GAMETE COMPATIBILITY, GAMETE TRAIT VARIATION AND THEIR EFFECT ON FERTILIZATION SUCCESS IN A NORTHWEST ATLANTIC BLUE MUSSEL (*Mytilus edulis* L. AND *Mytilus trossulus* GOULD) HYBRID ZONE

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By

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Abstract

Mytilus edulis and *M. trossulus* coexist and form a natural hybrid zone along the Atlantic Canada coast, which provides an excellent model to study marine invertebrate evolution, speciation and especially reproductive isolation barriers that maintain species integrity. Gamete traits and their role in determining fertilization success among *M. edulis*, *M. trossulus* and their hybrids were investigated in the present study. Sperm of all three genotypes (the term genotypes was used throughout the thesis to include *M. edulis*, *M. trossulus* and their hybrids) conducted circular movement in a two dimensional plane, which may represent a trait that increases fertilization success under sperm limitation on a small spatial scale. Neither egg water nor egg presence elicited sperm chemotaxis in our experimental set-up, which suggests that sperm chemotaxis may not be necessary for successful fertilization of marine invertebrate species with dense aggregations, synchronized broadcast spawning and high gamete output. Although no significant difference in gamete output was detected among the different genotypes, sperm velocity differed among *M. edulis*, *M. trossulus* and their hybrids, F_1 hybrids producing the slowest swimming sperm. Sperm of *M. edulis* had faster VAP (average path velocity) and angle change rate than those of *M. trossulus*. Most of the variation in sperm velocity was attributed to variation within individuals, which may be caused by differences in stage of maturity among individual sperm. Both sperm velocity and sperm half life (T_{50}) decreased as temperature increased. F_1 hybrids had the shortest sperm longevity and *M. trossulus* was least temperature sensitive, which may be an adaptation to a longer spawning period, resulting in exposure to higher temperature fluctuations. *M. edulis* produced larger eggs than *M. trossulus* and F_1 hybrids, providing a larger target for sperm-egg interaction. Both homospecific and heterospecific fertilization success differed among parental combinations and may be due to the combined effects of gamete compatibility and gamete trait variation on fertilization success. Sperm velocity, egg size and especially

the combination of these two gamete traits were positively correlated with fertilization success, supporting the hypothesis that fertilization success is partially determined by these gamete traits. The three parameters (β, β_0 and β/β_0) of the fertilization kinetics model differed significantly among the different crosses. The small β/β_0 detected for the heterospecific crosses between *M. edulis* and *M. trossulus* suggests strong reproductive isolation between these species and can explain the rarity of F_1 hybrids. In general, the poor gamete quality (small egg size, slow swimming speed and short life span of sperm) of F_1 hybrids can lead to reduced fertilization success and may help to explain the genetic composition, especially the bimodal structure, of the blue mussel hybrid zone in Atlantic Canada. The present study shows that gamete trait variation (sperm velocity, sperm longevity and egg size) and gamete compatibility are crucial factors affecting both homospecific and heterospecific fertilization success, and provides a better understanding of prezygotic reproductive isolation between and among broadcast spawning species.

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List of Abbreviations

ALH	=	Amplitude of Lateral Head movement
ANOVA	=	Analysis of Variance
BCF	=	Beat Cross Frequency
ca	=	Circa
CASA	=	Computer assisted or aided sperm analysis
DUI	=	Doubly Uniparental DNA Inheritance
E	=	*Mytilus edulis*
E_0	=	Egg concentration
F_1	=	First filial generation hybrid
F_{20}	=	Sperm concentration required to fertilize 20% of the eggs
F_{50}	=	Sperm concentration required to fertilize 50% of the eggs
FSW	=	Filtered Seawater
HMT	=	Hamilton Throne sperm analyzer
LDV	=	Laser Doppler Velocity
LIN	=	Linearity of sperm track
MAD	=	Mean Angular Displacement
MEP	=	Multiple-exposure photography
Ni	=	Number of individuals
Ns	=	Number of sperm
S.D.	=	Standard deviation
S.E.	=	Standard error
S_0	=	Sperm concentration
sec	=	Seconds
STR	=	Straightness of sperm track
T	=	*Mytilus trossulus*

T_{50}	=	Sperm half life
TEM	=	Time-exposure photomicrography
VAP	=	Average path sperm velocity
VCL	=	Curvilinear sperm velocity
VHS	=	Video Home System
VSL	=	Straight-line sperm velocity
WOB	=	Wobble, side to side movement of sperm head
β	=	Fertilization rate constant
β_0	=	Collision rate constant

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Chapter 1

Introduction

1.1 Speciation, reproductive isolation and hybrid zones

Speciation is a fundamental issue in evolutionary biology, but it is both fascinating and frustrating since the process is in general too slow to be observed directly and the investigator must therefore rely on rigorous inference. It is generally accepted that the majority of speciation events occur when two populations are geographically isolated and diverge genetically through natural selection and/or genetic drift, eventually resulting in intrinsic reproductive barriers between the populations (Dobzhansky 1937; Mayr 1963). Diverging populations may come into contact again and form a natural hybrid zone. Since reproductive isolation is often incomplete between populations that hybridize, there can be ongoing gene flow between populations, and therefore natural hybrid zones can be maintained by reproductive isolation barriers that counteract the homogenization effect of gene flow. Therefore, hybrid zones provide excellent opportunities for studying the evolution of reproductive isolation barriers and the speciation process (Arnold 1997; Barton 2001; Barton and Hewitt 1985, 1989; Berrieman et al. 2005; Harrison 1993; Jones et al. 2006; Orr 1996; Vines et al. 2003).

Unlike most species with internal fertilization, marine broadcast spawning organisms with external fertilization may lack the complex mating behaviors that often play an important role in reproductive isolation (Arita 1979; Boughman 2002; Gomez and Serra 1995; Price and Boake 1995; Yamada et al. 2008). Furthermore, in many marine broadcast spawning species, high dispersal by planktonic larvae is often

associated with only limited genetic differentiation over large spatial scales (Palumbi 1994). Therefore, it is not only important but also challenging to study the evolution of reproductive isolation barriers and the speciation process in marine broadcast spawning species. As has been documented for many terrestrial species, natural hybrid zones offer a unique opportunity to investigate variation in reproductive isolation experimentally. Mussels in the *Mytilus edulis* complex form hybrid zones whenever two species come into contact and can therefore serve as a useful model system for studying speciation in marine broadcast spawning species.

1.2 Systematic status, distribution and importance of mussels

Mussel populations have a world-wide distribution in both northern and southern temperate and cold oceans (Blanchette and Gaines 2007; Hilbish et al. 2002; Moreau et al. 2005; Vuorinen et al. 2002; Wonham 2004). There are several species in the genus *Mytilus* including *Mytilus californianus*, *M. chilensis*, *M. platensis*, *M. planulatus*, *M. desolationis*, *M. edulis*, *M. edulis aoteanus*, *M. galloprovincialis* and *M. trossulus* (Gardner 2004; Gosling 1984; Hilbish et al. 2000; Koehn 1991; Mcdonald et al. 1991; Seed 1992; Wonham 2004), but the taxonomic status has yet to be resolved for some species. It is generally accepted that *M. chilensis*, *M. platensis*, *M. planulatus*, *M. desolationis* and *M edulis aoteanus,* which are only found in the southern hemisphere, probably originated from northern Atlantic populations and should be considered as sister taxa to *M. edulis* and *M. galloprovincialis* (Hilbish et al. 2000; Mcdonald et al. 1991; Toro 1998). Although *M. californianus* is found in the northern hemisphere, like *M. edulis, M. trossulus* and *M. galloprovincialis*, and has a distribution along the Pacific coast from Alaska to southern Baja California, it is more distantly related to these species (Koehn 1991; Seed 1992). Furthermore, although *M. edulis, M. trossulus* and *M. galloprovincialis* are closely related species, based on both morphological and genetic differences, *M. trossulus* is considered to be more distantly

related to *M. edulis* and *M. galloprovincialis* (Beynon and Skibinski 1996; Gardner 1992; Innes and Bates 1999; Koehn 1991; Seed 1992).

Mussel hybrid zones occur along the coasts of the Atlantic and Pacific Oceans and the Baltic and Mediterranean Seas, wherever the distributions of *M. edulis, M. trossulus* and *M. galloprovincialis* overlap (Bierne et al. 2003b; Comesaña and Sanjuan 1997; Comesaña et al. 1999; Gilg and Hilbish 2000, 2003; Gilg et al. 2007; Gosling et al. 2008; Hilbish et al. 2002; Innes and Bates 1999; McCartney and Lima 2006; Riginos and Cunningham 2005; Riginos et al. 2002; Toro et al. 2004, 2006; Väinölä and Hvilsom 1991; Wonham 2004). Marine mussel hybrid zones in which viable hybrids are produced naturally provide an important model system for understanding speciation and present a paradox for the biological definition of a species, usually defined as "a population of actually or potentially interbreeding individuals that produce fertile offspring" (Mayr 1942). According to this definition both parental forms are one species, as they can produce fertile offspring. However, the two populations remain identifiably different in their morphology and genetics (Diz and Skibinski 2007; Heath et al. 1995; Innes and Bates 1999; Inoue et al. 1995), conforming to an alternative definition of species as "taxa that retain their identity despite gene flow" (Barton and Hewitt 1989).

In general, studying mussel hybridization and reproductive isolation is important for understanding speciation in marine broadcast spawning species and can also contribute to a better understanding of how heterospecific variation in morphological and growth characteristics can assist mussel aquaculture. Furthermore, mussel species, especially *M. edulis*, *M. trossulus* and *M. galloprovincialis*, are not only an important component of the intertidal and subtidal communities in terms of biomass and production but also economically important for aquaculture.

1.3 The blue mussel hybrid zone in Newfoundland, Canada

M. edulis, *M. trossulus* and their hybrids coexist along Newfoundland coasts (Bates and Innes 1995; Innes and Bates 1999). The blue mussel hybrid zone in Newfoundland has a different spatial structure to most hybrid zones. In most cases, two divergent populations come into contact to form a hybrid zone of varying width, producing a clinal change in the frequency of mixed hybrid genotypes (Barton and Hewitt 1985, 1989). In contrast, *M. edulis*, *M. trossulus* and hybrid individuals are found along the entire shore of Newfoundland, forming a mosaic distribution pattern consisting of mixtures of both parental species and various hybrid genotypes (Bates and Innes 1995). According to studies that used five genetic markers, most adult individuals from the Northwest Atlantic blue mussel hybrid zone are pure *M. edulis* or *M. trossulus* (Comesaña et al. 1999; Saavedra et al. 1996; Toro et al. 2004), forming a bimodal genetic composition (most individuals are pure parental species with few hybrids). Most hybrids appear to be the result of intercrossing among hybrid individuals and backcrossing to both parental species, and only about 1~2.5% of the population is F_1 hybrids. The number of diagnostic genetic markers available to distinguish among the two species and hybrids is limited. Two commonly used diagnostic genetic markers were employed in the present study: GLU, a nuclear DNA marker that amplifies different parts of the gene that encodes the mussel polyphenolic adhesive protein produced by the pedal gland (Comesaña et al. 1999; Rawson et al. 1996), and ITS, a nuclear marker that amplifies the internal transcribed spacer regions that lie between the 18s and 28s nuclear rDNA coding regions (Comesaña et al. 1999; Heath et al. 1995). However, this small number of genetic markers limits the accuracy of any genotype determination of individuals scored as either one of the two species or as hybrids (Boecklen and Howard 1997). Newfoundland blue mussels produce viable hybrids, suggesting incomplete reproduction isolation between *M. edulis* and *M. trossulus*, which is consistent with studies of various organisms (Barton and Hewitt

1985, 1989; Harper and Hart 2005; Milne and Abbott 2008). However, the low frequency of F_1 hybrids suggests that although reproductive isolation between *M. edulis* and *M. trossulus* may be incomplete, it is sufficient to maintain the genetic integrity of the two species in the hybrid zone.

1.4 Reproductive isolation barriers in mussel hybrid zones

Knowledge of reproductive isolation barriers is not only crucial to understanding how natural hybrid zones are maintained but also particularly important to the biological species concept. Reproductive isolation barriers can act at different life history stages and are generally divided into two groups, prezygotic (or premating) isolation mechanisms and postzygotic (or postmating) isolation mechanisms (Nei et al. 1983). The former prevent fertilization and zygote development, and include temporal isolation, spatial isolation, behavioral isolation, mechanical isolation and gamete incompatibility, whereas the latter cause inviability or sterility of hybrids at different life-history stages.

Although *M. edulis* and *M. trossulus* in Newfoundland show some differences in spawning time, the spawning periods overlap (Toro et al. 2002). Temporal difference in spawning time may not provide strong reproductive isolation barriers in Newfoundland because of the relatively short summer season. However, there is some evidence for habitat segregation between the two species that may reduce opportunities for hybridization. For example, *M. edulis* from the Nova Scotia hybrid zone prefers low salinity and less wave-exposed environments (Gartner-Kepkay et al. 1983). Similarly, Bates and Innes (1995) found that more than 90% of the individuals at two wave-exposed sites in Newfoundland were *M. trossulus*, suggesting a potential for habitat segregation between the two species. *Mytilus edulis* from a Mediterranean hybrid zone also preferred more sheltered habitats for settlement compared with *M. galloprovincialis* (Bierne et al. 2003a). However, this trend of habitat segregation for

different mussel species was not observed in hybrid zones in the Baltic Sea, southwest England and the Irish Sea (Coghlan and Gosling 2007; Gilg and Hilbish 2003; Riginos et al. 2002).

Generally, *M. edulis* and *M. trossulus* individuals are morphologically similar, although they do differ in shell shape and size range (Innes and Bates 1999). Although *M. edulis* produces larger eggs than *M. trossulus* (Toro et al. 2002), sperm head size does not differ between the two species (Miranda 2004). Thus, it is unlikely that any gamete incompatibility between *M. edulis* and *M. trossulus* is due to size or morphology alone.

Gamete incompatibility between *M. edulis* and *M. trossulus* has been reported from the hybrid zone in Maine, where a 100~700 fold higher F_{20} (the sperm/egg concentration ratio required to fertilize 20% of the eggs) was reported for inter-species fertilization than for intra-species fertilization (Rawson et al. 2003). A similar study found that the degree of gamete incompatibility did not differ significantly among heterospecific crosses involving *M. edulis* females that were either from populations where both species coexisted (sympatric) or from populations consisting of *M. edulis* only (allopatric) (Slaughter et al. 2008).

There is also evidence for postzygotic reproductive isolation between *M. edulis* and *M. trossulus* in the Newfoundland mussel hybrid zone. For example, a higher rate of abnormal larval development and mortality in hybrids than in parental species was reported from a limited number of crosses by Toro et al. (2006). Similar results were obtained by Miranda (2004), who used a larger number of crosses but recorded considerable variation in larval development and survival among heterospecific crosses.

1.5 Gamete traits and reproductive isolation in free spawning organisms

Unlike copulating species, free spawning species such as mussels release their gametes directly into the water column, where external fertilization takes place. There may be a lack of behavioral reproductive isolation barriers because there are no courtship or copulation behaviors. Fertilization success for free spawning organisms such as *Mytilus* species depends mainly on gamete interactions (Vogel et al. 1982). Therefore, gamete traits such as sperm velocity, sperm longevity, sperm temperature tolerance and egg size are important for determining fertilization success in free spawning organisms (Levitan 1996, 2000b, 2006, 2008). Furthermore, because gamete concentration can affect fertilization success (Levitan et al. 1991), releasing gametes into an open, mixing water system may also lead to other factors affecting fertilization success, such as sperm competition (Ball and Parker 1996, 1997; Parker 1982) and sperm limitation (Levitan and Petersen 1995). Generally, different traits will be selected under different conditions, e.g. higher sperm velocity favoured when there is a higher probability of sperm competition and sperm with a longer life span favoured when fewer sperm are present (Levitan 2004a). According to the fertilization kinetics model proposed by Vogel et al.(1982), gamete traits determine fertilization success in free spawning organisms. Therefore, heterospecific differences in gamete traits may lead to reduced success for heterospecific fertilizations. Hence gamete trait differences between species can have the potential to act as reproductive isolation barriers.

1.6 Objectives

To date only a few studies (Rawson et al. 2003; Slaughter et al. 2008; Toro et al. 2002) have investigated reproductive isolation in the *M. edulis* - *M. trossulus* hybrid

zone. There is little information on the role that gamete traits, especially sperm velocity, sperm longevity, sperm chemotaxis and egg size, play in determining fertilization success among *M. edulis*, *M. trossulus* and their hybrids. Differences in gamete traits between hybridizing sympatric species can affect fertilization success through processes such as sperm competition, which may lead to prezygotic reproductive isolation between species.

The main objective of the present study was to use the Newfoundland *M. edulis* - *M. trossulus* hybrid zone as a model to provide a better understanding of how the gamete traits of free spawning organisms affect reproductive success and to determine whether gamete trait differences between species can act as a reproductive isolation barrier.

The following are the specific objectives addressed:

Chapter 2: Conduct a quantitative analysis of plane, non-linear circular movement for free-swimming sperm of the blue mussels *M. edulis*, *M. trossulus* and their hybrids by

(1) using computer assisted sperm velocity video analysis and

(2) designing appropriate parameters to describe and verify the sperm movement pattern using software (Image-J) that may be widely applicable to other organisms.

Chapter 3: Determine the effect of egg water and egg presence on sperm velocity in the blue mussels *M. edulis*, *M. trossulus* and their hybrids:

(1) Investigate the effect of egg water and egg presence on sperm velocity.

(2) Determine whether there is species-specific sperm activation and sperm chemotaxis that can affect both homospecific and heterospecific fertilization success and that may influence the degree of reproductive isolation between the genotypes *M. edulis*, *M. trossulus* and hybrids.

Chapter 4: Investigate the role of sperm competition as a post-spawning prezygotic reproductive isolation barrier in the blue mussels *M. edulis*, *M. trossulus* and their hybrids:

(1) Estimate gamete output in a natural *M. edulis* and *M. trossulus* hybrid zone, which plays an important role in determining the degree of sperm competition.

(2) Determine whether sperm velocity differs among genotypes and, if so, how sperm velocity differences within each group and within individuals contribute to the total variation in sperm velocity.

(3) If sperm velocity does differ among genotypes then estimate how sperm velocity differences among genotypes affect fertilization success through heterospecific sperm competition by calculating the gamete collision rate according to Vogel's fertilization kinetics model.

Chapter 5: Determine the effect of temperature on sperm longevity in blue mussels *M. edulis*, *M. trossulus* and their hybrids:

(1) Investigate how temperature and sperm age affect sperm velocity in each genotype.

(2) Determine whether there are differences in sperm longevity among genotypes and discuss how such differences affect fertilization success, especially during sperm limitation.

(3) Calculate the trade-off between sperm velocity and sperm longevity and test whether the energy restraint hypothesis holds true between species.

Chapter 6: Assess gamete compatibility and the effect of gamete traits on fertilization success in an Atlantic Canada blue mussel hybrid zone:

(1) Investigate variation in egg size among *M. edulis*, *M. trossulus* and hybrids, which may affect both homo- and heterospecific fertilization success by increasing gamete collision rate.

(2) Investigate the effect of sperm velocity, egg size and the combination of these gamete traits on fertilization success.

(3) Determine how different parental combinations affect fertilization success.

(4) Estimate the effect of gamete contact time on fertilization success in *M. edulis*.

(5) Estimate β (the fertilization rate constant), β_0 (the gamete collision rate constant) and β/β_0 (the proportion of the egg surface that is fertilizable) for homospecific and heterospecific fertilization by integrating all the available data (sperm velocity, sperm longevity, egg size, gamete concentration and fertilization success) within Vogel's fertilization kinetics model.

(6) Determine the degree of gamete incompatibility for homospecific and heterospecific fertilization among *M. edulis*, *M. trossulus* and hybrids by comparing F_{20} (the sperm/egg concentration ratio required to fertilize 20% of the eggs) and the parameters that define the fertilization kinetics.

(7) Discuss and compare the two methods (F_{20} and Vogel fertilization kinetics model) used to estimate gamete incompatibility.

Chapter 2

Quantitative analysis of plane non-linear circular movement in the sperm of the blue mussels *Mytilus edulis*, *M. trossulus* and their hybrids

2.1 Introduction

2.1.1 History of sperm velocity analysis

Sperm velocity is an important determinant of fertilization success and has been investigated in many invertebrate and vertebrate species, including polychaetes (Kupriyanova and Havenhand 2002), sea urchins (Levitan 1996, 2000b), salmon (Gage et al. 2004), red deer (Malo et al. 2005), rats (Moore and Akhondi 1996) and humans (Auger et al. 1994; Jouannet and Serres 1998; Sallam et al. 2001a, b; 2003; Van den Bergh et al. 1998). In the last few decades, advances in technology, especially in computer analysis, have made it possible to measure sperm velocity by various methods. Although each technique has its own advantages and disadvantages, CASA (computer aided sperm analysis) has become more popular than other methods. The methods can be grouped as follows:

(1) Methods that measure sperm velocity indirectly.

Early sperm velocity studies depended on passage counting, in which the number of sperm that crossed a certain line or area in a certain period of time is used to estimate the average sperm velocity for the whole semen sample (Bartak 1973; Hynie 1962; Levin et al. 1984).

Spectrophotometry and laser doppler velocimetry (LDV) measure sperm velocity based on physical characters other than movement itself. In the former method the turbidity change in the sperm medium can be detected in a spectrophotometer and the proportion of rapidly moving sperm and average sperm velocity can be calculated (Halangk and Bohnensack 1986; Levin et al. 1984; Sokoloski et al. 1977). This technique has recently been used to estimate the vertical velocity of the sperm by detecting the turbidity changes in different layers of the medium in which the sperm are swimming (Saha et al. 2007).

Laser doppler velocimetry is based on the principle of the doppler effect, and detects the changes in the frequency of light caused by sperm movement. The proportion of motile sperm, the velocity distribution and the instantaneous velocity can be estimated by LDV (Jouannet et al. 1977; Mortimer et al. 1984).

All these methods are relatively simple and rapid, but they provide limited information and the measurements are indirect, which means that it is impossible to show the sperm movement track and describe movement patterns for individual sperm.

(2) Methods that measure sperm velocity directly.

Microcinematography (Mortimer et al. 1984), timed-exposure photomicrography (TEM) (Chan et al. 1991), multi-exposure photography (MEP) (Oliva et al. 1993), videomicrography (Gottlieb et al. 1991; Tessler and Olds-Clarke 1985) and computer assisted sperm analysis or computer aided sperm analysis (CASA) (Betancourt et al. 2006; King et al. 2000; Mortimer 2000; Toth et al. 1995) are all methods that measure sperm velocity directly. All share some similar steps and follow similar principles, so it is hard to separate them very clearly. For example, CASA is usually carried out on the raw data from videomicrography, hence it is difficult to separate completely CASA from videomicrography. In general, these methods are based on the reconstruction of the sperm movement track, regardless of whether the data are derived from film images, video sequences or digital images. Various parameters can

be calculated from the reconstructed sperm track and unlike in the first group of methods, sperm velocity is measured directly and therefore much more movement information can be obtained, as will be discussed in detail below.

2.1.2 CASA (computer assisted sperm analysis or computer aided sperm analysis) and the application of Image-J in CASA

CASA was first applied to measure rat sperm velocity in the 1980s (Working and Hurtt 1987) and since then this technique has been widely used for diverse organisms and purposes, especially human clinical studies. A number of commercial systems have been developed, including the CellSoft System, the Hamilton Throne sperm analyzer (HTM system), the MedeaLab CASA sperm analyzer, Motion Analysis Corporation's CellTrak system and the VideoTesT-Sperm computer assisted sperm analyzer (Amann and Katz 2004; Mortimer 1997).

Although CASA is the most popular and powerful method, it is not perfect and there are certain limitations that should be taken into consideration, depending on the specific purpose of the study. One issue that limits the use of CASA is the high price of commercial CASA systems. In addition, they were originally designed for clinical use on humans, which may be problematic when they are applied to other organisms. For example, the parameters obtained from these systems may not be biologically meaningful for sperm with a circular movement pattern. Furthermore, caution is required when comparing sperm velocity results obtained from different systems or even from the same system because of the differences in sample preparation, parameter settings, algorithms and the sperm track reconstruction method (Chan et al. 1991; Gottlieb et al. 1991; Levine et al. 1989; Mortimer et al. 1995; Mortimer 2000; Mortimer and Swan 1999; Rijsselaere et al. 2003).

Image-J is a powerful Java-based image processing program developed by the National Institutes of Health (http://rsb.info.nih.gov/ij/, Rasband W., NIH). Different

Java plug-ins can be developed using the built-in editor and Java compiler for specific purposes. Recently a sperm tracking or velocity analysis function has been added to the Image-J program, and a manual tracking plug-in developed for cell movement tracking was used in the present study (http://rsb.info.nih.gov/ij/plugins/track/track.html, Fabrice P. Cordelières, Institut Curie). With this plug-in, both the coordinate values and the total velocity (curvilinear velocity or VCL) data can be obtained. Another plug-in is available (http://rsb.info.nih.gov/ij/plugins/docs/CASAinstructions.pdf, Jonas Wilson-Leedya and Rolf L. Ingermann, University of Idaho, Moscow) to interface CASA analysis functions with the Image-J program. By taking advantage of this open source software, Image-J CASA analysis can now be conducted at low cost (Wilson-Leedy and Ingermann 2007). Furthermore, new descriptive parameters for specific movement patterns can also be easily developed from the original coordinate data.

2.1.3 Commonly used sperm velocity parameters

Many parameters have been applied to describe sperm velocity, such as curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head movement (ALH), mean angular displacement (MAD) and beat cross frequency (BCF) (Kawaguchi et al. 2004; Mortimer 1997). VCL, VAP and VSL are the most commonly used parameters for sperm velocity analysis, and most of the others are calculated from these three. Although different CASA instruments may use different algorithms for various sperm parameter calculations, generally these parameters can be interpreted and calculated as outlined below (see Fig.2.1a):

Curvilinear Velocity: VCL, also known as total velocity, is calculated by dividing the total point to point curvilinear distance by the time taken for the sperm to travel this distance.

Average Path Velocity (VAP): A mathematically smoothed path is reconstructed and VAP is calculated by dividing the total smoothed distance by the time taken for the sperm to travel this distance.

Straight Line Velocity: VSL, a parameter measuring the rate of net space gain of the sperm, is calculated by dividing the distance from the start point to the end point by the time taken for the sperm to travel this distance.

Linearity (LIN) and Straightness (STR): LIN is calculated by taking the percentage ratio of VSL to VCL to describe the path curvature (LIN=VSL/VCL*100%). STR is calculated by taking the percentage ratio of VSL to VAP to describe the path straightness (STR=VSL/VAP*100%). Neither LIN nor STR can exceed 100%. The circling movement gives low LIN and STR values and will be discussed later in this chapter.

Wobble: WOB is calculated as the ratio of VAP to VCL and describes the side to side movement of the sperm head (WOB=VAP/VCL*100%). The value of WOB is less than 100% and gives information regarding how well the smoothed path fits the raw path.

Beat Cross Frequency: BCF is defined as the frequency at which the VCL path crosses the VAP average smoothed path.

Other parameters such as ALH and MAD are also used to describe the sperm head movement pattern. However, since it is the sperm velocity that is believed to affect fertilization success directly, VCL, VSL and VAP are used more often than any of the other parameters.

2.1.4 Objective and importance of this study

The blue mussel species *Mytilus edulis* and *M. trossulus* coexist in Atlantic Canada, forming a natural hybrid zone (Bates and Innes 1995; Innes and Bates 1999; Toro et al. 2002, 2004, 2006). Genetic studies have shown that F_1 hybrids represent

less than 2.5% of the individuals in sympatric natural populations of these species (Comesaña et al. 1999; Saavedra et al. 1996). The present study is focused on the prezygotic hybridization barrier formed by homospecific differences in gamete traits that affect fertilization success, in order to gain more information to explain the low frequency of F_1 hybrids observed in natural populations and to understand how this natural hybrid zone is maintained. To investigate how sperm traits affect fertilization success, it is important to choose appropriate parameters to measure movement in sperm from *M. edulis*, *M. trossulus* and their hybrids. The objective of the study described in this chapter is to describe the movement pattern for sperm from *M. edulis*, *M. trossulus* and their hybrids, to formulate appropriate parameters to quantify the circular movement of mussel sperm, and to discuss the biological meaning of this movement pattern. The results will contribute to an understanding of variation in sperm velocity in mussels and will also be useful for relating sperm velocity to fertilization success. In addition, the parameters can be used to describe circular movement in sperm from other organisms.

2.2 Materials and methods

2.2.1 Collection and holding of mussels

Adult blue mussels were collected twice a month from a mussel farm at Trinity, Trinity Bay, Newfoundland, Canada from late May to early August 2005 and were housed in flowing, 10 °C seawater until spawning was induced by thermal shock (Everett et al. 2004). The mussels were fed daily with Commercial Instant Shellfish Diet 1800® (Reed Mariculture, Inc) to keep them in spawning condition according to the instructions provided. After spawning (details in 2.2.2 below) individuals were genotyped using GLU and ITS nuclear markers to identify the two species and hybrids following the methods of described by Health and Inoue et al. (1995) and Inoue et al.

(1995). Those individuals shown to be hybrids by both genetic markers were classified as F_1 hybrids and those shown to be hybrids by only one of the genetic markers were classified as backcross hybrids.

2.2.2 Spawning and gamete collection

Spawning was induced by thermal shock following the methods of described by Jha et al. (2008) and Everett et al. (2004). Each mussel was rinsed with filtered seawater three times and placed in a separate container (total volume about 1.5 liters) that contained 1 liter filtered seawater (FSW) warmed to 20 °C. FSW was obtained by passing the seawater through a series of filters (100μm, 30μm, 10μm and 1μm). Each individual male was allowed to spawn for 30 min once the spawning had started. The sperm suspension was then passed through a 20μm sieve (within a few minutes). About 30μl of filtered sperm suspension was used to make a wet mount (depth approximately 0.25 mm) for the sperm movement study following Everett et al. (2004).

2.2.3 Video data collection and preparation

Sperm movement was viewed with a 100X objective lens on an Axiovert inverted light microscope (Carl Zeiss, Inc.) fitted with a video camera. A one minute video sequence was recorded for each individual on VHS videotape (30 frames per second) with a freshly made sperm solution wet mount. (Based on a preliminary experiment, blue mussel sperm can live and swim for several hours. Therefore, time was less of an issue than in studies with fish sperm, for example). The images were digitized with the video capture function in Adobe Premiere Pro 3.0. The digital video data was cut into small video clips using Windows Movie Maker. These clips were

transformed into uncompressed old format avi files withVirtualDub in order to satisfy the format requirement of Image-J.

2.2.4 Calibration and raw data collection with Image-J manual tracking plug-in

Digitized video clips of sperm motion were input into Image-J by the avi reader plug-in (Daniel Marsh, http://rsb.info.nih.gov/ij/plugins/avi-reader.html). Image stack data was analyzed with the Image-J manual tracking plug-in. Before each run of the analysis, a calibration was conducted by measuring a known distance horizontally and vertically with a stage micrometer. By comparing the horizontal and vertical distance results from Image-J output, the x/y calibration setting in the manual tracking plug-in was calculated. After calibration, the Image-J reconstructed moving track was used for movement pattern analysis and all the coordinate data were collected from the Image-J output for sperm movement track reconstruction and movement pattern analysis.

2.2.5 Data analysis and statistics

The sperm movement track was quantitatively reconstructed with raw sperm head coordinate data using equations in Microsoft Excel (See appendix 1 in the attached CD for equations and calculation details; Wilson-Leedy and Ingermann 2007). To verify whether there was constant movement along the x axis, a binomial test was conducted by comparing the total number of track centers moving along the x axis in each direction ($dx>0$ versus $dx<0$) using the the "R" program (R-Development CoreTeam 2008). The same analysis was applied for dy to verify whether there was a constant track center movment along the y axis. One-way ANOVA was performed to detect the effect of swimming velocity on radius and to compare mean angle change rate in different genotype groups using the "R" program. Pairwise mean comparisons

were conducted (Tukey test in OriginPro 7.5) in order to evaluate the differences in mean angle change rate.

2.3 Results

2.3.1 The movement pattern of blue mussel sperm

All sperm from *M. edulis*, *M. trossulus* and hybrids exhibited circular movement in a two dimensional plane (Fig. 2.2, Video 2.1) as reported in various free spawning invertebrate species (Table 2.1). The motion was always clockwise (Video 2.1).

2.3.2 Parameters developed for circular movement of blue mussel sperm

In addition to VCL and VAP, three new parameters were developed using the raw coordinate values of the position of a moving sperm (Fig. 2.3). From three sequential positions of the tracking sperm head, we can determine the locus $O(x_0, y_0)$ (the center of the circular track), R (the radius of the circle movement path) and θ (angle change per frame or angle change rate per 1/30 sec). Here θ is different from the angle of the sperm direction (which is calculated by atan(dy/dx)).

All sperm head position points A, B and C are from the same circular path, so their coordinate values fit the equation (1)

$$(x - x_o)^2 + (y - y_o)^2 = R^2 \qquad (1)$$

When we substitute the coordinate values of $A(x_1, y_1)$, $B(x_2, y_2)$ in (1), we obtain (2) and (3)

$$(x_1 - x_o)^2 + (y_1 - y_o)^2 = R^2 \qquad (2)$$

$$(x_2 - x_o)^2 + (y_2 - y_o)^2 = R^2 \qquad (3)$$

$R^2 = R^2$, therefore, we then obtain (4)

$$x_1^2 + x_o^2 - 2x_1x_o + y_1^2 + y_o^2 - 2y_1y_o = x_2^2 + x_o^2 - 2x_2x_o + y_2^2 + y_o^2 - 2y_2y_o \quad (4)$$

(4) can be simplified as (5)

$$x_1^2 - 2x_1x_o + y_1^2 - 2y_1y_o = x_2^2 - 2x_2x_o + y_2^2 - 2y_2y_o \quad (5)$$

solving for x_o, we obtain (6)

$$x_o = \frac{y_1 - y_2}{x_2 - x_1} \cdot y_o + \frac{1}{2} \cdot \frac{y_2^2 - y_1^2 + x_2^2 - x_1^2}{x_2 - x_1} \quad (6)$$

When we substitute the coordinate values of $B(x_2, y_2)$ and $C(x_3, y_3)$ in equation (1) and follow the same steps we obtain (7)

$$x_o = \frac{y_2 - y_3}{x_3 - x_2} \cdot y_o + \frac{1}{2} \cdot \frac{y_3^2 - y_2^2 + x_3^2 - x_2^2}{x_3 - x_2} \quad (7)$$

$x_o = x_o$, therefore, from (6) and (7), we obtain (8)

$$\frac{y_1 - y_2}{x_2 - x_1} \cdot y_o + \frac{1}{2} \cdot \frac{y_2^2 - y_1^2 + x_2^2 - x_1^2}{x_2 - x_1} = \frac{y_2 - y_3}{x_3 - x_2} \cdot y_o + \frac{1}{2} \cdot \frac{y_3^2 - y_2^2 + x_3^2 - x_2^2}{x_3 - x_2} \quad (8)$$

Solving for y_o, we obtain (9)

$$y_0 = \frac{1}{2} \cdot \left(\frac{y_3^2 - y_2^2 + x_3^2 - x_2^2}{x_3 - x_2} - \frac{y_2^2 - y_1^2 + x_2^2 - x_1^2}{x_2 - x_1}\right) / \left(\frac{y_1 - y_2}{x_2 - x_1} - \frac{y_2 - y_3}{x_3 - x_2}\right) \quad (9)$$

from (6) and (9), therefore, we then obtain (10)

$$x_o = \left[\frac{1}{2} \cdot \frac{y_1 - y_2}{x_2 - x_1} \cdot \left(\frac{y_3^2 - y_2^2 + x_3^2 - x_2^2}{x_3 - x_2} - \frac{y_2^2 - y_1^2 + x_2^2 - x_1^2}{x_2 - x_1}\right)\right] / \left(\frac{y_1 - y_2}{x_2 - x_1} - \frac{y_2 - y_3}{x_3 - x_2}\right) + \frac{1}{2} \cdot \frac{y_2^2 - y_1^2 + x_2^2 - x_1^2}{x_2 - x_1} \quad (10)$$

All the coordinate values for the three points $A(x_1, y_1)$, $B(x_2, y_2)$ and $C(x_3, y_3)$ are known from sperm velocity manual tracking. Following steps (1) to (10) the center position coordinate values (x_0, y_0) of the circular path were calculated. When we substitute (x_0, y_0) in equation (2), we obtain (11)

$$R = \sqrt{(x_1 - x_o)^2 + (y_1 - y_o)^2} \quad (11)$$

From Fig.3, angle change (θ), distance between A, B (AB) and the radius (R) are described by equation (12)

$$Sin(\frac{\theta}{2}) = (\frac{1}{2} \cdot AB) / R \quad (12)$$

AB is the distance between point A (x_1, y_1) and point B (x_2, y_2), which can be calculated by equation (13)

$$AB = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2} \quad (13)$$

From (11), (12) and (13) the angle change rate can be calculated using equation (14)

$$\theta = 2 \cdot a\sin(\frac{1}{2} \cdot \frac{\sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}}{\sqrt{(x_1 - x_o)^2 + (y_1 - y_o)^2}}) \quad (14)$$

2.3.3 Verification of the circular movement pattern

From every three sperm head positions in a row, the center of the circular path can be calculated by equations (9) and (10). A series of center position coordinate values $O_t(x_t, y_t)$ (center position at tracking time t), which is obtained from all the sperm head position coordinates, can be used for circular path verification. For any two center positions in a row, $O_1(x_1, y_1)$ and $O_2(x_2, y_2)$, the horizontal and vertical coordinate changes (dx and dy) can be calculated from equations (15) and (16)

$$dx = x_2 - x_1 \quad (15)$$

$$dy = y_2 - y_1 \quad (16)$$

The number of times that dx and dy satisfies $dx>0$; $dx<0$ and $dx=0$ or $dy>0$; $dy<0$ and $dy=0$ was counted respectively.

Although the data for the horizontal and vertical movements of $O_t(x_t, y_t)$ show that the position of $O_t(x_t, y_t)$ is always moving, the counts ratios for net gain and net loss regardless of position along the x or y axis are not significantly different (binomial test (Hollander and Wolfe 1973), Table 2.2.). Therefore, statistical analysis provides evidence that mussel sperm exhibit a circular movement pattern without a consistent net gain or loss in any direction.

2.3.4 Radius, angle change rate and their relationship with sperm velocity

The mean radius of the sperm circular movement track for *M. edulis*, *M. trossulus*, F_1 hybrids and back cross hybrids was 149.28±16.75(SD), 149.76±27.51, 175.70±38.62 and 150.95±21.78 μm respectively (Table 2.3). There was no significant difference between genotypes, and no effect of swimming velocity (ANOVA, Table 2.4).

The mean angle change rate per frame for *M. edulis*, *M. trossulus*, F_1 hybrids and back cross hybrids was 0.41±0.15(S.D.), 0.33±0.16, 0.23±0.14 and 0.42±0.16 radian changes per frame, respectively (Table 2.3). The radian change rate was significantly different among genotypes (ANOVA, Table 2.5). F_1 hybrids had the significantly lowest radian change rate compared to both parental species and backcross hybrid groups (Tukey test, Table 2.3).

2.4 Discussion

Sperm circular movement in a two dimensional plane has been reported in many organisms (Table 2.1) including corals (Morita et al. 2006), ascidians (Miller 1982), sea urchins (Bohmer et al. 2005; Gibbons 1980; Rikmenspoel and Isles 1985; Wood et al. 2005, 2007), starfish (Bohmer et al. 2005; Shiba et al. 2006b), the urochordate

Oikopleura dioica (Miller and King 1983), several teleost species (Geffen 1999; Lahnsteiner et al. 1996a, b, 1997, 1998; Ravinder et al. 1997), rats (Ban et al. 2001; Kaneto et al. 1999) and rabbits (Suarez et al. 1983). Since little attention has been paid to the study of sperm circular movement, it is highly likely that sperm from more organisms show a similar pattern but have not been investigated. For example, of the few studies that have examined blue mussel sperm velocity, none have considered this movement pattern. Two studies have considered the relationship between mitochondrial DNA polymorphisms and sperm velocity in *M. edulis* (Everett et al. 2004; Jha et al. 2008). Instead of investigating the sperm movement pattern before analyzing sperm velocity data, the authors assumed that the sperm swim in a helical three-dimensional movement pattern. Fitzpatrick et al. (2008) studied the effect of copper on *M. trossulus* sperm velocity and larval development but the sperm velocity data were used without any information about the sperm movement pattern.

There are several reasons for the lack of attention to this movement pattern. First, circular movement in sperm may be a rare phenomenon in some organisms. Kaneto et al. (1999) showed that only a small proportion of sperm (1-5%) conducted circular swimming pattern in rat sperm. Similarly, because of the low frequency of occurrence of this movement pattern, human sperm have been grouped into rapid progressive swimming sperm (velocity $\geq 25\mu m\ s^{-1}$), slow progressive swimming sperm (velocity 5-24 $\mu m\ s^{-1}$), nonprogressive swimming sperm (velocity $< 5\ \mu m\ s^{-1}$) and immotile sperm without any consideration of the circular movement pattern (NAFA and ESHRE-SIGA 2002). Secondly, since sperm velocity is correlated with fertilization success in several species (Gage et al. 2004; Levitan 2000b; Moore and Akhondi 1996; Sallam et al. 2001b; Van den Bergh et al. 1998), many researchers have focused on sperm velocity as the most important sperm trait and have not considered the sperm movement pattern. However, recent work on sea urchin sperm movement pattern

(Farley 2002) suggests that ignoring this variable may compromise the interpretation of data on fertilization success.

The paucity of information on sperm movement pattern may also be partially attributable to technical difficulties. For example, a study on the zebra mussel and the quagga mussel (Miller et al. 1994) reconstructed only a part of the sperm movement track, which made it appear to be curvilinear rather than circular. Without a quantitative analysis, it is often hard to distinguish circular movement from similar movement patterns such as a two dimensional spiral movement. Although most sperm circular movement patterns in blue mussels can be easily determined from a reconstruction of the circular track as shown in Fig. 2.2, quantitative verification may be required when investigating sperm movement pattern in other organisms. Morita et al. (2006) studied coral sperm (Fig. 2.4) and provided a good example of both a clear circular track and a planar spiral track. The difference between a circular and a spiral track may be ambiguous if the center of the spiral track moves along the x or y axis at very low speed. The lack of appropriate quantitative descriptive parameters may also complicate the study of sperm circular movement. It is not surprising that some of the most commonly used parameters in sperm velocity studies become less informative or useful when sperm swim in a circular movement pattern. Taking VSL (straight line velocity) as an example, low values will be found for circular swimming sperm because the maximum net space gain is limited to the diameter of the track (Fig. 2.1.b). Since VSL is calculated as net space gain divided by sperm swimming time, as the sperm swimming time increases the net space gain at given points from the circular path does not change, which leads to a constant decreasing trend for the VSL value. In this situation, VSL is not very useful for describing the sperm movement pattern. LIN and STR are derived from VSL and describe the linearity and straightness respectively, which will both be extremely low as a consequence of the low VSL and therefore become less useful for describing a circular movement pattern. It is also impossible to

distinguish a circular movement pattern from other non-linear movement using only the LIN or STR values.

Considering the universality of sperm circular movement in different organisms, it is important to develop appropriate parameters to verify and describe this movement pattern quantitatively to meet the methodological and technical requirements for sperm velocity studies. The methodology of the quantitative verification of the sperm movement pattern will also be useful to detect sperm movement pattern changes which are usually investigated by human judgment in sperm chemotaxis studies (Bohmer et al. 2005; Friedrich and Julicher 2007).

To verify the sperm circular movement pattern, changes in the position of the center of the circular path were analyzed statistically. If sperm exhibit a helical or spiral movement pattern in a two dimensional plane as shown in Fig 2.4B, the real time track center will show a constant movement trend in a certain direction. In this case, the significant difference between the total number of values of dx that satisfies $dx<0$ and $dx>0$ and/or significantly different between the total number of values of dy that satisfies $dy<0$ and $dy>0$, which indicates constant move of sperm track center along x and y axis respectively, should be detected. The binomial test showed no difference between these values, which means that although the center of the circular path keeps moving the sperm swimming pattern is more circular than helical or spiral in the two dimensional plane.

The radius of the overall sperm track has been used to describe the circular movement pattern of sperm in several studies (Geffen 1999; Suarez et al. 1983). However, no clear definitions or instructions for the calculation of circular movement have ever been provided and no real-time radius has ever been measured. The calculation of the "real time" radius (R) based on the smoothed sperm movement track

coordinate was conducted in this study with the Image-J manual tracking plug-in, making it possible to measure the track radius at different time points. Instead of VSL, angle change rate per frame (θ) was developed to directly describe how fast the sperm conduct the circular movement cycle. In a circular movement track, if the angle change rate φ is constant (radians s^{-1}, which can be calculated as $\varphi = \theta$/frame frequency), VSL can be calculated as $VSL = 2 \cdot R \cdot \sin(\frac{\varphi \cdot t}{2}) / t$, which clearly demonstrates why VSL decreases as sperm swimming time increases.

The radius of the circular track showed no significant difference among *M. edulis*, *M. trossulus* and their hybrids (overall mean value about 150 μm). The radius of the circular path of mussel sperm is much larger than that of other species, e.g. 13.2 ± 2.8 μm in the sea urchins *Strongylocentrotus droebachiensis* and *S. purpuratus* (Riedel et al. 2005), 18.5 μm in starfish (Shiba et al. 2006a), approximately 20 μm in the rabbit (Suarez et al. 1983), less than 20 μm in the rainbow trout (Lahnsteiner et al. 1996b,1998), 15-50 μm in the herring *Clupea harengus* (Geffen 1999) and 13.7±9.2 to 38.5±8.9 μm in the rat (Ban et al. 2001). As suggested by Farley (2002), the increased radius of the sperm track may increase egg effective cross section area from πR^2 to $\pi R^2 + \pi r^2$ (R and r are the radii of the egg and the circular or spiral sperm track, respectively). Therefore, the larger radius found in the present study may be a trait that increases fertilization success by increasing gamete interaction rate according to Vogel's fertilization kinetics model (Vogel, 1982) at a small scale with high gamete concentration. Considering differences in collection, preparation, treatment and measurement of sperm, caution must be taken when comparing the swimming radius from different studies.

F_1 hybrid sperm take longer to complete one complete circular path than those of *M. edulis*, *M. trossulus* and other hybrids, as evidenced by the significantly lower

angle change rate. VAP was positively related to angle change rate because the latter is calculated by dividing VAP by radius, which is fairly constant among the blue mussel species. In this particular situation, angle change rate can be used as an indicator of sperm velocity, which may directly affect fertilization success (Vogel et al. 1982).

Why sperm of several organisms exhibit circular movement in a two dimensional plane is an interesting but complicated issue that can be discussed at different levels. One possible explanation arises from a technical concern in which the interaction of the sperm head and a solid surface within a close distance (ca. 10 sperm body length) may create a hydrodynamic trapping of the cells close to the solid surface (Gee and Zimmer-Faust 1997; Lauga et al. 2006). A circular track can be obtained when a helical path is compressed lengthwise from three dimensions to two dimensions (Gray 1955) if sperm swim towards the solid glass surface. In this case, the radius of a two dimensional circular track will be related in some manner to the radius of the helical track in three dimensions. Since it has been suggested that the radius of a sperm helical track will affect gamete collision rate by increasing the effective egg cross-section area (Farley 2002), the radius of a two dimensional circular track will be an important parameter associated with fertilization success. However, the interaction of a sperm head with a solid surface may be species specific in terms of working distance and strength, which should be taken into consideration when interpreting the sperm swimming pattern. Circular and helical movement of sperm in close proximity to the glass surface (≤100μm) and helical movement of sperm distal from the glass surface (5mm) have been reported by Gee and Zimmer-Faust (1997) in the study of the sea urchin *Arbacia punctulata*, which fits Gray's hypothesis (Gray 1955). However, a study of rabbit sperm movement did not show any swimming-pattern differences between sperm closer (25μm) and more distant (100μm) from a glass surface (Suarez et al. 1983). Similar results for carp sperm showed that the movement

pattern did not differ between 10μm and 130μm depth in a testing chamber (Ravinder et al. 1997). In addition, circular movements of sperm close to a solid glass surface were not always detected in all species, suggesting that the presence of a glass surface may not always lead to circular movement of sperm.

Physiologically and physically, sperm velocity is a direct result of flagellar action, apart from nematode sperm, which crawl (Bottino et al. 2002; Sadler and Shakes 2000). This circular movement pattern can also be explained by the asymmetry of the flagellar beat, which is caused by one or more of the peripheral fibers on one side of the axoneme having limited force-producing ability (Gibbons and Gibbons 1984; Okuno and Brokaw 1981; Rikmenspoel and Isles 1985). Therefore, sperm circular movement can also be a natural phenomenon having nothing to do with the interaction between the sperm head and solid surfaces. The phenomenon that all blue mussel sperm swim in a clockwise circular pattern (video 2.1) can also be interpreted in physical terms. In the sea urchins *Strongylocentrotus droebachiensis* and *S. purpuratus*, it is the strong hydrodynamic interaction force between sperm (ca. 0.03 piconewtons), rather than any chemical signal, that creates this self-organized vortex array (Riedel et al. 2005).

Although it is still unknown whether it is the interaction of the sperm head and solid surfaces or flagellar beat asymmetry that leads to circular movement pattern in two dimensions in most species, the detection of a circular swimming pattern indicates that the sperm swim in circular or helical path naturally. Firstly, with the same total velocity (VCL), less sperm net space gain will be obtained by circular and helical swimming compared to linear swimming. Evolutionarily, most characters, including both morphological and behavioral traits, of an organism are determined by natural selection. In this context, sperm swimming pattern is directly affected by the organism's reproductive ecology and mating behavior. Therefore, the two dimensional

circular movement pattern of sperm in all species examined (Table 2.1) may illustrate the evolutionary importance of less net space gain by sperm. Sperm circular movement is rare in copulating mammals. Only sperm of rats and rabbits show circular movement pattern, although few sperm (1~15% and 40% in rats and rabbits, respectively) actually swim in a circular path. Most sperm circular movement patterns have been reported in fish and aquatic invertebrates that release a large number of gametes into the water column during external fertilization, which takes place after gamete contact. In addition, the proportion of sperm exhibiting circular movement appears to be dependent on gamete density. For teleost fishes, which release clouds of sperm close to the eggs and create high gamete concentrations, various proportions of the sperm (<100%) exhibit circular movement. In contrast, in sessile aquatic invertebrates such as corals, sea urchins and starfish, whose gametes are released at relatively low densities, all sperm swim in a circular pattern. Since eggs are deposited at one end of the female tract in internal fertilizing species, selection should favour a faster linear swimming pattern and chemotaxis mechanisms that guide sperm towards eggs. Therefore, a circular swimming pattern in a two dimensional plane is not expected to be prevalent in mammalian sperm regardless of whether it is a natural phenomenon or a compressed helical swimming pattern, since both swimming patterns will result in less net space gain (VSL) towards the egg than a linear swimming pattern. Since circular movement patterns and chemotaxis are often reported simultaneously in sperm of fish and aquatic invertebrates (Bohmer et al. 2005; Miller 1982; Miller and King 1983; Morita et al. 2006; Shiba et al. 2006b), the prevalent two dimensional circular movement in aquatic organisms may suggest a mechanism to save sperm energy and avoid swimming further away from the egg before the sperm detects the chemoattractant gradient. In species that exhibit external fertilization, eggs can be deposited in any direction from the sperm. Before the sperm detects the chemoattractant gradient and changes to a faster linear swimming pattern towards the egg, it will be more efficient for the sperm to remain in a certain location

with less net space gain in any direction. In addition, differences in relative gamete density can lead to different selection pressures on sperm traits. When gametes occur at higher densities, selection favours sperm traits such as a short lifespan and fast swimming, which maximize fertilization success under sperm competition (Levitan 1996). Similarly, when gametes are more dispersed, traits which maximize the probability of gamete contact, such as energy conservation by producing long lived and slow swimming sperm, will be selected (Levitan 1993, 2004a; Levitan and Petersen 1995; Yund 2000). Therefore, a circular movement pattern in two dimensions that is dependent on gamete density can be interpreted as a sperm energy saving mechanism which is favored under sperm limitation situations (Levitan 1993, 2004a; Yund 2000). Furthermore, a circular or helical sperm movement pattern covers a larger area than a linear movement pattern on a small scale and subsequently can improve fertilization success by increasing gamete interaction rate (Farley, 2002). The sperm circular movement pattern can also be viewed as a favorable trait for free spawning invertebrates producing high gamete densities.

2.5 Summary

Sperm of blue mussels, *M. edulis, M. trossulus* and their hybrids, exhibit circular movement in a two dimensional plane. No heterospecific differences were found in the radius of the circular movement track in these laboratory experiments. However, the sperm of F_1 hybrids had a lower angle change rate than those of *M. edulis* and *M. trossulus,* which may affect fertilization success. Specially designed parameters based on the open source software Image-J can be used for both verification and description of circular movement in future studies. Circular movement is more prevalent in sperm of aquatic broadcast spawning species than in those species in which spawning is associated with mating behavior or in species with internal fertilization. A two dimensional circular movement pattern in sperm may, therefore, represent a trait that increases fertilization success in broadcast spawning species at both low and high gamete concentrations.

Table 2.1: Sperm circular movement pattern in different organisms.

(Percentages of circular movement were obtained or calculated from data from the cited literature)

Organisms	Mating behavior and fertilization type	Percentage of circular movement	Reference
Corals	free spawning and external fertilization	100%	Morita 2006
Ascidians	free spawning and external fertilization	NA (<100%)	Miller 1982
Sea urchins	free spawning and external fertilization	100%	Gibbons 1980; Rikmenspoel and Isles 1985; Wood 2005,2007; Bohmer 2005
Starfish	free spawning and external fertilization	100%	Bohmer 2005; Shiba 2006
Urochordates	free spawning and external fertilization	NA (<100%)	Miller and King1983
Teleosts	sperm are released nearby eggs, external fertilization	~20% (burbot) ~15-70% (rainbow trout) <100% (Atlantic herring) ~10-80% (carp)	Lahnsteiner 1996a,1996b, 1997,1998;Ravinder 1997; Geffen 1999
Rats	copulation and internal fertilization	1~15%	Kaneto 1999; Ban 2001
Rabbits	copulation and internal fertilization	~40%	Suarez 1983

Fig. 2.1.a

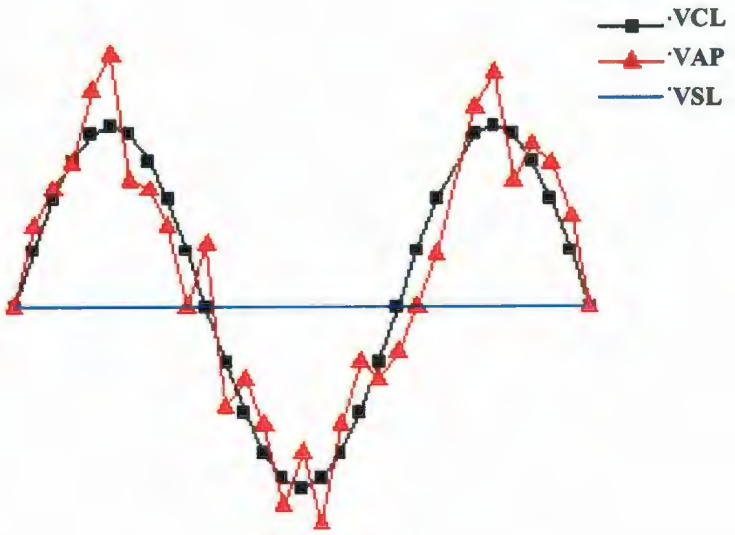


Fig. 2.1.b

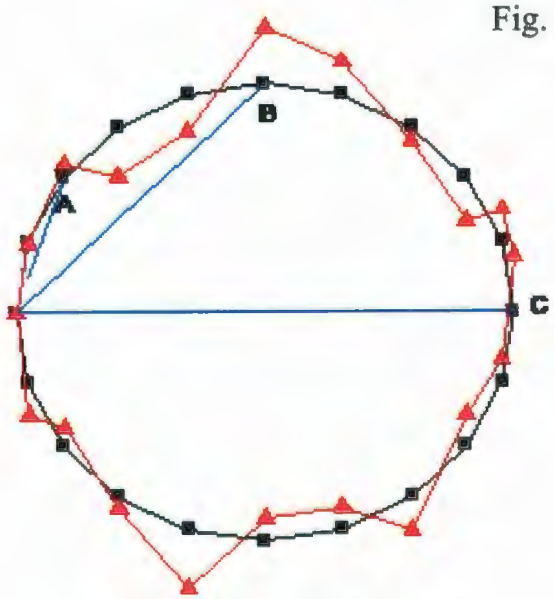


Fig. 2.1. Main sperm velocity parameters calculated in common and circular sperm movement tracks (sperm start swiming from the left end). Fig. 2.1.a. Common movement track; Fig. 2.1.b. Circular movement track. VAP (average path velocity); VCL (curvilinear velocity); VSL (straight line velocity). VSL values depend on the final position (A, B and C) on the circular track. The first time the sperm head reaches the opposite end of the circular track position C will give the greatest VSL, and VSL will be zero when the sperm moves back to its origin.

Fig. 2.2. Image-J reconstructed blue mussel sperm movement track based on a one minute video sequence appears to be circular.

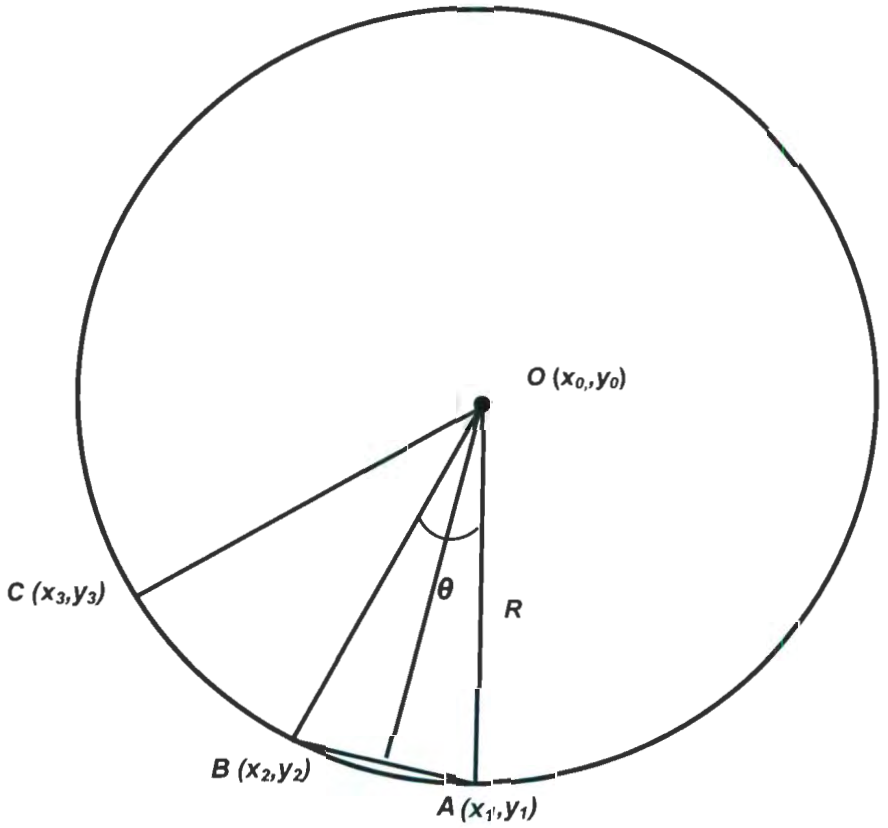


Fig. 2.3. Schematic diagrarn illustrating the calculation of new descriptive parameters for sperm movement.

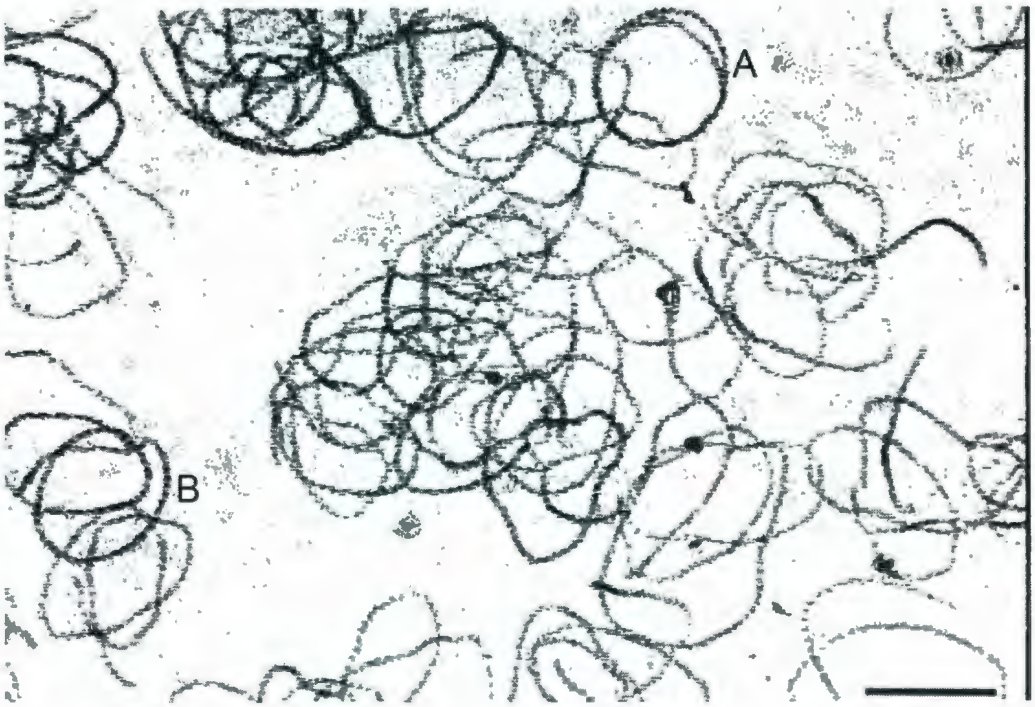


Fig. 2.4. Sperm swimming tracks of coral, *Acropora digitifera*, in egg and N_a-free artificial seawater containing 20 mmol L^{-1} NH_4Cl (Morita et al. 2006) demonstrate the sperm movement pattern. A: clear circular track; B: planar spiral track.

Table 2.2: Example of a binomial test of the horizontal and vertical movement of the circular center $O_t(x_t, y_t)$ conducted with 20 sperm tracks taken from one individual.

Sperm No.	Horizontal				Vertical			
	$dx>0$	$dx<0$	$dx=0$	p	$dy>0$	$dy<0$	$dy=0$	p
1	47	52	0	0.6879	45	54	0	0.4215
2	49	51	0	0.9204	51	49	0	0.9204
3	47	54	0	0.5507	54	47	0	0.5507
4	51	48	0	0.8408	53	46	0	0.5467
5	49	51	0	0.9204	51	49	0	0.9204
6	50	54	0	0.7688	51	53	0	0.9220
7	49	47	0	0.9188	45	51	0	0.6101
8	49	51	0	0.9204	49	51	0	0.9204
9	49	51	0	0.9204	53	47	0	0.6173
10	43	57	0	0.1933	45	55	0	0.3682
11	51	49	0	0.9204	48	52	0	0.7644
12	48	52	0	0.7644	50	50	0	1.0000
13	49	52	0	0.8424	52	49	0	0.8424
14	48	51	0	0.8408	47	52	0	0.6879
15	51	49	0	0.9204	48	52	0	0.7644
16	48	52	0	0.7644	47	53	0	0.6173
17	49	51	0	0.9204	56	44	0	0.2713
18	50	50	0	1.0000	55	45	0	0.3682
19	47	53	0	0.6173	49	51	0	0.9204
20	55	45	0	0.3682	52	48	0	0.7644

Table 2.3: R (radius) and θ (radian change frame^{-1}) for *Mytilus edulis, M. trossulus* and their hybrids (mean±S.D.). n: number of individuals; N: number of sperm.

Parameters	*M. edulis* (n=23) (N=374)	*M. trossulus* (n=16) (N=190)	F_1 hybrids (n=5) (N=59)	Backcross hybrids (n=18) (N=264)
R (um)	149.28±16.75	149.76±27.51	175.69±38.62	150.95±21.78
θ (radian change/frame)	0.41±0.15	0.33±0.16	0.23±0.14[1]	0.42±0.16

Notes:

[1]: Radian change rates (θ) of *Mytilus edulis, M. trossulus* and backcross hybrid sperm are significantly greater than that of F_1 hybrids (Tukey test, $P < 0.01$).

Table 2.4: ANOVA of the effect of VAP on sperm circular movement radius in different genotype groups.

Response: R (radius of circular track)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Genotypes	3	2507.1	835.7	1.5621	0.2087
VAP	1	107.6	107.6	0.2010	0.6556
Residuals	56	29958.8	535.0		

Table 2.5: ANOVA of the effect of VAP and radius on sperm angle change rate in different genotype groups (linear model was constructed as $\theta = \alpha + \beta_0 \cdot \frac{VAP}{R} + \beta_1 \cdot Genotypes + error$).

Response: Angle change per frame

	Df	sum sq	mean sq	F value	Pr(>F)
Genotypes	3	0.18085	0.06028	9.3209	4.287e-05
VAP/Radius	1	0.71223	0.71223	110.1210	7.742e-15
Residuals	56	0.36219	0.00647		

Chapter 3

Effect of egg water and egg presence on sperm movement in the blue mussels *Mytilus edulis, M. trossulus* and their hybrids

3.1 Introduction

3.1.1 Egg water (ovarian fluid) and fertilization study

Fertilization success is crucial for organisms that undergo sexual reproduction. Free spawning aquatic organisms release their gametes directly into the water column, where external fertilization takes place following gamete contact. This process means exposure of gametes to a changing and unpredictable physicochemical environment, in contrast to the gametes of internally fertilizing organisms. Furthermore, spawning in many coexisting species is triggered by similar temperatures, food supply or lunar activity, which can result in a degree of spawning synchrony. Therefore, gametes from free spawning organisms must also minimize interference from heterospecific gametes to achieve a high homospecific fertilization success. For these reasons, free spawning organisms provide excellent models for studying the dynamics of fertilization and especially for investigating the effects of egg water and egg presence on sperm velocity, which in some species appears to be one of the key factors affecting fertilization success (Gage et al. 2004; Levitan and Petersen 1995; Moore and Akhondi 1996; Sallam et al. 2001b; Van den Bergh et al. 1998).

Egg water and ovarian fluid have sometimes been identified as different components associated with spawned eggs (Elofsson et al. 2003b; Hathaway 1963; Isaka and Ikemori 1980; Lahnsteiner 2002; Litvak and Trippel 1998; Urbach et al. 2005; Wojtczak et al. 2007), but in most cases it is difficult to distinguish between them. Free spawning organisms such as blue mussels release their gametes directly into the water column, and the water containing materials that have diffused from the eggs is referred to as egg water or egg-derived products (Hathaway 1963; Heck and Laskin 2003). Salmonid fishes release a considerable amount (10-30% of the egg mass) of the ovarian or coelomic fluid (Scott and Baynes 1980) that bathed the oocytes before spawning (Wojtczak et al. 2007). Initially, both ovarian fluid and egg derived products diffuse into the surrounding water column, so that the egg water contains materials other than those that have diffused from the eggs. For the purposes of the present study, the terms egg water and ovarian fluid will be used interchangeably.

3.1.2 Direct effects of egg water or ovarian fluid on sperm movement

Early studies (Lillie and Just 1924) classified the direct effects of egg water on sperm as activation, aggregation and agglutination. Activation of sperm in an inactive state (as in the testis), or from a less active state to a higher level of activity, by egg water has been demonstrated in several sea urchin species, based on oxygen consumption data (Gray 1928; Hathaway 1963). Similar results have been reported in more recent studies on Atlantic cod (*Gadus morhua*) (Litvak and Trippel 1998), Arctic charr (Turner and Montgomerie 2002; Wojtczak et al. 2007) and three-spined stickleback *Gasterosteus aculeatus* (Elofsson et al. 2003a), demonstrating that the presence of ovarian fluid results in an increase in sperm velocity, sperm longevity and the percentage of motile sperm.

Both sperm aggregation (the phenomenon that sperm aggregate to a high concentration in the presence of egg secretions; Cornman 1941) and agglutination (the phenomenon that sperm clump in the presence of egg secretions; Glabe and Lennarz 1979; Tyler 1940) are caused by sperm chemotaxis (Kaupp et al. 2008), by which sperm alter their swimming path and move up the signal gradient to the egg cell in the presence of eggs or egg water. To date, sperm chemotaxis has been demonstrated in ascidians (Miller 1982; Shiba et al. 2006a; Yoshida et al. 1993), cnidarians (Carre and Sardet 1981; Miller 1979a, b; Morita et al. 2006), echinoderms (Miller 1985, 1997), molluscs (Riffell et al. 2002; Zatylny et al. 2002), urochordates (Miller and King 1983), amphibians (AlAnzi et al. 1997), teleosts (Amanze and Iyengar 1990) and mammals (Jaiswal et al. 1999; Koyama et al. 2006; Sun et al. 2005).

3.1.3 Biological and evolutionary consequences of sperm activation and sperm chemotaxis caused by egg water or ovarian fluid

Several aspects of the interaction between egg water and sperm have important biological and evolutionary consequences. First, the effect of egg water or ovarian fluid on sperm velocity and sperm chemotaxis are important biological features that directly increase fertilization success (Riffell et al. 2003, 2004). Sperm chemotaxis helps to bring sperm closer to eggs to increase the sperm-egg encounter rate and therefore increase fertilization success, as demonstrated in the red abalone, *Haliotis refescens*, by Riffell et al.(2004). In addition, according to Vogel's fertilization model (Vogel et al. 1982) higher sperm velocity, in the presence of egg water or eggs, can increase fertilization success by increasing both the sperm-egg collision rate and the proportion of sperm-egg collisions that result in fertilization. Furthermore, the sperm swimming pattern, especially an increase in the radius of the circular (two dimensional) or helical (three dimensional) path, can increase the effective size of the egg, which will also increase fertilization success (Farley 2002). Laboratory

experiments showed that sperm chemotaxis in *Oikopleura dioica* increased the gamete collision rate by about 4-15 times by increasing sperm velocity, and that the sperm in the center of the chemoattraction gradient swam in larger concentric circles than those at the periphery (Coll et al. 1995; Miller and King 1983).

Second, ovarian fluid or egg water consists of many components, including sodium, potassium, calcium, chloride, glucose, fructose, lactate, cholesterol, phosphatidylcholine, lysophosphatidylcholine, choline, free amino acids, and various enzymes, including alkaline phosphatase, lactate dehydrogenase, beta-D-glucuronidase, proteases and acid phosphatase. It varies in pH and osmolality (Heerden et al. 1993; Lahnsteiner et al. 1995), and may act as an excellent buffer to protect gametes deposited in unfavorable environments. For example, ovarian fluid prolongs the longevity of active sperm in the three-spined stickleback, whose gametes can be released into both seawater and freshwater (Elofsson et al. 2003a, 2006).

Third, the physical and physiological characters of ovarian fluid or egg water differ among species and individuals depending on egg quality and the physiological status of the female (Lahnsteiner 2000; Lahnsteiner et al. 1999a). This difference between species makes it possible for sperm velocity to increase in response to species-specific egg presence or egg water and for sperm chemotaxis to occur, thereby increasing fertilization success. For example, significant differences in ovarian fluid composition among species have been reported in the salmonids rainbow trout (*Oncorhynchus mykiss*), charr (*Salvelinus alpinus*), lake trout (*Salmo trutta lacustris*) and Danube salmon (*Hucho hucho*) (Lahnsteiner et al. 1995). Similarly, eggs from different species can secrete different sperm activating and sperm attracting factors which affect sperm flagellar movement and lead to species-specific sperm velocity effects or sperm chemotaxis (Coll et al. 1995; Matsumoto et al. 2003; Oda et al. 1995, 1998; Ohtake 2003; Oishi et al. 2004; Pousette et al. 1993; Shiba et al. 2005; Tatsu et al. 2002). Sperm chemotaxis may act as a reproductive isolating mechanism which greatly increases the chances of homospecific fertilization by orienting sperm to eggs

of the same species or by changing sperm swimming patterns to make heterospecific fertilizations much less likely, especially when combined with sperm competition and sperm limitation caused by dilution (Levitan and Petersen 1995). In addition, intraspecific differences in the physical characters of ovarian fluid may also affect fertilization success at the individual level (Heerden et al. 1993; Litvak and Trippel 1998). For example, ovarian fluids from different female Arctic charr, *Salvelinus alpinus*, can affect sperm motility differently, depending on individual female-male interactions, which suggests a potential mechanism for cryptic female choice (Urbach et al. 2005).

3.1.4 Objective and importance of this study

As outlined in chapter 2, a natural hybrid zone between *Mytilus edulis* and *M. trossulus* exists along the Atlantic coast of Canada with a very low frequency of F_1 hybrids (Bates and Innes 1995; Innes and Bates 1999; Saavedra et al. 1996; Toro et al. 2002, 2004, 2006) and provides a good model to study reproductive isolation. A few studies (Everett et al. 2004; Jha et al. 2008) have investigated sperm velocity in blue mussel species, but none have considered sperm activation, the chemoattractive effect of eggs or egg water (or ovarian fluid), or the potential effect of eggs and ovarian fluid on sperm movement pattern, fertilization success and reproductive isolation. The present study was designed to answer the following questions: First, do egg water (or ovarian fluid) and eggs cause sperm velocity change and chemotaxis in blue mussels? Second, do mussels release species-specific sperm chemoattractants? Third, what are the consequences of these processes for reproductive success and the evolution of reproductive isolation?

3.2 Materials and methods

3.2.1 General

Adult blue mussels were collected from Trinity, Newfoundland, Canada from late May to early August in 2006 and 2007, and were kept in 10 °C flowing seawater until spawning was induced (Everett et al. 2004). They were fed Commercial Instant Shellfish Diet 1800® (Reed Mariculture, Inc.) following the company instructions. All spawned individuals were genotyped using the GLU and ITS genetic markers following Heath et al. (1995) and Inoue et al. (1995). Experiments investigating the effect of egg water on sperm velocity were conducted in 2006 and those investigating the effects of egg presence were undertaken in 2007. Thermal shock was used to induce spawning in the laboratory as described in Chapter 2 (2.2.2).

3.2.2 Effect of ovarian fluid and egg presence on sperm movement pattern

Mature individuals were induced to spawn by thermal shock following Everett, (2004) and Jha et al. (2008) as described in Chapter 2. Eggs were collected 30 minutes after spawning by pouring the egg suspension through 50 µm and 20 µm sieves. Egg water was sequentially filtered through 250 µm and 100 µm sieves. 15ml supernatant fluid without egg cells was added to 35ml fresh filtered sperm suspension for the egg water effect study. A control was set up by adding 15ml filtered seawater to the duplicate 35ml sperm suspension to eliminate the effect of sperm concentration on sperm velocity traits. To study the effect of egg presence on sperm movement pattern on a small scale in a small volume of water, 10 µl filtered egg water containing about 20 eggs was mixed with 20 µl sperm suspension on a glass slide. A control was prepared by mixing 10 µl filtered seawater with 20 µl sperm suspension. Data were

obtained from both video (VHS) and Image-J tracking analysis of the sperm movement pattern as described in Chapter 2.

Another experiment was set up to study the effect of egg presence on the direction of sperm movement on a larger scale, to simulate the situation that may occur in nature (Fig. 3.1). Furthermore, this larger scale can help to reduce any effects of wall-sperm head interaction on the sperm movement pattern that may occur on a smaller scale, as discussed in Chapter 2. After the initial sperm concentration was recorded with a Multisizer™ II, a diluted, homogenized sperm suspension was poured into three identical 2000 ml graduated cylinders, forming one control group (no eggs) and two experimental groups with eggs present at the top and at the bottom of the cylinder, respectively. The filtered egg suspension was passed again through a 20 μm sieve and concentrated egg cells collected with a pipette. A concentrated suspension of eggs was prepared by adding 50 ml egg water to this egg concentrate, and the final egg concentration determined with a Multisizer™ II. About 20 ml of this egg suspension containing about 1000 eggs was poured into a 20 ml plastic chamber with a 20 μm sieve at the side which allowed sperm to swim through while retaining eggs inside the chamber. The sperm concentration at various depths was determined with a Multisizer™ II after 1.5 hours treatment.

3.2.3 Video data collection, preparation and calibration

The protocols described in Chapter 2 (2.2.3 and 2.2.4) were followed for video data collection, preparation and calibration.

3.2.4 Data analysis and statistics

Parameters for sperm plane circular movement were calculated from raw coordinate data following the methods described in Chapter 2 (2.3.2 and 2.3.3) using

equations in a Microsoft Excel spreadsheet (See appendix 1 in the attached CD for equations and calculation details, Wilson-Leedy and Ingermann 2007). The binomial tests of the horizontal and vertical movement of the sperm circular movement track were conducted with the "R" program (R-Development-Core-Team 2008). ANOVAs of sperm concentration at different depths with homospecific or heterospecific eggs at the top or at the bottom were also conducted with the "R" program using a linear model constructed as: y (sperm concentration) = $a + b$*treatment + b_1*groups + b_2*depth + b_3*treatment*groups + b_4* treatment*depth + b_5* groups*depth + residuals. Treatments were: control, eggs deposited at top and eggs deposited at the bottom; groups were homospecific eggs and heterospecific eggs).

3.3 Results

3.3.1 Effect of egg water (ovarian fluid) on sperm movement

Sperm did not change their circular movement pattern when treated with egg water. Regardless of the source of the sperm (*M. edulis*, *M. trossulus,* hybrids) or whether the egg water was obtained from the same species or a different species, the binomial test (Clopper and Pearson 1934) showed that the centre of the circular path had no detectable horizontal or vertical deflection (Table 3.1.).

Although the sperm from the two species and hybrids still conducted a circular movement pattern when treated with egg water, sperm movement parameters did respond differently to egg water from the same species or different species (Table 3.2.). For *M. edulis* sperm with homospecific egg water, the radius of the circular movement path was significantly smaller than that of the sea water control group. Although no significant difference was detected in average path velocity for *M. edulis* sperm treated with homospecific or heterospecific egg water, VCL for *M. edulis* sperm was significantly higher in the presence of egg water from *M. edulis* females

than with egg water from *M. trossulus*. No significant radius, VCL and VAP differences were found among sperm from *M. trossulus* and hybrids treated with homospecific or heterospecific egg water. Heterospecific egg water did not significantly affect sperm velocity in F_1 and backcross hybrids.

3.3.2 Effect of egg presence on sperm movement

To investigate the potential sperm chemoattraction effect of egg presence, 1000 eggs were used as the chemoattractant at one end of the apparatus. If a chemoattraction gradient is created by these 1000 eggs and the sperm can swim straight along the gradient, then sperm at the far end swimming with a limited mean velocity of 100 μm sec^{-1} (for example about half the measured VCL) will take about 1 hour to travel the whole distance (about 40 cm) and reach the eggs. As a result, differences in sperm concentration among control and treatment groups at different positions in the gradients should be detected after about 1.5 hours. The results showed that regardless of whether homospecific or heterospecific eggs were positioned at the top or at the bottom of the treatment groups, there was no significant difference between control and treatment groups, i.e. sperm did not respond to egg presence in such a way as to cause a significant change in sperm concentration along the potential sperm chemoattraction gradient (Tables 3.3 and 3.4). However, *M. edulis* sperm concentration did differ significantly among depths (Table 3.4), generally showing a decrease with increasing depth (Table 3.3 and Fig. 3.2). Additional evidence for the absence of sperm chemoattraction behavior was the lack of response of sperm to the presence of egg cells as measured on a microscope slide using a video camera (Video 3.1).

3.4 Discussion

One of the main effects of egg water or ovarian fluid on sperm velocity in all free spawning species is sperm activation, including the initiation of sperm movement and increases in the proportion of motile sperm and in sperm velocity (Elofsson et al. 2003a; Gray 1928; Hathaway 1963; Litvak and Trippel 1998; Ohtake 2003; Shiba et al. 2005; Turner and Montgomerie 2002; Wojtczak et al. 2007; Yoshida 1972; Yoshino et al. 1990). In the present study, *M. edulis* sperm had a significantly greater mean total velocity in the presence of homospecific egg water than did the control and the heterospecific egg water groups (1.22 and 1.46 times greater than control and inter-species egg water groups, respectively). However, no significant effect was found for sperm from *M. trossulus* and hybrids in the present study. Sperm from hybrids was not tested with egg water from hybrids.

The results suggest that the degree to which egg water stimulates sperm velocity is highly variable among species, which is consistent with data from other organisms. Various effects of egg water (ovarian fluid) or egg extract on sperm velocity activation have been reported, such as a 1.25-1.3 times increase in sperm velocity when activated by egg water or egg extract in Atlantic cod (*Gadus morhua*) (Litvak and Trippel 1998); about a 2 times increase in Arctic charr (*Salvelinus alpinus*) (Turner and Montgomerie 2002); a 1.5 times increase in the urochordate *Oikopleura dioica fol* (Miller and King 1983); but no effect in the fifteen-spined stickleback (*Spinachia spinachia*) (Elofsson et al. 2003b), the zebra mussel *Dreissena polymorpha* and the quagga mussel *D. bugensis* (Miller et al. 1994).

The observation that *M. edulis* sperm can only be activated by homospecific egg water suggests that the sperm activation effect of egg water in blue mussels may be species specific. Various molecules influence sperm velocity or cause sperm

chemotaxis in different species, including calcium and Cyclic-AMP in the ascidian *Ciona savignyi* (Yoshida et al. 1994, 1995), (3R, 4R, 7R, 25S)-3, 4, 7, 26-tetrahydroxycholestane-3, 26-disulfate in the ascidian *C. intestinalis* (Oishi et al. 2003, 2004), about 80 different peptides in the phyla Cnidaria and Echinodermata (Kaupp et al. 2006), proteins from eggs in Pacific herring *Clupea pallasii* (Griffin et al. 1996; Oda et al. 1995,1998;. Ohtake 2003) and nitric oxide in the human (Miraglia et al. 2007). In the well-studied class Echinoidea (Eisenbach 1999, 2004; Hildebrand and Kaupp 2005; Kaupp et al. 2006, 2008), for example, several sperm activation and attraction molecules and mechanisms have been reported for different species (Kamei 2004; Kamei and Glabe 2003). Although there have been no other studies on egg water components in the blue mussels *M. edulis*, *M. trossulus* and their hybrids, it is possible that sperm activation molecules and mechanisms differ among them, accounting for the species-specific sperm activation effect in *M. edulis* found in present study.

Another important effect of egg water or egg presence on sperm velocity is sperm chemotaxis, which has been reported in many taxa (Ishikawa et al. 2004; Morita et al. 2006; Riffell et al. 2002; Shiba et al. 2005; Sun et al. 2005). In this study two aspects of sperm chemotaxis were investigated in blue mussels, the effect of egg water or egg presence on the sperm movement pattern and the effect of egg presence on the spatial distribution of sperm. Furthermore, the effects of egg presence on sperm movement were investigated at both smaller (microscopic level with about 30 μl total volume) and larger spatial scales (2 liter total volume). Sperm from *M. edulis*, *M. trossulus* and hybrids did not change their movement pattern in the presence of eggs or egg water. The same circular sperm movement pattern was observed under these conditions as in the filtered sea water control group, suggesting the absence of sperm chemotaxis in blue mussel species. Unlike data from Arctic charr (*Salvelinus alpinus*) and various species of corals (*Acropora digitifera*, *A. gemmifera* and *A. tenuis*), in

which the ovarian fluid or egg presence significantly increased the linearity index, indicating that sperm swam in a straighter trajectory than in the control group (Morita et al. 2006; Turner and Montgomerie 2002), our results were consistent with those from *Oikopleura dioica fol*, Atlantic cod (*Gadus morhua*), zebra mussel (*Dreissena polymorpha*) and quagga mussel (*Dreissena bugensis*) (Litvak and Trippel 1998; Miller and King 1983; Miller et al. 1994). The presence of eggs did not affect path linearity in sperm of Atlantic cod, *Gadus morhua* (Litvak and Trippel 1998). In *Oikopleura dioica fol,* once the sperm reached the centre of the chemoattractant gradient they still swam in a circular path, but with a greater radius (Miller and King 1983). Similarly, oocyte extracts did not influence sperm movement parameters in zebra mussels and quagga mussels (Miller et al. 1994).

Both ecological and evolutionary explanations have to be taken into consideration when discussing the absence of sperm chemotaxis in blue mussels. Chemotaxis mechanisms that guide sperm to swim closer to eggs and, therefore, increase fertilization success may only evolve when sperm-egg interaction rate is low. Sperm chemotaxis has only been reported in sessile aquatic invertebrates that have a dispersed distribution in which the spawned gametes are far apart from each other, such as has been found in ascidians, corals, siphonophores, holothurians, ophiuroids and echinoderms (Ishikawa et al. 2004; Kaupp et al. 2008; Miller 1997; Morita et al. 2006; Shiba et al. 2005). In contrast, *M. edulis* and *M. trossulus* live in clusters and attach to each other and the substrate by means of byssal threads. Clustering of mussels greatly reduces the total travel distance for the sperm to reach the egg, compared with other more dispersed sessile invertebrates, which might explain the lack of a sperm chemotaxis mechanism in mussels. However, species-specific sperm chemotaxis that acts as a reproductive isolation barrier may only evolve when two or more closely related species occur in close proximity and have overlapping spawning periods. For example, Miller (1997) demonstrated species specific sperm activation

and chemotaxis by egg or ovarian extracts in 24 holothurian and 22 ophiuroid species from the Great Barrier Reef. Since about 1500 fish species, 5000 mollusc species, 400 coral species and dozens of holothurian and ophiuroid species coexist on the Great Barrier Reef, most of them sharing the same spawning season (CRC Reef Research Centre Ltd.), species specific sperm chemotaxis is essential to reduce the interference of gametes from other species. Similarly, in mass spawnings of the corals, *Acropora digitifera*, *A. gemmifera* and *A. tenuis*, recently released normal immotile sperm are only activated by, and attracted to, eggs from the same species (Morita et al. 2006).

In contrast to broadcast spawning aquatic invertebrates, the primary reproductive isolation barriers for organisms with internal fertilization are based on mate-choice behaviour prior to copulation and sperm transfer. In this case, sperm chemotaxis primarily facilitates fertilization by guiding sperm to egg cells, and there is no need for sperm chemotaxis to evolve species specificity in order to avoid heterospecific fertilization. For example, *in vitro* experiments have shown that human and rabbit sperm respond similarly to human, rabbit and bovine egg-related factors (Sun et al. 2003). For aquatic broadcast spawning species, species-specific chemotaxis would be expected to evolve when there is a high probability that homospecific gametes may mix with heterospecific gametes. Based on laboratory experiments, there was no evidence for individuals aggregating into clusters consisting of single species in blue mussels *M. edulis* and *M. trossulus* (own unpublished data and data from Erin Stapleton, Biology Department, Memorial University of Newfoundland). Therefore, the formation of mixed-species clusters of mussels should favour the evolution of species-specific recognition mechanisms. However, mixed aggregations of blue mussels can also produce a dense suspension of gametes from different species during spawning events and lead to considerable heterospecific gamete interaction merely by random collision, even if there is species-specific sperm chemotaxis. Therefore, species-specific sperm chemotaxis is not likely to be a completely effective

reproductive isolation barrier in blue mussel species. Like abalone and sea urchin, blue mussels possess a gamete recognition mechanism based on the lysin protein in the sperm and receptors in the egg which has the potential to act as a strong hybridization barrier between the species once gametes make contact (Clark et al. 2007; Galindo et al. 2003; Kresge et al. 2001; Riginos and McDonald 2003; Riginos et al. 2006). Therefore, there is no need for blue mussel species to evolve species-specific sperm chemotaxis, and gamete interaction over short distances can be effected by hydrodynamic forces and random collisions.

Certain problems involving technique and experimental design issues must be taken into consideration when interpreting sperm chemotaxis data for mussels. For example, unlike blue mussel species the freshwater mussels *Dreissena polymorpha* (zebra mussel) and *D. bugensis* (quagga mussel), which also live in mixed-species clusters, exhibit completely species-specific sperm attraction by oocyte extracts (Miller et al. 1994). Considering that the concentration of egg water or ovarian fluid used in such experiments can have a significant affect on sperm velocity, it is very likely that the contrast in results is attributable to different concentrations of egg water or ovarian fluid. As shown in Arctic charr by Turner and Montgomerie (2002), a low concentration of ovarian fluid (5%) reduces sperm velocity while a high concentration (>5%) increases it. Although species-specific sperm chemotaxis has been reported for several free spawning invertebrates, including freshwater mussel species, the experiments were conducted with egg extractions or purified chemoattractants rather than live eggs (Coll et al. 1995; Miller 1979a, 1985, 1997; Miller et al. 1994). Such high concentrations of egg extract or chemoattractants are unlikely to be found in nature, especially in view of the dilution effects of the water (Levitan and Petersen 1995), which can reduce the egg water or ovarian fluid concentration very quickly. Therefore, although sperm chemotaxis can be observed in the presence of concentrated egg extracts or chemoattractants in laboratory experiments, it may not

occur at the lower concentrations characteristic of natural conditions. Because this is the first study of the effects of egg water on sperm velocity in blue mussels, no information is available on the range in concentration of egg water that can affect sperm velocity. The 30% (volume percentage) of egg water used in the present study was based on the concentration used for Arctic charr and Atlantic cod (Elofsson et al. 2003b; Turner and Montgomerie 2002), which is more likely to approximate the natural situation than do artificially concentrated egg extracts or chemoattractants.

Interpreting how egg water or egg presence affects fertilization success among blue mussel species is complex. In this study, sperm activation by homospecific egg water was found in *M. edulis*. The higher velocity of *M. edulis* sperm in the presence of conspecific egg water resulted in a gamete collision rate for intra-species fertilizations which was about 22% or 46% greater than was observed in the control and heterospecific egg water groups respectively, thereby improving the fertilization success rate according to Vogel's fertilization kinetics model (Vogel et al. 1982). However, the reduction in the radius of the circular movement track observed in *M. edulis* sperm in the presence of intra-species egg water has not been reported in any other species (Litvak and Trippel 1998; Miller and King 1983; Miller et al. 1994; Morita et al. 2006; Turner and Montgomerie 2002), further complicating the interpretation of the direct effects of egg water on fertilization success. According to Vogel's fertilization kinetics model, the collision rate of a gamete (β_0) is directly related to sperm velocity (v) and egg cross-sectional area (σ) following the equation $\beta_0 = v \cdot \sigma$ (Vogel et al. 1982). An increase in the radius of the sea urchin sperm helical swimming path enhances the effective egg cross section area by increasing $\sigma = \pi(radius_{egg})^2$ to $\sigma = \pi(radius_{egg})^2 + \pi(radius_{sperm})^2$, thereby improving fertilization success (Farley 2002). However, the degree to which a change in the circular radius affects fertilization success in *M. edulis* remains unknown,

because there is no information available on the relationship between the radii of the circular and helical sperm movement patterns. Furthermore, as suggested by previous authors, egg water or egg extracts can also increase the proportion of motile sperm (Miller 1997), which may also increase fertilization success. Without further data on the effect of egg water on the proportion of motile sperm, its precise effect on fertilization success in *Mytilus* species remains to be determined. Nevertheless, the effect of species-specific sperm activation in *M. edulis* on fertilization success due to increased sperm velocity, a reduced circular path radius or a change in the percentage of motile sperm should be the same for both homospecific and heterospecific fertilizations. Although sperm activation in this study only resulted from homospecific components of the egg water, according to Vogel's fertilization model it will affect the ability of *M. edulis* sperm to fertilize eggs from all blue mussel species rather than homospecific eggs only, by increasing the gamete collision rate for both homospecific and heterospecific crosses.

3.5 Summary

Neither egg water nor egg presence changed the circular movement pattern of sperm from *M. edulis, M. trossulus* and hybrids. Sperm chemotaxis may therefore not be necessary for high fertilization efficiency or reproductive isolation in blue mussel species. The aggregation habit of mussels, together with synchronized spawning, ensures a high encounter rate between sperm and eggs without sperm chemotaxis. Furthermore, under conditions of relatively high gamete density, mechanisms acting at the gamete surface may be more efficient in creating or maintaining reproductive isolation than species-specific sperm chemotaxis.

Species-specific sperm activation by egg water was only found in *M. edulis*, whose sperm exhibited faster circular swimming, with a reduced radius, in the

presence of homospecific eggs compared to the control and heterospecific treatment groups. How this sperm activation effect directly influences fertilization success remains unknown. Nevertheless, this effect does not act as a reproductive isolating barrier favouring homospecific over heterospecific fertilizations. However, species-specific sperm activation in *M. edulis* may provide a competitive advantage when there is competition among sperm in populations with mixtures of *M. edulis, M. trossulus* and hybrids.

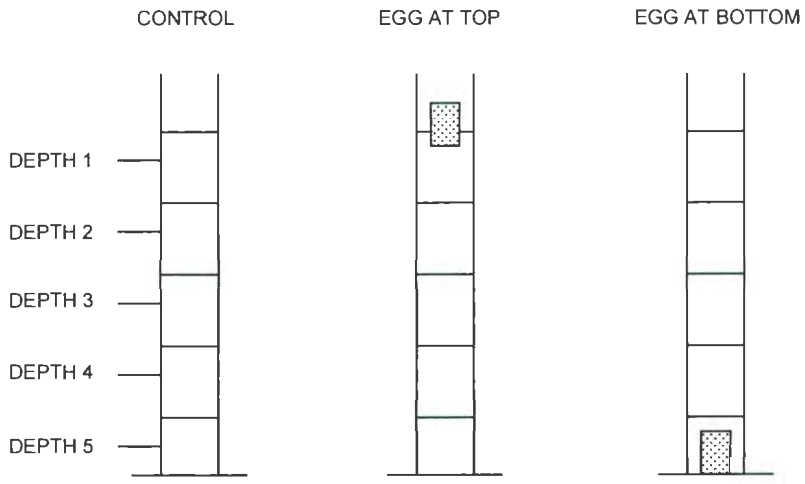


Fig. 3.1. Schematic diagram of experimental setup to study the effect of egg presence on the direction of sperm movement.

Table 3.1: Binomial test of the horizontal and vertical movement of the centre of the circle $O_t(x_t, y_t)$ conducted with 20 sperm tracks in the presence of egg water.

Sperm No.	Horizontal				Vertical			
	dx=1	dx=-1	dx=0	p	dy=1	dy=-1	dy=0	p
1	50	50	0	1.0000	48	52	0	0.7644
2	48	57	0	0.4351	57	48	0	0.4351
3	50	52	0	0.9212	55	47	0	0.4884
4	51	49	0	0.9204	46	54	0	0.4841
5	48	52	0	0.7644	51	49	0	0.9204
6	55	47	0	0.4884	47	55	0	0.4884
7	56	48	0	0.4926	56	48	0	0.4926
8	51	50	0	1.0000	47	54	0	0.5507
9	50	49	0	1.0000	47	52	0	0.6879
10	49	54	0	0.6937	52	51	0	1.0000
11	51	49	0	0.9204	48	52	0	0.7644
12	54	48	0	0.6208	48	54	0	0.6208
13	51	51	0	1.0000	48	54	0	0.6208
14	48	52	0	0.7644	49	51	0	0.9204
15	50	49	0	1.0000	52	47	0	0.6879
16	47	53	0	0.6173	50	50	0	1.0000
17	49	51	0	0.9204	52	48	0	0.7644
18	55	46	0	0.4262	53	48	0	0.6908
19	48	51	0	0.8408	50	49	0	1.0000
20	47	53	0	0.6173	51	49	0	0.9204

Table 3.2: Sperm velocity parameters for homospecific and heterospecific egg water treatments tested against the sea water control. (R: radius of circular movement pattern; VCL: sperm curvilinear velocity; VAP: sperm average path velocity).

Groups	N	R (μm)	VCL (μm/sec)	VAP (μm/sec)
M. edulis				
Control group	31	261.16±95.54	214.38±68.70	145.33±49.57
Intra-species group	12	183.23±23.84**[1]	262.39±71.67*[2]	159.45±28.80
Inter-species group	19	312.72±133.80	179.78±36.99*[3]	122.59±39.80
M. trossulus				
Control group	8	188.48±89.41	182.21±129.91	130.59±48.12
Intra-species group	5	200.36±94.32	129.84±94.92	116.24±47.86
Inter-species group	3	172.25±71.24	246.91±78.86	159.05±3.39
F_1 hybrids				
Control group	3	126.53+50.13	130.98±78.22	141.24+35.86
Intra-species group			NA	
Inter-species group	3	167.70+7.17	91.82±28.20	110.65+80.55
Backcross hybrids				
Control group	34	235.33+120.70	222.88±85.65	148.27+40.82
Intra-species group			NA	
Inter-species group	34	260.38+117.23	207.03±68.83	145.33+34.69

One way ANOVAs to compare treatments with control

**[1]: $F_{1,41}=7.69$, $P<0.01$

*[2]: $F_{1,41}=4.13$, $P<0.05$

*[3]: $F_{1,48}=4.07$, $P<0.05$

Table 3.3: Relative sperm concentrations (final sperm concentration/original sperm concentration) at different depths with no eggs (control), eggs at top and eggs at bottom for blue mussels *M. edulis*, *M. trossulus* and their hybrids. Hybrids (both F_1 and backcrosses) genotyped by ITS and GLU genetic markers.

	Relative sperm concentration					
Species and Treatment	Top	Depth 2	Depth 3	Depth 4	Bottom	N
Control	1.0409±0.0958	0.9998±0.0633	1.0166±0.0698	0.9723±0.0747	0.9703±0.0893	15
Mytilus edulis						
Homospecific eggs at top	1.0600±0.0439	1.0131±0.0393	0.9922±0.0356	0.9719±0.0153	0.9628±0.0214	4
Homospecific eggs at bottom	1.0022±0.0147	0.9885±0.0479	0.9803±0.0313	0.9533±0.0540	1.0758±0.1255	4
Heterospecific eggs at top	1.0390±0.0316	1.0233±0.0021	0.9939±0.0111	0.9623±0.0073	0.9816±0.0334	2
Heterospecific eggs at bottom	0.9991±0.0066	1.0115±0.0251	1.0012±0.0130	0.9999±0.0223	0.9884±0.0538	2
Mytilus trossulus						
Homospecific eggs at top	1.0975	1.0679	1.0372	0.9367	0.8607	1
Homospecific eggs at bottom	1.1090	0.9980	0.9763	0.9583	0.9584	1
Heterospecific eggs at top	1.0439±0.0932	1.0062±0.0242	1.0318±0.0747	0.9569±0.0053	0.9613±0.0480	2
Heterospecific eggs at bottom	0.9708±0.0325	0.9850±0.0218	1.0014±0.0563	1.0273±0.0225	1.0156±0.0230	2
Hybrids						
Heterospecific eggs at top	1.0777±0.0713	1.0275±0.0570	0.9879±0.0395	0.9410±0.0842	0.9660±0.0757	3
Heterospecific eggs at bottom	0.9957±0.0800	0.9933±0.0466	1.0006±0.0217	0.9945±0.0090	1.0160±0.0573	3

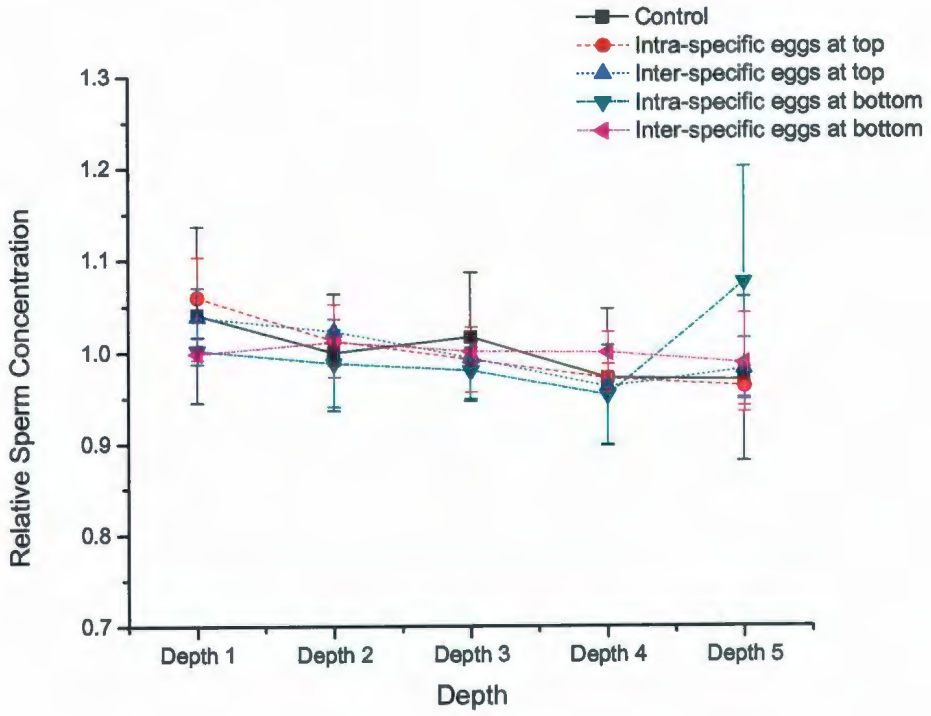


Fig. 3.2. Relative concentrations (mean ± S.D.) of *Mytilus edulis* sperm at increasing depths in control and experimental groups. Linear regression for controls, R^2=0.68, p=0.083, y=1.0608-0.01562x; for homospecific eggs at top, R^2=0.91, p=0.012, y=1.08379-0.02149x; for heterospecific eggs at top, R^2=0.97, p<0.01, y=1.10921-0.0287x; for homospecific eggs at top, R^2=0.36, p=0.282, y=1.01954-0.00914x; for heterospecific eggs at bottom, R^2=0.02, p=0.804, y=0.99887+0.0004x.

Table 3.4: ANOVA of sperm concentration at different depths with homospecific or heterospecific eggs at the top or at the bottom.

Response: Relative sperm concentration					
Sperm genotype	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Mytilus edulis					
Treatment[1]	2	1.567e-32	7.834e-33	4.703e-30	1.0000
Group[2]	1	1.064e-31	1.064e-31	6.386e-29	1.0000
Depth	4	0.027411	0.006853	4.1143	0.0049[3]
Treatment*Group	1	4.049e-32	4.049e-32	2.431e-29	1.0000
Treatment*Depth	8	0.030276	0.003785	2.2722	0.0329
Group*Depth	4	0.010484	0.002621	1.5737	0.1919
Treatment*Group*Depth	4	0.001467	0.000367	0.2202	0.9262
Residuals	65	0.108263	0.001666		
Mytilus trossulus					
Treatment	2	1.164e-3	5.824e-4	0.0683	0.9342
Group	1	1.477e-3	1.477e-3	0.1733	0.6809
Depth	4	0.047156	0.011789	1.3830	0.2695
Treatment*Group	1	1.396e-3	1.396e-3	0.1638	0.6893
Treatment*Depth	8	0.021981	0.002748	0.3223	0.9494
Group*Depth	4	0.017555	0.004389	0.5149	0.7255
Treatment*Group*Depth	3	0.004125	0.001375	0.1613	0.9213
Residuals	24	0.204578	0.008524		
Hybrids[4]					
Treatment	2	4.543e-32	2.271e-32	3.376e-30	1.0000
Depth	4	0.046246	0.011562	1.7186	0.1649
Treatment*Depth	8	0.036748	0.004594	0.6828	0.7038
Residuals	40	0.269090	0.006727		

1. Treatment including control, egg at top and egg at bottom
2. Group including homospecific egg or heterospecific egg
3. $p<0.01$
4. Hybrids included both F_1 and backcross hybrids. No egg from homospecific individual was used in the experiment of hybrid sperm

Chapter 4

The contribution of sperm velocity and gamete output to sperm competition and its role as a post-spawning prezygotic reproductive isolation barrier in blue mussels *Mytilus edulis*, *M. trossulus* and their hybrids

4.1 Introduction

4.1.1 Sexual selection and postcopulatory selection

The success of an organism is measured by two components of fitness: the number of offspring produced and the quality or probable success of the offspring. Genes associated with traits that confer higher fitness will be disproportionately represented in subsequent generations by the process of selection. A distinction is generally made between natural selection and sexual selection. When first proposed by Charles Darwin, sexual selection was described as the effect of the "struggle between the individuals of one sex, generally the males, for the possession of the other sex" (Darwin 1871). "The sexual struggle is of two kinds: in the one it is between the individuals of the same sex, generally the males, in order to drive away or kill their rivals, the females remaining passive; while in the other, the struggle is likewise between the individuals of the same sex, in order to excite or charm those of the opposite sex, generally the females, which no longer remain passive, but select the more agreeable partners" (Darwin 1871). The "male to male combat" (intrasexual selection) and "mate choice" (intersexual selection) lead to the selection of secondary sexual characteristics such as "weapons" (traits like horns, antlers etc.

that evolved for male to male combat) and "ornaments" (traits that evolved due to mate choice).

Darwin's sexual selection theory is prevalent and most studies on the subject have focused on intrasexual and intersexual selection processes (Atema 1986; Contreras-Garduno and Cordoba-Aguilar 2006; Mateos 1998; Roulin and Bize 2007). However, there are important limitations to Darwin's sexual selection theory. First, his work was based on organisms with internal fertilization, so his theory may be difficult to apply to free spawning organisms with external fertilization. Second, he assumed that all females are monogamous, so his theory is exclusively precopulatory. Considering that females from many species with internal fertilization are now known to be polyandrous, and the gametes from different individuals of free spawning organisms are often mixed and compete to fertilize the limited number of opposite sex gametes, there is the potential for postcopulatory selection (or post-spawning selection in the case of free spawning organisms) to occur.

4.1.2 Postcopulatory (post-spawning) selection and adaptations

About 100 years after Darwin proposed the concept of sexual selection, Parker (1970) noted the occurrence of sperm competition ("the competition between sperm of two or more males for the fertilization of an ovum") in insects. There is the potential for sperm competition to occur whenever sperm from more than one male compete with each other to fertilize a limited number of female reproductive cells (Mesterton-Gibbons 1999; Parker 1982, 1990, 1993; Parker and Begon 1993). In free spawning organisms gametes from several individuals are often mixed and sperm compete with each other to fertilize a limited number of egg cells (Bode and Marshall 2007; Harper and Hart 2005; Marshall and Evans 2005).

Many reproductive strategies or adaptations have evolved either for more successful competition or for avoiding competition as a response to postcopulatory (or post-spawning) selection. For example, behavioral adaptations such as postcopulatory mate guarding (Tsubaki et al. 1994), morphological adaptations such

as genital modification that remove sperm from competitors or act as mating plugs (Fincke 1984; Foellmer 2008; Mikheyev 2003), physiological adaptations and even psychological adaptations (Shackelford et al. 2005) have been reported in different organisms. However, sperm trait adaptations are directly related to sperm competition, which is similar to a lottery in that the more tickets (or sperm) that an individual can produce the greater the chance to win (pass genes more frequently than competitors) (Parker 1990). ESS (evolutionarily stable strategy) models have shown that individuals are selected to produce more sperm when the intensity of sperm competition increases (Parker 1998), and have been supported by experimental data from various organisms (Baker and Bellis 1989; Simmons and Kotiaho 2002; Stockley et al. 1997). However, since sperm are costly to produce and the energy of an individual is finite, additional sperm trait adaptations, other than merely increasing the sperm number, have evolved as a response to sperm competition. These adaptations include producing less costly ejaculates that contain significant amounts of parasperms which act as a cheap filter, eliminating space for sperm of other competitors, or help with transportation of real functional sperm (Buckland-Nicks 1998; Hayakawa 2007; Oppliger et al. 2003; Swallow and Wilkinson 2002), and improving sperm quality by increasing sperm velocity (Levitan 2000b) or producing larger sperm (LaMunyon and Ward 1999, 2002).

4.1.3 Sperm velocity and sperm competition in free spawning organisms

Several ESS models have been developed to evaluate sperm competition for species with external fertilization (Ball and Parker 1996, 1997, 1998b, 2003; Mesterton-Gibbons 1999). In broadcast spawning species there is the possibility of polyspermy, in which one egg cell is fertilized by more than one sperm, generally resulting in a nonviable zygote (Levitan et al. 2007). Polyspermy may be a negative outcome of sperm having evolved to be too efficient at reaching and fertilizing eggs due to the selection pressures of sperm competition. Therefore, a revised model has

been suggested for sperm competition in marine sessile invertebrates (Bode and Marshall 2007).

Sperm swimming velocity is a crucial character which is directly associated with fertilization success in many species, ranging from invertebrates to mammals (Auger et al. 1994; Gage et al. 2004; Jouannet and Serres 1998; Kupriyanova and Havenhand 2002; Levitan 2000b; Malo et al. 2005; Van den Bergh et al. 1998). Therefore, in addition to greater numbers of sperm, increasing sperm velocity can also enhance sperm competitiveness (Ball and Parker 1996). Although sperm velocity is one of the key variables in these models, few studies have focused on the effect of sperm velocity on sperm competition (Levitan 2000b). A study on the sea urchin *Lytechinus variegates* showed that males with faster swimming sperm fertilized more eggs than did males with slower swimming sperm, suggesting a role for sperm competition in the evolution of fast swimming sperm within a species (Levitan 2000b). A study of two sea urchin species, *Heliocidaris erythrogramma* and *Holopneustes purpurescens*, showed that mating sequence can also affect fertilization success and fitness of offspring. The first sperm that contacts the egg suspension increases the opportunity to fertilize the largest egg and consequently increases offspring fitness (Marshall et al. 2004). Furthermore, Harper and Hart (2005) showed that sperm competition can affect paternity and hybridization between the sympatric sea stars *Asterias forbesi* and *A. rubens*.

4.1.4 Objective and importance of this study

Knowledge of reproductive isolation barriers is important for understanding speciation and the maintenance of hybrid zones for free-spawning species such as mussels. Reproductive isolation at the gamete stage (post-copulation or post-spawning reproductive isolation that occurs after copulation or spawning but before zygogenesis) may be an important factor in preventing gene flow between coexisting, closely related species (Eady 2001), and heterospecific sperm competition may be an important component of reproductive isolation. Sperm

velocity and gamete output are two of the main factors that affect sperm competition (Ball and Parker 1996; Parker 1990, 1998), and therefore differences in these traits between sympatric hybridizing species may affect fertilization success and contribute to prezygotic reproductive isolation between species. The sympatric blue mussel hybrid zone in Newfoundland where *Mytilus edulis* and *M. trossulus* coexist and produce natural hybrids provides an excellent model for studying prezygotic reproductive isolation. Although extensive studies have been conducted on mussel hybrid zones, they have mainly focused on earlier (prespawning) or later (postzygotic) events and there is little information on the role of gamete traits, especially variation in sperm swimming velocity, for reproductive isolation among *M. edulis*, *M. trossulus* and their hybrids. Furthermore, the contribution of variation in sperm velocity to sperm competition within species remains unclear.

This study focuses on: (1) determining whether there are differences in sperm velocity and gamete output within and among *M. edulis*, *M. trossulus* and their hybrids from sympatric populations in Newfoundland, Atlantic Canada, (2) investigating variation in sperm velocity within and between species, (3) estimating the effect of sperm velocity differences on fertilization success according to a fertilization model (Vogel et al. 1982) and (4) understanding the origin of variation in sperm velocity and how sperm competition and reproductive isolation influence the relative frequencies of the two species and hybrids in the population.

4.2 Materials and methods

4.2.1 Spawning, gamete collection and gamete output

The same parent mussels and methods were used as described in Chapter 2 (2.2.1 and 2.2.2). After 30 minutes of spawning, the number of gametes released was determined for individual mussels with a Multisizer™ II (Beckman Coulter). In total, 47 *M. edulis*, 22 *M. trossulus* and 10 F_1 hybrid males and 28 *M. edulis*, 45 *M.*

trossulus and 6 F_1 hybrid females were used in this experiment to estimate sperm and egg output respectively.

4.2.2 Sperm velocity

After recording the initial sperm concentration with a Multisizer™ II, 2.5 ml homogenous sperm suspension (with a sperm concentration of about 10^7 ml^{-1}) was used for the sperm velocity study following the same methods as described in Chapter 2 (2.2.3 and 2.2.4). In total, 23 *M. edulis* (374 sperm), 16 *M. trossulus* (190 sperm), 5 F_1 hybrids (59 sperm) and 18 backcross hybrid males (264 sperm) were used.

4.2.3 Data analysis and statistics

Equality of variances of the mean gamete output for different individuals were confirmed by Levene's test before an ANOVA was carried out to compare the mean gamete output between species during a 30 minutes spawning period ("R" program, R Development Core Team 2008). The sperm movement track was quantitatively reconstructed with raw sperm head coordinate data using equations in Microsoft Excel (for details see Excel appendix 1 in CD file). One way ANOVA was conducted using the "R" program to compare the mean sperm velocity between species (a linear model was constructed as $Velocity = a + \beta_0 * Species + error$) after variance equality was confirmed for the mean sperm velocity of different individuals. Nested analysis of variance was carried out in SPSS to partition variance components for sperm velocity among sources including between genotypes, between individuals and between sperm. Pairwise mean comparisons were conducted for VCL (curvilinear velocity), VAP (average path velocity) and angle change rate of all genotypes (Tukey's test in OriginPro 7.5). Mean sperm velocity

was calculated by taking the average of mean individual sperm velocity (mean velocity of all individual sperm) to construct Table 4.6.

4.3 Results

4.3.1 Gamete output during 30 minutes spawning in the laboratory

Most mussels finished spawning within 30 minutes of the start of spawning. Although individuals released different numbers of gametes, there was no significant difference in gamete output among species and hybrids (Table 4.1).

4.3.2 Sperm velocity variations among genotypes and its effect on gamete collision rate

Significant differences in sperm velocity were found among individuals and among *M. edulis*, *M. trossulus* and hybrids (Tables 4.2, 4.3, 4.4, 4.5 and 4.6). Most (60-70%) of the variance in sperm velocity was attributable to variation among sperm of an individual male, most of the remainder to differences among individuals within genotypes (Table 4.3). The VCL (curvilinear velocity) showed the lowest (2 %) variation among genotypes while for both VAP (average path velocity) and angle change rate about 20 % of the total variation was among genotypes (Tables 4.4 and 4.5).

F_1 hybrids produced sperm with a lower mean velocity (Table 4.6) than that of sperm from pure *M. edulis* and pure *M. trossulus* (Tables 4.7, 4.8 and 4.9). F_1 hybrid sperm had a significant smaller VAP and angle change rate (θ) than backcross hybrid sperm, but there was no significant difference in VCL between sperm from F_1 hybrids and backcross hybrids (Table 4.7). Pure *M. edulis* and pure *M. trossulus* sperm did not differ significantly in VCL, but pure *M. edulis* sperm had a significantly greater VAP and angle change rate (Table 4.7, 4.8 and 4.9). There was

no significant difference between pure *M. edulis* and backcross hybrid sperm in VCL, VAP and angle change rate (Tables 4.7, 4.8 and 4.9). The sperm velocity differences may be crucial to fertilization success under sperm competition between the two species and hybrids. According to Vogel's fertilization model (Vogel et al. 1982), fertilization success can be calculated as:

$$\varphi_{\infty} = 1 - \exp\{-\frac{\beta \cdot S_0}{\beta_0 \cdot E_0}(1 - e^{-\beta_0 E_0 \tau})\} \qquad (1)$$

where:

φ_{∞}: the proportion of eggs fertilized;

S_0 and E_0: the sperm and egg concentration respectively;

β ($mm^3 s^{-1}$): the fertilization rate constant, the measure of how many collisions result in fertilization;

β_0 ($mm^3 s^{-1}$): the collision rate constant, the measure of how often gamete collisions occur;

τ : gamete contact time;

also, $\beta_0 = v \cdot \sigma_0$ (2)

where v is sperm velocity and σ_0 is egg cross-sectional area.

Sperm velocity is directly related to the collision rate constant and consequently affects fertilization success. Given the sperm velocity differences observed, the collision constant differences among genotypes can be estimated. Based on VAP, the probability of an F_1 hybrid sperm colliding with an egg cell (either intra-species or inter-species) was about 65.30%, 79.11% and 67.35% of that for pure *M. edulis*, pure *M. trossulus* and backcross hybrid sperm, respectively. Based on VCL, this probability was about 81.58%, 78.82% and 88.48% of that for the same three genotypes.

4.4 Discussion

Both gamete number and sperm velocity increase the probability of successful fertilization (Parker 1970, 1990). However, accurate direct measurements of fecundity are difficult to obtain due to the technical difficulties of determining total gamete output for individuals over the entire spawning season. My data did not show significant differences among species in gamete output during a 30 minute spawning event in the laboratory, but individual variation was high. Variation within a species might be caused by differences in age, size or physiological condition, none of which were controlled in the present study in order to represent as a natural situation as possible. Despite this variation, the gamete output per mussel is consistent with the results of previous fecundity studies (Pronker et al. 2008; Thompson 1979).

The absence of significant differences in gamete output among species and hybrids can be used to assess the potential for sperm competition, assuming a mixed species and hybrid population that spawns simultaneously during the overlapping spawning seasons. Considering the short contact time required for fertilization reported in various species (Grubert et al. 2005; Kupriyanova 2006; Rosenthal et al. 1988), sperm-egg interaction and fertilization should occur within minutes at high gamete density during synchronized spawning events. Even if only a few individuals are still releasing gametes after 30 minutes, these individuals will probably face the problem that most of the gametes from other individuals are already fertilized and any fertilization success after 30 minutes of spawning will only account for a small proportion of the total fertilization. Therefore, only sperm released within 30 minutes are likely to compete, assuming synchronous release of both eggs and sperm. It is highly likely that sperm competition among *M. edulis*, *M. trossulus* and hybrids will occur in nature. Mussels attach to rocks or other hard substrates by strong byssal threads and form clumps with hundreds of individuals of each species clustered together. When fully ripe, gametes are liberated via the exhalent siphon directly into

the water column, where external fertilization occurs. Males generally start to spawn first, and the presence of sperm in the water stimulates the females to spawn (Newell 1989). Considering the overlap in the spawning season between the species (Toro et al. 2002), the synchrony of spawning, the high density of individuals and the very large numbers of sperm that individual blue mussels produce (Thompson 1979, 1984a), there is high potential for sperm competition among *M. edulis*, *M. trossulus* and hybrids. Under these conditions, sperm velocity is likely to play an important role in determining the outcome of sperm competition for broadcast spawning blue mussels. However, *M. edulis* is the only blue mussel species for which sperm velocity data have been published, mean VCL being about 120~200 μm sec^{-1} and mean average VAP about 100~120 μm sec^{-1} for *M. edulis* (Everett et al. 2004; Jha et al. 2008). In the present study, *M. edulis* sperm mean velocity was 285.07±76.93 μm sec^{-1} and 158.46±45.37 μm sec^{-1} (mean ± standard deviation) for VCL and VAP, respectively, both being greater than the estimates in the published studies, which employed a more dilute sperm suspension with only about 20 or 50 sperm per field of view, a procedure that may lead to a decrease in sperm velocity due to a dilution effect (Benzie and Dixon 1994). The higher concentration (about 10^7 sperm ml^{-1}) of sperm used in the present study provided hundreds of sperm per field of view, which may account for the higher sperm velocities observed according to dilution effect (Oliver and Babcock 1992). Furthermore, the coefficient of variation (CV) for the published mean velocity data was about 1.2~1.8% (Jha et al. 2008) and 13.9~22.4% (Everett et al. 2004) compared with 27.0~28.6% for *M. edulis* in the present study. Sample size differences between the studies may explain the difference in variation. Jha et al. (2008) used 68 and Everett et al. (2004) used 18 *M. edulis* males (14 standard and 4 recently masculinized individuals in the latter case). The present study used 23 *M. edulis* males without differentiating mtDNA genotypes, which may explain why the CV is similar to that of Everett's study and much greater than that of Jha's study. In addition, comparing sperm velocity results from different laboratories is difficult, owing to differences in sample preparation, parameter settings,

algorithms and the method used to reconstruct the sperm track (Chan et al. 1991; Gottlieb et al. 1991; Levine et al. 1989; Rijsselaere et al. 2003).

Sperm velocity differed significantly among *M. edulis*, *M. trossulus* and hybrids because values for sperm of F_1 hybrids were much lower than for sperm of the two parental species, although no significant differences were found between *M. edulis* and *M. trossulus*. Consequently, heterospecific sperm competition may be an important component of sperm fertilization success that shapes the genetic composition of mussel populations. Firstly, although *M. edulis, M. trossulus* and F_1 hybrids release similar quantities of gametes per individual during 30 minute spawning events, natural populations are dominated by *M. edulis* and *M. trossulus* with only a small proportion of F_1 hybrids (Comesaña et al. 1999; Saavedra et al. 1996; Toro et al. 2004). Thus, *M. edulis* and *M. trossulus* together produce more gametes than the F_1 hybrids, and their sperm therefore enjoy a velocity and total gamete output advantage over those of F_1 hybrids. Consequently, both the rate and the total number of gamete collisions involving F_1 hybrid sperm will be lower in nature during a single spawning event, because it is more likely that the low numbers of slow swimming F_1 hybrid sperm have to compete with large numbers of faster swimming sperm from *M. edulis* and *M. trossulus*. Therefore, lower fertilization success would be predicted for F_1 hybrid males in nature as a result of sperm competition, which may contribute to a post-spawning prezygotic reproductive isolation barrier in the mussel hybrid zone by limiting genetic introgression between the species.

Secondly, *M. edulis* eggs or egg water can significantly increase *M. edulis* sperm velocity, unlike *M. trossulus* and hybrid egg or egg water (Chapter 3). Therefore, *M. edulis* sperm appear to swim faster than *M. trossulus* sperm because *M. edulis* have a faster VAP and angle change rate when surrounded by homospecific eggs or egg water. There are two alternative means for organisms to deal with sperm competition: either respond defensively, i.e. avoiding it, or invest more energy in it.

Therefore, according to the sperm competition ESS model, *M. edulis* sperm should hold the advantage when competing with *M. trossulus* sperm during a spawning event (Ball and Parker 1996; Parker 1990). However, *M. trossulus* allocates more energy to reproduction than *M. edulis* (Lowen 2008), which supports the hypothesis that males in species where the risk of sperm competition is high should invest more in reproduction than related species in which the risk is low (Ball and Parker 1998a; 1998b; Parker 1998). Moreover, compared with *M. edulis*, *M. trossulus* has a prolonged spawning season from late spring to early autumn (Toro et al. 2002) and releases gametes more frequently (Lowen 2008). Similar to the prediction for ejaculate expenditure, this difference may also be an adaptation to sperm competition (Ball and Parker 1997; Parker et al. 1996), in which the investment is spread over more spawning events, especially when there are fewer competitors in each spawning event.

Nevertheless, even if there were a significant sperm velocity difference between the two species, this factor would probably not result in strong reproductive isolation between them because of the very large numbers of mixed gametes produced during spawning events. Gamete recognition proteins (Vacquier 1998; Vacquier et al. 1990) may provide a more effective mechanism for reproductive isolation within mussel hybrid zones. M7 lysin, a sperm acrosomal protein that dissolves the egg vitelline envelope during fertilization and is associated with gamete recognition, has been reported in mussel species and may play a role in reproductive isolation between *M. edulis* and *M. trossulus* (Riginos and McDonald 2003; Riginos et al. 2006). Therefore, although large numbers of gametes from both *M. edulis* and *M. trossulus* mix during spawning events, few F_1 hybrids are produced due to the increased frequency of homospecific fertilizations controlled by gamete recognition proteins. Furthermore, considering that F_1 hybrid sperm have a greater chance to collide with *M. edulis* or *M. trossulus* eggs and F_1 hybrid eggs have a greater chance to be fertilized by *M. edulis* or *M. trossulus* sperm, owing to the greater proportion of gametes from *M. edulis* and *M. trossulus* released during spawning events, a greater

proportion of backcross hybrids will be produced than F_1 hybrids. Under these conditions, blue mussel populations are expected to be composed of a high frequency of *M. edulis* and *M. trossulus* individuals, with some backcross individuals and very few F_1 hybrids, which is in accordance with published data on the structure of mussel hybrid zones (Comesaña et al. 1999; Saavedra et al. 1996; Toro et al. 2004).

In addition to differing among *M. edulis*, *M. trossulus* and hybrids, sperm velocity can also vary within species and individuals, accounting for 10%~27% and >60% of the total variation, respectively. Velocity differences among sperm from a single mussel may be a result of variation in sperm maturity. Since *M. edulis*, *M. trossulus* and hybrids have a relatively long spawning season (at least 2~3 weeks during the summer) (Toro et al. 2002), it is likely that gametogenesis continues throughout the spawning season. Therefore, when spawning is induced in the laboratory using thermal shock, males may release sperm at different stages of maturity. Relatively fresh mature sperm may swim faster than immature sperm, or than overmature sperm that have lost some of their velocity due to energy depletion caused by a long period of metabolism before spawning. It is theoretically possible that an individual may produce different kinds of sperm that differ in velocity, such as the parasperms and normal sperm reported in other organisms, as an adaptation to sperm competition (Bigatti et al. 2008; Buckland-Nicks 1998; Hayakawa 2007). It is not likely that variation in sperm velocity within individuals in blue mussels is due to swimming pattern or morphological differences, since there is no variation in these variables among sperm within individual males (Chapter 2 of this thesis; Miranda 2004).

Differences in sperm velocity among individuals within a species could be a result of genetic variation among individuals. Mitochondrial DNA inheritance in the *Mytilus edulis* complex, termed doubly uniparental inheritance (DUI), differs from that which occurs in most other organisms (Stewart et al. 1995; Zouros et al. 1992).

In the DUI system *Mytilus* males transmit their male type mtDNA (M-type) to their sons, whereas females pass on their female type mtDNA (F-type) to both sons and daughters. Consequently, *Mytilus* males contain both M-type and F-type mtDNA. However, F-type mtDNA sometimes changes their inheritance route into male type mtDNA one. These recently masculinized male types (Hoeh et al. 1997) may comprise up to about 20~25% of *M. edulis* males (Everett et al. 2004). A study comparing sperm velocity in different mtDNA types in *M. edulis* showed that the recently masculinized M-type sperm had significantly higher VCL and VAP than standard M-type sperm (Jha et al. 2008). This mtDNA difference among individuals within species may partly explain sperm velocity differences observed in the present study.

Variation in sperm morphology, both within and among species, may also explain variation in sperm velocity. Variation in sperm morphology, particularly total length and sperm head characteristics, has been reported within many species (Shackelford et al. 2005; Ward 1998). Furthermore, a correlation between sperm velocity and sperm length has been reported in the red deer *Cervus elaphus hispanicus* (Malo et al. 2006). However, the relationship between sperm morphology and sperm velocity remains controversial (Gage and Freckleton 2003; Gage et al. 2002; Gomendio and Roldan 1991) and it is not known whether sperm morphological variation contributes to sperm velocity variation within species. Based on a limited sample size (14 *M. edulis*, 11 *M. trossulus* and 3 hybrid individuals), no significant differences in acrosome length, head length and nucleus width were found among *M. edulis*, *M. trossulus* and hybrids by Miranda (2004). Therefore, it is unlikely that sperm velocity variation among *M. edulis*, *M. trossulus* and hybrids is caused by morphological differences.

4.5 Summary

No significant differences in gamete output were detected among *M. edulis*, *M. trossulus* and hybrids of various sizes and ages induced to spawn in the laboratory. However, significant differences in sperm velocity were observed among *M. edulis*, *M. trossulus*, F_1 hybrids and backcross hybrids, sperm from F_1 hybrids having the lowest velocity, suggesting a lower sperm-egg collision rate and consequently a lower fertilization success. These results suggest that the fertilization success of hybrids may be lower than that of each parent species, which may contribute to reproductive isolation at the post-spawning prezygotic level. Most of the variation in sperm velocity (>60%) was within individuals, which might be caused by differences in stage of maturity of sperm. Generally, in nature F_1 hybrids produce fewer, slower swimming sperm than do parental species, which may lead to reproductive isolation through sperm competition and partially explain the genetic structure of the blue mussel hybrid zone in Newfoundland.

Table 4.1: Reproductive output (total numbers of sperm or eggs) of blue mussels during one spawning event (30 minutes spawning) in the laboratory. (aE + n means a*10^n).

Male output			Female output		
M. edulis	*M. trossulus*	F_1 hybrids	*M. edulis*	*M. trossulus*	F_1 hybrids
1.34E+09	3.99E+09	3.10E+09	3.42E+06	8.82E+05	5.00E+04
1.54E+09	2.18E+09	7.82E+09	9.91E+06	5.58E+05	6.26E+05
3.25E+09	1.63E+09	2.14E+10	1.62E+06	1.56E+05	1.82E+05
6.63E+08	4.65E+09	8.50E+09	5.70E+05	1.29E+06	3.33E+06
1.03E+09	5.73E+09	2.43E+09	2.76E+05	3.38E+05	1.52E+06
1.14E+09	5.38E+09	4.71E+08	2.44E+05	7.76E+05	1.87E+06
1.72E+09	6.92E+09	7.24E+09	1.15E+06	1.20E+06	
1.13E+09	1.27E+10	4.03E+09	5.82E+05	1.17E+06	
1.08E+09	9.37E+09	9.46E+09	1.24E+06	7.50E+05	
2.70E+09	1.00E+10	3.45E+09	1.04E+05	9.79E+06	
2.79E+09	1.42E+10		1.18E+07	1.26E+07	
1.65E+10	1.22E+10		7.44E+05	2.78E+06	
3.23E+09	3.86E+09		1.60E+06	1.18E+06	
6.14E+09	6.73E+09		5.58E+05	4.74E+06	
3.86E+09	1.48E+10		1.84E+05	9.14E+05	
4.04E+09	8.58E+09		4.56E+05	1.37E+06	
9.03E+08	2.83E+09		7.78E+05	1.85E+06	
9.26E+08	7.86E+09		7.14E+06	4.69E+06	
2.17E+09	1.56E+10		3.23E+06	1.94E+06	
5.94E+10	3.84E+09		2.47E+06	5.86E+06	
6.13E+10	1.03E+10		7.47E+06	2.06E+05	
1.34E+11	3.06E+09		1.63E+06	7.06E+06	
2.27E+10			1.75E+06	4.94E+06	
5.80E+09			1.67E+06	6.90E+06	
3.08E+08			3.73E+06	6.50E+05	
1.03E+10			1.55E+06	4.50E+05	
1.52E+10			1.16E+06	5.34E+05	
4.15E+10			9.58E+06	1.27E+06	
1.39E+10				1.15E+06	
7.27E+09				7.88E+05	
4.73E+09				7.15E+06	
1.72E+09				7.12E+05	
4.03E+10				1.64E+06	
1.40E+10				2.12E+06	
6.05E+09				2.21E+06	
5.75E+09				8.84E+05	

	4.64E+09				1.38E+06	
	2.93E+09				1.61E+06	
	1.33E+10				1.24E+06	
	4.70E+09				1.24E+06	
	8.84E+09				2.41E+06	
	1.46E+10				1.58E+06	
	6.04E+09				4.34E+06	
	1.16E+10				4.10E+06	
	3.44E+10				1.78E+06	
	3.00E+09					
	6.71E+09					
CV.	1.75	0.56	0.83	1.18	1.06	0.90
N	47	22	10	28	45	6
Mean	1.30E+10	7.56E+09	6.70E+09	2.74E+06	2.52E+06	1.26E+06
SE	3.35E+09	9.20E+08	1.87E+09	6.21E+05	4.03E+05	5.08E+05
	Levene's Test	$F_{2,76}$=0.79, p=0.46			$F_{2,76}$=0.84, p=0.44	
	One way ANOVA	$F_{2,76}$=0.66, p=0.52			$F_{2,76}$=0.94, p=0.40	

Table 4.2: Mean sperm velocity in *Mytilus edulis*, *M. trossulus*, F_1 hybrid and backcross hybrid individuals.

Variable	Individual identity	Total sperm number (Ns)	Mean±S.E.
VCL (μm sec^{-1})			
M. edulis			
	1	20	191.41±10.01
	2	20	351.50±7.76
	3	20	317.72±10.07
	4	20	228.54±9.80
	5	20	331.86±9.77
	6	20	328.25±8.08
	7	20	248.18±7.07
	8	9	193.48±17.27
	9	20	237.11±6.77
	10	17	255.64±3.63
	11	19	238.35±6.75
	12	17	330.05±14.51
	13	9	114.78±8.48
	14	3	270.52±14.13
	15	19	355.32±9.75
	16	20	374.10±20.81
	17	10	295.59±13.65
	18	3	352.04±14.23
	19	19	251.03±12.60
	20	9	230.83±6.49
	21	21	242.87±5.60
	22	20	306.76±10.08
	23	19	399.60±14.80
M. trossulus			
	1	15	206.21±7.54
	2	6	200.20±7.17
	3	10	146.68±9.73
	4	20	226.45±4.23
	5	9	83.49±6.98
	6	20	499.80±17.18
	7	17	414.26±10.98
	8	18	407.37±23.63
	9	9	589.07±19.52
	10	7	189.59±21.68

	11	8	110.31±11.59
	12	20	213.60±7.47
	13	20	310.97±8.13
	14	4	173.12±22.22
	15	2	176.59±18.46
	16	5	320.18±16.10
F_1 hybrids			
	1	10	142.85±12.04
	2	9	99.27±16.25
	3	22	209.28±8.70
	4	17	382.49±20.55
	5	1	292.44
Backcross hybrids			
	1	21	236.36±5.42
	2	20	228.09±3.68
	3	11	209.31±12.20
	4	20	251.90±6.59
	5	20	373.97±10.73
	6	4	291.83±31.19
	7	16	465.58±19.19
	8	7	233.06±7.48
	9	20	169.13±10.35
	10	20	250.59±4.72
	11	20	280.69±7.15
	12	11	119.70±8.08
	13	6	81.30±5.69
	14	7	211.76±5.52
	15	16	453.05±30.83
	16	20	14.00±0.22
	17	5	176.04±17.12
	18	20	454.48±25.23
VAP ($\mu m\ sec^{-1}$)			
M. edulis			
	1	20	135.41±7.25
	2	20	258.38±6.35
	3	20	192.72±7.41
	4	20	155.81±4.09
	5	20	132.68±5.57
	6	20	111.25±4.58
	7	20	169.09±7.25
	8	9	141.41±13.10
	9	20	185.44±5.36

	10	17	182.43±5.39
	11	19	173.45±7.09
	12	17	157.15±5.30
	13	9	158.69±14.11
	14	3	191.26±4.86
	15	19	172.34±8.81
	16	20	180.33±9.45
	17	10	197.59±13.62
	18	3	162.98±5.62
	19	19	162.49±7.25
	20	9	109.32±5.26
	21	21	140.87±6.87
	22	20	126.13±7.42
	23	19	117.72±10.87
M. trossulus			
	1	15	164.69±10.07
	2	6	169.44±8.35
	3	10	84.02±13.49
	4	20	147.54±6.48
	5	9	41.04±3.11
	6	20	112.36±6.98
	7	17	129.35±6.91
	8	18	96.68±8.63
	9	9	93.52±7.20
	10	7	151.56±16.76
	11	8	78.69±6.89
	12	20	164.52±3.75
	13	20	154.06±3.97
	14	4	145.41±17.51
	15	2	149.51±16.27
	16	5	246.35±7.89
F_1 hybrids			
	1	10	86.42±12.22
	2	9	38.19±8.08
	3	22	137.97±7.32
	4	17	105.90±8.52
	5	1	61.57
Backcross hybrids			
	1	21	183.07±4.26
	2	20	161.66±2.91
	3	11	159.64±10.79
	4	20	174.74±6.40
	5	20	151.03±9.55

6	4	222.32±18.08
7	16	84.79±4.53
8	7	162.66±6.39
9	20	146.31±3.94
10	20	185.98±3.87
11	20	172.64±6.50
12	11	135.45±6.81
13	6	114.43±11.73
14	7	165.84±3.55
15	16	132.54±9.46
16	20	176.14±4.05
17	5	141.12±12.19
18	20	102.16±7.96

θ (Angle change per frame, radian sec^{-1})

M. edulis

1	20	0.35±0.02
2	20	0.61±0.02
3	20	0.45±0.02
4	20	0.45±0.02
5	20	0.35±0.02
6	20	0.29±0.03
7	20	0.43±0.03
8	9	0.36±0.03
9	20	0.49±0.02
10	17	0.46±0.02
11	19	0.46±0.03
12	17	0.42±0.04
13	9	0.45±0.05
14	3	0.64±0.06
15	19	0.48±0.04
16	20	0.46±0.04
17	10	0.55±0.07
18	3	0.47±0.03
19	19	0.49±0.04
20	9	0.26±0.02
21	21	0.38±0.04
22	20	0.35±0.03
23	19	0.28±0.04

M. trossulus

1	15	0.43±0.04
2	6	0.40±0.04
3	10	0.18±0.05
4	20	0.39±0.02

	5	9	0.06±0.01
	6	20	0.26±0.02
	7	17	0.33±0.02
	8	18	0.21±0.10
	9	9	0.19±0.02
	10	7	0.39±0.05
	11	8	0.18±0.05
	12	20	0.43±0.03
	13	20	0.42±0.02
	14	4	0.29±0.04
	15	2	0.47±0.08
	16	5	0.67±0.02
F_1 hybrids			
	1	10	0.19±0.03
	2	9	0.06±0.02
	3	22	0.34±0.02
	4	17	0.23±0.03
	5	1	0.11
Backcross hybrids			
	1	21	0.50±0.03
	2	20	0.45±0.02
	3	11	0.41±0.05
	4	20	0.50±0.03
	5	20	0.41±0.03
	6	4	0.56±0.07
	7	16	0.17±0.01
	8	7	0.50±0.05
	9	20	0.51±0.03
	10	20	0.53±0.02
	11	20	0.46±0.03
	12	11	0.34±0.03
	13	6	0.27±0.03
	14	7	0.52±0.04
	15	16	0.32±0.04
	16	20	0.52±0.02
	17	5	0.33±0.02
	18	20	0.24±0.03

Table 4.3: Nested analysis of variance for VCL (four genotype groups: *M. edulis*, *M. trossulus*, F_1 hybrid and backcross hybrid).

Source of variation	df	MS	F	P	Percentage of variance components
Genotype	3	85285	34.155	<0.0001	2.05
Individual	58	174253	69.785	<0.0001	27.29
Sperm	825	2497			70.66

Table 4.4: Nested analysis of variance for VAP (four genotype groups: *M. edulis*, *M. trossulus*, F_1 hybrid and backcross hybrid).

Source of variation	df	MS	F	P	Percentage of variance components
Genotype	3	74087	87.376	<0.0001	22.21
Individual	58	18306	21.590	<0.0001	15.65
Sperm	825	848			62.14

Table 4.5: Nested analysis of variance for angle change rate (four genotype groups: *M. edulis*, *M. trossulus*, F_1 hybrid and backcross hybrid).

Source of variation	df	MS	F	P	Percentage of variance components
Genotype	3	2.7241	87.376	<0.0001	20.00
Individual	58	9.4767	21.590	<0.0001	10.00
Sperm	825	11.7713			70.00

Table 4.6: Mean sperm velocity in *M. edulis*, *M. trossulus*, F_1 hybrids and backcross hybrids.

Parameter	Individual Number (Ni)	Total sperm Number (Ns)	Mean±SE
VCL (μm sec^{-1})			
M. edulis	23	374	285.07±3.98
M. trossulus	16	190	295.06±10.57
F_1 hybrids	5	59	232.56±15.48
Backcross hybrids	18	264	262.83±8.45
VAP (μm sec^{-1})			
M. edulis	23	374	158.46±2.35
M. trossulus	16	190	130.79±3.50
F_1 hybrids	5	59	103.48±6.21
Backcross hybrids	18	264	153.64±2.44
θ (Angle change per frame, radian sec^{-1})			
M. edulis	23	374	0.41±0.01
M. trossulus	16	190	0.33±0.01
F_1 hybrids	5	59	0.23±0.02
Backcross hybrids	18	264	0.42±0.01

Table 4.7: Pairwise comparisons of VCL in various genotype groups using Tukey's test.

Comparison groups	Difference between means	Simultaneous confidence intervals		Significant at 0.01 level
		Lower limit	Upper limit	
M. edulis: *M. trossulus*	-10.00	-42.65	22.65	No
M. edulis: Backcross hybrids	22.24	-7.22	51.70	No
M. edulis: F_1 hybrids	52.51	1.17	103.85	Yes
M. trossulus: Backcross hybrids	32.23	-2.63	67.10	No
M. trossulus: F_1 hybrids	62.50	7.88	117.13	Yes
Backcross hybrids: F_1 hybrids	30.27	-22.50	83.05	No

Table 4.8: Pairwise comparisons of VAP in various genotype groups using Tukey's test.

Comparison groups	Difference between means	Simultaneous confidence intervals		Significant at 0.01 level
		Lower limit	Upper limit	
M. edulis: *M. trossulus*	27.67	15.24	40.09	Yes
M. edulis: Backcross hybrids	4.81	-6.40	16.02	No
M. edulis: F_1 hybrids	54.98	35.45	74.51	Yes
M. trossulus: Backcross hybrids	-22.86	-36.12	-9.59	Yes
M. trossulus: F_1 hybrids	27.31	6.53	48.09	Yes
Backcross hybrids: F_1 hybrids	50.17	30.09	70.25	Yes

Table 4.9: Pairwise comparisons of angle change rate in various genotype groups using Tukey's test.

Comparison groups	Difference between means	Simultaneous confidence intervals		Significant at 0.01 level
		Lower limit	Upper limit	
M. edulis: *M. trossulus*	0.0893	0.0461	0.1324	Yes
M. edulis: Backcross hybrids	-0.0074	-0.0463	0.0315	No
M. edulis: F_1 hybrids	0.1814	-0.1427	0.2492	Yes
M. trossulus: Backcross hybrids	-0.0967	0.1135	-0.0506	Yes
M. trossulus: F_1 hybrids	0.0921	0.0199	0.1643	Yes
Backcross hybrids: F_1 hybrids	0.1888	0.1190	0.2585	Yes

Chapter 5

Sperm longevity and the effect of temperature on sperm longevity in the blue mussels *Mytilus edulis*, *M. trossulus* and their hybrids

5.1 Introduction

5.1.1 Theoretical effect of sperm longevity on fertilization success

"An individual spermatozoon retains its capacity to fertilize only for a certain time after extraction from the testis. The average of these times over the spermatozoa of a given male is called life span of sperm of this male" (Vogel et al. 1982). At the same time that Vogel formulated this definition of sperm life span, he also proposed sperm longevity or life span as another important sperm character, in addition to sperm velocity, that can affect fertilization success.

Furthermore, Vogel et al. (1982) developed a fertilization model based on experimental data from the free spawning sea urchin *Paracentrotus lividus*:

$$\varphi_{\infty} = 1 - \exp\{-\frac{\beta \cdot S_0}{\beta_0 \cdot E_0}(1 - e^{-\beta_0 E_0 \tau})\}$$

where:

φ_{∞}: the proportion of eggs fertilized;

S_0 and E_0: the sperm and egg concentration respectively;

β ($mm^3 s^{-1}$): the fertilization rate constant, the measure of how many collisions result in fertilization;

β_0 ($mm^3 s^{-1}$): the collision rate constant, the measure of how often gamete collisions occur;

τ : gamete contact time or sperm half life;

also, $\beta_0 = v \cdot \sigma_0$

where v is sperm velocity and σ_0 is egg cross-sectional area.

According to the fertilization kinetics model, an increase in sperm half-life (τ) increases the time available for gamete interactions and consequently leads to higher cumulated fertilization success.

As sperm age, they gradually lose the ability to fertilize eggs, as has been demonstrated in the sea urchin *Lytechinus variegates* (Levitan 2000b) and the starfish *Acanthaster planci* (Benzie and Dixon 1994). Sperm longevity has been investigated in different species for various purposes (Anon 1993; Arnaud et al. 2001; Johnson and Yund 2003; Litvak and Trippel 1998; Pasquini et al. 2008; Watson and Nikolakopoulos 1996). For example, Pasquini (2008) studied the effect of three different cryopreservation procedures on sperm longevity after thawing; Watson (1996) studied sperm longevity in the equine uterus; Litvak and Trippel (1998) studied how egg presence and ovarian fluid affect sperm longevity in the Atlantic cod *Gadus morhua*; and Johnson and Yund (2003) studied sperm longevity in the colonial ascidian, *Botryllus schlosseri*. Makler (1979) first suggested that sperm longevity could be a useful index for human sperm quality.

5.1.2 Sperm limitation or sperm competition?

In contrast to theoretical predictions from the simple fertilization kinetics model, the effect of sperm longevity on fertilization success is empirically complicated and depends on species-specific reproductive strategy, lifestyle and environmental conditions. In contrast to internal fertilization, free-spawning organisms release sperm directly into the water column, which is a complex natural environment. Whether sperm longevity following spawning affects fertilization success in free spawning species is still a subject for debate. For example, some researchers believe that sperm longevity in organisms that release both sperm and

eggs is irrelevant to fertilization success because dilution of sperm below fertilizable concentrations occurs before the sperm become incapable of fertilizing the egg (Levitan 1993; Levitan and Petersen 1995; Levitan et al. 1991). Dilution of sperm limits the effective sperm concentration within a very short distance of spawning males. Data from *Strongylocentrotus droebachiensis* showed that even with several males synchronously spawning, less than 40% of the eggs were fertilized at distances greater than 2 meters downstream (Pennington 1985). Similarly, the *in situ* fertilization of hydroid eggs greatly decreased beyond 3 meters of the spawning male(s) (Yund 1990). In the colonial ascidian *Botryllus schlosseri* fertilization success decreases as the distance between colonies increases (Grosberg 1987). It is generally believed that a certain minimal sperm concentration is required for fertilization to occur in free spawning organisms. Pennington (1985) showed that fertilization success in *S. droebachiensis* requires relatively dense sperm concentrations (>10^3 sperm ml^{-1} to fertilize 50% of the eggs). Similar results have been reported in other species, e.g. concentrations greater than 10^3 sperm ml^{-1} were also required to fertilize 50% of the eggs in *S. franciscanus*,(Levitan et al. 1991) and about 10^5-10^6 sperm ml^{-1} were needed to maximize fertilization success in 4 coral species during synchronous spawning events (Oliver and Babcock 1992).

Based on the observations described above, it has been suggested by many authors that many eggs remain unfertilized due to sperm limitation and that sperm longevity is not a determinant of fertilization success in free spawning species (Levitan 1993; Levitan and Petersen 1995). However, other researchers consider that sperm limitation may not be as severe as is often stated, due to various adaptations to increase fertilization success (Yund 2000) and to the role of hydrodynamics in fertilization (Babcock et al. 1994; Denny et al. 2002). The complexity of hydrodynamic forces has to be taken into account when considering fertilization success in free spawning species. On the one hand, shear force may increase the chance of gamete contact, counteracting any sperm limitation. On the other hand, shear force may decrease gamete concentration and prevent sperm from attaching to

the egg surface. It is also possible that a high shear force could damage the sperm, resulting in sperm limitation.

Denny and Shibata (1989) and Mead and Denny (1995) suggested that turbulence may rapidly dilute gametes of *S. purpuratus*. Low shear stress can improve fertilization success, probably by increasing the chance of gamete contact, but high shear stress will decrease fertilization success, probably by interfering with gamete contact. Similarly, in the red abalone *Haliotis rufescens* fertilization success was greatest at a low shear stress of 0.1 s^{-1} and decreased at values above 1.0 s^{-1} (Riffell and Zimmer 2007). Furthermore, gamete damage was not responsible for the reduced fertilization success observed under high shear force in the red abalone. Realizing the limitations of their earlier study, Denny et al. (2002) revised the experimental design and found that fertilization success can exceed 80% even when the dissipation energy is higher than is likely to be encountered in breaking waves. Therefore, rather than limiting sperm and reducing fertilization success, a higher shear force may result in a greater fertilization success. Clearly, more research is required to clarify how hydrodynamic forces control gamete contact.

Although fertilization rates from field surveys are usually below 100%, the average fertilization success is often >85% when most individuals in the population spawn (see Table 1 in Yund 2000), which suggests that there are reproductive adaptations such as synchronous spawning which reduce sperm limitation effects (Yund 2000). Synchronous spawning has been reported in many species in which a high concentration of gametes is produced, which may decrease the dilution effect (Baird et al. 2001; Gosselin 2004; Guest et al. 2002; Harrison et al. 1984; Mangubhai et al. 2007; Morita et al. 2006; Scott and Harrison 2005; Takemura 2004). Oliver and Babcock (1992) suggested that synchronous spawning of broadcast-spawning corals during periods of low water motion and the production of buoyant gamete bundles that accumulate at the surface could be adaptations to minimize gamete dilution effects. Similarly, sperm limitation was not observed in reproduction of the alga *Fucus distichus*, which only released gametes when water movement was low, and fertilization success was almost 100% (Pearson and Brawley 1996; Pearson et al.

1998). Furthermore, in organisms such as *S. droebachiensis* the release of gametes into viscous body fluids may also be an adaptation, together with synchronous spawning, to reduce dilution effects (Meidel and Yund 2001).

In free spawning species that release both male and female gametes, eggs are generally viable for a longer period than sperm. For example, according to field observations *S. droebachiensis* eggs can be successfully fertilized up to 48 hours from release (Meidel and Yund 2001). Similar results have been reported in two polychaete and one asteroid species, in which the eggs were viable for long periods (up to 36 hours in *Nereis virens*, more than 72 hours in *Arenicola marina* and about 14 hours in *Asterias rubens*) (Williams and Bentley 2002). Considering the remarkably long life span of egg cells, even if there is sperm limitation in the short term there is a high probability that eggs will be exposed to sperm during long spawning events, and consequently fertilization success can be integrated over time (Meidel and Yund 2001).

Like synchronous spawning, aggregation before spawning will increase the local population density and decrease the distance between mates, which minimizes the gamete dilution effect (Coma and Lasker 1997; Levitan 1991b; Levitan and Ferrell 2006; Pennington 1985; Yund and McCartney 1994). Although sperm limitation is more likely to occur in widely spaced individuals, high fecundity may overcome it and ensure high fertilization success (Babcock et al. 1994).

In contrast to free-spawning species, which are more common, other species release only sperm into the water column and retain the eggs inside the body or within the shell, which is referred to as brooding or spermcast mating (Bishop and Pemberton 2006). Brooding organisms can use feeding structures to filter and extract diluted sperm efficiently for internal fertilization (Pemberton et al. 2003). A very long life span for sperm at low concentrations has been reported in several spermcasting species, which has a considerable impact on fertilization success in these taxa. For example, sperm of the colonial ascidian *Botryllus schlosseri* can fertilize eggs at remarkably low concentrations (10 sperm ml^{-1}), and have a much longer life span (16~26 hours) than sperm of other marine invertebrates (Johnson

and Yund 2004). The half-life of the sperm of the ascidian *Diplosoma listerianum* recorded by Bishop (1998) was 8 hours, but a substantial number of fertilizations occurred even with 24-hour-old sperm. A longer life span for sperm in dilute suspensions, and the existence of specialized sperm capture structures that extract them, make it unlikely that sperm limitation is an acute problem in these spermcast species (Bishop 1998; Bishop and Pemberton 2006). Furthermore, a longer sperm life span is required for high fertilization success in species releasing sperm at low concentrations (Johnson and Yund 2004).

Life history, reproductive strategy and the hydrodynamic environment are unique for each species, and there is considerable debate about the degree of sperm limitation, if it exists at all (Levitan and Petersen 1995; Yund 2000; Yund and McCartney 1994). Although there is the potential for sperm limitation in free spawning species, sperm availability may be sufficient for sperm competition to occur under natural conditions. The evolution of a wide variety of mechanisms preventing polyspermy (multiple sperm fertilizing a single egg) in free spawning organisms is indirect evidence against sperm limitation, suggesting that sperm competition may be a more important component of reproductive success than sperm limitation (Bode and Marshall 2007; Togo et al. 1995; Yund and McCartney 1994).

5.1.3 Selection pressure on sperm traits under conditions of sperm competition and sperm limitation

In free spawning species the number of sperm produced by each individual is several magnitudes greater than the number of eggs, and there is a potential for sperm competition (Ball and Parker 1996; Gage et al. 2004) to occur. However, as discussed in 5.1.2, there is also potential for sperm limitation due to dilution effects. Taking blue mussel *Mytilus* species as an example, and considering their aggregation behavior (Newell 1989), synchronous spawning (Gosselin 2004) and high gamete output (Gosselin 2004; Thompson 1979, 1984a), sperm competition is more likely to

occur within relatively short distances of the cluster of spawning parents, where a dense cloud of gametes can form. In contrast, sperm limitation is more likely when gametes originate from different clusters of spawning mussels separated by a distance sufficiently great for gamete dilution to play a role in determining fertilization success.

There have been many studies of selection pressure on sperm traits under conditions of sperm competition and sperm limitation (Ball and Parker 2007; Engqvist and Reinhold 2007; Levitan 1993, 1998, 2000a; Malo et al. 2006; Parker 2000). Sperm longevity, which is considered an important parameter in sperm competition models (Ball and Parker 1996; Parker 1970), is negatively correlated with sperm velocity in the sea urchin *Lytechinus variegates* (Levitan 2000b). In the Atlantic salmon, *Salmo salar*, sperm velocity is positively, and sperm longevity negatively, associated with sperm success in fertilization (Gage et al. 2004). Considering the gamete contact time required for successful fertilization at high sperm concentration is usually only a few minutes (Grubert et al. 2005; Kupriyanova and Havenhand 2005; Levitan 1991b; Rosenthal et al. 1988), which is significantly shorter than sperm half-life, this suggests that sperm velocity is a critical trait during sperm competition. Therefore, higher energetic capacity and initial swimming speed is crucial for sperm of sneaker males to obtain a high fertilization success during sperm competition in the bluegill, *Lepomis macrochirus* (Burness et al. 2004). Field studies in three sea urchin species showed that *Strongylocentrotus purpuratus,* which had the highest adult density, exhibited the fastest sperm velocity and shortest sperm life span. *S. droebachiensis,* which was the least densely distributed, had the slowest sperm velocity but the longest sperm life span, whereas *S. franciscanus* exhibited intermediate levels of adult density and gamete traits (Levitan 1998). Thus when sperm are competing for eggs natural selection favours faster swimming sperm with a short life span, whereas when the availability of sperm is limited long lived, slow swimming sperm are favoured (Levitan 1993, 1998, 2000b).

5.1.4 Effect of temperature on sperm traits

Free spawning species release gametes into the water column, in which the temperature often fluctuates (Becker and Pauly 1996; Mallet et al. 1999). Temperature affects sperm performance both physiologically and physically. First, temperature can affect the metabolic rate of sperm (Hammerstedt and Hay 1980; Mansour et al. 2003; Nashed et al. 1964). An increase in temperature leads to an increase in sperm velocity and metabolic rate (Alavi and Cosson 2005; Esfandiari et al. 2002). Secondly, an increase in seawater temperature leads to a decrease in viscosity and therefore an increase in the velocity of moving objects such as sperm (Podolsky and Emlet 1993). Larval velocity in the sand dollar *Dendraster excentricus* increases with seawater temperature, as a result of both physiological (biochemical reaction rate) and physical (viscosity) changes (Podolsky and Emlet 1993). Regardless of the mechanism responsible for this increase in velocity at higher temperature, sperm possess a finite energy resource, and therefore sperm that swim faster should exhibit reduced longevity (Levitan 1993, 2000b).

5.1.5 Objectives and importance of this study

The blue mussel hybrid zone in Atlantic Canada provides a good model to investigate reproductive isolation barriers in free spawning species. Differences in sperm traits between *Mytilus edulis, M. trossulus* and their hybrids may influence heterospecific fertilization success and are important for understanding the mechanisms that maintain this hybrid zone. However, no study has investigated how differences in sperm longevity among parental and hybrid species contribute to reproductive isolation within the zone and how sperm longevity is affected by temperature.

In this chapter, both the relative sperm longevity (half-life or T_{50}) and the effect of temperature on sperm longevity were investigated in *M. edulis, M. trossulus* and their hybrids from sympatric populations in Newfoundland, Atlantic Canada. The

trade-off between sperm velocity and longevity was also estimated for these genotypes.

5.2 Materials and methods

5.2.1 Collection of mussels, spawning and sampling of gametes

The same individual mussels and methods were used as described in Chapter 2 (2.2.1 and 2.2.2).

5.2.2 Effect of temperature on sperm velocity

After recording the initial sperm concentration with a Multisizer™ II, the sperm suspension was stirred gently with a clean glass rod. 50ml of mixed sperm suspension (concentration approximately 10^7 sperm ml^{-1}) was delivered into each of three identical 60ml glass jars and incubated at 4, 8 and 20 °C respectively. Initial sperm velocity was measured in the suspensions just before the incubations began. Sperm velocity measurements were made for 623 sperm of different ages (t) (1 to 6 h) from 44 mussels (23 *M. edulis*, 16 *M. trossulus* and 5 F_1 hybrids) at one hour intervals from the beginning of the temperature treatment, using the same methods as described in Chapter 2 (2.2.3 and 2.2.4).

5.2.3 Data analysis and statistics

A 3- dimensional figure was constructed using OriginPro 7.5 to show the combined effects of increasing temperature and sperm age on sperm velocity. Relative velocity was calculated for sperm of different ages by dividing sperm velocity at age t (υ_t) by the initial velocity (υ_0), and the data fitted to an exponential decay model in OriginPro 7.5. Relative sperm longevity was then calculated by

solving for sperm age at a relative sperm velocity of 0.5 (Fig 5.1). Equality of variances was confirmed by Levene's test before an ANOVA was carried out ("R" program; R Development Core Team, 2008) to determine the effects of temperature and genotype on sperm relative longevity. Pearson correlation coefficients were calculated to show the relationship between mean velocity and mean longevity of all sperm from one individual at various temperatures.

5.3 Results

5.3.1 Effects of temperature and sperm age on sperm velocity

Sperm velocity decreased as temperature and sperm age increased in both mussel species and their hybrids (Fig 5.2). For *M. edulis,* as the sperm aged (2~6 hours) the lowest relative sperm velocity was found at higher temperatures (Fig 5.3.a). A similar pattern of decrease in sperm velocity with age and increasing temperature was also observed for sperm from *M. trossulus* and, to a lesser degree, from hybrids (Fig 5.3.b and 5.3.c).

5.3.2 Genotypic variation in sperm half life and the effect of temperature on sperm half life

The mean sperm half life was significantly different among genotypes and among temperatures (Table 5.1). Both mussel species and hybrids had a significant longer mean sperm half life (T_{50}) at lower temperature (Table 5.2). F_1 hybrid sperm had the shortest mean life span at all three temperatures. Sperm of *M. edulis* had the longest mean half life, 1.81~2.27 times longer than that of sperm of F_1 hybrids. Sperm of *M. trossulus* had an intermediate mean life span, 1.23~1.99 times as long as that of sperm of F_1 hybrids (Table 5.2).

5.3.3 Relationship between sperm velocity and sperm longevity

Sperm longevity was negatively correlated with initial sperm velocity in all genotypes at all temperatures (Fig. 5.4.a, b and c).

5.4 Discussion

Sperm velocity (VCL, curvilinear velocity) of mussels decreased as sperm age increased, which is in accordance with previous studies. For example, in the giant cuttlefish, *Sepia apama*, sperm velocity decreased as sperm age increased (Naud and Havenhand 2006). A similar negative relationship between velocity and age has been shown in sperm of the sea urchin *Lytechinus variegates* (Levitan 2000b). Similar trends have also been found in several fish species. For example, in the carp all three sperm velocity parameters, VCL, VAP (average path velocity) and VSL (straight line velocity), decreased as sperm age increased (Ravinder et al. 1997), whereas in the fifteen-spined stickleback both VCL and VSL decreased (Elofsson et al. 2003b). In Arctic charr, VCL also decreased with sperm age (Turner and Montgomerie 2002). Herreros et al. (2005) showed that VCL, VAP and VSL of boar sperm decreased as sperm aged in different incubation media.

As sperm age, not only velocity but also the fertilization ability of sperm decreases, as has been reported in various invertebrate species including *Arenicola marina* and *Nereis virens* (Williams and Bentley 2002), *Asterias rubens* (Williams and Bentley 2002), *Haliotis tuberculata* (Liu et al. 1991), *Laternula elliptica* (Powell et al. 2001), *Nacella concinna* (Powell et al. 2001), *Ciona intestinalis* (Bolton and Havenhand 1996), *Ascidiella aspersa* (Bolton and Havenhand 1996), *Paracentrotus lividus* (Vogel et al. 1982) and *Acanthaster planci* (Benzie and Dixon 1994).

Since sperm velocity and fertility are both correlated with sperm age, there are two methods to determine sperm longevity (half-life, T_{50}). The first, which is commonly used, is to estimate sperm longevity from the well known relationship between fertilization success and sperm age (Bolton and Havenhand 1996; Johnson and Yund 2004; Levitan 2000b; Vogel et al. 1982), sperm half-life being derived by solving for the time at which fertilization success drops to half its initial value. This method is advantageous because it is directly related to fertilization success. However, although freshly spawned eggs were used in most experiments testing the effect of sperm age on fertilization success (Bolton and Havenhand 1996; Williams and Bentley 2002), other egg traits (Levitan 1996, 2006), sperm-egg compatibility (Takamura and Miyajima 1999) and cryptic choice by females (Nordeide 2007), as well as sperm traits, will also contribute to fertilization success.

The second method, which was used in the present study, is to determine sperm half-life from the relationship between sperm longevity and velocity. Although this approach does not provide a direct measure of sperm fertilization ability, it does avoid the errors associated with the first method. Furthermore, because sperm velocity is correlated with fertilization success in many species (Gage et al. 2004; Kupriyanova and Havenhand 2002; Levitan 1996; Malo et al. 2005; Moore and Akhondi 1996), it can still provide an indirect estimate of sperm fertilization ability. If the decrease in sperm velocity at time t is always proportional to sperm velocity at time t, then the following equation will hold true:

$$\upsilon_t = \upsilon_0 \cdot e^{-\alpha \cdot t}$$

So the relative sperm velocity at time t will be:

$$\upsilon_{relative} = \frac{\upsilon_t}{\upsilon_0} = \frac{\upsilon_0 \cdot e^{-\alpha \cdot t}}{\upsilon_0} = e^{-\alpha \cdot t}$$

where υ_0, υ_t and $\upsilon_{relative}$ are initial sperm velocity, sperm velocity at time t and relative sperm velocity, respectively, and α is a constant that reflects how fast the sperm velocity decreases as sperm age increases.

Thus sperm relative velocity fits the exponential decay model well, and sperm half-life (T_{50}) can then be estimated by solving for the time at which relative sperm velocity is 0.5. Estimating sperm longevity from the relationship between sperm velocity and age has been done in other organisms such as the giant cuttlefish *Sepia apama* (Naud and Havenhand 2006) and the fifteen-spined stickleback (Elofsson et al. 2003b), but the present study provides the first quantitative estimate of sperm half-life derived from sperm velocity and longevity.

In the present study, sperm longevity was negatively correlated with sperm initial velocity in all mussel genotypes investigated, which is consistent with observations on the sea urchin *Lytechinus variegatus* (Levitan 2000b). The negative relationship between initial sperm velocity and sperm longevity probably indicates a trade-off due to a finite amount of energy. With finite energy reserves, sperm can either swim faster but die sooner or swim slower but live longer. Some studies have suggested that any energy savings in swimming will prolong sperm longevity, even if it is by intermittent swimming (Bishop 1998) or by fast swimming only when chemo-attractants are present (AlAnzi et al. 1997; Carre and Sardet 1981; Miller 1979a, 1981, 1982, 1985, 1997; Miller and King 1983; Miller et al. 1994; Morita et al. 2006; Yoshida et al. 1993). However, when comparing sperm longevity among species the assumption that faster swimming sperm have a shorter life span may not always be true. The sperm of *M. edulis* has a higher initial velocity and a longer half-life than those of *M. trossulus* and hybrids (Fig 5.2 and Table 5.2), probably because the sperm of *M. edulis* has more energy reserves. Differences among sperm can play an important role in maintaining the hybrid zone because under conditions of sperm competition or limitation, sperm from F_1 hybrids, which exhibit the slowest

swimming speed and shortest half-life, will have the poorest chance to fertilize eggs of both species, which will lead to a reduced fitness of F_1 hybrids.

The temperature of the water column into which free-spawning species release their gametes can affect sperm velocity and longevity, and therefore fertilization success. Variance in thermal tolerance among gametes may also reduce hybridization in broadcast spawning marine invertebrates (McClary and Sewell 2003). In this study we investigated sperm velocity and longevity at 4 °C (an extreme low temperature during summer), 8 °C (the approximate seawater temperature during July, when the natural spawning starts; Thompson 1984b), and 20 °C (an extremely high temperature that intertidal individuals may encounter). Higher temperatures led to higher sperm velocity in recently spawned sperm (1~2 hours) (Fig 5.3.a, b, c), probably owing to increased metabolic rate and lower water viscosity. On the other hand, relative velocity was lower at higher temperature in older sperm (3~6 hours), which may be attributable to a decrease in remaining energy reserves. With finite energy reserves, higher metabolic rate leads to a shorter life span, which may explain the reduced sperm longevity at higher temperatures (Table 5.1 and Fig 5.3.a, b, c). Regardless of temperature, sperm of *M. edulis* and F_1 hybrids had the longest and shortest half lives, respectively (Table 5.1). The half life of *M. trossulus* sperm was the least affected by temperature, the greatest sperm longevity (4 °C) being only 1.52 times that of the least (20 °C), compared with 1.96 and 2.47 times for *M. edulis* and F_1 hybrids, respectively. Considering that *M. trossulus* has a longer spawning season than *M. edulis* in Atlantic Canada (Toro et al. 2002), the higher temperature tolerance of *M. trossulus* sperm may be an adaptation to a wider range of temperatures during the long spawning season.

5.5 Summary

Sperm velocity decreased as temperature and sperm age increased in the blue mussels *M. edulis*, *M. trossulus* and their F_1 hybrids. Both temperature and genotype had a significant effect on sperm half life (T_{50}). Sperm longevity decreased as seawater temperature increased in all genotypes. *M. edulis* had the greatest sperm longevity and F_1 hybrids the least. *M. trossulus* sperm were less temperature sensitive than those of *M. edulis* or hybrids.

The results suggest that F_1 hybrids may have lower fitness than the parental species due to the poorer quality of the sperm. Selection will therefore favour the parental species over the hybrids under conditions of sperm competition or sperm limitation. Sperm longevity and velocity were negatively correlated within species, probably due to energy constraints. However, this relationship may not hold true among species, because allocation of energy to each sperm is probably a species specific trait. Finally, the high temperature tolerance of *M. trossulus* sperm may be an adaptation to its longer spawning period, which results in exposure to a greater temperature fluctuation.

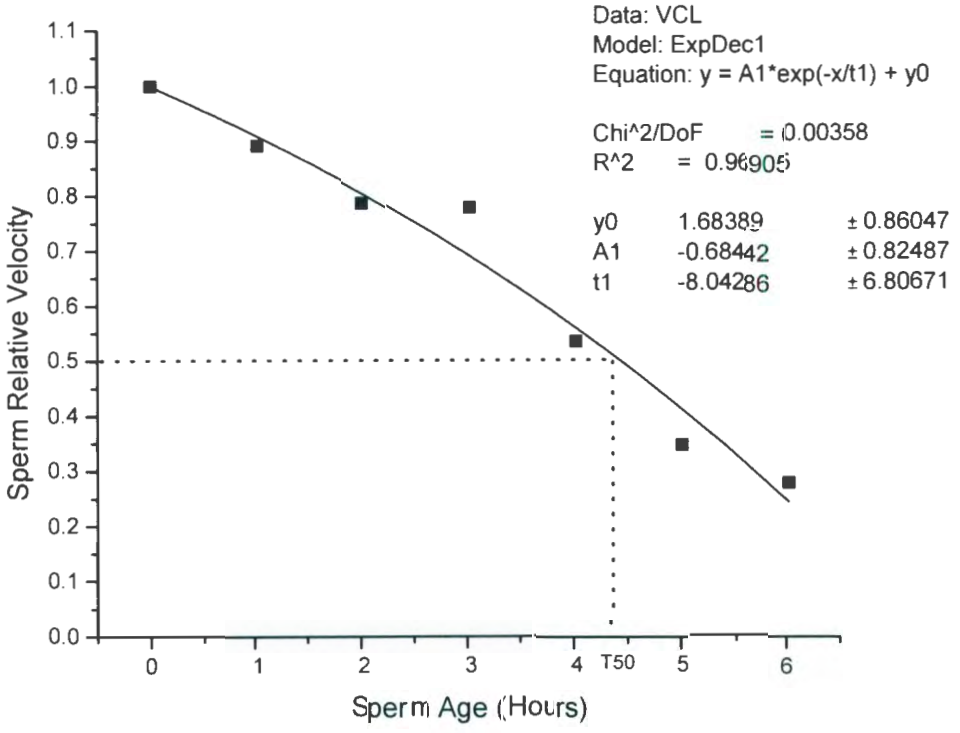


Fig 5.1. Example of the calculation of the relative longevity of a sperm cell from one *Mytilus edulis* individual at 8 °C. Solid dots are the relative sperm velocity data points; solid line is the fitted exponential decay model; dashed line shows the interpolation of sperm half life.

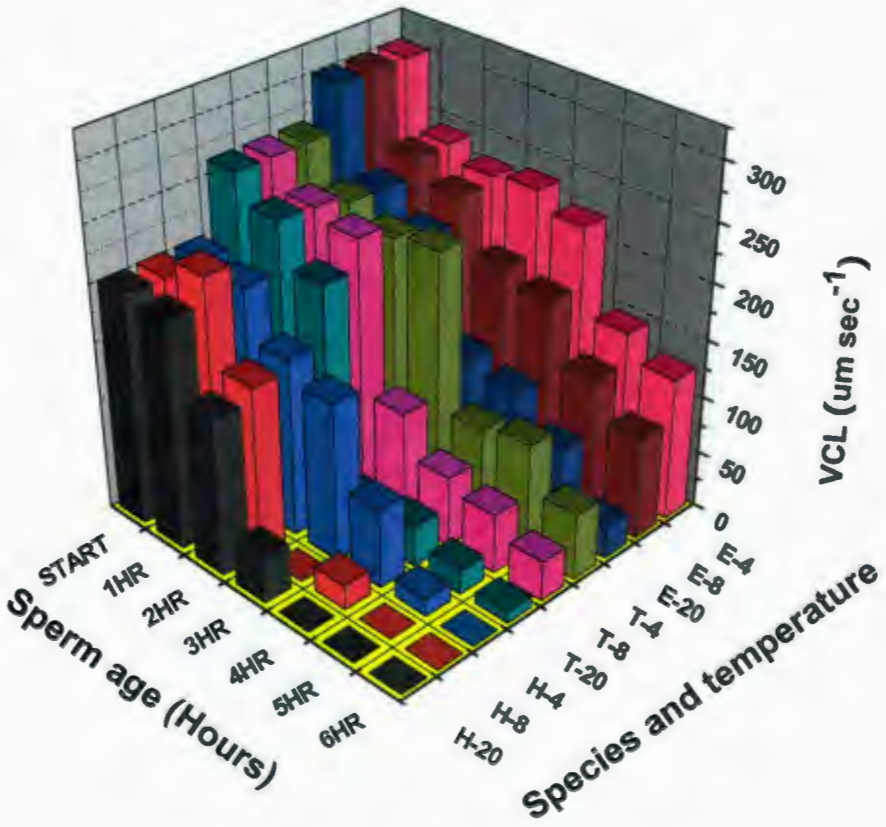


Fig 5.2. Effects of sperm age (0~6 hours) and temperature (4, 8 and 20 °C) on mean sperm velocity (VCL, curvilinear velocity). E: *Mytilus edulis*; T: *M. trossulus*; H: F_1 hybrids. 4, 8 and 20 represent 4, 8 and 20 °C respectively.

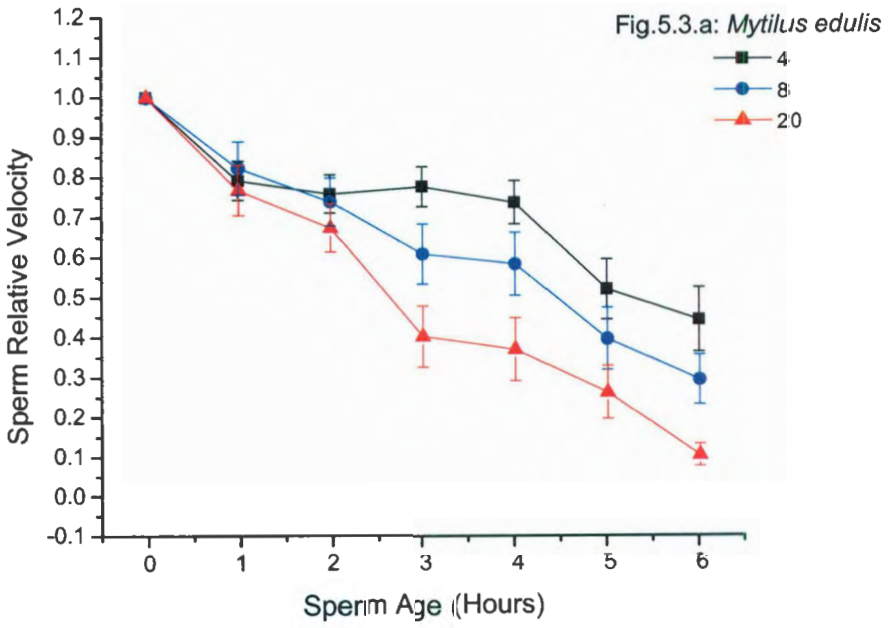
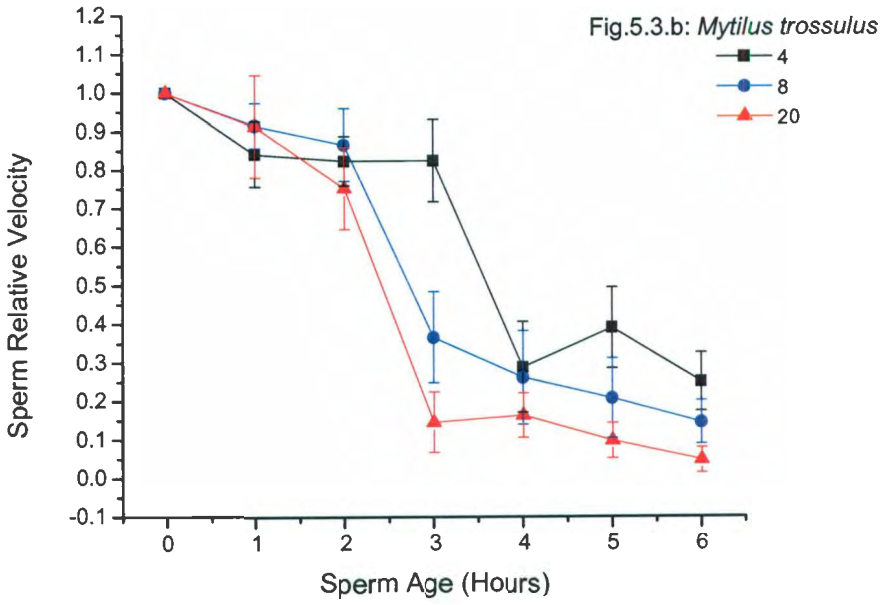


Fig.5.3.a: Mytilus edulis
4
8
20
Sperm Relative Velocity
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
0
1
2
3
4
5
6
Sperm Age (Hours)



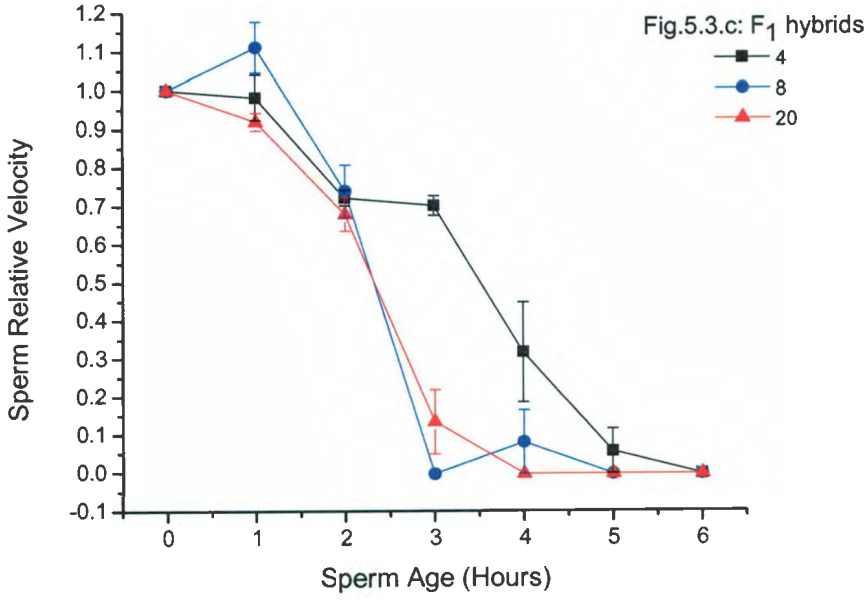


Fig 5.3. Effects of sperm age (0~6 hours) and temperature (4, 8 and 20 °C) on sperm velocity (VCL, curvilinear velocity) in blue mussel genotypes. Fig 5.3.a: *Mytilus edulis*; Fig 5.3.b: *M. trossulus*; Fig 5.3.c: F_1 hybrids.

Table 5.1: ANOVA: effect of temperature and species on sperm half life (T_{50}).

Response: T_{50}					
	df	Sum SQ	Mean SQ	F value	Pr(>F)
TEMP	2	82.18	41.09	10.121	8.831e-05 ***
SPECIES	2	105.39	52.70	12.980	8.107e-06 ***
TEMP*SPECIES	4	10.12	2.53	0.623	0.647
RESIDUALS	117	474.99	4.06		

Table 5.2: Relative sperm longevity (T_{50}) at different temperatures in the blue mussel, *Mytilus edulis*, *M. trossulus* and their hybrids (mean $\pm$ S.D.), with ANOVAs.

Species	T_{50} (half-life in hours)			ANOVA	
	4 °C	8 °C	20 °C	F	P
M. edulis	6.07±3.14	4.49±1.96	3.09±1.59	$F_{2,63}$=9.25	<0.001****
M. trossulus	4.12±2.13	3.28±1.78	2.71±1.42	$F_{2,43}$=2.48	0.096*
F_1 hybrids	3.36±1.20	2.03±0.71	1.36±0.29	$F_{2,12}$=7.67	<0.01***
ANOVA					
F	$F_{2,39}$=3.61	$F_{2,38}$=4.62	$F_{2,41}$=2.92		
P	<0.05**	<0.05**	0.065*		

Notes:
Levene's tests were conducted before ANOVA; variances were homogenous for all comparisons.

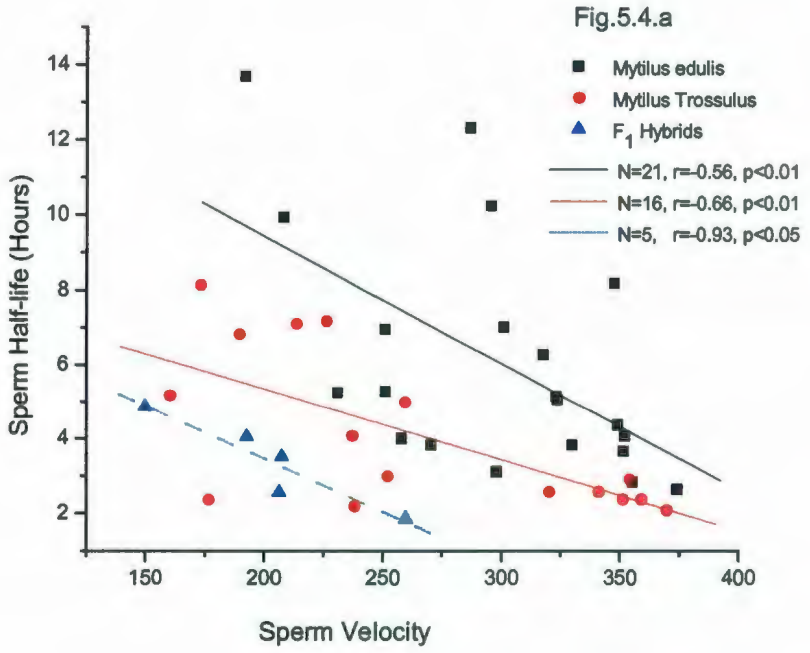


Fig.5.4.a
Mytilus edulis
Mytilus Trossulus
F1 Hybrids
N=21, r=-0.56, p<0.01
N=16, r=-0.66, p<0.01
N=5, r=-0.93, p<0.05
Sperm Half-life (Hours)
Sperm Velocity
14
12
10
8
6
4
2
150
200
250
300
350
400

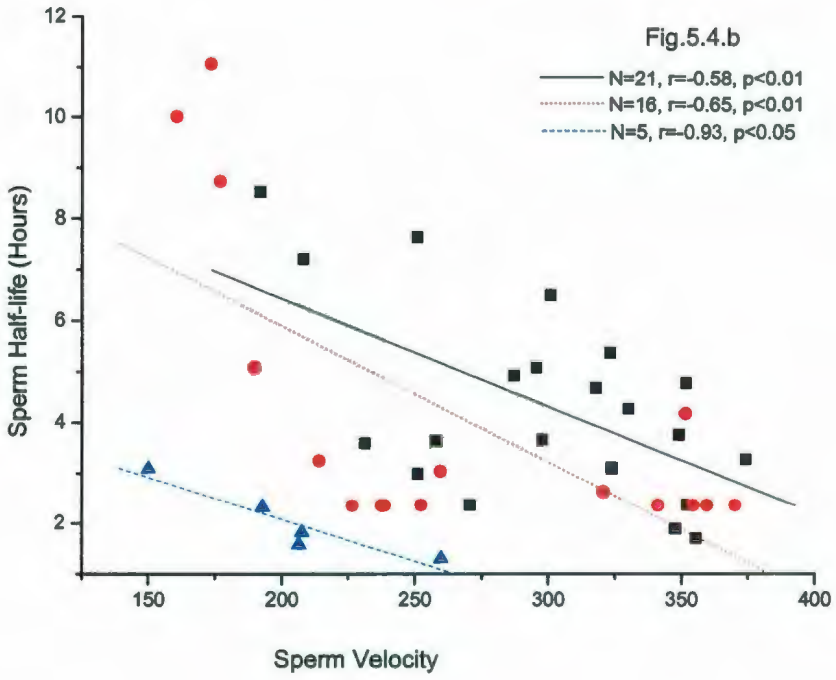


Fig.5.4.b
N=21, r=-0.58, p<0.01
N=16, r=-0.65, p<0.01
N=5, r=-0.93, p<0.05
Sperm Half-life (Hours)
Sperm Velocity
12
10
8
6
4
2
150
200
250
300
350
400

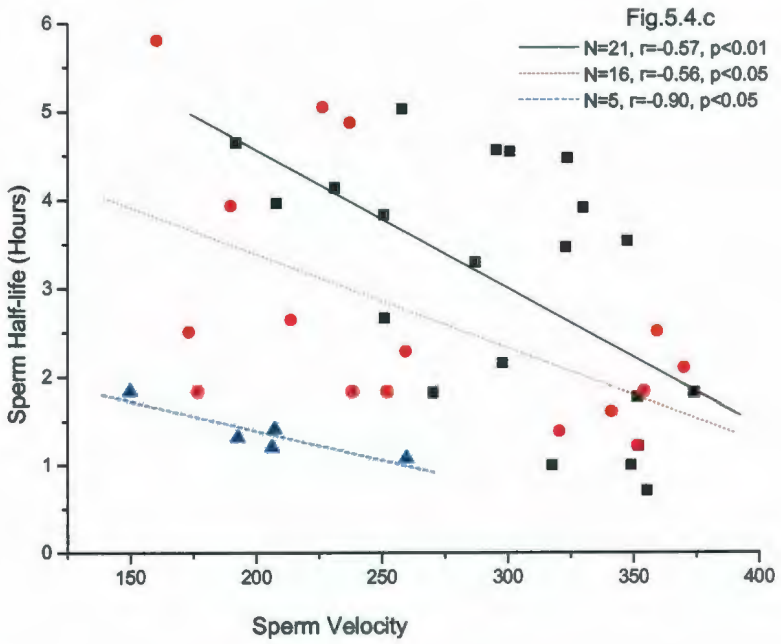


Fig 5.4. Trade-off between sperm half-life and sperm velocity (VCL, μm sec^{-1}) in blue mussels. 5.4.a: 4 °C; 5.4.b: 8 °C; 5.4.c: 20 °C.

Chapter 6

Gamete compatibility and the effect of gamete traits on fertilization success in a blue mussel hybrid zone in Atlantic Canada

6.1 Introduction

6.1.1 Gamete incompatibility in the sea

In free spawning invertebrates, gametes are released into the water column and fertilization success depends primarily on direct gamete interactions. Following contact, gamete compatibility can vary among individuals within a species or between species. Gamete incompatibility is defined as the failure of sperm to fertilize eggs following sperm-egg contact. The degree of gamete compatibility may vary from complete (for most cases of homospecific fertilization) to gamete incompatibility between gametes from different species. Although homospecific crosses usually have a greater fertilization success than heterospecific crosses, heterospecific fertilization success can vary and in some cases incompatibility between gametes of different species is incomplete. For example, partial heterospecific gamete incompatibility has been reported in cnidarians (Buss and Yund 1989; Knowlton et al. 1997; Levitan et al. 2004; Szmant et al. 1997), polychaetes (Marsden 1992; Pernet 1999), mollusks (Banks et al. 1994; Bierne et al. 2002; Gaffney et al. 1993; Grant et al. 1998; Slaughter and McCartney 2003; Slaughter et al. 2008; Warwick et al. 1990) and echinoderms (Aslan and Uehara

1997; Harper and Hart 2005; Levitan et al. 2007; McCartney and Lessios 2002; Metz et al. 1994; Metz and Palumbi 1988; Palumbi and Metz 1991; Rahman and Uehara 2004; Rahman et al. 2004). Gamete incompatibility can lead to reproductive isolation between species, thereby maintaining the integrity of species (Palumbi 1994; Wiese and Wiese 1977; Wiese et al. 1976). However, if gamete incompatibility is incomplete there can be a limited degree of gene flow between species through hybridization (McClary and Sewell 2003; Rawson et al. 2003). Furthermore, gamete incompatibility between species is often asymmetric, which means that gamete compatibility differs between reciprocal crosses. This asymmetry of gamete compatibility has been reported in species from various taxa including hydroids (Buss and Yund 1989), corals (Levitan et al. 2004), polychaetes (Pawlik 1988), molluscs (Buss and Yund 1989; Rawson et al. 2001) and echinoderms (Aslan and Uehara 1997; Lessios and Cunningham 1993; McCartney and Lessios 2002; Metz et al. 1994).

Both the degree and the directionality of gamete compatibility affect gene flow, genetic differentiation and speciation (Rawson et al. 2003; Wiese and Wiese 1977; Wiese et al. 1976). Although how gamete incompatibility occurs and why it is often asymmetric are still not totally understood, studies on gamete recognition proteins suggest that changes in a small number of genes associated with fertilization may lead to gamete incompatibility between species (Babcock 1995; Brewis and Wong 1999; Geyer and Palumbi 2003; Hemachand et al. 2002; Kim and Fritz 1993; Landry et al. 2003; McCartney and Lima 2006; Palumbi 1999; Springer and Crespi 2007; Vacquier 1998). Furthermore, gamete recognition genes involved in sperm attachment or the acrosome reaction appear to evolve rapidly (Landry et al. 2003; Vacquier 1998), and selection on gamete recognition proteins depends on sex, density and genotype frequency (Levitan and Ferrell 2006). In addition to gamete recognition proteins, other gamete traits such as sperm velocity and egg size, which are directly related to fertilization success (Levitan 1996, 2000a, 2004b; Vogel et al. 1982), differ between species and may also contribute to heterospecific fertilization success.

6.1.2 The effect of gamete traits on fertilization success

The effect of gamete traits on fertilization success has been well studied in several species, especially vertebrates such as fishes (Arnaud et al. 2001; Casselman et al. 2006; Hirano et al. 2001; Robertson 1996; Tvedt et al. 2001) and humans (Auger et al. 1994; Hirano et al. 2001; Liu et al. 1991; Mak et al. 1994; Ng et al. 1986; Pusch 1987). In free spawning invertebrates mating behavior is absent and gamete traits play an even more important role in the gamete interactions that affect fertilization success.

According to Vogel's fertilization model (Vogel et al. 1982), which has been supported by studies on various free spawning invertebrate species (Au et al. 2002; Kupriyanova 2006; Levitan 2000b; Oliver and Babcock 1992), both increased swimming velocity and increased longevity of sperm result in increased fertilization success. The model also predicts that egg size, especially effective egg size (the cross-sectional area of an egg that presents a target for sperm-egg interaction), plays an important role in fertilization success, as has been demonstrated in echinoderms (Levitan 2004b, 2006, 2008; Podolsky and Strathmann 1996; Vogel et al. 1982). Larger eggs provide a bigger target for sperm-egg collision (Farley and Levitan 2001; Levitan 1991a, 1996, 2004b, 2006; Levitan and Irvine 2001; Podolsky and Strathmann 1996; Styan 1998). A larger egg also possesses more energy than a smaller one, which probably leads to higher fitness of individual offspring (Fischer et al. 2003; Lerner and Gunns 1952; Santo et al. 2001; Silva et al. 2007). Owing to finite energy resources individuals can produce a large number of small eggs or a small number of larger eggs, so the species-specific optimal egg size should be that which leads to the greatest parental fitness (Levitan 2000a).

6.1.3 Fertilization ecology and gamete incompatibility in the blue mussel hybrid zone

The presence of hybrids in areas where *Mytilus* species coexist indicates that reproductive isolation is incomplete. The existence of natural hybrid zones of the blue mussel *M. edulis* complex therefore provides an opportunity to study speciation and the evolution of reproductive isolation barriers that maintain species integrity (Comesaña et al. 1999; Gilg et al. 2007; Heath et al. 1995; McCartney and Lima 2006; Riginos and McDonald 2003; Toro et al. 2002; Wonham 2004). In order to understand the evolution of reproductive isolation mechanisms, especially prezygotic isolation in a natural hybrid zone, it is important to understand reproductive ecology and the influence of gamete traits on fertilization success and gamete compatibility in coexisting *Mytilus* species.

Reproductive ecology, including reproductive cycles, fecundity and reproductive effort have been well studied in *Mytilus* species (Brousseau 1983; Thompson 1979, 1984a; Toro et al. 2002, 2006; Yakovlev 1986). Furthermore, several studies have focused on gamete incompatibility in blue mussels that could lead to strong prezygotic isolation. In Europe, where *M. edulis* and *M. galloprovincialis* coexist and produce natural hybrids, strong assortative fertilization has been reported (Bierne et al. 2002). A study in Maine, USA, where an *M. edulis* and *M. trossulus* natural hybrid zone exists, showed that about 100~700 fold higher sperm concentrations were required for heterospecific than for homospecific fertilization (Rawson et al. 2003). Furthermore, gamete incompatibility in Maine mussels appeared to be asymmetric, *M. edulis* eggs being more receptive to heterospecific sperm than *M. trossulus* eggs (Rawson et al. 2001). The reinforcement model for reproductive isolation predicts a greater degree of incompatibility between heterospecific gametes from populations where species are sympatric compared with crosses involving gametes from allopatric populations of each species (Dobzhansky 1937). However, the degree of gamete incompatibility between *M. edulis* and *M. trossulus* does not differ between *M. edulis* from sympatric and allopatric

populations (Slaughter et al. 2008). Little is known about gamete incompatibility in the blue mussel hybrid zone in Newfoundland, Canada, where *M. edulis* and *M. trossulus* coexist. The only previous study of gamete incompatibility in Newfoundland mussels showed that in general conspecific crosses had a higher average fertilization success than heterospecific crosses, based on experiments using a sperm : egg ratio of 100 : 1 (Miranda 2004). However, a significant proportion (about half) of heterospecific crosses also resulted in a fertilization success rate as high as that of conspecific crosses. Furthermore, some individual females showed high fertilization success with both conspecific and heterospecific sperm, suggesting that gamete trait variation such as egg size and sperm velocity may also contribute to fertilization success and should be taken into consideration when interpreting variation in fertilization success.

6.1.4 Objective and importance of this study

Two blue mussel species (*M. edulis* and *M. trossulus*) coexist and hybridize along the coast of Newfoundland (Bates and Innes 1995). Explaining the coexistence of the two species and hybrids requires information on the factors that affect hybridization, such as fertilization success. Fertilization incompatibility may be an important reproductive isolating mechanism to maintain species integrity (Wiese et al. 1976). Many factors such as gamete incompatibility and gamete traits (specifically sperm velocity and egg size) affect fertilization and have been investigated in free-spawning invertebrates such as sea urchins (Levitan 1993, 1996; McCartney and Lessios 2002). Many studies have focused on blue mussel hybrid zones, although most have emphasized genetic variation (Bates and Innes 1995; Comesaña et al. 1999; Heath et al. 1995; Saavedra et al. 1996) or reproductive ecology (Brousseau 1983; Thompson 1979, 1984a; Toro et al. 2002, 2006; Yakovlev 1986), and only a few have considered gamete incompatibility (Rawson et al. 2001, 2003; Slaughter and McCartney 2003; Slaughter et al. 2008). Furthermore, no study to date has investigated how gametes of F_1 hybrids perform during fertilization

events. The traditional method for evaluating gamete incompatibility from the relationship between sperm : egg concentration ratio and fertilization success (McCartney and Lessios 2002; Rawson et al. 2003) may be affected by differences in gamete traits. Therefore, a comprehensive quantitative study of post-spawning prezygotic reproductive isolation in the Newfoundland mussel hybrid zone is required, including measurement of gamete traits (for both sperm and eggs) that affect fertilization success and thus individual fitness and separation of the effects of gamete traits and gamete incompatibility on fertilization success.

The purpose of the present study was to measure the degree of gamete incompatibility between blue mussel species, including hybrids, to estimate the effect of different parental combinations on fertilization success, and to determine how gamete traits affect fertilization success in a blue mussel hybrid zone in Newfoundland. In order to eliminate the effect of gamete traits (sperm velocity and egg size) on the estimation of gamete incompatibility, three parameters (β, β_0 and β/β_0) derived from Vogel's fertilization kinetics model were used to separate and estimate the effects of gamete traits and gamete incompatibility on fertilization success.

6.2 Materials and methods

6.2.1 Sample collection, maintenance of mussels and species identification

Adult *M. edulis*, *M. trossulus* and their hybrids, with shell lengths of 62.47±9.96 mm for females and 62.74±10.75 mm for males, were collected at two-week intervals from late May to early August 2005 from Trinity Bay, Newfoundland, where Comesaña et al. (1999) reported the distribution of genotypes to be approximately 34% *M. edulis*, 40% *M. trossulus* and 26% hybrids. Mussels were held in the laboratory as described in Chapter 2 (2.2.1). Genotyping was

conducted after spawning using two genetic markers, ME and ITS (Heath et al. 1995; Inoue et al. 1995).

6.2.2 Spawning and gamete collection

The same methods as described in Chapter 2 (2.2.2) were used for spawning induction and gamete collection. Both sperm and egg concentrations were measured with a Multisizer™ II (Beckman Coulter).

6.2.3 Experimental crosses and fertilization success

After gamete concentrations were measured, homospecific and heterospecific crosses were conducted in 50 ml plastic containers at 8 different sperm : egg ratios ranging from 10^{-4} to 10^{3}. About 10^{3} eggs were used for each trial and no gametes older than 2 hours were used, in order to minimize any effect of gamete age on fertilization success.

Three hours after the gametes were mixed, embryos were collected from the bottom of each container and a wet mount prepared. At least three replicate photographs were taken of 200 or more embryos from each cross at a total magnification of 100X using a Zeiss 3030 inverted light microscope (Carl Zeiss, Inc.) . Fertilization success was then established by recording the presence of polar bodies or cell cleavage in the images (Harper and Hart 2005) (Fig 6.1) and a mean value for fertilization success estimated from the three replicate photographs.

6.2.4 Gamete contact time and fertilization success

To evaluate the effect of gamete contact time on fertilization success, freshly spawned (within 1 hour) *M. edulis* sperm and eggs were mixed (100 : 1). After a contact time of 5, 10, 15, 20, 25, 30, 35 or 55 seconds the embryos and unfertilized

eggs were retained on a 50 μm mesh screen and washed with filtered seawater to remove residual sperm. The fertilization ratio was estimated as described in 6.2.3.

6.2.5 Sperm velocity and egg size measurement

The methods described in chapter 4 (4.2.2) were used for sperm velocity investigations. The mean diameter of the eggs from each parent was determined with a Multisizer™ II.

6.2.6 Data analysis and statistics

After a check for equality of variance by Levene's test, ANOVA was performed to compare egg size among genotypes using the "R" program (R Development Core Team 2008). Pairwise mean comparisons (Bonferroni test) of egg size (individual average value) were then conducted with OriginPro 7.5.

Although high fertilization success (>50%) was found in most crosses when the sperm concentration was high (sperm-egg ratio 10^3), the *M. trossulus* x *M. edulis* and reciprocal crosses were exceptions, exhibiting a fertilization success below 50%. Therefore, the F_{20} value (the sperm concentration required to fertilize 20% of the eggs) for each cross was used to indicate gamete incompatibility (Harper and Hart 2005; Levitan 2002; McCartney and Lessios 2002; Rawson et al. 2003; Slaughter et al. 2008). The sigmoid relationship between fertilization success and sperm-egg ratio was logit transformed and linearized as logit(P)=ln(P/1-P), where P is fertilization success (%) (Harper and Hart 2005; Levitan 2002; McCartney and Lessios 2002; Rawson et al. 2003; Slaughter et al. 2008). F_{20} was calculated from the linear regression equation. To correct for inequality of variances, treatments (crosses) were compared by one-way ANOVA of logit transformed F_{20} values (Harper and Hart 2005; Slaughter et al. 2008). Pairwise mean comparisons of log(F_{20}) were then made using the Bonferroni test in OriginPro 7.5.

F_{20} is not appropriate for investigating the effects of different parental combinations on fertilization success in homospecific crosses, because fertilization success at most sperm:egg ratios was greater than 50%. Instead, F_{50} was calculated from the linear regression for each cross by determining the sperm concentration at which logit(0.5) = 0 (McCartney and Lessios 2002). Furthermore, F_{20} is not appropriate for demonstrating the effects of parental combinations on fertilization success for heterospecific crosses due to the large variation observed. Log (F_{20}) was therefore used instead of F_{20} to illustrate the effects of parental combination on heterospecific fertilization success.

Pearson correlation coefficients were calculated to show the relationship between single gamete traits (velocity for sperm and egg size for eggs) or the combined effects of gamete traits and F_{50} (lower F_{50} indicates higher fertilization success) for all *M. edulis* homospecific crosses.

To evaluate the effect of gamete contact time on fertilization success, linear regression was performed between contact time and the fertilization ratio.

According to the fertilization kinetics model, fertilization success can be derived from the following equation (Vogel et al. 1982).

$$\varphi_{\infty} = 1 - \exp\{-\frac{\beta \cdot S_0}{\beta_0 \cdot E_0}(1 - e^{-\beta_0 E_0 \tau})\} \qquad (1)$$

φ_{∞}: proportion of eggs fertilized;

S_0 and E_0: sperm and egg concentration respectively;

β ($mm^3\ s^{-1}$): fertilization rate constant, the measure of how many collisions result in fertilization;

β_0 ($mm^3\ s^{-1}$): collision rate constant, the measure of how often gamete collision occurs;

τ : gamete contact time or sperm half life;

also, $\beta_0 = v \cdot \sigma_0$ (2)

where v is sperm velocity and σ_0 is egg cross-sectional area.

Unlike previous studies (Rawson et al. 2003; Slaughter and McCartney 2003; Slaughter et al. 2008), comprehensive information on fertilization success, sperm velocity (VCL, data from Chapter 4), egg size, gamete concentration and sperm half life (T_{50}, data from Chapter 5) was obtained in this study, so the three fertilization kinetic parameters (β,β_0 and β/β_0) could be estimated for each individual by fitting all the variables into Vogel's fertilization kinetics model. Only the fertilization data with a sperm : egg ratio of 100 : 1 were used, to minimize the effect of sperm dilution on sperm traits. Although the experiment involving gamete contact time on fertilization success showed that more than half the eggs were fertilized within 60 seconds in the *M. edulis* homospecific crosses, complete fertilization success was not achieved in all experimental groups, so it is possible that excess sperm that were still alive may have been able to fertilize the remaining unfertilized eggs given more contact time. Therefore, species specific sperm half life (T_{50}) was used instead of a fixed time (McCartney and Lessios 2002). β_0 values were estimated directly from equation (2) and β values were obtained by putting all variables, including β_0, into equation (1). After a check for equality of variances by Levene's test, one-way ANOVAs were performed to compare β,β_0 and β/β_0 among crosses. Pairwise comparisons were conducted with the Bonferroni test in OriginPro 7.5.

6.3 Results

6.3.1 Egg size

ANOVA showed a significant effect of genotype on egg size (diameter) (Table 6.1). Pairwise Bonferroni tests showed that there was no significant difference in egg size between *M. trossulus* and F_1 hybrids (Table 6.2), which produced smaller eggs than *M. edulis* (Fig. 6.2).

6.3.2 Gamete incompatibility (F_{20}) in various crosses

Significant differences in logit transformed F_{20} were found among crosses (Fig 6.3). Generally, inter-species crosses had a higher log (F_{20}) than intra-species crosses and backcrosses (Fig 6.3, Table 6.3 and Table 6.4). The *M. edulis* (♀) x F_1 hybrid (♂) cross had the smallest log (F_{20}) of all the crosses (the reciprocal cross was not performed).

6.3.3 The effect of parental combinations on homospecific and heterospecific fertilization success

F_{50} (the sperm: egg ratio required to obtain a 50% fertilization rate) for homospecific crosses and log (F_{20}) for heterospecific crosses differed among parental combinations, suggesting a combined effect of gamete quality and gamete incompatibility on fertilization (Table 6.5, 6.6, 6.7 and 6.8). First, there were gamete compatibility effects as shown by crosses 4, 5, 7 and 8 (Table 6.5). A lower sperm : egg ratio was needed to achieve 50% fertilization success when female 7.4F1 was crossed with male 7.4M4 than when the same female was crossed with male 7.4M3. However, when another female (7.4F2) was crossed with the same males, the F_{50} showed an opposite trend which cannot be explained by gamete quality alone. This result suggested that some gamete combinations (such as crosses 5 and 7, Table 6.5) were more compatible than others (such as crosses 4 and 8, Table 6.5). Second, the extremely high F_{50} detected in crosses 10 and 13 (Table 6.5) ($t_2 = -7.50$, $p<0.01$) may also have been caused by poor sperm quality, since eggs from the same females with same egg diameter did not show any quality problems when crossed with other males (crosses 9 and 14 in Table 6.5). When different males 7.26M1 and 7.26M3 were crossed with same females (7.26F1, 7.26F2, 7.26F3 and 7.26F5, Table 6.6) ($t_6 = -2.09$, $p<0.05$), poor sperm quality of 7.26M3 may explain the observation that F_{50} of all of the crosses that male 7.26M1 involved were smaller than that of male 7.26M3. Similarly, when different females (females 7.7F1 and 7.7F2 in Table 6.6)

were crossed with the same males (males 7.7M1 and 7.7M3), the F_{50} of all the crosses involving female 7.7F2 were higher than that of female 7.7F1 ($t_2 = -6.96$, $p<0.05$), which may be due to poor quality of eggs from 7.7F2.

6.3.4 The effect of gamete traits on fertilization success in *M. edulis*

In order to determine how gamete traits affect fertilization, the correlation between fertilization success and gamete traits was investigated in *M. edulis*. Both sperm velocity and egg size were individually correlated with fertilization success in the *M. edulis* intra-species crosses (Fig 6.4.a and Fig 6.4.b). The strongest negative correlation was found between fertilization success and log (VCL*egg cross section area), which represents the combined effects of egg size and sperm velocity (Fig 6.4.c).

6.3.5 The effect of gamete contact time on fertilization success in *M. edulis*

Fertilization success (P = fertilized egg number/total egg number) increased with contact time in *M. edulis* (Fig 6.5). About half the eggs were fertilized within 60 seconds at a sperm : egg ratio of 100 : 1.

6.3.6 Gamete incompatibility comparison using fertilization kinetic parameters for different crosses

The results presented in both 6.3.3 and 6.3.4 suggested that the commonly used F_{20} comparison, which does not take into account the effects of gamete traits on fertilization success, was not an accurate estimate of gamete incompatibility. Therefore three fertilization kinetics parameters (β, β_0 and β/β_0) were calculated to better estimate gamete incompatibility between homospecific and heterospecific crosses and to estimate the effect of gamete incompatibility on fertilization success.

All three were significantly different among homospecific and heterospecific crosses (Table 6.9). The significantly lower values of these parameters from heterospecific crosses suggested a high degree of reproductive isolation between *M. edulis* and *M. trossulus*, caused by both a low chance of gamete collision (effect of gamete quality) (Table 6.10) and a low proportion of gamete collisions resulting in fertilization (the combined effects of gamete incompatibility and gamete quality) (Table 6.11).

6.4 Discussion

The egg diameter of blue mussels measured in the present investigation (60~70 μm) is in accordance with previous studies (Bayne et al. 1978; Toro et al. 2002). Eggs of *M. edulis* were significantly larger than those of hybrids and *M. trossulus*, which is also consistent with a previous study (Toro et al. 2002).

The effect of natural selection on sperm velocity and egg size is determined by the life history of a species and environmental conditions. Comprehensive studies on various echinoderm species have suggested that to cope with sexual conflicts at the gamete level, males of species with high population densities and a high degree of sperm competition produce fast swimming sperm with a short life span, whereas the females release a large number of smaller, selective eggs (see below). On the other hand, males of species with lower population densities, in which gametes are often diluted and sperm limitation occurs, produce longer lived, slower swimming sperm and the females larger, more compatible eggs (Levitan 1991a, 1991b, 1993, 1996, 1998, 2000b, 2004b, 2006, 2008; Levitan and Irvine 2001; Levitan and Petersen 1995; Yund 2000; Yund and McCartney 1994). *M. edulis* produces not only faster swimming, longer lived sperm but also larger eggs than *M. trossulus* and hybrids. This combination of gamete traits appears to conflict with the general pattern

expected under conditions of sperm competition or sperm limitation, but makes more sense when the differences in reproductive strategies between the two blue mussel species are taken into consideration. *M. trossulus* allocates more energy to reproduction than *M. edulis* and has a longer spawning season (Toro et al. 2002), which means that *M. trossulus* may achieve similar fitness as *M. edulis* by producing relatively low quality gametes (smaller eggs, slowly swimming and short lived sperm) while spawning more often. The relatively shorter spawning season in *M. edulis* means that the greater number of eggs fertilized during limited spawning events will probably lead to higher fitness. Therefore, producing larger eggs that are easier to fertilize rather than smaller, more selective eggs might be an adaptation to maximize fitness for *M. edulis* to cope with the short spawning season.

Significantly different sperm : egg ratios were required to fertilize 20% of the eggs among the various crosses. For intra-species crosses involving *M. edulis* and *M. trossulus*, a smaller F_{20} was observed than in inter-species crosses, suggesting strong gamete incompatibility between these species. Similar results were reported for homospecific crosses by Rawson et al. (2003), although the F_{20} values from the present study were much smaller than theirs, which may be a consequence of the different methods used for measuring fertilization success. Rawson et al. (2003) expressed fertilization success as the proportion of eggs that reached the 8-16-cell stage after 12.3 hours exposure to sperm. In the present study polar body extrusion and early cell division were used for estimating fertilization success after only 3 hours exposure time (Harper and Hart 2005), because a longer period of exposure to sperm may bias estimates of fertilization success. Basing the measurement on the multi-cell stage only may underestimate fertilization success, since this procedure excludes some fertilized eggs with polar body extrusion that may not have been able to continue cell division due to post-fertilization incompatibility.

The sperm : egg ratios required for fertilizing 20% of the eggs in the reciprocal crosses between *M. trossulus* and F_1 hybrids were not significantly different from

those required for intra-species crosses, suggesting that gametes are more compatible than in the inter-species crosses between *M. edulis* and *M. trossulus*. Furthermore, *M. edulis* eggs were more easily fertilized in heterospecific crosses than in reciprocal crosses involving *M. trossulus* eggs. Interpreting these results requires that the effect of gamete compatibility and gamete trait differences be taken into consideration. For example, there are significant egg size differences among species. The large eggs, which provide a larger target for gamete interaction, are probably more easily fertilized, as has been suggested in echinoderm species (Levitan 1991a, 2008). *M. edulis* eggs, which are larger, also provide a bigger target for sperm-egg collisions than those of *M. trossulus*, and may, therefore, be more easily fertilized by sperm of either species. Thus, the egg size difference in these closely related mussel species may partially explain the differences in fertilization success for different crosses and the asymmetry of the gamete incompatibility observed between the species. Furthermore, Rawson et al. (2003) suggested that gamete incompatibility should become more prominent with increasing genetic distance among blue mussel species *M. edulis*, *M. trossulus* and *M. galloprovincialis*, although this was not demonstrated experimentally. The present study provided some evidence that this may also hold true in the *M. edulis*, *M. trossulus* hybrid zone, where more compatibility was found between backcrosses compared with homospecific crosses, and may be a consequence of the greater genetic similarity between F_1 hybrids and both *M. edulis* and *M. trossulus*. Recent studies on gamete recognition proteins also suggest that the M7 lysin, which is involved in the acrosomal reaction during fertilization in blue mussels, plays an important role in fertilization success and reproductive isolation of blue mussel species (Riginos and McDonald 2003; Riginos et al. 2006). It is possible that F_1 hybrids have M7 lysin alleles that are compatible with those of *M. edulis* and *M. trossulus*. However, studies relating M7 lysin genotypes to fertilization success have not yet been conducted in *Mytilus* species.

Fertilization success differed among various intra-species parental combinations, which could be due to the combined effects of gamete quality, genetic

compatibility (compatibility in both genetic background and gamete recognition proteins) and cryptic female choice. Several factors may contribute to this difference. First, as discussed in Chapter 4, since *M. edulis*, *M. trossulus* and their hybrids have a relatively long spawning season (at least 2~3 weeks during summer) (Toro et al. 2002), it is probable that gametogenesis continues throughout the spawning season. Therefore, when thermal shock is used to stimulate spawning in the laboratory, males may release sperm at different stages of maturity, which may affect fertilization success. Furthermore, gametes of differing quality can be produced naturally due to differences in physiological condition among males (Lahnsteiner 2000; Lahnsteiner et al. 1999b). Thus, low quality gametes lead to lower fertilization success, which may partially explain why some individuals always have a lower fertilization success than others, even when crossed with the same mates. Second, the observation that gametes from some individuals are more compatible with those of certain mates suggests that gamete compatibility varies among individual combinations, possibly as a result of cryptic female choice in which the females (eggs) may choose among sperm from two or more males spawning at the same time (Eberhard 1996; Olsson et al. 1996; Thornhill 1983). Data from other species suggest that the cryptic female choice mechanism is based on genetic compatibility that differentiates fertilization success (Clark et al. 1999; Wilson et al. 1997). Thus, blue mussel eggs may be able to distinguish among sperm from different males based on subtle individual differences in gamete recognition proteins, resulting in differences in gamete compatibility among pairings. Just as no two individuals are identical, there are differences in quality among gametes from different species or individuals. Blue mussels tend to aggregate, many individuals being attached to one another with byssal threads. This habit increases the possibility of polyandry during synchronic spawning events and may increase fertilization success and fitness of individual mussels through mate choice by eggs during polyandrous matings (Colegrave et al. 2002; Ivy 2007; Marshall and Evans 2005; Tregenza and Wedell 2000).

This is the first study to investigate the effect of gamete traits on fertilization success in blue mussels. Previous studies showed that both sperm and egg traits are correlated with fertilization success in marine broadcast spawning invertebrates, such as corals (Oliver and Babcock 1992), polychaetes (Kupriyanova 2006) and sea urchins (Au et al. 2002; Levitan 1996, 2000b, 2008). My results show that although fertilization success is positively correlated with sperm velocity and egg size, it is the combination of sperm and egg traits that shows the strongest correlation with fertilization success. As reported in other species (Au et al. 2002; Auger et al. 1994; Levitan 1996, 2000b, 2008; Vogel et al. 1982), mussels attain a higher fertilization success with faster swimming sperm and larger eggs.

Contact time is an important variable in Vogel's fertilization kinetics model (Vogel et al. 1982), and has been studied in several taxa, including polychaetes (Kupriyanova 2006), sea urchins (Levitan et al. 1991), abalones (Ebert and Houk 1984; Grubert et al. 2005) and fish (Rosenthal et al. 1988). My data showed that intra-species fertilization success in *M. edulis* increased with contact time, which is consistent with previous studies on other organisms (Babcock and Keesing 1999; Ebert and Houk 1984; Levitan et al. 1991). In addition, high fertilization success (>60%) in *M. edulis* intraspecies crosses was obtained with a concentration of 10^7 sperm ml^{-1} and a sperm : egg ratio of 100 : 1 in a short time (<60 seconds), as in other species in which high rates of fertilization have been reported after several seconds to less than 15 minutes in various species (Ebert and Houk 1984; Kupriyanova 2006; Levitan et al. 1991; Rosenthal et al. 1988). Considering the high aggregation (Newell 1989), synchronous spawning behaviour (Gosselin 2004) and high gamete output (Newell 1989) in blue mussels, my data suggest that an *M. edulis* sperm will attach very rapidly to the surface of an *M. edulis* egg when the gametes are in close proximity (Kupriyanova 2006). In all experimental pair-mating groups with sufficient sperm present, most eggs were fertilized within 60 seconds, although some were left unfertilized (about 35%, based on estimates using the regression equation). This suggests that there are differences in egg quality or gamete

compatibility within a clutch, the larger, more easily fertilized, eggs being fertilized first. A previous study in sea urchins showed that there can be egg size differences within clutches and that smaller eggs are often left unfertilized when sperm are limiting (Levitan 1996).

Considering that both sperm and egg quality are strongly correlated with fertilization success, the F_{20} and F_{50} values may not be an accurate estimate of gamete incompatibility because the effect of gamete quality on fertilization success can also affect the F_{20} or F_{50} values calculated. For example, a high F_{50} will be calculated for some crosses (such as crosses 10 and 13 in Table 6.5; crosses 5 and 6 in Table 6.6) in which the low fertilization success may be caused by poor quality of gametes rather than gamete incompatibility. Therefore, interpreting high values of F_{20} or F_{50} as poor fertilization success caused by gamete incompatibility is inappropriate. The fertilization kinetic model provides a better means of partially separating the effects of gamete quality and gamete incompatibility on fertilization success. Although fertilization kinetics has been well studied in many organisms (Kupriyanova 2006; Levitan et al. 1991; Styan 1998; Vogel et al. 1982), this is the first investigation to provide detailed estimates of fertilization kinetics in blue mussels. My data differ from the only reported β and β_0 values available for a marine invertebrate, 3.8×10^{-6} mm^3 s^{-1} and 3.3×10^{-4} mm^3 s^{-1} respectively, recorded for sea urchins (Harper and Hart 2005; Vogel et al. 1982). This comparison suggests that β and β_0 values are species specific, since different species produce gametes with different traits. For example, as there are significant sperm velocity and egg size differences among *M. edulis*, *M. trossulus* and F_1 hybrids, it is not surprising that the gamete collision rate constants also differ significantly among crosses between different genotypes.

The three parameters (β, β_0 and β/β_0) in the fertilization kinetic model can be used to estimate the influence of gamete quality, gamete incompatibility and their

combined effects on fertilization according to their biological meaning. The collision rate constant (β_0), which is only directly affected by gamete quality and is calculated from the gamete trait data, is strongly correlated with fertilization success (Figure 6.4.3), and, can, therefore, be used as an indicator to show how gamete traits contribute to fertilization success through increased collision rate. Similarly, β/β_0, which estimates the fertilizable egg surface ratio, is a specific constant for a specific cross, is irrelevant to gamete quality and can be used as an indicator to show the effect of gamete incompatibility alone on fertilization success. In addition to being affected by gamete incompatibility, the fertilization rate constant (β) can also be influenced by gamete traits. For example, faster swimming sperm may increase fertilization success not only by increasing gamete collision rate but also by an increased ability to penetrate the egg envelope due to a greater contact force. Hence the fertilization rate constant can be used as an indicator to show the combined effects of both fertilization capacity and gamete incompatibility on fertilization success. However, additional information on sperm velocity, sperm longevity and egg size will be required for a precise estimation of β, β_0 and β/β_0.

The homospecific crosses of *M. edulis*, which has the largest eggs and fastest swimming sperm, have higher rates of gamete collision than *M. trossulus* (♀) x *M. trossulus* (♂) and *M. edulis* (♀) x *M. trossulus* (♂) crosses. *M. edulis* (♀) x *M. trossulus* (♂) crosses have a smaller β than *M. trossulus* and *M. edulis* intra-species crosses, which suggests strong reproductive isolation between *M. edulis* (♀) and *M. trossulus* (♂), due to both gamete incompatibility and differences in gamete fertilization capacity. According to my β/β_0 data, about 2.49% of the surface of an *M. edulis* egg is fertilizable by *M. edulis* sperm and only 0.45% fertilizable by sperm of the other genotypes, suggesting strong gamete incompatibility between *M. edulis* females and *M. trossulus* males.

6.5 Summary

M. edulis, *M. trossulus* and F_1 hybrids produced eggs of different sizes (*M. edulis* > F_1 hybrids and *M. trossulus*). Both homospecific and heterospecific fertilization success differed among parental combinations, which suggests an effect of both gamete quality and compatibility on fertilization success. Fertilization success in *M. edulis* was positively correlated with sperm swimming speed and egg size, especially the combination of these two gamete traits, which supports the hypothesis that fertilization success is partially determined by gamete traits.

Data for the sperm : egg ratio required to fertilize 20% of the eggs showed that there is strong reproductive isolation between *M. edulis* and *M. trossulus*. However, F_1 hybrids were more compatible with both *M. edulis* and *M. trossulus* than *M. edulis* were with *M. trossulus* or *M. trossulus* with *M. edulis*. Heterospecific gamete compatibility was slightly asymmetrical, *M. edulis* eggs being more easily fertilized by *M. trossulus* sperm than *M. trossulus* eggs by *M. edulis* sperm, which might be partially explained by the larger egg size in *M. edulis*.

Gamete contact time and the three parameters (β, β_0 and β/β_0) of the fertilization kinetics model were investigated and will be useful for future fertilization studies in blue mussels. Fertilization success increases with gamete contact time, and most of the sperm attachment process takes place within one minute of gamete contact in *M. edulis* homospecific crosses. Collision rate constant (β_0), penetrable egg surface (β/β_0) and fertilization rate constant (β) may give better estimates than F_{20} or F_{50} of how gamete traits, gamete incompatibility and a combination of both affect fertilization success in different crosses. Significant fertilization parameter differences were found among heterospecific crosses, suggesting that the strong reproductive isolation between *M. edulis* and *M. trossulus*

results from reduced fertilization success due to both gamete incompatibility and gamete trait differences.

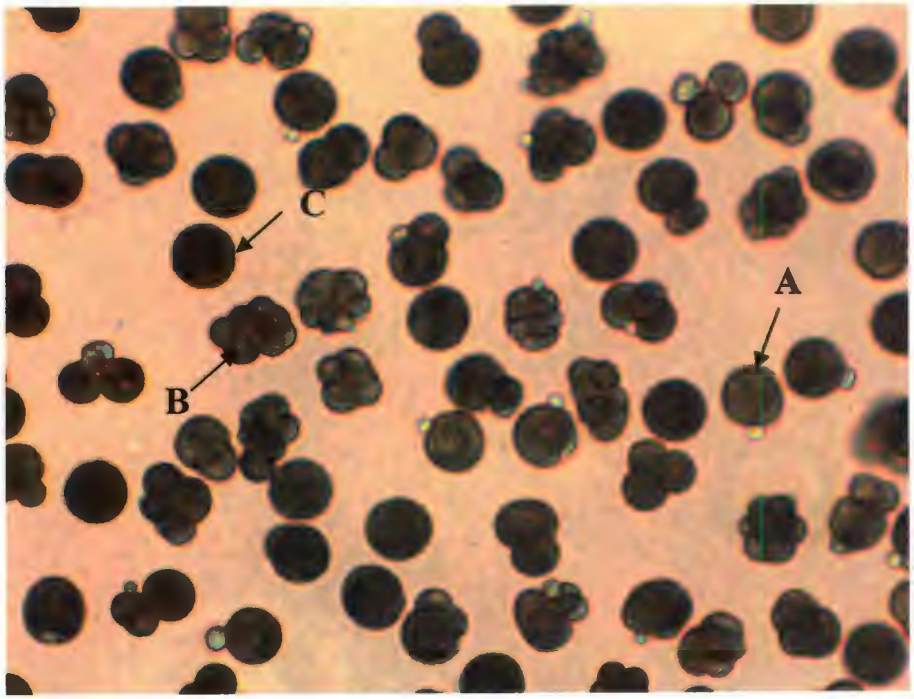


Fig 6.1. Examples (sperm:egg ratio=1000:1, *M. edulis* x *M. trossulus*) of fertilized embryos and unfertilized eggs used to calculate fertilization ratio. A: fertilized egg with polar body; B: fertilized multi-cellular embryo; C: unfertilized egg.

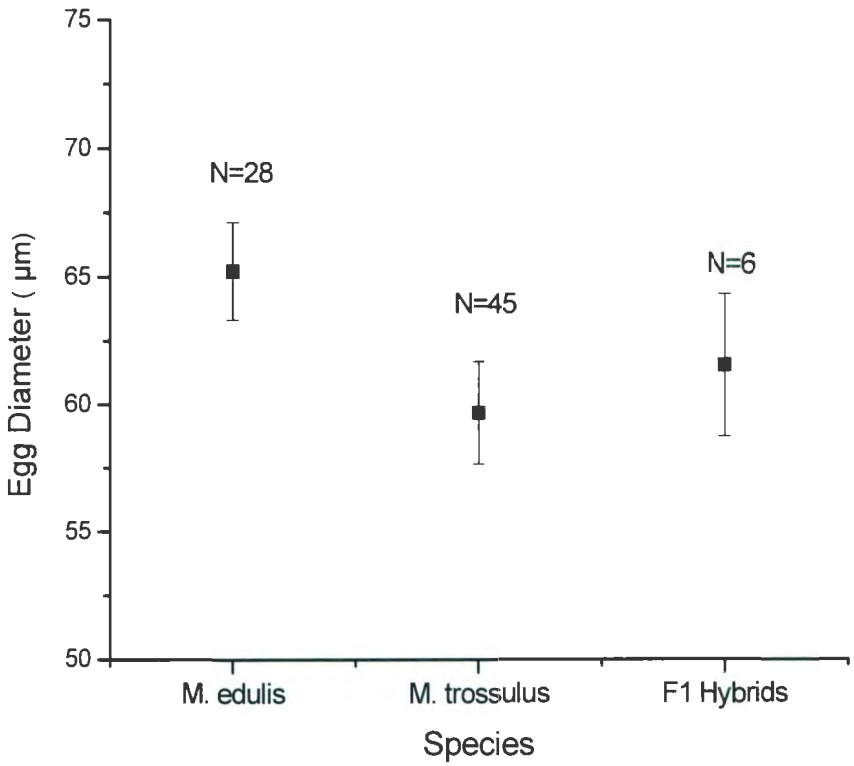


Fig 6.2. Egg diameter (mean ± S.D.) in blue mussel species, *Mytilus edulis*, *M. trossulus* and hybrids.

Table 6.1: One way ANOVA for egg diameter in blue mussels, *Mytilus edulis*, *M. trossulus* and hybrids.

Response: Egg diameter				
	df	Mean SQ	F value	Pr(>F)
Genotype	2	267.35	64.54	<0.001 ***
Residuals	76	4.14		

Table 6.2: Pairwise comparisons of egg diameters in blue mussels, *Mytilus edulis*, *M. trossulus* and hybrids (Bonferroni test).

Comparison groups	Difference between means	Simultaneous confidence intervals		Significant at 0.01 level
		Lower limit	Upper limit	
M. edulis: *M. trossulus*	5.56	4.08	7.05	Yes
M. edulis: F_1 hybrids	3.68	0.91	6.46	Yes
M. trossulus: F_1 hybrids	-1.88	-4.56	0.80	No

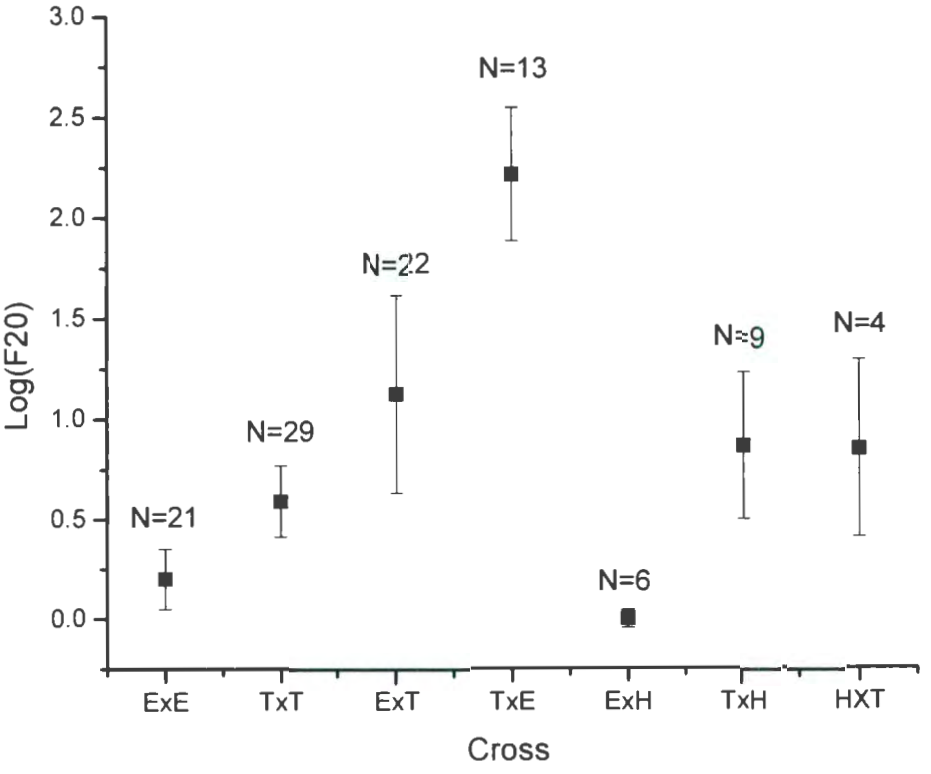


Fig 6.3. Log (F_{20}) (mean ± S.D.) in various blue mussel crosses. E: *Mytilus edulis*, T: *M. trossulus*; H: F_1 hybrids. The first character in crosses stands for the genotype of the female and the second for the male. For example, ExT means the cross *M. edulis* ♀ x *M. trossulus* ♂. Log (F_{20}) differ significantly among crosses (one way ANOVA, $F_{6,97}$=6.74, $p<0.01$)

Table 6.3: Comparison of log (F_{20}) for interspecific, homospecific and backcrosses in blue mussels (one way ANOVA).

Response: Log (F_{20})					
	df	Sum SQ	Mean SQ	F value	Pr(>F)
Type of cross	2	41.91	20.96	14.09	<0.01***
Residuals	101	150.25	1.49		

Table 6.4: Pairwise log (F_{20}) comparisons of interspecific, homospecific and backcrosses in blue mussels (Bonferroni test).

Comparison groups	Difference between means	Simultaneous confidence intervals		Significant at 0.01 level
		Lower limit	Upper limit	
Homospecific: Heterospecific	-1.38	-2.19	-0.57	Yes
Homospecific: Backcross	-0.16	-1.15	0.83	No
Heterospecific: Backcross	1.22	0.18	2.27	Yes

Table 6.5: Effect of different male-female combinations on fertilization success (estimated by F_{50}: the sperm concentration required to fertilize 50% of the eggs) in *Mytilus edulis* homospecific crosses. Individual ID was recorded as date and individual number.

Cross No.	Female ID (*M. edulis*)	Male ID (*M. edulis*)	F_{50}
1	6.24F1	6.24M1	38.54
2	6.24F2	6.24M1	19.18
3	7.4F1	7.4M2	41.52
4	7.4F1	7.4M3	38.19
5	7.4F1	7.4M4	4.68
6	7.4F2	7.4M2	41.56
7	7.4F2	7.4M3	16.57
8	7.4F2	7.4M4	44.81
9	7.6F2	7.6M1	57.07
10	7.6F2	7.6M4	253.70
11	7.8F2	7.8M2	22.64
12	7.8F2	7.8M3	7.42
13	7.13F4	7.13M2	209.89
14	7.13F4	7.13M3	23.87
15	7.14F6	7.14M3	63.29
16	7.14F6	7.14M4	45.30
17	7.14F6	7.14M5	72.02
18	7.25F2	7.25M1	7.02
19	7.25F2	7.25M3	37.98
20	7.26F4	7.26M2	31.02
21	7.26F4	7.26M4	10.12

Table 6.6: Effect of different male-female combinations on fertilization success (estimated by F_{50}: the sperm concentration required to fertilize 50% of the eggs) in *Mytilus trossulus* homospecific crosses.

Cross No.	Female (*M. trossulus*)	Male (*M. trossulus*)	F_{50}
1	6.28F1	6.28M1	42.34
2	6.28F2	6.28M1	54.54
3	7.7F1	7.7M1	25.85
4	7.7F1	7.7M3	56.46
5	7.7F2	7.7M1	336.1
6	7.7F2	7.7M3	772.66
7	7.7F3	7.7M1	41.51
8	7.7F3	7.7M3	212.91
9	7.7F4	7.7M1	232.34
10	7.7F4	7.7M3	280.94
11	7.8F1	7.8M1	57.31
12	7.8F1	7.8M4	64.66
13	7.12F1	7.12M1	27.68
14	7.18F5	7.18M1	55.68
15	7.18F5	7.18M2	61.07
16	7.18F5	7.18M4	50.57
17	7.21F2	7.21M3	102.31
18	7.21F2	7.21M5	123.97
19	7.25F1	7.25M2	22.28
20	7.26F1	7.26M1	20.08
21	7.26F1	7.26M3	177.38
22	7.26F2	7.26M1	38.15
23	7.26F2	7.26M3	60.64
24	7.26F3	7.26M1	26.38
25	7.26F3	7.26M3	75.39
26	7.26F5	7.26M1	30.37
27	7.26F5	7.26M3	49.03
28	7.27F1	7.27M1	37.32
29	7.27F2	7.27M1	35.08

Table 6.7: Effect of different male-female combinations on fertilization success (estimated by Log (F_{20}): the log transformed sperm concentration required to fertilize 20% of the eggs) in *Mytilus edulis* (♀) x *M. trossulus* (♂) interspecific crosses.

Cross No.	Female (*M. edulis*)	Male (*M. trossulus*)	Log (F_{20})
1	7.6F2	7.6M2	0.354
2	7.8F2	7.8M1	1.682
3	7.8F2	7.8M4	2.285
4	7.14F6	7.14M1	0.635
5	7.14F6	7.14M2	0.649
6	7.15F1	7.15M1	2.012
7	7.15F1	7.15M2	0.984
8	7.15F1	7.15M4	1.934
9	7.25F2	7.25M2	0.350
10	7.26F4	7.26M1	0.103
11	7.26F4	7.26M3	0.449
12	7.27F3	7.27M1	1.892
13	7.27F4	7.27M1	1.240

Table 6.8: Effect of different male-female combinations on fertilization success (estimated by Log (F_{20}): the log transformed sperm concentration required to fertilize 20% of the eggs) in *Mytilus trossulus* (♀) x *M. edulis* (♂) interspecific crosses.

Cross No.	Female (*M. trossulus*)	Male (*M. edulis*)	Log (F_{20})
1	7.4F3	7.4M2	2.060
2	7.4F3	7.4M3	1.239
3	7.4F3	7.4M4	2.678
4	7.8F1	7.8M2	2.504
5	7.8F1	7.8M3	5.116
6	7.12F1	7.12M2	2.023
7	7.13F1	7.13M2	1.944
8	7.13F1	7.13M3	2.224
9	7.13F2	7.13M2	6.289
10	7.13F5	7.13M2	1.572
11	7.13F5	7.13M3	0.435
12	7.13F6	7.13M2	2.960
13	7.13F6	7.13M3	1.099
14	7.13F7	7.13M2	0.807
15	7.13F7	7.13M3	0.146
16	7.19F2	7.19M2	1.994
17	7.19F2	7.19M3	4.294
18	7.21F2	7.21M2	3.974
19	7.25F1	7.25M1	1.032
20	7.25F1	7.25M3	0.385
21	7.26F3	7.26M2	2.262
22	7.26F3	7.26M4	1.678

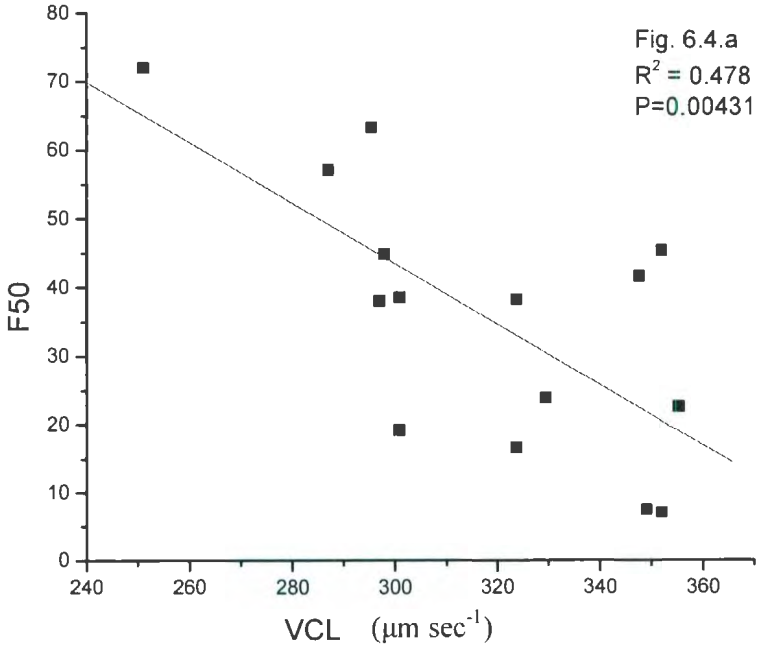


Fig. 6.4.a
R^2 = 0.478
P=0.00431
F50
VCL (μm sec^{-1})
0
10
20
30
40
50
60
70
80
240
260
280
300
320
340
360

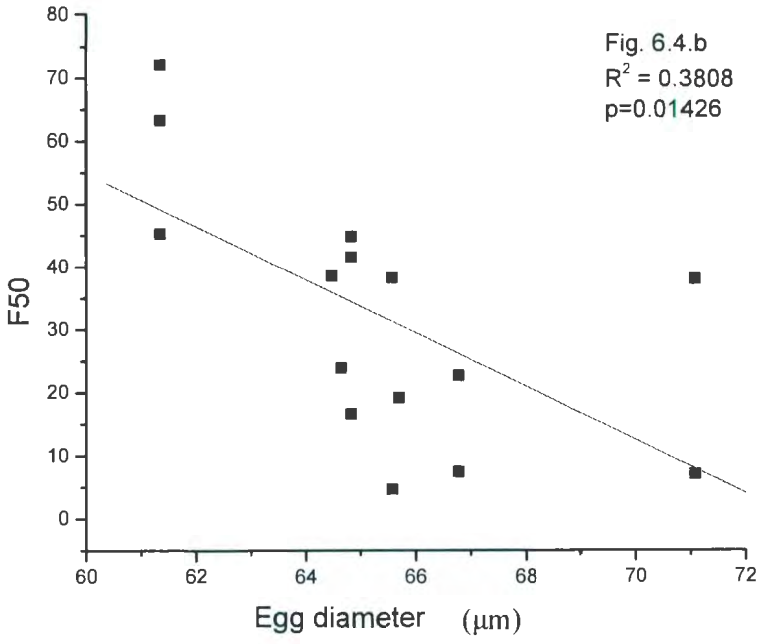


Fig. 6.4.b
R^2 = 0.3808
p=0.01426
F50
Egg diameter (μm)
0
10
20
30
40
50
60
70
80
60
62
64
66
68
70
72

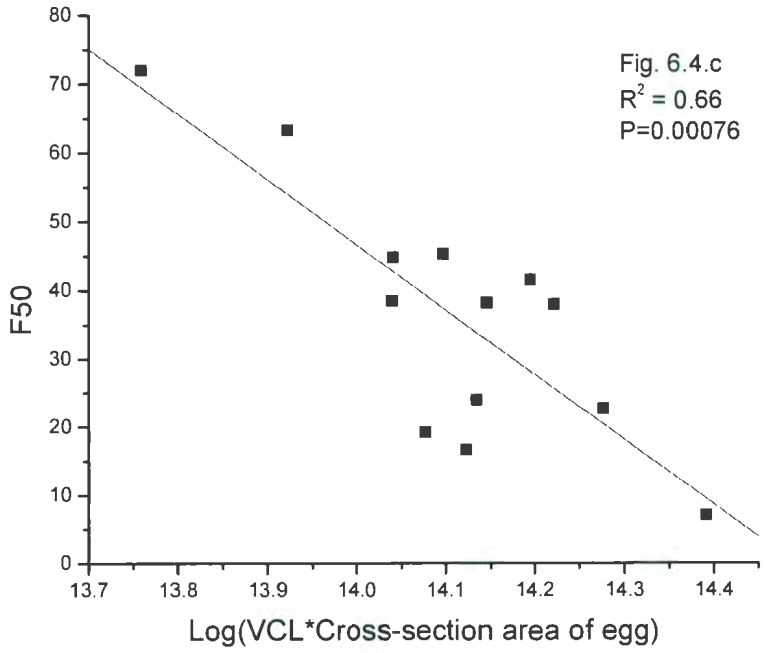


Fig 6.4. Effect of sperm velocity (Fig 6.4.a), egg size (Fig 6.4.b) and gamete traits (log(VCL*egg cross section area), which equals log(β_0)) (Fig 6.4.c) on F_{50} in all *Mytilus edulis* individual crosses.

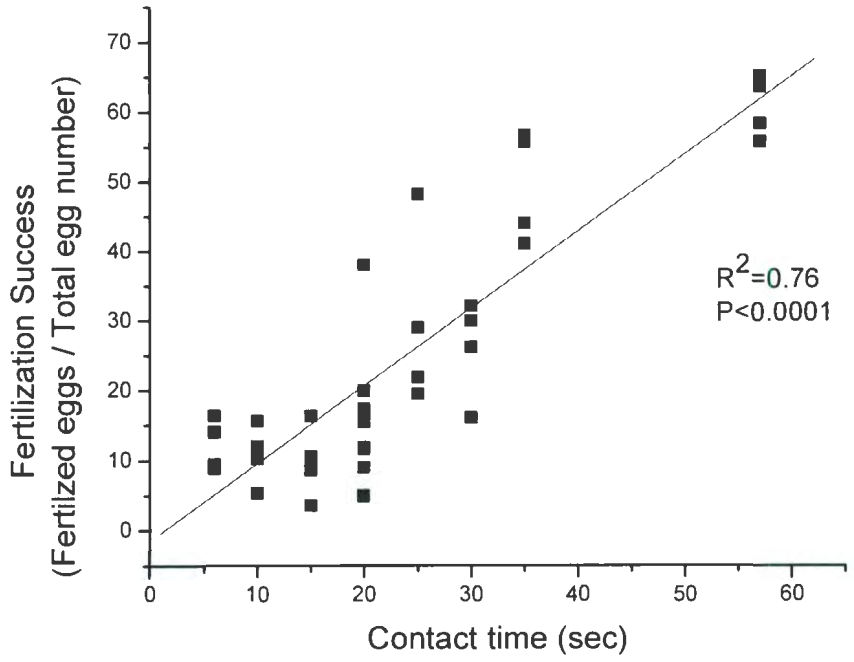


Fig 6.5. Effect of contact time on fertilization success in *Mytilus edulis* crosses.

Table 6.9: Estimation and one way ANOVA of β, β_0 and β/β_0 in different types of cross (mean $\pm$ S.D.). *E = M. edulis*; *T = M. trossulus*.

Cross	N	β ($mm^3\ s^{-1}$)	β_0 ($mm^3\ s^{-1}$)	β/β_0
E(♀) x *E*(♂)	19	$27.1\pm17.7\times10^{-6}$	$10.8\pm1.47\times10^{-4}$	$2.49\pm1.50\times10^{-2}$
T(♀) x *T*(♂)	6	$11.5\pm5.98\times10^{-6}$	$7.79\pm2.19\times10^{-4}$	$1.45\pm0.49\times10^{-2}$
E(♀) x *T*(♂)	6	$3.55\pm2.42\times10^{-6}$	$7.75\pm2.46\times10^{-4}$	$0.45\pm0.22\times10^{-2}$
ANOVA		$F_{2,28}=7.19$, $p<0.01$	$F_{2,28}=10.12$, $p<0.01$	$F_{2,28}=6.79$, $p<0.01$

Table 6.10: Pairwise comparisons of β for A: *Mytilus edulis* (♀) x *M. edulis* (♂), B: *M. trossulus* (♀) x *M. trossulus* (♂) and C: *M. edulis* (♀) x *M. trossulus* (♂) in blue mussels using the Bonferroni test.

Comparison groups	Difference between means	Simultaneous confidence intervals		Significant at 0.01 level
		Lower limit	Upper limit	
A : B	1.555×10^{-5}	-6.137×10^{-6}	3.723×10^{-5}	No
A : C	2.351×10^{-5}	1.822×10^{-6}	4.519×10^{-5}	Yes
B : C	7.960×10^{-6}	-1.878×10^{-5}	3.470×10^{-5}	No

Table 6.11: Pairwise comparisons of β_0 for A: *Mytilus edulis* (♀) x *M. edulis* (♂), B: *M. trossulus* (♀) x *M. trossulus* (♂) and C: *M. edulis* (♀) x *M. trossulus* (♂) in blue mussels (Bonferroni test).

Comparison groups	Difference between means	Simultaneous confidence intervals		Significant at 0.01 level
		Lower limit	Upper limit	
A : B	3.004×10^{-4}	2.627×10^{-5}	5.746×10^{-4}	Yes
A : C	3.049×10^{-4}	3.069×10^{-5}	5.790×10^{-4}	Yes
B : C	4.421×10^{-4}	-3.336×10^{-4}	3.424×10^{-4}	No

Chapter 7

General discussion and conclusions

The distributions and spawning seasons of marine broadcast spawners often overlap those of closely related species. Due to the lack of complex mating behavior, large numbers of gametes from closely related species may come into contact, increasing the chance of interbreeding. Considering the high potential for gene flow, an understanding of the speciation process requires knowledge of how species that make secondary contact and form natural hybrid zones maintain their identities. Previous studies have shown that differences in spawning time, mate recognition, environmental tolerance and gamete compatibility can act individually or together as reproductive isolation barriers to restrict gene flow between sympatric species. However, very little is known about how heterospecific variation in gamete traits contributes to reproductive isolation through its effect on intra- and heterospecific fertilization success. The present study of the *Mytilus* hybrid zone in Newfoundland serves as a model to reveal important aspects of evolution and speciation in free spawning organisms at the gamete level.

Sperm velocity is an important determinant of fertilization success in many species. However, little attention has been paid to the movement pattern of the sperm. In this study, sperm of *M. edulis, M. trossulus* and their hybrids all exhibited circular movement in a two dimensional plane, with no heterospecific differences in the radius. Use of specifically designed new parameters based on the open source software Image-J not only verified the circular movement pattern but also allowed a more precise description of sperm velocity than had hitherto been obtained. A comprehensive comparison of circular movement found in the sperm of all species

examined to date suggests that this pattern may be natural and more universal than has been reported previously, that it is more prevalent in aquatic free spawning species than in others, and that it may be a means of energy conservation, decreasing the chances of sperm swimming further away from eggs before chemical cues can be perceived. Furthermore, this sperm movement pattern can be advantageous at high gamete densities on small spatial scales by increasing the effective egg cross-sectional area. Considering the universality of the sperm circular movement pattern, the specifically designed parameters in the present study could also be adapted and applied to other species.

Although species-specific sperm activation by egg water was found in *M. edulis*, there was no evidence for sperm chemotaxis in mussels in the *M. edulis* complex in Newfoundland. This suggests that sperm chemotaxis may not always be necessary for free spawning organisms to achieve high fertilization success, which can simply result from high gamete collision rates under conditions of high gamete concentration. Furthermore, under these conditions species-specific sperm chemotaxis alone may not be an effective reproductive isolating mechanism.

Although no differences in gamete output among genotypes were found, sperm of F_1 hybrids swam slower than those of parental species, which may have led to a lower sperm-egg collision rate and consequently a lower fertilization success due to sperm competition. The observation that sperm of F_1 hybrids have a shorter life than those of the parental species also suggests that F_1 hybrids produce low quality sperm which will have low fertilization success not only under sperm competition but also under sperm limitation. Therefore, heterospecific differences in sperm velocity in closely related free spawning organisms such as blue mussels may contribute to reproductive isolation between species at a post-spawning prezygotic level under both sperm competition and sperm limitation, providing a better understanding of the bimodal genetic structure of the blue mussel natural hybrid zone in Newfoundland.

Sperm velocity decreased as sperm aged and temperature increased in *M. edulis*, *M. trossulus* and F_1 hybrids, although *M. trossulus* sperm were less sensitive to higher temperatures, which may be an adaptation to increased temperatures during the latter part of the relatively long spawning period.

Sperm longevity and velocity were negatively correlated within species. However, sperm of *M. edulis* swam faster and lived longer than those of *M. trossulus* and hybrids, which suggests that sperm of closely related species may differ in energy reserves according to the species' reproductive strategies. Different reproductive strategies may evolve in free spawning species such as *M. edulis* and *M. trossulus* to either enhance or avoid heterospecific sperm competition and consequently achieve higher fertilization success.

Strong but incomplete gamete incompatibility between *M. edulis* and *M. trossulus* based on the F_{20} comparison suggests considerable reproductive isolation between them at the gamete level. However, F_1 hybrids were more compatible with parental individuals than with inter-species crosses. Therefore, although F_1 hybrids produce poor quality sperm that may lead to a lower fitness, they have the advantage of being compatible with both parental species, which account for most of the individuals in this natural hybrid zone. The interaction between gamete incompatibility and gamete trait variation may contribute to an understanding of the maintenance of the natural mussel hybrid zone and explain the genetic composition of the mixed genotype group that consists primarily of backcross genotypes.

Although both sperm (sperm velocity) and egg (egg size) traits can affect fertilization success within *Mytilus* species, it is the combination of these gamete traits that is strongly correlated with fertilization success, which suggests that they are important for fertilization success in free spawning organisms and that gamete trait differences between the species may contribute to understanding their reproductive isolation and the asymmetry of gamete compatibility between them.

Different parental combinations result in different fertilization success due to the combined effects of gamete incompatibility and gamete traits. Therefore, estimates of gamete incompatibility by F_{20} comparison without taking the effect of gamete trait variation on fertilization success into account are inappropriate. Using information on sperm velocity, sperm longevity, egg size, gamete concentration and fertilization success, key parameters (β, β_0 and β/β_0) can be estimated from Vogel's fertilization kinetics model. Since the proportion of the egg surface that can be penetrated by sperm (β/β_0) is specific to each cross and is independent of gamete quality, this parameter can be widely used as a precise indicator to show the effect of gamete incompatibility on fertilization success for different crosses. The significant differences in β, β_0 and β/β_0 among crosses suggests strong reproductive isolation between *M. edulis* and *M. trossulus* that results from a difference in fertilization success due to both gamete incompatibility and differences in gamete traits.

In general, differences in gamete traits can influence both intra- and heterospecific fertilization success in free spawning marine species by affecting both gamete collision rate and fertilization rate. Since gametes from different species are often mixed during overlapping spawning periods, strong post-spawning prezygotic factors may result in reproductive isolation between or among closely related marine broadcast spawning species that form a natural hybrid zone, restricting gene flow between species and maintaining species identity. Differences in gamete traits and gamete incompatibility between species may contribute to this postspawning prezygotic isolation mechanism. The process of reinforcement may also play a role in the maintenance of a natural hybrid zone at the gamete level if F_1 hybrids produce less fit sperm that are selected against through sperm competition.

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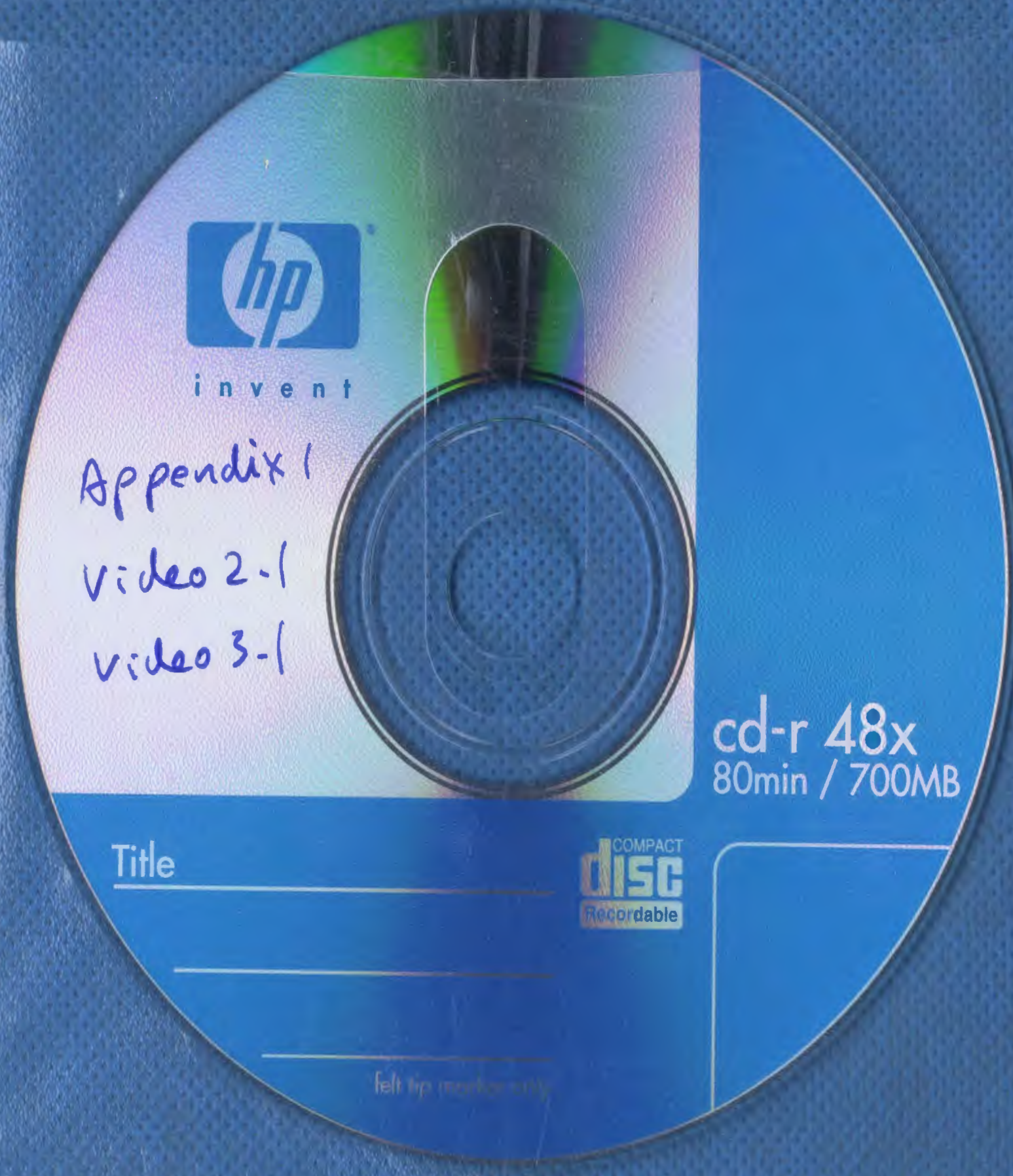
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