the laboratory (Fig. 6 in Stephano and Gould 1988). Franke *et al.* (2002) recorded up to 76% polyspermy at $6 \times 10^6$ sperm ml$^{-1}$ for experimental *in situ* spawnings and up to 40% in laboratory experiments (Chapter 3) in *E. chloroticus*. However, for all these studies, contact times were possibly higher (no data for contact times given in Desroisers *et al.* 1996), 10 min in Clotteau and Dubé (1993), 10-15 min in Stephano and Gould, 2-10 min in Franke *et al.* 2002 and similar contact times to this study in Chapter 3).

The experimental conditions, which led to the frequent occurrences of polyspermy were likely to be similar to conditions during naturally occurring spawnings possibly for this as well as
other species. Concentrations of $10^6$ sperm ml$^{-1}$ and above have been measured in the field during natural spawning events (Hamel and Mercier 1996, Williams et al. 1997) and in many species spawning has been shown to extend over the course of hours (e.g. Lamare and Stewart 1998, Chapter 4) and days (e.g. Babcock et al. 1986, Meidel and Yund 2001); hence, the potential for high sperm concentrations to occur and in turn to lead to polyspermy exists and thus it may exert sufficient evolutionary pressure to affect aspects of reproductive ecology, such as egg size, spawning aggregation and synchrony as well as molecular evolution.

**Comparison of Methods**

**Staining vs. Envelope data assessing Fertilization**

Comparison among the egg samples that were stained with Ethidium homodimer-1 for different gamete ages indicate that at levels of fertilization ranging between 0 to 75%, envelope samples generate higher values of fertilization. This may be due to the conservative estimate of sperm entry, i.e. only sperm inside the egg were counted as a fertilizing sperm, whereas sperm attached to the egg periphery were not. When stained fertilization levels were above 75%, then the staining method gave higher fertilization values compared to envelope samples. This may be due to failure of eggs experiencing severe polyspermy to raise their envelope, resulting in their being categorized as unfertilised (cf. Chapter 3). This has been observed in other urchin species (Levitan pers. com.) and represents thus a feasible explanation for this phenomena.

**Staining vs. Cleavage data assessing Fertilization, Polyspermy and Monospermy**

As results from regression analysis showed, the same results were generated whether fertilization was assessed via staining or cleavage. This is interesting, because for *E. chloroticus*, this result was only obtained when monospermy was assessed (Chapter 3). However, *E. chloroticus*’ eggs were fixed much more rapidly after fertilization (2 - 3 min
compared to 15 min in *C. muricata*). Hence, sperm entry into the egg may not have been completed in *E. chloroticus*, which would underestimate fertilization when eggs were stained, compared to *C. muricata*, where sperm entry into the egg would have been completed 15 min after fertilization.

However, in agreement with results for *E. chloroticus* (Chapter 3), a poor comparison between staining and cleavage samples existed for polyspermy. These results differ from those for the oyster *C. gigas* (Stephano and Gould 1988) where a similar comparison generated the same result in their study. The differences between our study and that of Stephano and Gould (1988) may be twofold. Firstly, Stephano and Gould (1988) used maximum sperm concentrations of $3 \times 10^7$ sperm ml$^{-1}$, whereas our maximum sperm concentration was $1 \times 10^8$ sperm ml$^{-1}$. Consequently, *C. gigas* eggs may not have experienced as severe polyspermy as eggs in our study. Eggs experiencing severe polyspermy may fail to raise their envelope completely, which we scored as unfertilized, and this may not have been the case for Stephano and Gould (1988). Secondly, Stephano and Gould (1988) equated abnormally and uncleaved eggs with polyspermy, whereas we only scored abnormally cleaved eggs as polyspermy. Eggs that remained at 1-cell stage (i.e. uncleaved but showing evidence of a fertilization envelope) were scored to be monospermic in our study. This was done, because *C. muricata* eggs that experienced low fertilization levels and low sperm concentrations also possessed 1-cell stage eggs, which when representative samples were stained, never exhibited any polyspermy. As a result, our estimate of polyspermy when analysing cleavage would have underestimated the degree of polyspermy compared to staining, particularly when polyspermy levels were high. In turn, monospermy levels would be overestimated in cleavage samples and generate consistently higher values compared to stained samples. This was found to be the case in our study.
Staining vs. Embryo data assessing Fertilization, Polyspermy and Monospermy

Comparison of staining and embryo samples did not generate a curve like the expected relationship $y=x$ for any of the categories examined. Fertilization was consistently higher when embryos were assessed similar to results for *E. chloroticus* (Chapter 3). Polyspermy did show a significant positive relationship between embryo and staining samples, which could not be demonstrated for *E. chloroticus*. However, the same pattern between staining and cleavage samples in this study as well as for *E. chloroticus*, i.e. higher levels of polyspermy in embryo samples when staining was low, and lower levels of polyspermy in embryo samples when staining was high, were also detected here. This supports the notion that eggs experiencing severe polyspermy fail to raise their envelope, and hence are scored as unfertilised eggs. Similar phenomena have been observed in other urchin species (Levitan per. com.) and thus provide a plausible explanation.

In contrast to results for *E. chloroticus*, where both staining and embryo methods of assessment generated the same result for monospermy, this study showed consistently higher levels for monospermy when embryo development is assessed. This may be due to some unfertilised eggs dissolving over the course of 24 hours (pers. obs.), and hence total fertilization levels in embryo samples may be arbitrarily higher than in staining samples. In addition, because only healthy larvae were equated with monospermy, and unfertilised eggs may have dissolved over the course of the 24 hours, this would arbitrarily increase the amount of monospermy as shown here.

Overall, comparisons of methods assessing monospermy seem to be systematically higher, when cleavage/embryo development is examined. This is most likely due to 1-cell- stage eggs being categorised as monospermy, whereas in fact some may be polyspermic.
Whatever the reason for the discrepancies we observed between methods, and even though staining may sometimes be difficult, because of failure to detect sperm when eggs are large and yolky (e.g. in sea star *Patiriella calcar*, Franke unpub. data), reliable assessment of polyspermy is only possible via staining. This is important, because even low levels of polyspermy may be of significant evolutionary importance (Gavrilets 2000). Furthermore, staining is also important if high levels of polyspermy are to be detected, because of the failure of eggs to raise their envelope when experiencing severe polyspermy.

**Modelling Fertilization**

Predicted levels of unfertilised eggs, polyspermy and monospermy for stained data based on Styan’s (1998) model showed a close fit to individual contact times at gamete age $T=0$. However, obtaining a high $r^2$ value in itself is not a sufficient test of the model, because a range of parameters can be fitted but still generate similar $r^2$ values (Styan and Butler 2000). Hence, observing how the predicted curve changes with changing contact time and its relative position within the generated data is a more appropriate test of the model (Styan, pers. com.).

In theory, $F_e$ values should not vary with contact time, because eggs between different batches of experiments are expected to be the same and hence their egg surfaces are expected to be just as “fertilizable” as each other. Therefore, the directional change of increasing fertilizability ratio ($F_e$ value) with increasing contact time is counterintuitive. This discrepancy may be a direct indication that longer gamete contact times allow time for fusion of less compatible sperm-egg combinations (e.g. Palumbi 1999), which is represented in the model as the eggs becoming “more fertilizable” if contact times are long. Furthermore, the estimated polyspermy block latency of 0.21 sec for contact times 10 sec to 1 min indicates a very fast block and lies an order of magnitude below the measured fast blocks to polyspermy (3 sec for sea urchins and thought to be similar for starfish, Jaffe and Gould 1985). For the
longest contact time (5 min) a polyspermy block latency of 2.17 sec was generated and this may be a more accurate reflection of reality. On the other hand, a block to polyspermy that takes longest at the highest contact time could also reflect the inability to effectively detect a polyspermy block, because of sperm swamping the eggs when contact times are long and sperm concentrations are high. Again though, eggs should not differ in their polyspermy block latencies, as all eggs came from the same species.

Differences in generated values of fertilization kinetics models have been observed elsewhere (Styan and Butler 2000), and even models that have been modified to improve their predictive ability still generate varying $F_e$ and $t_b$ values with different gamete contact times for the same species (Gribben and Millar in prep.). These differences however, were not directional as in this study, i.e. an increase in contact time did not lead to an increase in $F_e$ or $t_b$ value each time (Gribben and Millar in prep.) and reasons for why this may be the case are still under speculation (Millar pers. com., Styan pers. com.).

Fertilization parameters, such as the occurrence of sperm chemotaxis, differing sperm swimming speeds at different sperm concentrations (Styan and Butler 2000) as well as individual differences in reproductive condition (Gribben and Millar in prep.) have been postulated as possible explanations as to why different values are obtained when modelling different gamete contact times. However, the effect of sperm chemotaxis should be the same across all eggs. Also, sperm swimming speeds have recently been shown not to vary with sperm concentration in scallops (Williams pers. com.), and replication should mask the effect of individual differences in reproductive condition.

Thus, the differences in generated $F_e$ and $t_b$ values could mean that the model is not appropriately allowing for the effect contact time *per se* has on fertilization. The model might
have to be mathematically amended to give appropriate weighing to the effect of contact time and/or mathematically decouple the relationship between $F_e$ and $t_b$ parameters. Reducing the predicted values of the model to only one parameter may help to clarify this issue. This could be achieved by empirically measuring the polyspermy block in $C. muricata$ eggs as done in urchins (e.g. Jaffe and Gould 1985, Schuel 1984). Alternatively, determining the amount of sperm/egg receptors on $C. calamaria$ eggs similar to studies conducted in urchins (see Gilbert 2000 for photograph of limited number of sperm binding to egg surface) and thus empirically calculating the fertilizable fraction of eggs, might also overcome the observed discrepancies. Ideally, empirical measurements of both, polyspermy block latency and sperm-egg receptors, should overcome problems with the model, and thus allow assessing any mathematical discrepancies.

None the less, Styan’s (1998) prediction of polyspermy occurring under sperm-limited conditions (i.e. less than 100% fertilization) has been confirmed by our data and the generated curves of prediction are consistent with the data generated.

**The importance of polyspermy in fertilization ecology**

**in broadcast spawning marine invertebrates**

The further demonstration of the frequent occurrence of polyspermy under sperm limited conditions in this study, and the close fit of the data and the model, suggests that polyspermy is not rare, but a frequent occurrence during spawning events, and that this phenomena is not restricted to urchins, but occurs at least in one other member of the echinoderms and possibly in many other organisms, because polyspermy blocks clearly exist within a wide range of taxa. Thus, the evolutionary changes and processes suggested to be driven in part by polyspermy, including spawning behaviour, egg size, larval type, molecular co-evolution of gamete recognition proteins and possibly even speciation, are very likely to be real.
However, we have yet to determine the occurrence of polyspermy during a truly natural spawning event in marine invertebrates. This is logistically difficult for various reasons. Firstly, spawning in many marine invertebrates is unpredictable (e.g. Brewin et al. 2000, Meidel and Yund 2001, Wahle and Gilbert 2002), which would make collections of gametes inherently difficult. Secondly, some organisms may spawn under turbulent conditions (e.g Chapter 4., Grange 1976), which would further complicate gamete collection.

Our results have shown if monospermy is to be increased, increasing gamete contact times may be just as effective as increasing sperm concentration. Hence, if organisms spawn over hours/days/weeks (e.g. Babcock et al. 1986, Lamare and Stewart 1998) and have long lived eggs (e.g., Meidel and Yund 2001, Wahle and Gilbert 2002), then sufficient larval production should occur, while minimizing the risk of polyspermy.

However, the trade-off between polyspermy and sperm limitation cannot be assessed in isolation. Sperm competition may occur just as frequently as sperm limitation (Levitan, 1998, Petersen and Warner 1998, Yund 2000). This will maintain selective forces that lead to higher sperm concentrations posing a risk of increased polyspermy; and generating the conditions for sexual conflict.
Figures

Figure 1
Eggs at $10^8$ sperm ml$^{-1}$, contact time 5 min, gamete age $T=0$, of Coscinasterias muricata when stained with Ethidium Homodimer-1 as viewed under the Confocal Lazer Microscope (x 25 oil). The scale bar indicates 50 µm. Arrows indicate sperm, which have entered the eggs. Eggs shown are monospermic (one sperm) and polyspermic (three sperm). The additional structure in the egg represents the condensing egg nucleus, which was also apparent in unfertilized eggs (not shown). The picture represents an overlay of three 2 µm thick slices, taken within the eggs.

Figure 2
Data from staining samples representing fertilization, polyspermy and monospermy (%) vs. sperm concentration ($\log_{10}$) and gamete age (min) for contact times 10 sec, 30 sec, 1 min, 2 min and 5 min. Error bars have been omitted for clarity. No polyspermy was detected below $10^5$ sperm ml$^{-1}$, either because samples showed less than 10% fertilization when envelope was assessed and/or no polyspermy was detected in corresponding samples experiencing an order of magnitude higher sperm concentration when stained. Hence data within the range of $10^5$-$10^8$ sperm ml$^{-1}$ are shown here. Numbers on contour graphs represent the raw means of fertilization parameters.

Figure 3
Raw means of fertilization (%) for envelope and staining samples are compared for all gamete ages ($T$). ● represents data for contact time 10 sec, ○ for 30 sec, ▲ for 1 min, ▼ for 2 min and ■ for 5 min. Data from all contact times were pooled to generate a linear regression line (▬). — indicates $y=x$, the expected relationship between the envelope and staining data. $r^2$ values are indicated on graphs for each gamete age time.
Figure 4
Comparison of staining and cleavage samples at gamete age T=0 assessing fertilization (F), polyspermy (P) and monospermy (M). The raw mean of the two replicates per contact time experiment were calculated for staining samples and compared with corresponding mean of cleavage samples. ● represents pooled data for contact times 2 min 5 min. to which a linear regression line (▬) was fit and * indicates significant regressions. – indicates y=x, the expected relationship between the staining and cleavage data. $r^2$ values are indicated on graphs for each fertilization parameter.

Figure 5
Comparison of staining and embryo samples at gamete age T=0 for fertilization (F), polyspermy (P) and monospermy (M). Again, raw data means of pooled replicates per contact time experiment were plotted against each other. ● represents pooled data for contact times 2 min 5 min. to which a linear regression line (▬) was fit and * indicates a significant regression. – indicates y=x, the expected relationship between the staining and cleavage data. $r^2$ values are indicated on graphs for each fertilization parameter.

Figure 6
Styan’s (1998) fertilization kinetics model was fitted to each individual contact time data of 10 sec, 30 sec, 1 min, 2 min and 5 min for gamete age T=0 using sperm swimming speed = 0.122 mm sec$^{-1}$, egg concentration = 0.1 μl$^{-1}$ and egg diameter = 0.15 mm. ● represents experimental data for contact time 10sec, ○ for 30 sec, ▲ for 1 min, ▼ for 2 min and ■ for 5 min. Solid lines indicate the model’s predictions. Calculated $r^2$ values, polyspermy block latency ($t_b$) and $F_e$ (fertilizability of eggs) values are summarized in Table 4.
Fig. 1
Fig. 2 A

Gamete Age (min)

Sperm Concentration (log_{10})

10 sec

30 sec

F

P

M
Fig. 2 B

Gamete Age (min)

Sperm Concentration (log_{10})
Fig. 3

Staining (%) vs Envelope (%) for different time points:
- T=0: $r^2=0.58^*$
- T=15 min: $r^2=0.46^*$
- T=30 min: $r^2=0.62^*$
- T=60 min: $r^2=0.63^*$
Fig. 4

Staining (%) vs. Cleavage (%)

- F: $r^2=0.88^*$
- P: $r^2=0.13^*$
- M: $r^2=0.4^*$
Fig. 5

Staining (%) vs Embryo (%)

- Graph F: $r^2=0.79^*$
- Graph P: $r^2=0.4^*$
- Graph M: $r^2=0.69^*$

Staining (%) vs Embryo (%)
Tables

Table 1
Overview of analysed samples. Time of fixation indicates the time between the completion of gamete interaction and fixation in 10% formaldehyde. Methods used to assess total fertilization, polyspermy and monospermy are indicated.

Table 2
Odds ratios generated from logistic regression analysis. Numbers in brackets indicate the percentage increase (if odds >1) or decrease (if odds <1) in the likelihood of the particular dependent variable with a unit increase in the independent variable. For example, there is a 257.7% chance for polyspermy to increase with a 10 Fold increase in sperm concentration and a 3.7% chance for polyspermy to decrease with a one minute increase in gamete age. Sperm concentration was found to be the most important predictor for all dependent variables examined, but for monospermy contact time was almost of equal importance to sperm concentration.

Table 3
Parameters generated from Styan’s (1998) fertilization kinetics model for all different contact times. High $r^2$ values are achieved for all contact times, but generated $F_e$ values range over four orders of magnitude and are increasing with increasing contact time. Generated $t_0$ values were in the same order of magnitude for contact times 10 sec to 2 min, but one order of magnitude higher for contact time 5 min.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Time of Fixation after Contact Time Experiment completed</th>
<th>Method of Assessment</th>
<th>Total Fertilization</th>
<th>Polyspermy</th>
<th>Monospermy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Envelope</td>
<td>15 min</td>
<td>Envelope elevation, eggs of samples showing ≥10% fertilization, were subsequently stained</td>
<td>Not possible</td>
<td>Not possible</td>
<td></td>
</tr>
<tr>
<td>Staining</td>
<td>15 min</td>
<td>Sperm stained with Ethidium Homodimer-1 inside an egg</td>
<td>&gt;1 sperm visible inside an egg</td>
<td>1 sperm visible inside an egg</td>
<td></td>
</tr>
<tr>
<td>Cleavage</td>
<td>2-3 hours</td>
<td>Regular and irregular cleavage</td>
<td>Irregular cleavage (3, 5,6,7 cell segments)</td>
<td>1 cell stage and regular cleavage</td>
<td></td>
</tr>
<tr>
<td>Embryo</td>
<td>24 hours</td>
<td>Larvae and cleaved cell bundles</td>
<td>Total fertilization - larvae</td>
<td>Late prism, early pluteus larvae</td>
<td></td>
</tr>
<tr>
<td>Fertilization parameters/Fertilization category</td>
<td>Sperm Concentration (log)</td>
<td>Contact Time (min)</td>
<td>Gamete Age (min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>--------------------------</td>
<td>--------------------</td>
<td>------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Fertilization</td>
<td>2.151 (115.1%)</td>
<td>1.541 (54.1%)</td>
<td>0.971 (2.9%)</td>
<td></td>
<td></td>
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<tr>
<td>Polyspermy</td>
<td>3.577 (257.7%)</td>
<td>1.51 (51.1%)</td>
<td>0.963 (3.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monospermy</td>
<td>1.258 (25.8%)</td>
<td>1.211 (21.1%)</td>
<td>0.993 (0.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gamete contact time/ model parameters</td>
<td>10 sec</td>
<td>30 sec</td>
<td>1 min</td>
<td>2 min</td>
<td>5 min</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>--------</td>
<td>--------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.91</td>
<td>0.93</td>
<td>0.73</td>
<td>0.82</td>
<td>0.87</td>
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<tr>
<td>$F_c$</td>
<td>0.00009</td>
<td>0.00005</td>
<td>0.009</td>
<td>0.006</td>
<td>0.0156</td>
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<tr>
<td>$t_b$</td>
<td>0.21</td>
<td>0.21</td>
<td>0.21</td>
<td>0.3</td>
<td>2.17</td>
</tr>
</tbody>
</table>
References


Rothschild L. and Swann M (1951) The fertilization reaction in the sea urchin. The probability of a successful sperm-egg collision. J. Exp. Biol. 28: 403-416


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Fertilization kinetics, polyspermy and its importance
in fertilization ecology for the seastar *Coscinasterias muricata*

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Keywords: New Zealand, Coscinasterias muricata, Fertilization kinetics, polyspermy, evolution

Table 3

Results generated from regression analysis comparing different methods of assessing fertilization, polyspermy and monospermy. * indicates a significant regression, and the corresponding $r^2$ value is given in brackets. n.a. indicates if a test was not applicable, because $b \neq 1$, and hence experimental data did not produce the same line as the expected line of $x=y$. t values are given where the slope was found to be significantly different from 1 at the significance level of $p=0.05$. Significant p values where the intercept was found to be equal to zero are represented in bold. Results in italics indicate the regression no different from the expected relationship $y=x$, i.e. $b=1$ and the intercept not significantly different from 0.
The studies of various aspects of echinoderm fertilization ecology described in this thesis have shown that polyspermy may be a reasonably common occurrence. For *Evechinus chloroticus*, spawning in shallow and calm waters extensive amounts of polyspermy can be produced even under sperm limiting conditions (Franke et al. 2002). Laboratory studies with both *E. chloroticus* and the sea star *Coscinasterias muricata* have also demonstrated that polyspermy occurred when gamete contact times were short (10-30 sec) as well as long (1-5 min), and when sperm concentrations were medium to high ($10^6$-$10^8$ sperm ml$^{-1}$). Again, polyspermy was detected under sperm limiting conditions (Chapter 3, Chapter 5).

All of these results were predicted by the mathematical fertilization kinetics model, which incorporates polyspermy (Styan 1998), and data from *E. chloroticus in situ and in vitro*, as well as *Coscinasterias muricata in vitro* confirmed this model. The verification of this model from three separate sets of data (Franke et al. 2002, Chapter 3 and Chapter 5), suggests that modeling can be useful in the attempt to describe fertilization events. However, predictions generated by the model have to be viewed with caution, because different values for polyspermy block latency ($t_b$) and fertilizability of eggs ($F_e$) were generated for different gamete contact times (Chapter 3). This was counterintuitive, because experiments were replicated in time and should have overcome problems of individual variability, which has been argued to account for observed differences in model-generated values elsewhere (Gribben and Millar submitted). Consequently, these predicted values are best measured empirically. Furthermore, incorporation of measured
polyspermy block times (e.g. Jaffe and Gould 1985, Schuel 1984), increased effective
diameter of eggs due to sperm chemotaxis (e.g. Miller and King 1983, Jantzen et al.
2001) and possible differences in sperm swimming speeds at different concentrations of
sperm (e.g. Chia and Bickell 1983, but see Williams in prep.) may refine models and
improve their predictive ability. None-the-less, as a result of such discrepancies in this
and other studies (e.g. Styan and Butler 2000, Gribben and Millar submitted) of model-
generated predictions, new and possibly more realistic models of fertilization events,
including polyspermy, have been developed (Millar and Anderson, in press; Gribben and
Millar submitted, Styan pers. com.).

Laboratory studies of *E. chloroticus* and *C. muricata* also highlighted the point that if
polyspermy is to be reliably detected, DNA staining of sperm is necessary. This is
important for future studies, because polyspermy cannot be reliably determined when
eggs experiencing severe polyspermy fail to raise fertilization envelopes and are thus
classified as unfertilized. Furthermore, correct assessment of polyspermy can sometimes
be difficult if this is equated with 1-cell stage eggs and/or abnormal development. There
are at least two reasons for this. Firstly, 1-cell stage eggs were often detected in samples
experiencing very low sperm concentrations (10³-10⁴ sperm ml⁻¹), but polyspermy was
never detected when representative samples were stained (Chapter 3 and Chapter 5). In
this case, single cell eggs are likely to be unfertilized. Secondly, abnormal development
can result from factors other than polyspermy, such as oxygen deprivation and/or
bacterial contamination (Bardach et al. 1972). It might be argued that the level of
technical difficulty and time costs imposed by this method outweigh the benefits of being
able to detect low levels of polyspermy, however, low levels of polyspermy (i.e. <5%) have been modeled to be of significant evolutionary importance (Gavrilets 2000, Frank 2000), thus staining is important to detect these.

It can be argued that high levels of polyspermy obtained from rockpool spawnings of *E. chloroticus* may be limited to these narrow ecological niches. However, simulated open-ocean spawnings, where water is deeper and sperm dilution is more likely to be rapid, have also shown that polyspermy occurs under sperm limiting conditions in *E. chloroticus* (Babcock and Franke in prep., not included in this thesis) as well as *Centrostephanus rodgersii* (Babcock *et al.* in prep.). Thus the risk of polyspermy is likely to be very real during most spawning events, and as a result represents an important evolutionary driving force.

**Polyspermy causing Sperm Limitation?**

The occurrence of polyspermy under a wide range of gamete contact times (Chapters 3 and 5), and at sperm concentrations as low as $10^5$-$10^6$ sperm ml$^{-1}$ (Franke *et al.* 2002) has not previously been demonstrated for marine invertebrates. These results may explain many phenomena in fertilization ecology as well as broader processes of evolution. This is important, because previous studies in fertilization ecology have focused on adaptations overcoming the occurrence of sperm limitation during spawning events ranging from cellular to population levels (reviewed in Levitan 1995, 1998). For example, it has been argued that attributes such as sperm chemotaxis (Miller 1985), enhanced viscosity of gametes (Thomas 1994), release of gametes under calm conditions
(e.g. Serrão et al. 1996), aggregation (Young et al. 1992, Hamel and Mercier 1995), synchronous spawning (e.g. Babcock et al. 1986, 1992) and the evolution of anisogamy and gender (Levitan 1996) are driven, at least in part, by sperm limitation. Furthermore, many of these phenomena, such as sperm chemotaxis (and other gamete properties), aggregation and release of gametes under calm condition have been demonstrated and/or modeled to increase fertilization success (e.g. Miller and King 1983, Levitan and Young 1995, Serrão et al. 1996, Babcock and Keesing 1999, Levitan 1998, Jantzen et al. 2001), thus providing evidence for their importance in overcoming sperm limitation. Although sperm limitation is undoubtedly an important evolutionary force, it is important to recognize that many phenomena exist that do not seem to enhance or to be related to enhancing fertilization success. For example, aggregation does not necessarily increase fertilization success per se in the brown surgeonfish (e.g. Kiflawi et al. 1998), but instead sperm competition appears to be the driving force for this behavior. Where sperm competition exists this is predicted to select for increased number of sperm in the vicinity of eggs. Again this poses the potential for high polyspermy occurrence. In fact, the occurrence of polyspermy per se has been recently argued to be a force exacerbating sperm limitation (Snook and Markow 2002) and it has been postulated that the risk of polyspermy may be a direct cause for sperm:egg ratios to be 1:1 shortly prior to fertilization in mammals (Hunter 1996). This low ratio is achieved by various physiological phenomena in the reproductive tracts and is thought to include countercurrent release of female reproductive hormones, which in turn would cause differential sperm transfer within the female tract thus causing low sperm:egg ratios during the early stages of fertilization (Hunter 1996).
If similar principles of reducing sperm concentrations occur, e.g. by limiting aggregations limiting the release of sperm, then this might explain, why sperm limitation occurs during natural (e.g. Babcock et al. 1992) and experimental (e.g. Pennington 1985, Levitan et al. 1992) fertilization events and in turn has been viewed as a dominant force during free and broadcast spawning events (e.g. Levitan and Petersen 1995, Levitan 1996).

**Polyspermy and Sperm Limitation are simultaneously acting Evolutionary Forces**

With the realization that polyspermy can be widespread in nature, many reproductive phenomena and evolutionary processes, ranging from cellular to population level, which at first appeared paradoxical, can now be explained. For example, on a cellular level, the co-existence of both polyspermy-driven polyspermy blocks (e.g. Jaffe and Gould 1985, Schuel 1985) and sperm-limitation-driven sperm chemoattractants in echinoids (e.g. Kaupp et al. 2003) can now be explained. Furthermore, spawning in response to storms and/or turbulent conditions (e.g. Grange 1976, Creese and Ballantine 1983, Keuskamp 1997, Chapter 4) may be a direct adaptation to reduce the risk of polyspermy during large scale-scale (epidemic) spawning events, even though post- fertilization benefits, such as enhance larval development and dispersal, may also be of adaptive value. Also, the fast polyspermy block present in algae (Brawley 1991) may have evolved in response to gametes being released under calm conditions (Brawley et al. 1999). Some polyspermy is produced under these conditions, but the polyspermy blocks appear to be highly effective unless decreases in salinity cause a decrease in efficiency of the Na⁺-dependent block (Brawley 1992).
Finally, evidence that polyspermy can occur under sperm limiting conditions provides support for previous hypotheses that high levels of variability in sperm-egg receptors and gamete compatibility (Palumbi 1999) have evolved as a polyspermy prevention mechanism (e.g. Vacquier et al. 1997, Galindo et al. 2003). This variability has profound implication for the potential for reproductive isolation and in turn speciation (e.g. Rice and Holland 1997, Rice 1998).

It is important to recognize however, that in many instances, neither polyspermy nor sperm limitation may be responsible for a particular reproductive phenomenon. For example, sperm chemotaxis may not have evolved purely from evolutionary pressures resulting from sperm limitation, but as a means of species-specific gamete recognition, particularly during multi-species spawnings (e.g. Babcock 1995, Coll et al. 1995). Here, the evolution of sperm-chemotaxis would have been driven by formation of non-viable hybrids resulting in loss of fitness.

This highlights the importance of considering each organism’s different ecological niche and life history characteristics. For example, differences in egg sizes, polyspermy block latencies and population densities may result in particular aspects of reproductive biology being more important in some organisms than in others. In E. chloroticus the combination of relatively high population densities and susceptibility to severe polyspermy under medium sperm concentrations in shallow and still waters (Franke et al. 2002) may explain why aggregations are not necessary for spawning (Dix 1969) and why
large parts of the population sometimes spawn synchronously under turbulent conditions (Chapter 4, Keuskamp 1997).

A further interesting example of the inter-dependent relationship between population and gamete characteristics and the relative importance of sperm limitation and sperm competition to fertilization success, is provided by three species of sea urchins (Strongylocentrotus sp.) co-existing in the North Pacific. The species that occurs at highest densities (S. purpuratus) also requires the highest sperm concentration to achieve the same fertilization success and the species occurring at the lowest density (S. droebachiensis) needs lowest sperm concentration. The third species (S. franciscanus), which is usually present at intermediate population densities requires intermediate sperm concentrations to achieve the same fertilization success as the other two species (Levitan 1993). Interestingly, this pattern is inversely related to egg size and sperm swimming speed, with S. purpuratus displaying the smallest egg, but fastest sperm, and S. droebachiensis having the largest eggs, yet, slowest sperm and S. franciscanus possessing intermediate egg size and intermediate swimming speed (Levitan 2002). Thus for S. purpuratus sperm competition is thought to be most important, whereas for S. droebachiensis sperm limitation has been implicated to be more significant during spawning events (Levitan 2002).

Future studies using these three urchin species will test the occurrence of polyspermy in situ, and it is predicted that S. droebachiensis will exhibit the highest amount of polyspermy at one particular sperm concentration, because eggs of this species may not
be as well adapted to excess sperm concentrations, i.e. their polyspermy block may not be as efficient as those of the other two species (Levitan and Franke, in progress). In addition, the occurrence of polyspermy will be related to the polymorphism that the sperm bindin protein exhibits, thus possibly allowing to test the extent of polyspermy driving the polymorphism in this gamete recognition protein (Levitan and Franke, in progress).

**CONCLUSION**

The demonstration of polyspermy and the fact that it occurs under a range of conditions of gamete concentrations, population densities and environmental conditions will hopefully contribute to the development of further reassessment and development of theories relating to fertilization ecology in broadcast spawning marine invertebrates. There is now good evidence to support the view that fertilization levels during *in situ* spawning events are not always low and that an excess of gametes is frequently of equal if not greater importance. Recently, the question of whether sperm limitation or sperm competition exists during natural fertilization events has been increasingly asked (Levitan 1998, Yund 2000) and fertilization levels *in situ* are now thought to underlie the continuum of sperm limitation to sperm competition (Levitan submitted). None-the-less, more evidence of polyspermy for different taxa under varying population and environmental conditions from natural spawnings is necessary, to determine its relative importance and large scale impact on ecological and evolutionary processes.
References


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APPENDIX