GENETIC AND ECOLOGICAL ASPECTS OF THE HYBRID ZONE BETWEEN THE MUSSELS *Mytilus edulis* AND *Mytilus trossulus* IN THE NORTHWEST ATLANTIC

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By

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Abstract

Two mussel species (*Mytilus edulis* and *Mytilus trossulus*) form a hybrid zone in the northwest Atlantic that is complex in its structure and dynamics. The study of *Mytilus* hybrid zones can reveal important aspects of marine evolution and speciation. A patchy distribution of *M. edulis* and *M. trossulus* around Newfoundland suggests that differential adaptation to environmental conditions may be important in determining the species relative frequency and fitness. The observation that *M. trossulus* is less tolerant to warm temperatures than *M. edulis* implies that physiological differentiation may contribute to reproductive isolation since discrete biogeographic distributions help to maintain the genetic identity of the species. Differences in the microgeographic distribution of the species indicate that habitat specialization may also contribute to reproductive isolation. The presence of pure *M. trossulus* populations in five sites sampled in Labrador likely result in a great larval input from this species into the hybrid zone through the Labrador Current. Poorer adaptation to the environmental conditions of Newfoundland would then result in a higher mortality of *M. trossulus* in the area. Evidence for differential survival was suggested by the observed decrease in the relative frequency of *M. trossulus* in the larger size classes at most sites in Newfoundland and by the greater mortality observed in field grow-out and temperature experiments. Differences in life-history strategies may also influence the observed frequency pattern since *M. trossulus* reproduces earlier, spawns smaller eggs and possibly has a shorter generation time than *M. edulis*. Gamete recognition and incompatibility are probably important isolating mechanisms between *M. edulis* and *M. trossulus*. Intrinsic postzygotic mortality likely represents an additional isolating mechanism. Reduced hybrid viability was observed mainly in the early stages of development, but higher mortality of hybrids also occurred at later stages. F1 hybrids and advanced backcrosses were the groups that showed greatest variance in fitness, suggesting that although most hybrids have reduced fitness, some hybrid combinations may be as fit or even fitter than the parent species. The use of several DNA markers revealed that the distribution of *Mytilus* genotypes in the northwest Atlantic hybrid zone

is strongly bimodal, showing that very few F1 hybrids are present and that most hybrids are advanced backcrosses. Although low levels of introgression were observed, the presence of few backcross individuals that were homozygous for alleles from the opposite species suggests that introgression can be a potential mechanism for adaptive evolution in *Mytilus*. Despite the overall lower fitness of hybrids, some hybrid individuals can express hybrid vigour and may be important for the introgression of genes between the species. Other intrinsic differences were observed between the species such as settlement behaviour, growth, survival, shell morphology and shell breakage resistance. Such differences likely play a role in maintaining the coexistence of both species. Information on species differences together with the increased performance of some hybrids can be used to help improve aquaculture production. As in other hybrid zones, the *Mytilus* hybrid zone in the northwest Atlantic is probably maintained by a combination of several factors. Pre-zygotic mechanisms (habitat specialization, gamete recognition and incompatibility) as well as post-zygotic mechanisms (reduced hybrid fitness) are critical factors responsible for reproductive isolation that were identified in the present study.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AMPL	=	Acetone mobile polar lipids
Anova	=	Analysis of Variance
AVC	=	Atlantic Veterinary College
Bp	=	Base pairs
BSC	=	Biological Species Concept
CA	=	Charles Arm
ca	=	Circa
CC	=	Cap Cove
CDNA	=	Coding DNA
COIII	=	Cytochrome oxidase c subunit III
CR	=	Clearance rate
CTD	=	Conductivity-Temperature-Depth
DB	=	Drac Bay
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleoside triphosphates
DUI	=	Doubly Uniparental Inheritance
E	=	*M. edulis*
EBMP	=	Shellfish Environmental and Biological Monitoring Program
EC	=	Enzyme code
EST	=	Esterase
ET	=	F1 hybrids from *M. edulis* female and *M. trossulus* male
F1	=	First generation
FAME	=	Fatty acid methyl esters
F-ed	=	*M. edulis* female mtDNA
FITC	=	Fluorescein isothiocyanate

FSW	=	Fresh seawater
F-tr	=	*M. trossulus* female mtDNA
GA	=	Goose Arm
GB	=	Gilbert Bay
GLU	=	Polyphenolic adhesive protein marker
h	=	Hours
HD	=	Hopedale
HhaI	=	Restriction enzyme from *Haemophilus haemolyticus*
ITS	=	Internal transcribed spacer
L	=	Liters
LAP	=	Leucine aminopeptidase locus
LH	=	Long Harbour
m	=	Meters
MAL	=	*Mytilus* Anonymous Locus
M-ed	=	*M. edulis* male mtDNA
mg	=	Milligrams
min	=	Minutes
MK	=	Makkovik
ml	=	Milliliters
mm	=	Millimeters
MPI	=	Mannose phosphate isomerase
MSR	=	Mate recognition system
MtDNA	=	Mitochondrial DNA
M-tr	=	*M. trossulus* male mtDNA
N	=	Number (sample size)
^{O}C	=	Celsius degrees
PCR	=	Polymerase chain reaction
PL	=	Phospholipids
PLII	=	Protamine-like sperm protein
ppt	=	Parts per thousand

Chapter 1

General introduction

1.1. Evolution, speciation and hybrid zones

Since Darwin presented his influential theory (Darwin, 1859), the study of evolution has become one of the most active areas of biology. Despite the title of his work (The Origin of Species), Darwin was notably unsuccessful in explaining the origin of species, mainly because he was not aware at the time of the mechanisms of inheritance. Only with the development of Mendelian genetics during the "Modern Synthesis" did Dobzhansky (1937) and Mayr (1942) uncover most of what we now know about how evolution occurs. Central to the understanding of the evolutionary process are the mechanisms responsible for differentiation within populations and species (microevolution) as well as the mechanisms that produce diversification of higher taxa (macroevolution). Speciation has received great attention because it provides a link between micro and macroevolution and therefore focuses on an important component of evolution, the diversification of species. However, even with a whole body of theory on the subject, there is very little agreement on what exactly defines a species. Over 20 different concepts of species have been formulated in order to classify groups of individuals, but the complex, slow and gradual process of evolution hampers a clear definition (Hey, 2001). The Biological Species Concept (BSC) has become the dominant definition of a species in which the central role of reproductive isolation makes the concept simple and clear. The BSC states that species are groups of actually or potentially interbreeding natural populations that are reproductively isolated from other such groups (Mayr, 1942). However, the BSC is not able to accommodate situations in which the process of speciation is ongoing and only partial reproductive isolation is observed. Other concepts such as the Evolutionary Species Concept (Wiley, 1978) or the Phylogenetic Species Concept (deQueiroz and Donoghue, 1990) are more embracing and allow

recently divergent groups like the blue mussels *Mytilus* to be recognized as different species. It is not within the scope of the present study to discuss what species are, mainly because one of the central focuses of the research is exactly the mechanisms of reproductive isolation and the process of speciation. Based on the fact that partial reproductive isolation is observed and genetic identity is maintained in the different groups of the *Mytilus edulis* complex, the present study will follow the approaches of Varvio et al. (1988), McDonald and Koehn (1988), Koehn (1991), McDonald et al (1991), Väinölä and Hvilson (1991), Sarver and Foltz (1993), Penney and Hart (1999) and Comesaña et al. (1999) to refer to the different groups as separate species. However, it is important to keep in mind that the species status is still arguable (Johannesson et al., 1990; Kautsky et al., 1990; Gosling, 1992a) and depends basically on how much divergence has occurred between the groups and on the species concept adopted.

As the evolutionary process is in general too slow to be documented, the study of speciation is usually based on inferences from living species. It is commonly accepted that speciation mostly occurs when two populations are geographically isolated and genetic divergence takes place through natural selection and/or genetic drift (Mayr, 1963; Palumbi, 1994). Although sympatric and parapatric speciation have been reported (reviewed in Via, 2001), the prevention of the homogenizing effect of gene flow in geographically separated groups allows the genetic differentiation that will ultimately result in intrinsic barriers to reproduction between the two groups. Since the geography and ecology of the Earth are constantly changing, groups undergoing divergence may come into contact again and, depending on the stage of divergence, interbreed to form hybrid zones. The great relevance of hybrid zones to speciation studies lies in the fact that patterns of gene flow, selection and reproductive isolation can be observed in nature, allowing for inferences about the evolutionary process (Harrison, 1990). Several models have been proposed to understand the structure and maintenance of hybrid zones. The greatest distinction between these models lies in the emphasis given to endogenous and exogenous factors that maintain hybrid zones. The tension zone model assumes that lower fitness of hybrids due to genetic incompatibilities followed by selection against these hybrids maintains the balance of hybrid zones (Barton and Hewitt, 1989; Barton and

Gale, 1993: Harrison, 1993). In this case the position of the hybrid zone may fluctuate, as the species interactions are independent of the environment. Environmental models predict that the parental populations are adapted to different environments and that hybrid zones are maintained due to the inability of the genotypes to function properly in suboptimal environments (Endler, 1977). Hybrids are then restricted to the zone of contact where a greater variability in hybrid fitness is usually observed and is commonly associated with disturbed or transitory environments. Arnold (1997) proposed a model in which some hybrid combinations would be favored in certain habitats while others would be selected against. Whether hybrids are more or less fit than their parents is not yet clear, but different classes of hybrids will probably show different levels of fitness relative to the parent species. The recent tendency to incorporate both endogenous and exogenous components in models to explain the maintenance of hybrid zones reflects the fact that many hybrid zones possess environmentally dependent features as well as genetic incompatibilities between the divergent genomes. In addition, some areas have been referred to as mosaic hybrid zones due to the patchy distribution of genotypes, often correlated with environmental conditions (Rand and Harrison, 1989; Ross and Harrison, 2002; Vines et al., 2003). Three different areas of hybridization between *Mytilus* species in the Atlantic and Pacific oceans have been described as mosaic hybrid zones (Bates and Innes, 1995; Suchanek et al., 1997; Penney and Hart, 1999; Bierne et al., 2002b, 2003b).

Independent from the structure and maintenance of hybrid zones, mechanisms promoting reproductive isolation are often classified into pre- and post-zygotic barriers to hybridization. Pre-zygotic barriers act before fertilization occurs, preventing gamete encounter (habitat specialization, mating preferences or asynchronous reproduction) or preventing fertilization (gamete incompatibility). Post-zygotic mechanisms reduce the fertility and/or viability of hybrids. Species may be isolated or partly isolated by both kinds of mechanisms, since pre- and post-zygotic barriers to reproduction commonly evolve independently of each other (Coyne and Orr, 1997). Understanding the relative importance of each mechanism can provide insights into the forces driving speciation.

The present study uses a *Mytilus* hybrid zone (*Mytilus edulis* and *Mytilus trossulus*) in the northwest Atlantic as a model system to understand mechanisms that

promote reproductive isolation in the ocean. Speciation in the ocean has been studied far less than speciation in the terrestrial environment (Palumbi, 1994). *Mytilus* mussels are an ideal group to study patterns of speciation since several different hybrid zones are well documented and a large amount of information has been collected on the biology of *Mytilus* species.

1.2. The *Mytilus edulis* complex

The systematic status of mussels in the genus *Mytilus* is controversial, but extensive studies in the Northern Hemisphere have clearly defined three distinct genetic groups that form the *Mytilus edulis* complex: *M. edulis* (Linneaus, 1758), *M. galloprovincialis* (Lamarck, 1819) and *M. trossulus* (Gould, 1850). Apart from the more distantly related *M. californianus* (Seed, 1992), a few other putative species in the genus are *M. chilensis* from Chile, *M. platensis* from Argentina, *M. planulatus* from Australia, *M. aoteanus* from New Zealand and *M. desolationis* from the Kerguelen Islands (McDonald et al., 1991). All the latter groups occur only in the Southern Hemisphere, probably originated from northern Atlantic populations during the Pleistocene, and can therefore be considered sister taxa to *M. edulis* and *M. galloprovincialis* (Hilbish et al., 2000). Part of the controversy about the systematic status of the species from the *Mytilus edulis* complex comes from the fact that whenever two species from the complex are found sympatrically, hybridization is observed (Gosling, 1992b). The morphological and ecological similarities between them also hamper their identification in areas of sympatry.

The sexes are separate in *Mytilus*, although a few hermaphrodites have been observed (Seed, 1976; Beaumont and Abdul-Martin, 1994; Toro, 1999). The reproductive cycle of temperate populations is closely related to environmental conditions and spawning usually occurs during the summer and spring (Seed, 1969; Gardner and Skibinski, 1990; Toro et al., 2002). Gametes are released in the water column and fertilization is followed by a larval period of 2 to 10 weeks, depending on environmental conditions (Seed and Suchanek, 1992). This potentially allows dispersal over great distances and genetic homogeneity among populations (Scheltema, 1971). The large

number of gametes produced (Thompson, 1979; Toro et al., 2002) also facilitates genetic variability among offspring. Great variation in growth has been observed among individuals that may reflect environmental conditions and/or genetic composition (Seed, 1976; Innes and Haley, 1977a; Rodhouse et al., 1986; Beaumont, 1991).

Mytilus edulis occurs predominantly in the North Atlantic, but the close genetic affinity of some populations in the Southern Hemisphere makes the precise distribution of the species unclear. In Europe *M. edulis* occurs from the Atlantic coast of France to very high latitudes that include the coasts of Iceland, Norway and Arctic Russia (McDonald at al., 1991; Varvio et al., 1988; Hummel et al., 2001). *M. galloprovincialis* occurs allopatrically in the Mediterranean, where the species presumably emerged during periods of geographic isolation in the Pleistocene (Barsotti and Meluzzi, 1968). Allopatric populations of *M. galloprovincialis* are also observed in the Black Sea and likely invaded the area from the Mediterranean (Ladoukakis et al., 2002). On the Atlantic coast of France and the British Isles, *M. edulis* hybridizes with *M. galloprovincialis*, forming a mosaic hybrid zone (Daguin et al., 2001; Bierne et al., 2002b, 2003b). In England, a substantial larval input by *M. edulis* is counterbalanced by a higher mortality of this species (Skibinski and Roderick, 1991; Gardner et al., 1993; Gardner 1994a; Wilhelm and Hilbish, 1998; Hilbish et al., 2002). Gilg and Hilbish (2003) demonstrated that the genetic composition of mussels in southwest England is highly influenced by patterns of ocean currents. Habitat specialization may also be an important factor responsible for species distribution, contributing to interspecific barriers to hybridization (Gosling and McGrath, 1990; Gardner, 1996; Comesaña and Sanjuan, 1997; Bierne et al., 2002b). Other hypotheses that may explain the maintenance of this mussel hybrid zone include physiological differentiation (Hilbish et al., 1994), differences in spawning time (Gardner and Skibinski, 1990), assortative fertilization (Bierne et al., 2002a) and differential mortality related to strength of attachment (Willis and Skibinski, 1992). All these factors may contribute to reproductive isolation. Despite the partial reproductive isolation, the high level of hybridization (Gardner, 1994a) allowed the introgression of nuclear (Bierne et al., 2003b) and mitochondrial DNA (Rawson and Hilbish, 1995b; 1998) in these areas.

European *M. edulis* also form a second hybrid zone at the entrance of the Baltic Sea, but in this area hybridization occurs with *M. trossulus*. This second zone is narrower than other *Mytilus* hybrid zones, possibly owing to the abrupt change in environmental conditions between the North Atlantic and the Baltic Sea (Väinölä and Hvilsom, 1991). The preferential colonization of the Baltic Sea by *M. trossulus* is likely related to physiological adaptation to lower salinity and the recent opening of the Baltic Sea to the ocean (Varvio et al., 1988). The hybrid zone is formed exactly at the entrance of the Baltic Sea, where a steep gradient in environmental conditions is observed. The most remarkable and well-studied feature of this area is the complete introgression of mitochondial DNA (mtDNA) from *M. edulis* into *M. trossulus* (Quesada et al., 1999; Zbawicka et al., 2003; Quesada et al., 2003). The unusual doubly uniparental inheritance (DUI, Zouros et al., 1994) in *Mytilus* adds a layer of complexity to understanding the introgression of mtDNA. In *Mytilus* there is the usual female mtDNA lineage that is inherited in all offspring but there is also a male mtDNA lineage that is transmitted only from fathers to sons (Zouros et al., 1994; Skibinski et al., 1994). Hybridization breaks down the DUI in some areas and is probably responsible for blocking mtDNA introgression in North America (Rawson et al., 1996b). Many exceptions to DUI have been reported, such as males homoplasmic for only female mtDNA and females heteroplasmic for both male and female mtDNA (Zouros et al. 1994; Stewart et al., 1995; Rawson and Hilbish, 1995a; Wenne and Skibinski, 1995; Quesada et al., 1996; Saavedra et al., 1997; Garrido-Ramos et al., 1998; Wood et al., 2003). The consequences of DUI and the occasional breakdown of DUI are not fully understood, nor is its relation to the maintenance of mussel hybrid zones.

On the Atlantic coast of North America, only *M. edulis* occurs from Cape Hatteras to Cape Cod or possibly to southern Maine (Koehn et al., 1984; Koehn, 1991; Seed 1992). From Maine to Newfoundland a mosaic hybrid zone is observed between *M. edulis* and *M. trossulus* (Bates and Innes 1995; Penney and Hart, 1999; Rawson et al., 2001). The northern limits of both species in the northwest Atlantic and the boundaries of the hybrid zone are not well established since no samples have been collected north of the island of Newfoundland. Asynchrony of spawning (Toro et al., 2002), habitat

specialization (Bates and Innes, 1995), differential mortality (Comesaña et al., 1999; Toro et al., 2004), thermal tolerance (Rawson et al., 2001), adaptation to salinity (Gardner and Thompson (2001) and gamete incompatibility (Rawson et al., 2003) may all be involved in maintaining this hybrid zone. The presence of allopatric populations of *M. edulis* in Hudson Bay and the presence of *M. trossulus* at the entrance of the bay (Koehn, 1991) suggest that another hybrid zone may exist in this area that has not yet been documented.

On the Pacific Coast of North America *M. trossulus* occurs from California to Alaska (McDonald and Koehn, 1988) and hybridizes with *M. galloprovincialis* in different areas from California to British Columbia (McDonald and Koehn, 1988; Sarver and Foltz, 1993; Geller et al., 1994; Heath et al., 1995; Suchanek et al., 1997). The discontinuity of these areas of hybridization may be related to the fact that *M. galloprovincialis* was recently introduced by human activity on various parts of the coast (Heath et al., 1995). The restriction of *M. galloprovincialis* to areas of introduction by shipping activities and/or aquaculture practices (Anderson et al., 2002) and the low level of introgression observed (Rawson et al., 1999) suggests that hybridization may be less frequent than in other *Mytilus* hybrid zones. Differential adaptation to salinity may affect the microgeographic distribution of the species (Sarver and Foltz, 1993). A macrogeographic distribution with *M. trossulus* occurring allopatrically from Puget Sound to Alaska and *M. galloprovincialis* in Southern California suggests that temperature tolerance may influence the distribution of each species (Suchanek et al., 1997). On the other side of the Pacific, at exactly the same latitude on the coast of Japan, the two species show a very similar pattern of displacement (Suchanek et al., 1997). Likewise, *M. galloprovincialis* has probably been introduced into the west Pacific by human activity (Wilkins et al., 1983) and the area of contact between *M. galloprovincialis* and *M. trossulus* has been poorly studied. The only report on hybridization in the west Pacific suggests that the hybrid zone is restricted to some areas of Japan (Inoue et al., 1997).

1.3. Objectives

The main objective of the present study is to provide a better understanding of evolution and speciation in the marine environment by examining pre- and postzygotic isolating mechanisms in the *Mytilus edulis* – *Mytilus trossulus* hybrid zone in the northwest Atlantic.

The following are the specific objectives to be addressed:

- Extend knowledge of the species distribution (mainly in Labrador) and of the boundaries of the hybrid zone.
- Make use of a static cohort analysis to determine the relative frequency of both species and their hybrids in different size classes that may suggest differential mortality between the species.
- Determine whether differential adaptation to temperature tolerance influences the distribution and fitness of both species and their hybrids.
- Investigate the microgeographic pattern of distribution of both species and hybrids.
- Determine other life-history parameters such as egg size, lipid content and age of reproduction that may contribute to the coexistence of both species in the same area.
- Provide a better understanding of the available genetic markers for *Mytilus* in order to optimize studies related to hybridization.
- Estimate the degree of natural hybridization and evaluate how the number of markers utilized in the analysis influences the results.
- Determine the frequency distribution of different types of hybrids with a hybrid index and investigate patterns of introgression.
- Investigate the inheritance of mitochondrial DNA in *Mytilus* and the disruption of doubly uniparental inheritance (DUI).
- Produce karyotypes from *M. trossulus* individuals from the northwest Atlantic and compare with those of other *Mytilus* populations.
- Compare fertilization success in intra-specific and inter-specific crosses.
- Estimate the viability and relative fitness of hybrids from the larval stage to maturity.
- Estimate the reproductive success of F1 hybrids and viability of F2 hybrids.

- Compare the fitness of *M. edulis* and *M. trossulus* in controlled laboratory and aquaculture conditions.
- Evaluate differences in shell morphometrics and breakage resistance between *M. edulis* and *M. trossulus*.
- Investigate differential patterns of larval settlement between *M. edulis* and *M. trossulus*.

Chapter 2

Biogeographic distribution and life history of *Mytilus edulis, M. trossulus* and their hybrids in Atlantic Canada

2.1. Introduction

Three genetically divergent groups of blue mussels form the *Mytilus edulis* complex and wherever two of them meet they hybridize (Gosling, 1992b). Although the species status is still contested by some authors due to the extensive hybridization observed in some areas (Johannesson et al., 1990; Kautsky et al., 1990; Gosling, 1992a), there is a general tendency to accept the three groups as distinct species (e. g. Varvio et al. 1988; McDonald and Koehn, 1988; Koehn, 1991; McDonald et al, 1991 Väinölä and Hvilson, 1991; Sarver and Foltz, 1993; Penney and Hart, 1999; Comesaña et al., 1999). Hybrid zones are particularly important to the study of speciation because they provide an insight into the interactions between divergent species and the mechanisms that maintain reproductive isolation (Hewitt, 1988). The hybrid zone between *Mytilus edulis* and *M. trossulus* in the northwest Atlantic is probably the result of secondary contact and shows a patchy distribution of both species and their hybrids from Newfoundland to Maine (Penney and Hart, 1999; Hilbish, 1996; Rawson et al., 2001). Mussels from the genus *Mytilus* probably first colonized the North Atlantic when the Bering Strait opened around 3.2 million years ago, connecting the North Pacific and Atlantic oceans for the first time and allowing a massive invasion of animals and plants, primarily from the Pacific to the Atlantic (Vermeij, 1991; Rawson and Hilbish, 1995b; Wares and Cunningham, 2001). Geographic isolation during the subsequent glacial periods allowed genetic divergence, giving rise to *M. edulis* in the Atlantic and *M. galloprovincialis* in the Mediterranean Sea (Barsotti and Meluzzi, 1968). When the two oceans were reconnected through the Arctic during interglacial periods or after the last glacial maximum, approximately 13 000 years ago, *M. trossulus* probably migrated from the Pacific into the Atlantic Ocean and encountered *M. edulis* populations, forming the present hybrid zone. Presently, *M. edulis*

is found in allopatry from Cape Hatteras to Maine, so the species is likely to have an advantage at the southern end of the hybrid zone. The northern distribution of both species and the boundaries of the hybrid zone are not well described, but high frequencies of *M. trossulus* observed on the Northern Peninsula, Newfoundland (Penney and Hart, 1999) suggest that this species may have an advantage at northern latitudes.

Adaptation to environmental conditions (e.g. temperature) may differentiate the species physiologically, therefore a latitudinal displacement may be expected on a macrogeographic scale of distribution. Thermal denaturation of proteins is probably responsible for a latitudinal displacement of *M. trossulus* and *M. galloprovincialis* in the Pacific Ocean (Hofmann and Somero, 1996). In this area, *M. trossulus* is less tolerant of high temperatures and has a more northern distribution (Sarver and Foltz, 1993; Geller et al. 1994; Rawson and Hilbish, 1995a). Summer mortality of mussels is a common phenomenon in the Canadian Maritimes (Dickie et al., 1984; Mallet et al., 1990; Myrand and Gaudreault, 1995, Tremblay et al., 1998 a, b), but to date no study has investigated differential mortality among the two species and their hybrids. The patchy distribution observed in the hybrid zone also suggests that adaptation to specific environments plays an important role in both species distribution.

Salinity is another important abiotic factor responsible for the distribution of marine species. In the Danish Belt Sea hybrid zone, tolerance for low salinity is apparently the factor that limits species distribution, *M. trossulus* being more tolerant than *M. edulis* to the low salinity of the Baltic Sea (Johannesson et al. 1990; Väinöla and Hvilsom, 1991). Salinity and temperature tolerance are usually the most important factors affecting bivalve distribution, but other factors may also be involved such as pH, wave exposure, strength of attachment, resistance to diseases and predators (Gardner, 1994a, b).

Bivalve distribution is also highly influenced by ocean currents because the planktonic larvae may be dispersed over hundreds of kilometers. The northwest Atlantic hybrid zone is situated in an area under strong influence of the Labrador Current, which flows southwards. Large numbers of larvae produced in Labrador may disperse into Newfoundland and other parts of the hybrid zone, but natural selection should act against these individuals if they are not well adapted to the environmental conditions where they

settle. A balance between high levels of immigration and selection against *M. edulis* has been proposed as a mechanism to maintain the genetic architecture of the hybrid zone formed with *M. galloprovincialis* in England (Wilhelm and Hilbish, 1998). In fact, selection is a major factor in the maintenance of hybrid zones. Environmental models that explain the dynamics of hybrid zones emphasize that the relative fitness of hybrids and parent genotypes is determined by geographic variation in the environment (Arnold, 1997). The bounded hybrid superiority model (Moore, 1977) and the mosaic model (Harrison, 1986; Howard, 1986) are also environmental models, since the fitness of individuals and the patchy distribution are determined by particular environmental conditions. On the other hand, the tension zone model implies that, regardless of the environment, hybrids are less fit than the parent species due to genetic incompatibilities of divergent genomes (Barton and Hewitt, 1985). Apart from selection against hybrids, one or the other species may also be at a disadvantage in parts of the hybrid zone, due to poorer adaptation to the local environment. A static cohort analysis from two wild populations in Newfoundland showed that the proportion of *M. trossulus* decreased with mussel size, suggesting that differential mortality may play an important role in maintaining this hybrid zone (Comesaña et al., 1999). The present study hypothesizes that an input of larvae from the Labrador Current, predominantly of *M. trossulus*, supplies juveniles to the hybrid zone, but most of these individuals are selected against due to poorer adaptation to the local environment compared with *M. edulis*. Alternatively, the lower frequency of *M. trossulus* in the larger size classes observed by Comesaña et al. (1999) may represent different life-history strategies, in which *M. trossulus* reproduces and dies earlier than *M. edulis*.

The *Mytilus* hybrid zone in the northwest Atlantic is complex in its structure and dynamics. The factors influencing the distribution patterns of both species in this area are not yet fully understood (Bates and Innes, 1995; Mallet and Carver, 1995). Several factors probably contribute simultaneously to the maintenance of partial reproductive isolation, thereby preventing the erosion of species identity associated with extensive hybridization. Toro et al. (2002) suggested that temporal displacement of the spawning peaks of both species in Newfoundland reduces the likelihood of hybridization. Furthermore, the

present study (Chapter 4) and Rawson et al. (2003) suggest that gamete incompatibility is an important factor responsible for reproductive isolation, and chapter 5 provides evidence of selection against hybrids, probably due to unfavorable genetic interactions. Finally, Bates and Innes (1995) observed some species-specific preferences for sheltered or exposed areas, suggesting that spatial displacement can potentially reduce the probability of gamete encounter and prevent extensive hybridization. Habitat specialization is probably an important factor that contributes to partial reproductive isolation between *M. edulis* and *M. galloprovincialis* in Europe (Bierne et al., 2002b, 2003a). Physiological differentiation may also be a component of this hybrid zone, since the two species differ in their tolerance of temperature stress (Hilbish et al., 1994). The macrogeographic pattern and patchy distribution observed in the northwest Atlantic hybrid zone suggest that adaptation to environmental conditions may play an important role in the dynamics of the hybrid zone.

The present study extends the knowledge of mussel species distribution by sampling areas in Labrador that have not been previously studied. Two laboratory experiments on temperature tolerance were used to investigate the basis of the macro- and microgeographic patterns of distribution of each species. A static cohort analysis was performed to search for differential mortality between the two species and differences in life-history strategies that may explain observed patterns of species size-frequency and spatial distributions. To investigate other possible life-history differences between the two species, egg size and lipid content were also measured. Finally, the first gonad development of a laboratory-produced cohort was followed to test the hypothesis that *M. trossulus* reproduces at an earlier age than *M. edulis*.

2.2. Materials and Methods

2.2.1. Distribution and size frequency

Farmed mussels were collected in June 2001 from Cap Cove (CC), Charles Arm (CA), Drac Bay (DB), Reach Run (RR) and Salmonier Cove (SC) (figure 2.1). Animals

were divided into 3 different size classes: 20 to 40 mm, 40 to 60 mm and larger than 60 mm. As animals were collected form mussel farms, the three size classes were known to correspond to different cohort years. Approximately 30 animals in each size class were genotyped, so the total sample size was 90 mussels for each site. Wild mussels were collected in July 2000 and August 2001 from Chance Cove and Bellevue. In 2000, mussels from Chance Cove were collected from a subtidal area (CS) at approximately 2 m depth. In 2001, mussels from Chance Cove were collected from 3 different areas; a shallow (80 cm deep) subtidal area (CT) 15 m from the 2000 collection area, and two intertidal areas (CL and CR) 30 m apart (figure 2.4A). Mussels from Bellevue were collected from a protected intertidal area (BP) and an exposed interdtidal area (BE), the same two locations sampled by Comesaña et al. (1999). During 2002 and 2003 samples from 6 sites in Labrador (Gilbert Bay, Makkovik, Hopedale, Anaktalak Bay, Kangeklukuluk Bay and Kangeklualuk Bay), one in Hudson Bay (Arviat) and another two mussel farms in Newfoundland (Long Harbour and Goose Arm) were analyzed to determine species frequency. Analysis of size class distribution was not possible for these samples with the exception of those from Goose Arm and Gilbert Bay. Samples from Chance Cove and Bellevue were genotyped with six diagnostic genetic markers (ME, ITS, MAL, COIII, MPI, EST-D; see chapter 3 for detailed information on genetic markers). Labrador, Long Harbour and Goose Arm samples were genotyped with ME and ITS and in most cases the remaining samples were genotyped only with ME.

In order to correlate species frequency and distribution pattern with environmental characteristics, temperature, salinity and chlorophyll *a* data were used to establish an environmental profile of each site. Temperature was recorded daily with a thermograph (Vemco Ltd.) for 2 years in Long Harbour, Salmonier Cove and Charles Arm. For the other mussel farms, seasonal data for temperature, salinity and chlorophyll *a* was provided by the Shellfish Environmental and Biological Monitoring Program (EBMP). Most of these data are also available in Nichols et al. (2002) for 1993 to 2001. For the sites where no environmental data was obtained (Labrador and non-farm sites in Newfoundland), information from the literature was used to infer basic environmental characteristics. Ocean current models developed for cod larvae (Davidson and deYoung,

1995; Colbourne et al., 1997) were also used to infer larval dispersal by the Labrador Current.

2.2.2. Responses to temperature stress

Two experiments were conducted to determine if temperature is a critical factor affecting species distribution and frequency. To test the hypothesis that the two species are physiologically adapted to different temperatures a series of clearance rate (CR) trials was performed in 2002. Mussels from both species and hybrids were acclimated at 4 or 12°C for at least 30 days and then suddenly moved to either 12°C (4°C acclimated mussels) or 20°C (12°C acclimated mussels). Clearance rate was measured one day before and one day after the temperature change and also 7 and 15 days after the change, when the animals should have acclimated to the new temperature (Widdows and Bayne, 1971). Trials consisted of placing individual mussels in separate beakers containing 1.5 L of static seawater filtered to 1 μm and UV sterilized. Microalgae (*Isochrysis galbana*, T-ISO) were added to each container at a density of $5x10^4$ cells ml^{-1}. Algal concentration was measured with a Coulter Multisizer at one hour intervals for a period of five hours. Two control containers were used in each trial with no algae added and the number of particles present was used as a standard background control for all containers. Background counts were then subtracted from all sample counts. All beakers were aerated to minimize algal deposition and one control beaker with algae but no mussel was always employed to determine if algal deposition or replication occurred during the trials.

Shell length of mussels used in the experiment ranged from 17 to 22 mm and mussels belonged to 19 families produced in the laboratory (offspring of pair-mating crosses). The parents of these mussels were genotyped with 6 genetic markers, so the identities of all individuals were known and they could be allocated to one of the following 5 genotypic groups: 1) *M. edulis* (4 families), 2) *M. trossulus* (3 families), 3) F1 hybrids from *M. edulis* female and *M. trossulus* male (ET group, 4 families), 4) F1 hybrids from *M. trossulus* female and *M. edulis* male (TE group, 2 families) and 5) advanced backcrossed individuals (6 families). Although all backcross families were

different from each other in their genetic composition, they were pooled to facilitate the analysis. In all trials one animal from each family was tested, so the total of mussels per trial was always 19. Mussels with the same genotype were used as replicates in each trial. For each temperature, 3 repetitions were performed with different mussels at each temperature and time. For example, when temperature was raised from 4°C to 12°C, a total of 12 trials was performed, 3 trials before the temperature shock, 3 trials one day after the shock, 3 trials after 7 days and another 3 trials 14 days after the shock. The same procedure was followed for the shock from 12°C to 20°C. No difference was observed between replicate trials at the same temperature (Anova, $p>0.05$), so replicates were pooled for the analysis, as were animals belonging to the same genotype group. Clearance rate was calculated from the formula: CR = V (log C1 – log C2) / t, where CR is clearance rate (L h^{-1}), V is volume (liters), C1 is initial algal concentration (cells ml^{-1}), C2 is final algal concentration and t is time (hours). A one-way Anova was performed on CR for each sampling date to determine differences between genotypes. For the trials at 20°C many individuals (mainly *M. trossulus* and backcrosses) did not survive the temperature shock long enough to permit measurement of CR. These animals were replaced with others from the same families that were kept under exactly the same conditions in case mortality occurred during the experiment.

A second experiment was conducted in August/September 2003 to investigate mortality related to temperature and physiological stress after the spawning season. Mussels belonged to the same laboratory produced cohort as the ones in the clearance rate trials, but these animals were kept at a mussel farm (Long Harbour) for 2 years and ranged from 26.4 to 56.1 mm (mean = 43.8 mm). Animals were brought from the field and spawned spontaneously in the laboratory when reintroduced into seawater at 15°C. The experiment started one week after the spawning event occurred. Two replicates of 17 mussels from each of the 4 genotypes (*M. edulis*, *M. trossulus* and F1 hybrids ET and TE) were kept in a common tank with 200 L. of static seawater at 24 ± 2°C for 36 days. Seawater was constantly aerated and replaced twice a week. At every water change dead animals were removed and microalgae added to the tank.

2.2.3. Egg size, egg composition and gametogenesis

To investigate the possibility of different reproductive strategies among genotypes, egg diameter was measured in 50 mussels that spawned in the laboratory. Mussels were collected from Chance Cove, Charles Arm and Long Harbour. Egg diameter was determined with a Coulter Multisizer and only samples with more than 500 eggs per female were used in the analysis. Mussels were genotyped with 6 diagnostic markers, with the exception of mussels from Charles Arm (almost entirely *M. edulis*) which were genotyped only with ME. Genotypes were grouped into 3 different classes, representing both parent species and hybrids (backcrosses). No putative F1 or F2 hybrids were observed, so all hybrids were advanced backcrosses.

The observation in a preliminary laboratory spawning that egg colour differed among some females at one site (Long Harbour) prompted an investigation to determine whether the lipid composition of the eggs could vary among the genotypes due to temporal displacement in gonad development or physiological adaptation to a specific environment. Approximately $5x10^4$ eggs from three *M. edulis* and three *M. trossulus* females were stored in 10ml vials (2 replicates per female) with 3ml chloroform. Each vial was flushed with nitrogen gas, capped and kept at –70°C until lipid extraction was carried out by a modified Folch method. The organic phase was isolated using a combination of chloroform, methanol and purified water and analyzed using thin-layer chromatography (TLC-FID) on a Chromarod-Iatroscan system to separate the lipid classes. Fatty acid methyl esters (FAME) were prepared by adding a mixture of BF_3/CH_3OH and hexane to a dried subsample of each extract and heating to 85°C for 90 minutes under nitrogen. Purified water and hexane were added and the resulting FAME derivatives separated and quantified with a Varian 3400 gas chromatograph equipped with a flame ionization detector. Percentages of the different lipid classes and fatty acids were transformed (arcsine square root) and a one-way Anova was performed to assess differences between genotypes.

The suggestion that *M. trossulus* reproduces earlier than *M. edulis* (Toro et al., 2002) was tested using a cohort of mussels produced in the laboratory in 2000. In August

2003 approximately 30 animals from the 18 families that survived in the laboratory were dissected to determine their sex and gonad development. Families had been monitored periodically and had never demonstrated any signs of gonad development until that reproductive season. Mussel length varied from 12.7 to 30.6 mm. Sex and gonad ripeness were determined by examining a squash of the gonad under a light microscope (40X) and classifying each preparation according to a table adapted from Seed (1969). Mussels with no visible gametes were scored as 0 and mussels with a completely full gonad were scored as 4 (figure 2.8, legend). Animals that had already partially spawned were scored as 5 since it was impossible to determine gonad condition before spawning occurred. Families were monitored from March to November to ensure that early or late gonad development had not occurred in those families that did not have any gametes in August.

2.3. Results

2.3.1. Distribution and size frequency

Species distribution in the study area was patchy, especially in Newfoundland, but *M. trossulus* showed higher frequencies in the northern populations (figure 2.1). *M. trossulus* also showed higher frequencies in the smaller size classes in Newfoundland, while *M. edulis* was predominant in the larger size classes (figure 2.2). Drac Bay (DB), on the Northern Peninsula, showed a high frequency of *M. trossulus* (80.6 %) in the smallest size class (20 to 40 mm), but the frequency decreased to 30.8 % in the largest (60 to 80 mm). Cap Cove (CC), Salmonier Cove (SC) and Reach Run (RR) also showed a considerable decrease in *M. trossulus* frequency in the largest size class (figure 2.2). *M. trossulus* was almost absent in all size classes in Charles Arm (CA). Size distribution analysis was not possible for most of the Labrador samples, but high frequencies of *M. trossulus* were observed at almost all sites. Although no mussels larger than 60 mm were collected from Gilbert Bay (GB), Labrador, the site showed a high proportion of *M. trossulus* (84.2 %) in the smallest size class (10 to 20 mm), decreasing to 46.2 % in the largest size class (40 to 60 mm) (figure 2.3). Goose Arm (GA) also contained only

mussels smaller than 60 mm, so the same size class distribution as Gilbert Bay was used and a decrease in *M. trossulus* frequency was again observed in the larger size classes (figure 2.3). The pattern of species frequency observed in Gilbert Bay was very similar to the most northern site sampled in Newfoundland (Drac Bay). In these two sites the relative frequency of *M. trossulus* in the largest size class was equivalent to that of *M. edulis*. The pattern observed in Goose Arm was similar to Reach Run, which lies approximately on the same latitude but at the opposite side of the island. Gilbert Bay (52°35'N, 55°55'W) was the most northern sampling site at which *M. edulis* was observed. Although shell length and sample size were small, only *M. trossulus* was recorded in Makkovik (7 mussels) and Hopedale (3 mussels). Shell length for these populations ranged from 12.4 to 36.8 mm (mean length 19.6 mm). The three sites around Voisey's Bay (Anaktalak Bay, Kangeklukuluk Bay and Kangeklualuk Bay) also had monospecific populations of *M. trossulus*. Sample size for these populations was relatively large (total of 125 mussels) and shell length ranged from 50.1 to 78.8 mm (mean length 62.1 mm). The mussel population at Arviat (Hudson Bay) was also monospecific for mussels ranging from 12.6 to 67.4 mm, but all mussels were *M. edulis* (N = 20).

Mussels collected in 2000 from the subtidal area at Chance Cove ranged from 28.5 to 62.2 mm (mean 41.1 mm), and a higher frequency of *M. edulis* (65.7 %) than *M. trossulus* (21.9 %) and hybrids (12.3 %) was observed (figure 2.4A). Only small mussels were collected in 2001 from Chance Cove (2.6 to 6.3 mm, mean 4.5 mm) and no difference was observed among the intertidal and the two subtidal collection sites, but species frequencies were the opposite of those recorded in 2000 (fig. 2.4A). *M. trossulus* was the most common species (mean 74.9 %) and *M. edulis* and hybrids showed mean frequencies of 4.5 % and 20.6 %, respectively. Samples from Bellevue also showed a contrasting pattern. The protected area exhibited a high frequency of *M. edulis* (62.8 %), whereas a high frequency of *M. trossulus* (76.6 %) was observed at the exposed area (fig 2.4B). However, shell length at the protected area (36.2 to 74.4 mm, mean 47.3 mm) was greater than at the exposed area (2.3 to 42.1 mm, mean 16.1 mm). Species frequencies at Long Harbour (LH) were similar for *M. edulis* (42.6 %) and *M. trossulus* (43.5 %), but a

second sample taken at different depths in 2003 exhibited marked differences in species frequency relative to depth (chapter 6).

The number of hybrid individuals identified in a given sample should be directly related to the number of markers used to genotype individuals (chapter 3). Nevertheless, hybrids were observed at all sites where both species co-occur regardless of the number of markers used. At most sites, hybrids showed a size frequency distribution similar to *M. trossulus*, although at Gilbert Bay and Drac Bay hybrids increased in frequency in the larger sizes.

A summary of the main environmental characteristics for the aquaculture sites is presented in table 2.1. Charles Arm (49°34'N, 55°27'W) is a semi-closed inlet where almost all mussels are *M. edulis*. The site has a soft bottom with an average depth of approximately 15 meters, and has several freshwater inputs. Salinity can reach as low as 16.9 ppt in the spring and the maximum temperature reaches around 18°C during the summer. Salmonier Cove (47°58'N, 55°78'W) is a very deep (over 200 m) inlet where *M. trossulus* is abundant in smaller size classes. This is the only site sampled in which significant ice cover does not occur, and the temperature rises more slowly during the spring than at the other sites. Cap Cove (48°37'N, 53°37'W) is a protected cove with several freshwater inputs. The site has a soft bottom and is relatively shallow (approximately 20m deep). Reach Run (49°41'N, 54° 68'W) is also shallow (15 m) with a soft bottom, but ocean currents are stronger in this area. Relatively low salinities (18.5 ppt) can be observed during the spring and high temperatures (20.3°C) occasionally occur during the summer. Drac Bay (51°17'N, 55°98W) is the most northern site sampled in Newfoundland and temperatures are relatively lower than at the other sites. Several freshwater inputs are present and salinity can drop to 25.4 ppt during the spring. Although the Shellfish Environmental and Biological Monitoring Program (EBMP) did not collect samples from Long Harbour regularly, the site is anecdotally known to contain high quantities of organic matter. Sewage from the Long Harbour community is released directly into the area, therefore the grower has a permit to collect spat, but not to grow mussels. The shallow water (approximately 15m), sustained high temperatures during the summer and organic deposition likely increase primary productivity in the area. A

thermograph deployed in 2001 and 2002 registered a maximum temperature of 19.2°C and a mean temperature of 13.3°C during the summer.

2.3.2. Responses to temperature stress

2.3.2.1. Clearance rate

An acute response was observed in which clearance rate (CR) greatly increased immediately (1 day) after transfer from 4°C to 12°C in all genotypes, but there was no significant difference among genotypes (Anova, $p > 0.05$). After one week at 12°C CR decreased for all genotypes and after two weeks animals were almost completely acclimated, CR being slightly higher than the initial values (figure 2.5A). *M. edulis* showed a significantly greater CR than *M. trossulus* after 7 days (Anova, $p < 0.05$). After 14 days at 12°C CR for *M. edulis* was greater than for backcrosses (Anova, $p < 0.05$), but no significant difference was observed among the other genotypes.

Trials in which mussels were transferred from 12°C to 20°C showed a different physiological response. Instead of an increase in CR after the temperature shock, a pronounced decrease was observed in *M. trossulus* and backcrosses (figure 2.5B). Although the apparent increase in CR observed in *M. edulis* and hybrid ET after 1 day at 20°C was not significantly different from the initial values, CR was significantly higher than in *M. trossulus* and backcrosses (Anova, $p < 0.05$). A gradual recovery was observed for *M. trossulus* and backcrosses at days 7 and 14, but values did not reach those observed before the temperature change. Clearance rate in *M. edulis* and the two kinds of F1 hybrids did not respond significantly to the temperature increase at any sampling date. All genotypes showed a consistent pattern in the several trials and repetitions performed. As animals of approximately the same size were chosen for the experiment, mean shell length and wet weight were very similar among the genotypes (Anova, $p > 0.05$), therefore there was no need to correct CR values for the tissue weight of each individual before analysis. Finally, 4 *M. trossulus*, 3 hybrids (TE) and 7 backcrosses died before the

measurements on day 14 at 20°C, but no mortality was observed for *M. edulis* in any of the treatments.

2.3.2.2. Mortality

Results for the experiment in which mussels were kept at 24°C after spawning were clear (figure 2.6). Mortality was observed in both kinds of F1 hybrids and *M. trossulus* a few days after the experiment initiated. Mortality occurred at a relatively constant rate for these groups and after 36 days very few animals were still alive. At the end of the experiment survival was only 11.4 % for *M. trossulus* and 31.4% and 37.1% for F1 hybrids ET and TE respectively (fig. 2.6). On the other hand, *M. edulis* suffered very little mortality and 94.3 % of *M. edulis* individuals were still alive after 36 days at 24°C.

2.3.3. Egg size, egg composition and gametogenesis

The egg diameter of *Mytilus edulis* (63.5 ± 2.6 μm, SE) was significantly greater than that of *M. trossulus* (59.4 ± 2.8 μm) (Anova, $p<0.01$, figure 2.7). Egg diameter in hybrids was not significantly different from that of *M. trossulus*, but the sample size was small (N= 5). There was no significant correlation between egg diameter and shell length of the female.

Lipid analysis produced a preliminary indication of differences between the two species. The most common lipid classes in all samples were triacylglycerol (TG), acetone mobile polar lipids (AMPL) and phospholipids (PL). Lipid class composition was similar for the two species, but *M. edulis* had significantly more TG, while *M. trossulus* had more AMPL (Anova, $p < 0.05$, table 2.2). Fatty acid composition showed similar results for both species. The major fatty acids were 16:0, 16:1w7 and the essential fatty acids 20:5w3 and 22:6w3 (table 2.3). Small differences in some fatty acid classes were observed between the species, but the polyunsaturated acid 22:6w3 was significantly more abundant in *M. trossulus* than in *M. edulis* (Anova, $p < 0.05$).

In the laboratory cohort in which gonad development was followed for 9 months, many more *M. trossulus* individuals than *M. edulis* underwent gametogenesis in the first reproductive season (fig. 2.8). Most *M. edulis* individuals (77.9 %) were classified as '0", meaning that no gametes were observed in these individuals, while most *M. trossulus* were classified as "3". Only 5.6 % of *M. trossulus* individuals did not show any signs of gonad development. A maternal effect in gonad development was evident in F1 hybrids. Hybrids from *M. edulis* mothers (hybrids ET) showed gonad development similar to *M. edulis* and hybrids TE were similar to *M. trossulus*. All animals had been kept in the laboratory and monitored periodically since the larval stage, so the summer of 2003 was certainly the first time that any individual produced gametes. In addition, 54 individuals were investigated between March and June and another 62 individuals between September and November, ruling out the possibility that *M. edulis* developed gametes outside the usual reproductive season (July and August).

2.4. Discussion

The study of *Mytilus* hybrid zones has been instrumental in clarifying several aspects of marine speciation. Although the area of contact between *M. edulis* and *M. trossulus* in the northwest Atlantic has been studied much less than other *Mytilus* hybrid zones, recent evidence has shown that the two species differ in genetics, geographic distribution, ecology, physiology and life-history traits (Bates and Innes, 1995; Mallet and Carver, 1995; Comesaña et al, 1999; Rawson et al., 2001; Toro et al., 2002). Physiological differentiation may be an important factor contributing to reproductive isolation, since discrete biogeographic distributions reduce the likelihood of hybridization (Hilbish et al., 1994). The present study provides evidence that temperature is an important factor affecting physiological activity and consequently partially determines both species distribution and interactions between species. Clearance rate was strongly depressed for *M. trossulus* and some hybrid individuals at 20°C, suggesting that this species is less adapted to warm environments than *M. edulis*. The lower capacity of *M. trossulus* for food intake at high temperatures likely reduces its energy balance.

Furthermore, the CR of *M. edulis* was greater than that of *M. trossulus* at 12°C, suggesting that more energy is available for growth if other components of scope for growth remain the same in both species. Although CR is an important component of individual production and scope for growth, other factors such as oxygen consumption, absorption efficiency and nitrogen excretion should also be considered for a better understanding of how the two species differ in their physiological response to temperature. Local adaptation to environmental changes within species may also be important to the distribution and fitness of species. Thompson and Newell (1985) observed that CR was considerably reduced at 25°C in a more northerly *M. edulis* population (Newfoundland), whereas mussels from a southern population (New York) were able to maintain a higher CR under these conditions. More comprehensive studies on the overall energy budget should clarify how the two species react physiologically to a temperature increase, but the present results provide clear evidence that they differ considerably at 20°C, at least for the variable measured. In addition, the high mortality observed only for *M. trossulus* and some hybrids under these conditions suggests that 20°C is close to the upper limit of temperature tolerance of these genotypes. Temperatures above 20°C are occasionally observed at sites around Newfoundland, and during the summer may remain above 18°C for several days at some of the mussel farms sampled. Semi-enclosed inlets and shallow waters often experience temperatures above average.

The second experiment, in which mortality at 24°C was observed for 36 days, clearly demonstrates that the two species are physiologically differentiated for their upper limit of temperature tolerance. Most *M. edulis* individuals survived under these conditions (94.3 %) whereas very low survival was observed for *M. trossulus* (11.4 %). The response of hybrids was intermediate to the parent species, a pattern observed for several other characteristics that have been studied and discussed in detail in chapters 4 and 5. In addition to the temperature shock induced in the laboratory, mussels were probably physiologically stressed during transportation from the mussel farms to the laboratory and by the spawning event that occurred one week before the experiment.

Temperature is probably not the only factor responsible for the differential mortality between genotypes and the observed geographic distribution. The combination

of temperature with other factors such as energy reserves, metabolic efficiency, life-history traits or even salinity may decrease survival and limit the establishment of stable populations in some areas. Summer mortality in *Mytilus* has been commonly observed in the Canadian Maritimes, but so far no study has directly compared differences in mortality between the two species. Tremblay et al. (1998a) observed differential mortality between two populations of *M. edulis* in the Magdalen Islands, proposing that the variation in resistance to summer mortality could be a consequence of differential metabolic response associated with the degree of heterozygosity (Tremblay et al., 1998 b, c). It has also been suggested that the low frequency of *M. trossulus* observed in the area (<10%) is partly explained by the shallow waters (3 to 7 m) and by high summer temperatures (>20°C) (Myrand and Gaudreault, 1995). Low frequencies of *M. trossulus* have also been reported from Prince Edward Island and the Northumberland Strait, where the shallow waters usually experience high temperatures during the summer (Mallet and Carver, 1995). On the Pacific coast of North America, *M. trossulus* also experiences considerable summer mortality, and Hofmann and Somero (1996) observed signs of stress at temperatures as low as 13°C. Differential temperature tolerance is probably responsible for the more northerly distribution and the geographic displacement of *M. trossulus* in relation to *M. galloprovincialis* (Sarver and Foltz, 1993; Geller et al. 1994; Rawson and Hilbish, 1995a). The metabolic efficiency of enzymes is strongly affected by temperature, and consequently enzymes work optimally within a specific temperature range (Freeman and Herron, 2001). Enzyme kinetic properties can be a species-specific characteristic, therefore if *M. trossulus* is better adapted to lower temperatures, the metabolic cost in warmer waters should increase as enzyme efficiency decreases. The intermediate fitness observed in hybrids can be explained by the production of "mixed enzymes" or both enzyme variants that could confer better adaptability than either of the parental variants alone.

The results for the static cohort analyses also support the hypothesis that the two species differ in their adaptation to the environmental conditions of Newfoundland. The decrease in frequency of *M. trossulus* observed in the larger size classes at most sites suggests that differential mortality may be responsible for the pattern observed. Although

static cohort analysis may mask real shifts in species frequency if larval recruitment changes from year to year, the consistent pattern observed for all sites and the close similarities to the results observed by Comesaña et al. (1999) point to a real life-history difference. However, differential mortality, although very likely, may not be the only mechanism accounting for the shift in species frequency as the cohort ages. The results for other life-history traits provide evidence that *M. trossulus* is more of an "r" strategist than *M. edulis*, reproducing earlier and spawning smaller eggs (figs. 2.7 and 2.8). Earlier reproduction and spawning smaller eggs are important characteristics of invasive and opportunistic species, and a shorter life span would represent a trade-off consistent with these reproductive traits (Ricciardi and Rasmussen, 1998). The strategy of reproducing earlier and dying earlier could allow the maintenance of a stable population with a shorter generation time, even in areas of intense competition with *M. edulis*, accounting for the lower frequency of *M. trossulus* in the larger size classes. Opposing this hypothesis is the fact that the shell length of *M. trossulus* from the 3 year-old cohort (60 – 80 mm size class) was very similar to that of *M. edulis*, suggesting similar growth and adult size in the two species. Also, monospecific populations of *M. trossulus* in Labrador (around Voisey's Bay) contained some very large individuals (above 75 mm), although age could not be determined. Finally, chapter 5 presents results from a laboratory produced cohort followed for 3 years at mussel farms, in which *M. trossulus* showed similar patterns of growth but higher mortality than *M. edulis* (figures 5.9 and 5.10). Mortality for both species in this experiment was very low until the first reproductive season, in which mortality increased in all genotypes, but *M. trossulus* and hybrids showed much greater mortality than *M. edulis* after spawning. The fact that no significant mortality occurred until the summer in which mussels were first reproductively active supports the hypothesis of differential mortality caused not necessarily by temperature stress alone, but possibly by a combination of temperature and other factors. However, these mussels were kept in pearl nets at low densities, which may explain the low mortality observed in the first two years, since these conditions prevented predation and interspecific competition for space and food. Differences in susceptibility to predators and strength of attachment to the substrate occur between *M. edulis* and *M. galloprovincialis* and may contribute to the

shift in species frequencies relative to size observed in Europe (Gardner, 1994a). Assuming that *M. trossulus* is more adapted to northern latitudes, where species diversity, competition and predation pressures are different, the species could be at a disadvantage when competing with *M. edulis* in the hybrid zone, especially if animals are already weakened by temperature stress.

The present results on temperature tolerance may partly explain the latitudinal displacement observed in the macrogeographic distributions of the two species. *M. trossulus* has a southern limit on the coast of Maine (or possibly Cape Cod) while *M. edulis* occurs further south to Cape Hatteras (Koehn et al., 1984; Koehn, 1991; Rawson et al., 2001). Defining the northern distribution of both species and the boundary of the hybrid zone will require more detailed study. However, the present observation that both species may be equally adapted to 4°C suggests that low temperatures may not limit the settlement of *M. edulis* population at northern latitudes. During the winter in Newfoundland and Labrador most sites experience sustained temperatures below 1°C for several weeks and physiological adaptation may be important to maintain a positive energy balance for prolonged periods. The fact that *M. edulis* in Europe occurs at very high latitudes on the coasts of Iceland, Norway and Arctic Russia (Varvio et al., 1988; Hummel et al., 2001) implies that low temperatures do not limit the species in these areas. However, *M. edulis* from the west Atlantic could differ physiologically from east Atlantic populations if these two populations were geographically isolated during part of the glacial periods and west Atlantic populations remained in a warmer refuge and adapted to warmer conditions. Discrete differences at allozyme loci between amphi-Atlantic populations suggest some level of genetic differentiation (reviewed in Gosling, 1992b), strengthening the hypothesis that the secondary contact in the northwest Atlantic occurred with an *M. edulis* population that was isolated somewhere in North America for at least part of the glacial periods. Wares and Cunningham (2001) used mtDNA and coalescent methods to demonstrate a long history of an *M. edulis* lineage in North America that was genetically disconnected from European populations during the glacial periods, although limited gene flow between the two populations may have occurred at some point. The authors also compared amphi-Atlantic populations that presumably occupied the Atlantic

after the first trans-Arctic invasion (see Vermeij, 1991), and concluded that the harsher environmental conditions in North America during the glacial events probably caused more extinction of North American species than European species. However, species with planktonic larvae may have preferentially persisted in North American refuges. Therefore, the northern limit of *M. edulis* and the position of the hybrid zone may reflect migrational stochasticity. The secondary contact likely occurred during an interglacial period or after the last glacial maximum, when *M. edulis* presumably expanded to the north from a temperate refuge in North America and *M. trossulus* expanded to the south from the Arctic Ocean when the Arctic route was reopened. It is also possible that the expansion of both species is still occurring (as discussed in chapter 6) but differential adaptation and competition prevent or slow down the establishment of permanent populations in areas where the other species is dominant. A very similar pattern is observed in the *Macoma balthica* complex, in which fragmented populations in the Atlantic probably reflect an initial Pliocene invasion when the Bering Strait first opened followed by repeated invasions during the Pleistocene and Holocene (Väinölä, 2003). In this complex, the recently re-invaded taxa usually occupies marginal environments such as the Baltic Sea, and hybridization occurs in areas of contact with previously established populations (Väinölä, 2003).

Data from the highest latitude site studied in Newfoundland (Drac Bay, Northern Peninsula) showed that the shift in species frequency is not as marked in this area and *M. trossulus* frequency in the largest size class (60 – 80 mm) is relatively high (30.8%) in comparison with sites further south. Penney and Hart (1999) reported even higher frequencies of *M. trossulus* in this area for commercial mussels (above 46 mm), and Innes et al. (1999) also observed a dominance of *M. trossulus* in a survey of 10 months old individuals. Goose Arm and Gilbert Bay, two other high latitude sites, also showed greater frequencies of *M. trossulus* and a pattern of size distribution similar to Drac Bay. The fact that Gilbert Bay was the most northerly site at which *M. edulis* was found, the other five populations sampled in Labrador being monospecific populations of *M. trossulus*, implies that the latter is predominant and probably has a competitive advantage

in the area. Ocean current patterns may also limit the expansion of *M. edulis* to the north and may play an important role in the distribution of both species, as discussed below.

The high frequency of *M. trossulus* in the smallest size class (20 – 40 mm) observed in almost all the sampled sites suggests that there is a considerable influx of larvae of this species in Newfoundland. There are basically two different and non-exclusive hypotheses to explain the pattern observed: local recruitment and dispersal by ocean currents. Assuming that *M. trossulus* indeed reproduces at an earlier age than *M. edulis* (figure 2.8) and has a greater reproductive investment (Toro et al., 2002), it is possible that the larval production of these smaller but more abundant *M. trossulus* could maintain a large population if larvae do not disperse too far. Toro et al. (2004) suggested that the species distribution observed in Trinity Bay, Newfoundland could be partly due to local recruitment, but immigration cannot be ruled out. The composition of a recruiting population should resemble the genetic composition of the source population, but the planktonic larval phase probably allows the mixture of larvae from several population sources, obscuring the relationship (Pedersen et al., 2000). If local recruitment were the main factor responsible for the species composition, then we would expect little variation in species composition between the established population and the recruiting population. Although a marked shift in species frequency with mussel size was observed at almost all sites, *Mytilus* shows an age dependent reproductive effort (Bayne et al., 1983) which should be balanced against the size structure of the effective population to compare local larval production with recruitment. Alternatively, the higher frequency of *M. trossulus* observed in the smaller size classes may be a consequence of immigration from outside the hybrid zone since larvae produced further north drift into Newfoundland with the Labrador Current. Allopatric populations of *M. trossulus* on the coast of Labrador (around Voisey's Bay) are abundant (Perron-Cashman, personal communication) and very large mussels (> 75 mm) are found in that area, suggesting that *M. trossulus* larval production is potentially very high in the region. The Labrador Current flows southward at a mean speed of 0.15 m s^{-1} (Colbourne et al., 1997, figure 2.9). Considering a larval period of at least 3 or 4 weeks (Bayne, 1976), larvae produced in Labrador could travel hundreds of kilometers to reach Newfoundland and other areas such as the Gulf of St. Lawrence. A

current model developed for cod larvae predicts that particles originating on the Hamilton Bank, off the coast of Labrador, will reach Newfoundland (Notre Dame, Bonavista and Trinity Bays) after 40 or 50 days (Davidson and deYoung, 1995). Assuming a similar larval period for mussels, the source populations providing at least part of the juveniles recruited in Newfoundland may well be from Labrador. However, the settlement of *M. trossulus* larvae in unsuitable areas where the species in not completely adapted may result in the high mortality observed and the formation of unstable populations. Wells and Gray (1960) observed a similar phenomenon, in which *M. edulis* larvae are carried south of Cape Hatteras and, as soon as summer temperatures reach about 27°C, these temporary populations suffer total mortality. The hybrid zone between *M. edulis* and *M. galloprovincialis* in England, in which a great larval input by *M. edulis* is counter-balanced by a higher mortality of this species (Skibinski and Roderick, 1991; Gardner et al., 1993; Hilbish et al., 2002) shows a very similar pattern to the one observed in the northwest Atlantic. A model developed to understand the origins of the larvae in the hybrid zone in southwest England suggests that 80 to 90% of the larvae are immigrants from pure population of *M. edulis* while only 10 to 20% originate inside the hybrid zone (Wilhelm and Hilbish, 1998). More recently, Gilg and Hilbish (2003) demonstrated that the genetic composition of mussel populations in southwest England is highly influenced by ocean currents.

The observation that samples from Arviat (Hudson Bay) contained only *M. edulis* raises the question whether this is an isolated population or if these mussels are genetically connected to other *M. edulis* populations. Koehn et al. (1984) reported a pure population of *M. edulis* close to Arviat in Hudson Bay, but samples from the Hudson Strait were all *M. trossulus*. It is possible that *M. edulis* has a patchy distribution along the coast of Labrador and the present study did not detect *M. edulis* because of the small sample sizes from Hopedale and Makkovik. Furthermore, the sites sampled around Voisey's Bay may represent areas where specific environmental characteristics favour *M. trossulus*. This area contains high concentrations of nickel (Veinott et al., 2001) and differences in tolerance to heavy metal concentration may benefit *M. trossulus*. Alternatively, and more likely, Hudson Bay contains isolated populations of *M. edulis*

that found an environmental refuge there after larval migration from Greenland or Iceland. The West Greenland Current forms a branch that enters the Hudson Strait (Chapman and Beardsley, 1989, figure 2.9) and may have brought, or may periodically bring, *M. edulis* larvae from Iceland or Greenland. Finally, accidental or intentional introduction could also explain the presence of *M. edulis* in Hudson Bay, as there is a shipping route from the port of Churchill during the summer. More data from northern Labrador, Greenland and the Hudson Strait should clarify if Hudson Bay represents a refuge for *M. edulis* or if the population is genetically related to European or North Atlantic populations. Apart from the origin of Hudson Bay populations, a second and unstudied hybrid zone may occur between *M. edulis* and *M. trossulus* in the Hudson Strait or somewhere inside Hudson Bay.

The patchy distribution of both species and their hybrids observed in the Canadian Maritimes may also be explained by microgeographic differences in environmental conditions and larval dispersal patterns. Adaptation to different environments is the primary explanation for the pattern observed in mosaic hybrid zones. The classic example of a mosaic hybrid zone is that of the cricket (*Gryllus pennsylvanicus* and *G. firmus*) in eastern North America, in which the distribution of both species is closely related to soil type (Rand and Harrison, 1989). Another well studied mosaic hybrid zone (the fire-bellied toads *Bombina bombina* and *B. variegata* in eastern Europe) shows a correlation between the presence of predators in certain environments and a patchy distribution due to the evolution of different strategies to prevent predation (Vines et al., 2003). In the *Mytilus* hybrid zone in the eastern Atlantic, Bierne et al. (2003a) observed that habitat specialization is an important factor controlling species distribution that also contributes to interspecific barriers to hybridization. Natural selection plays an important role in maintaining very structured populations, even in the ocean where gene flow is facilitated by larval dispersal. Koehn and collaborators (Koehn et al., 1980; Koehn, 1983; Hilbish and Koehn, 1985), in a very influential study on *Mytilus edulis*, demonstrated that despite extensive gene flow due to larval migration, selection at the leucine aminopeptidase locus (LAP) produced a steep cline in allele frequency in Long Island Sound which was closely correlated with a salinity gradient. Although Gardner and

Thompson (2001) did not find any significant difference between *M. edulis* and *M. trossulus* in their physiological response to salinity in laboratory experiments, the authors suggested that other environmental variables should be studied in association with salinity. The great shift in species frequency over a few meters observed in the present study (fig. 2.4A and B) suggests that the microgeographic distributions of the species may be influenced by some environmental factor. Furthermore, intermediate fitness of hybrids is incompatible with endogenous selection models. Although chapter 5 provides evidence of lower fitness for some kinds of hybrids, these generally showed a *M. trossulus*-like genetic background and behaved like *M. trossulus*, and in several instances hybrid fitness was intermediate to that of the parent species. Therefore, in addition to other pre and postzygotic barriers to hybridization, habitat specialization may also play an important role in maintaining the hybrid zone and probably contributes to the observed patchy distribution.

The results of the present study suggest that the species differ considerably in the life-history traits examined. The production of smaller eggs by *M. trossulus* may be under genetic control, although it could also be the result of specific exogenous factors in the area. A higher energetic cost of maintenance as a consequence of physiological stress and reduced adaptation to the local environment may result in less energy available for reproduction and consequently the production of smaller eggs. Bayne et al. (1983) showed that egg size did not differ significantly for *M. edulis* populations in relation to several natural environmental conditions studied, although Bayne et al. (1978) observed that stressed females in the laboratory spawned smaller eggs with lower lipid and protein content than non-stressed mussels. Samples from areas where *M. trossulus* seems to be better adapted than *M. edulis*, such as Voisey's Bay, should clarify if the species produces smaller eggs than *M. edulis* regardless of the environment, or if the results observed in the present study are applicable only to Newfoundland. The production of different sized eggs may also reflect character displacement in the hybrid zone. Character displacement, defined as the evolution of alternative strategies to meet competition where two similar species co-occur, is commonly observed in hybrid zones (Grant, 1972). In this case, sampling *M. trossulus* populations outside the hybrid zone would again clarify if the

production of smaller eggs is an intrinsic characteristic of the species or part of a life-history strategy to counter *M. edulis* competition. Although possible, character displacement is less likely, since the high gene flow facilitated by larval dispersal would eventually erode trait differences that had evolved within the hybrid zone. Toro et al. (2002) also observed smaller eggs in *M. trossulus* from Trinity Bay, an area where both species show a similar frequency, therefore the differences observed in the present study may indeed represent long term evolutionary processes rather than a response to local conditions.

Although the correlation between female length and egg diameter was not significant, the observation that the four largest mussels used were from Charles Arm and they produced the largest eggs invites further research. The considerable difference between the number of eggs spawned by 2 year old mussels cultured in the laboratory and 3 year old mussels from a mussel farm (chapter 5, figure 5.11) indicates that age and physiological condition may determine the number of eggs a female can produce. Fecundity and reproductive effort in *M. edulis* increase with age (Bayne, 1976; Bayne and Worrall, 1980, Bayne et al., 1983, Thompson 1984), but no relationship between egg size and female size has been established. Coincidentally, the four mussels that spawned the largest eggs were all collected from Charles Arm, a site where *M. edulis* is predominant, therefore specific environmental conditions and/or a positive energy balance could induce some individuals to produce more or larger eggs. However, the fact that very few large *M. trossulus* individuals were spawned limits the conclusions that can be drawn. Further studies are required to investigate how the age of mussels and environmental conditions affect egg size for both species. Also, the adaptive significance of smaller eggs in *M. trossulus* requires more attention, since this is a typical characteristic of invasive and opportunistic species that may be critical for a successful life-history strategy and the maintenance of the hybrid zone.

The differences in egg composition between the two species also suggest that the species differ in reproductive aspects that may affect life-history traits. Although only preliminary results are presented here and the energy invested per egg could not be quantified, the observation that eggs from both species were qualitatively different may

reflect distinct reproductive strategies. A temporal displacement in gonad development could result in the exploitation of different species of algae as a source of energy that optimize reproductive effort for each species. Toro et al. (2002) observed that *M. edulis* has a marked spawning peak whereas *M. trossulus* spawns throughout the whole summer in Newfoundland. This could not only prevent extensive hybridization, but also allow *M. trossulus* a competitive advantage during the settlement period, a critical stage that requires optimum conditions in order to guarantee survival. Alternatively, different egg composition may reflect distinct modes of energy utilization. If *M. trossulus* is better adapted to colder and nutrient poorer environments, its resources for reproduction are more restricted. Therefore, the differences in lipid composition may represent evolutionary divergence in genetic adaptation as well as a reduced energy balance for *M. trossulus* in areas where the species is less well adapted to the local environmental conditions.

The observation that *M. trossulus* reproduces at a younger age is another important characteristic of a fugitive species that may be critical to the persistence of *M. trossulus* in the hybrid zone. Clearly, many more *M. trossulus* individuals than *M. edulis* underwent gametogenesis after 3 years under laboratory conditions (figure 2.8). These laboratory results may not reflect development in the field but, comparatively, they show important differences in life histories of the species. Mussels kept continuously submerged at mussel farms usually undergo gametogenesis in the second summer after they settle, although wild mussels may take longer if they are periodically exposed to air, wave action or any other stress factors. As mussels usually reproduce only once a year, earlier reproduction in *M. trossulus* may represent a whole year of advantage depending on the environmental conditions. Therefore, early reproduction may be an important means of maintaining *M. trossulus* populations and should provide a mechanism for coexistence with *M. edulis* in the hybrid zone.

The *Mytilus* hybrid zone in the northwest Atlantic has a more complex structure and dynamic than we understand at the moment, but the present study adds important information on the geographic distribution and on physiological and life-history traits that may influence the observed pattern. As emphasized by Arnold (1997), the dynamics of

hybrid zones may be the result of interaction between endogenous and exogenous factors. Endogenous factors are likely present as gamete incompatibilities (chapter 4 and Rawson et al., 2003) or detrimental genetic interactions that reduce hybrid fitness (chapter 5). Wave exposure (Bates and Innes, 1995), spawning behaviour (Toro et al., 2002) and environmental characteristics (Mallet and Carver, 1995) have also been suggested as important factors in the maintenance of this mussel hybrid zone. Possibly, all these factors, as well as others yet unknown, are important for maintaining the hybrid zone structure. In addition to the interactions between the species where they hybridize, it is also very important to understand the intrinsic differences between the two species that will ultimately define species fitness in areas of contact and help to define the dynamics of this hybrid zone.

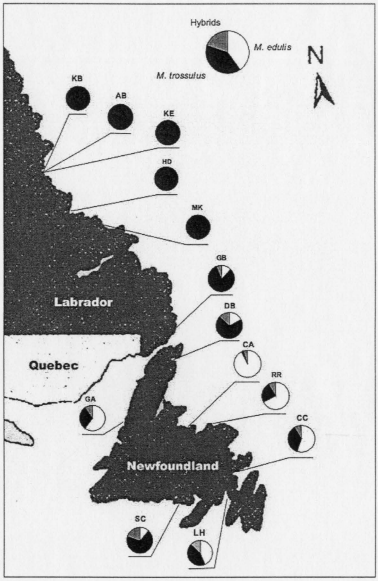

Figure 2.1. Frequencies of *M. edulis*, *M. trossulus* and their hybrids at sampling sites in Newfoundland and Labrador. N = 30 mussels per site, shell length 40 to 60 mm, except for Labrador samples. For site codes see text.

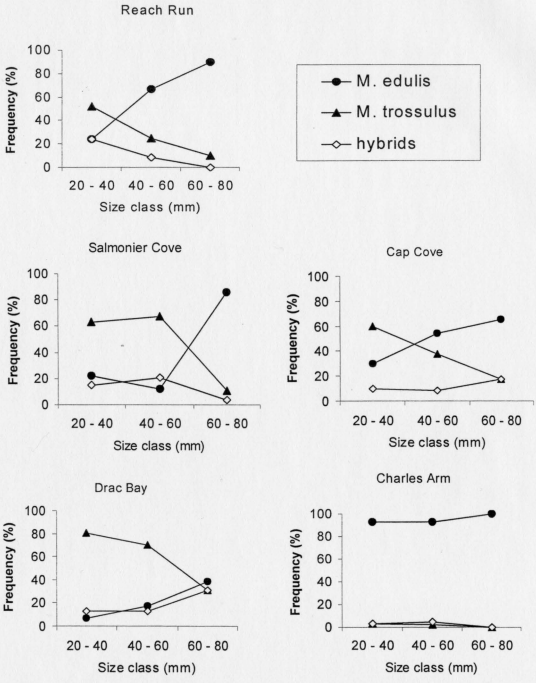

Figure 2.2. Frequencies of *M. edulis*, *M. trossulus* and their hybrids at five aquaculture sites. Mussels divided into three shell length classes. N = 30 mussels per size class for each site.

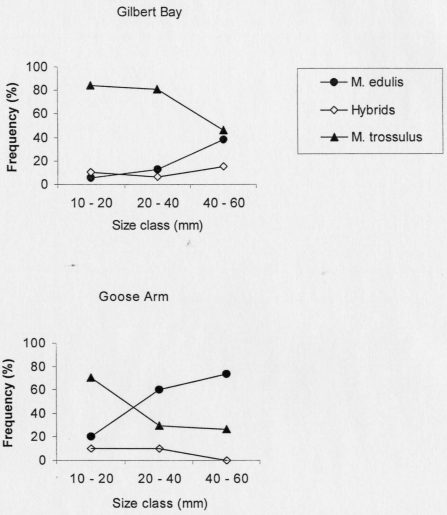

Figure 2.3. Frequencies of *M. edulis*, *M. trossulus* and their hybrids at Gilbert Bay and Goose Arm. Mussels divided into three shell length classes. N = 20 mussels per size class for each site.

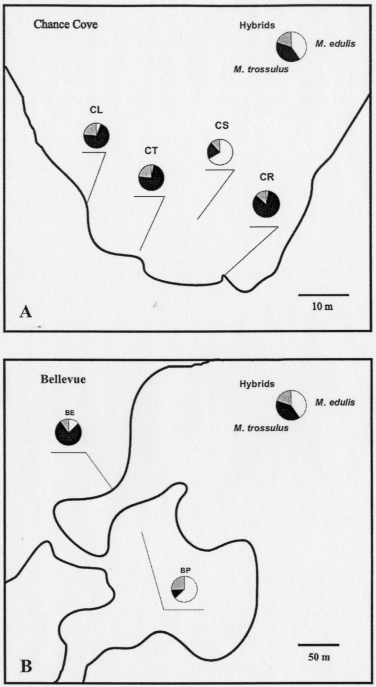

Figure 2.4. Microgeographical distributions of *M. edulis*, *M. trossulus* and their hybrids in Chance Cove and Bellevue. N = 120 mussels in Chance Cove (30 per site) and 60 mussels in Bellevue (30 per site). See results for differences in shell lengths.

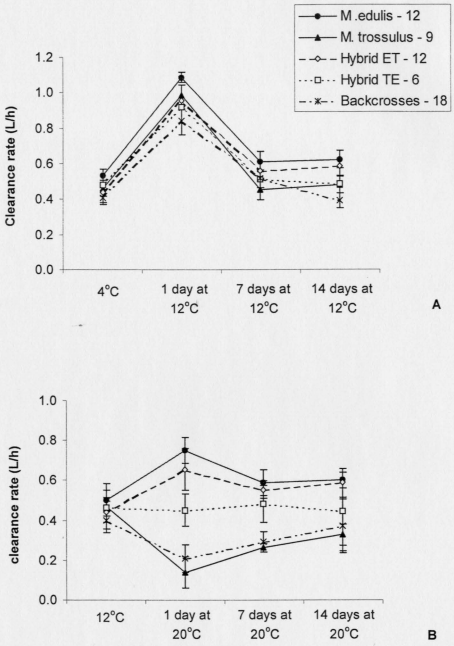

Figure 2.5. Clearance rate (liters per hour) of *M. edulis*, *M. trossulus* and their hybrids when moved from 4°C to 12°C (A) and from 12°C to 20°C (B). Error bars represent standard error. Number after genotypes represent sample size for each sampling date.

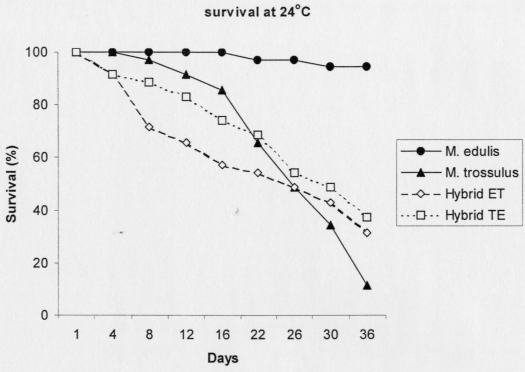

Figure 2.6. Survival of *M. edulis*, *M. trossulus* and their hybrids exposed to 24°C for 36 days after the spawning season (August/September). N = 35 mussels for each genotype.

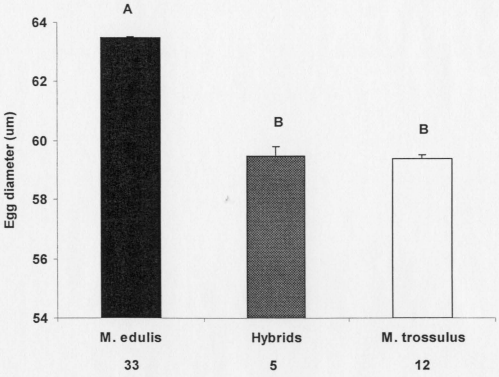

Figure 2.7. Egg diameter for *M. edulis*, *M. trossulus* and their hybrids. Different letters above columns represent significant differences (Anova, $p < 0.05$), error bars represent standard error and numbers below genotypes represent number of females.

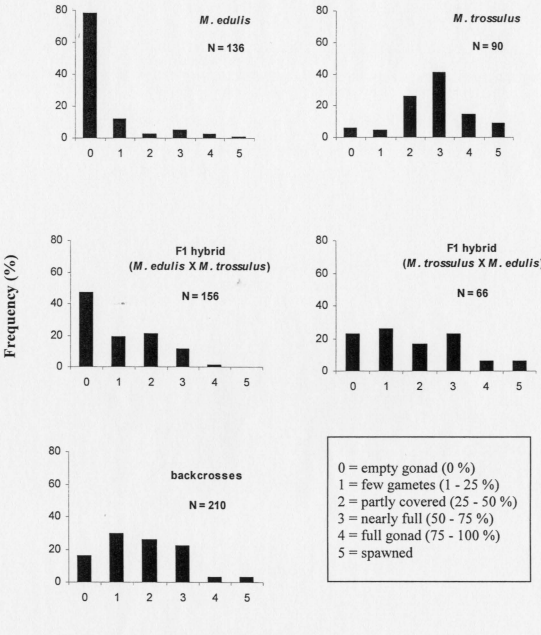

Figure 2.8. Gonad development in laboratory produced mussels after 3 years. Mussels dissected in August 2003. Average shell length 20.25 mm.

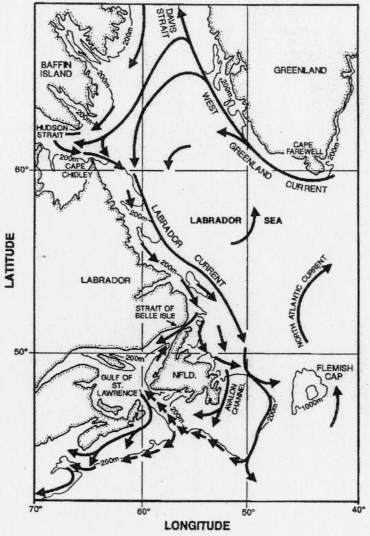

Figure 2.9. Main ocean current systems and current directions in the northwest Atlantic (from Colbourne et al., 1997).

Table 2.1. Seasonal minimum, maximum and mean temperature, salinity and chlorophyll-a for 5 aquaculture sites in Newfoundland. N represents number of samples collected from 1993 to 2001.

			Temperature (°C)				Salinity (ppt)				Chlorophyll-a (ug/L)			
season	site	N	min	max	mean	SD	min	max	mean	SD	min	max	mean	SD
spring	Cap Cove	2	-0.5	12.7	5.1	5.3	30.0	32.4	31.3	0.9	0.3	1.8	0.8	0.5
	Charles Arm	24	-0.9	11.1	2.7	3.1	16.9	32.4	30.2	2.2	0.3	10.0	1.8	1.8
	Drac Bay	11	-1.1	6.2	2.0	1.8	25.4	32.2	30.1	1.4	0.3	8.3	2.2	2.2
	Reach Run	27	-1.3	15.0	4.7	5.0	18.5	30.4	26.6	4.4	0.5	6.7	2.4	1.7
	Salmonier Cove	3	1.4	6.4	4.5	2.0	30.9	32.1	31.6	0.5	0.3	7.9	1.8	2.4
summer	Cap Cove	6	1.4	16.2	10.5	3.9	29.4	32.6	30.7	0.8	0.6	7.4	2.3	2.2
	Charles Arm	36	1.2	17.9	11.4	4.1	23.6	31.4	28.9	1.0	0.0	10.0	3.2	2.0
	Drac Bay	22	3.1	15.3	9.0	2.9	27.9	31.7	30.2	0.6	0.6	5.6	1.9	1.2
	Reach Run	23	8.7	20.3	16.1	2.4	24.2	29.6	28.2	0.9	0.7	15.9	2.8	2.0
	Salmonier Cove	5	10.4	18.3	14.9	2.7	30.0	31.6	31.0	0.5	1.4	3.5	2.6	0.7
fall	Cap Cove	4	2.5	10.7	6.3	3.0	30.1	32.5	30.8	0.8	0.5	2.8	1.1	0.7
	Charles Arm	22	1.4	12.8	6.8	3.3	29.0	31.2	30.1	0.5	0.2	5.1	1.9	0.9
	Drac Bay	11	1.3	10.2	5.3	2.5	29.3	32.2	30.8	0.7	0.5	4.2	1.2	0.9
	Reach Run	23	0.7	14.3	6.6	4.0	27.5	31.5	28.6	0.8	0.6	4.9	2.5	1.2
	Salmonier Cove	4	6.5	13.0	10.4	2.5	30.9	31.4	31.1	0.2	0.5	4.5	2.0	1.6
winter	Cap Cove	1	-0.8	0.4	-0.1	0.0	31.6	31.9	31.7	0.0	1.4	2.7	2.1	0.0
	Charles Arm	9	-1.5	0.6	-0.9	0.6	21.5	32.5	31.5	2.1	0.4	10.0	3.1	3.3
	Reach Run	8	-1.7	0.2	-1.2	0.6	23.6	32.1	29.6	3.0	0.5	3.4	1.3	0.9

Table 2.2. Lipid class composition of eggs from *M. edulis* and *M. trossulus* (mean ± standard deviation). Anova, ** $p < 0.01$. N = 3 females for each species.

Lipids	*M. edulis*	*M. trossulus*
Hydrocarbons	2.56 ± 1.24	2.19 ± 0.77
Steryl Esters/Wax Esters	0.81 ± 0.87	0.47 ± 0.36
Methyl Esters	3.74 ± 1.27	3.69 ± 1.81
Ethyl Ketones	0.04 ± 0.10	0.00 ± 0.00
Methyl Ketones	0.00 ± 0.00	0.00 ± 0.00
Glycerol Ethers	0.00 ± 0.00	0.15 ± 0.31
Triacylglycerol **	41.44 ± 3.06	33.39 ± 1.59
Free Fatty Acids	0.62 ± 0.97	0.67 ± 0.86
Alcohols	0.86 ± 2.10	0.00 ± 0.00
Sterols	2.80 ± 1.65	1.75 ± 0.51
Diacylglycerols	1.14 ± 1.93	1.19 ± 1.16
Acetone Mobile Polar Lipids **	19.64 ± 2.99	29.23 ± 1.18
Phospholipids	26.34 ± 3.20	27.25 ± 1.71

Table 2.3. Fatty acid composition (weight % of total fatty acid) of eggs from *M. edulis* and *M. trossulus* (mean ± standard deviation). Anova, * $p < 0.05$. N = 6.

	M. edulis	*M. trossulus*
14:0	3.43 ± 0.49	2.83 ± 0.24
15:0	0.59 ± 0.06	0.41 ± 0.28
16:0	20.09 ± 3.06	17.99 ± 2.44
16:1w5	0.33 ± 0.21	0.19 ± 0.13
16:1w7 *	9.78 ± 1.12	7.86 ± 0.86
16:2w4	0.51 ± 0.16	0.29 ± 0.20
16:4w1	0.03 ± 0.07	0.03 ± 0.05
17:0 *	0.60 ± 0.11	1.14 ± 0.32
17:1	2.95 ± 1.28	2.41 ± 1.12
18:0	1.89 ± 0.47	2.20 ± 0.65
18:1w11	0.31 ± 0.05	0.28 ± 0.20
18:1w5	0.24 ± 0.27	0.20 ± 0.23
18:1w6	0.10 ± 0.16	0.21 ± 0.24
18:1w7	2.70 ± 0.57	2.19 ± 0.46
18:1w9	3.04 ± 1.81	1.81 ± 0.22
18:2w4	0.24 ± 0.12	0.21 ± 0.14
18:2w6	2.01 ± 1.09	1.47 ± 0.14
18:3w3	1.70 ± 0.45	1.98 ± 0.44
18:3w4	0.07 ± 0.11	0.07 ± 0.14
18:4w1	0.15 ± 0.13	0.04 ± 0.09
18:4w3	4.91 ± 2.21	6.63 ± 3.10
19:0	0.05 ± 0.08	0.03 ± 0.05
20:0	0.00 ± 0.00	0.15 ± 0.30
20:1w11	0.96 ± 0.24	0.92 ± 0.18
20:1w7	0.96 ± 0.13	1.03 ± 0.19
20:1w9	3.43 ± 1.54	3.41 ± 0.31
20:2w6	0.64 ± 0.29	0.55 ± 0.37
20:3w3	0.11 ± 0.12	0.06 ± 0.11
20:3w6	0.03 ± 0.08	0.00 ± 0.00
20:4w3	0.53 ± 0.22	0.72 ± 0.28
20:4w6	1.17 ± 0.49	0.83 ± 0.05
20:5w3	19.64 ± 4.89	20.65 ± 2.93
21:5w3	0.24 ± 0.18	1.00 ± 1.02
22:0	0.00 ± 0.00	0.00 ± 0.00
22:1w11	0.08 ± 0.11	0.05 ± 0.09
22:1w7	1.21 ± 0.33	1.30 ± 0.07
22:1w9	0.05 ± 0.08	0.04 ± 0.08
22:2NIMDa	0.00 ± 0.00	0.11 ± 0.08
22:5w3	1.14 ± 0.11	1.16 ± 0.31
22:6w3 *	12.15 ± 2.48	15.62 ± 0.69

Chapter 3

Genetic identification, inheritance and introgression in a *Mytilus* hybrid zone

3.1. Introduction

Hybrid zones play a central role in evolutionary studies as they provide excellent opportunities to observe the interactions between divergent species (Arnold, 1997). In addition to providing insights into reproductive isolation and speciation, hybrid zones can also reveal patterns of inheritance, introgression, linkage disequilibrium and selection. A detailed understanding of hybrid zones depends on the availability of genetic markers that distinguish the species involved and reveal endogenous and exogenous factors responsible for maintaining genetic integrity. The application of enzyme electrophoretic techniques has allowed a better understanding of several hybrid systems, including the *Mytilus edulis* complex. Previously thought to be only one species, the *M. edulis* complex is now known to include two other genetically distinct taxa, *M. trossulus* and *M. galloprovincialis*. The resolution of this species complex was accomplished primarily from allozyme analysis carried out on mussels sampled from around the world (e.g. Koehn et al., 1984; Varvio et al., 1988; McDonald et al., 1991).

The extensive hybridization observed between *M. edulis* and *M. galloprovincialis* in Europe (Gardner, 1994b) led to several studies in other areas where two *Mytilus* species overlap, making the *Mytilus edulis* complex an important model system to study marine speciation. Enzyme-based studies on *Mytilus* populations in the northwest Atlantic have provided a general understanding of the population structure (Bates and Innes, 1995, Penney and Hart, 1999) but an accurate identification of hybrids has been hampered by the low resolution of allozyme markers. Among several enzyme systems, only mannose phosphate isomerase (Mpi) and esterase (Est-D) are considered diagnostic between *M. edulis* and *M. trossulus* (Varvio et al., 1988; Penney and Hart, 1999). In addition, some allozyme systems may not be neutral markers since selection has been observed for at least one extensively studied locus (Lap) in *M. edulis* populations

(Koehn et al., 1980; Koehn, 1983; Hilbish and Koehn, 1985). Allozymes may also produce redundant results since the most important loci that differentiate *Mytilus* species may belong to a single linkage group (Beaumont, 1994) and some of these loci may be under epistatic control (Hilbish et al., 1994). Therefore, the development of molecular markers that distinguish the species and their hybrids is critical for a further understanding of *Mytilus* hybrid zones. The correct choice of the available markers to answer specific questions is also an important point to be considered as the genotyping effort, expense and desired resolution will vary according to the number and resolving power of the markers used.

Although great effort has been devoted to the development of novel PCR-based markers for *Mytilus* (Inoue et al., 1995; Heath et al., 1995; Rawson et al., 1996a, b), only the combined use of several markers can provide a detailed description of hybridization and reliable discrimination of advanced backcross individuals. The number of markers necessary to identify precisely the genetic composition of individuals from the hybrid zone is an important factor in determining genotyping effort and the desired diagnostic resolution (Boecklen and Howard, 1997). Floate et al. (1994) suggested that up to 50 markers are necessary to differentiate adequately among advanced backcrossed individuals, although Boecklen and Howard (1997) concluded that around 4 markers can provide a coarse classification of individuals in hybrid zones. The use of the few markers available to distinguish *Mytilus* species should then be optimized in order to answer various questions about the species interactions in the different hybrid zones. The frequency of hybrids reported in the northwest Atlantic varies from 5 % (Mallet and Carver, 1995) to an average of 26 % (Comesaña et al., 1999), suggesting that the number and resolving power of markers may be critical in determining the number of hybrids identified. Although Comesaña et al. (1999) reported a higher frequency of hybrids than did previous studies, the authors used a combination of 4 markers to determine that most of the hybrids were backcrosses and only 1.25 % were putative F1 hybrids. To discriminate between the several kinds of hybrids, a hybrid index has been commonly applied which has been highly recommended if several markers are available (Arnold and Hodges, 1995; Jiggins and Mallet, 2000). Only by using several markers can patterns of

inheritance, linkage and introgression be revealed. In addition, the relationship between nuclear and cytoplasmic DNA may have special relevance in *Mytilus* as the unusual doubly uniparental inheritance (DUI, Zouros et al., 1994) may increase the occurrence of cyto-nuclear incompatibilities and therefore influence the dynamics of hybrid zones. In *Mytilus*, there is a male lineage of mtDNA transmitted only from fathers to sons in addition to the usual female lineage inherited by all offspring (Zouros et al., 1994; Skibinski et al., 1994). Hybridization usually breaks down the DUI in some areas (Zouros et al., 1994; Rawson et al. 1996b) and is probably responsible for blocking mtDNA introgression in North America (Rawson et al., 1996b). In contrast, mtDNA introgression has been observed in the hybrid zones between *M. edulis* and *M. galloprovincialis* in Europe (Rawson and Hilbish, 1995a; 1998) and between *M. edulis* and *M. trossulus* in the Baltic Sea (Quesada et al., 1999; Zbawicka et al., 2003; Quesada et al., 2003). Many exceptions to DUI have been reported. Males homoplasmic for only female mtDNA and heteroplasmic females with both male and female mtDNA have been frequently observed (Zouros et al. 1994; Stewart et al., 1995; Rawson and Hilbish, 1995a; Wenne and Skibinski, 1995; Quesada et al., 1996; Saavedra et al., 1997; Garrido-Ramos et al., 1998; Wood et al., 2003). The consequences of DUI and the occasional breakdown of DUI are not fully understood, nor is its relation to the maintenance of mussel hybrid zones.

In addition to allozyme and molecular markers, cytogenetic studies may also contribute to a better understanding of *Mytilus* hybrid zones. Recent studies have shown that *M. trossulus* from the Pacific and *M. edulis* from Europe differ in chromosomal arrangement (Martínez-Lage et al., 1997; González-Tizón et al., 2000), although the karyotypes of northwest Atlantic populations have not been compared. However, if the same situation occurs chromosome differentiation may play an important role in hybridization and the maintenance of the hybrid zone in the northwest Atlantic. Although chromosome hybrid zones have been reported in other species (Rieseberg, 2001), differences in chromosome arrangements between Atlantic *M. trossulus* and *M. edulis* would add another layer of complexity to the dynamics of this hybrid zone. On the other hand, if *M. trossulus* from the Atlantic shows the same chromosomal arrangement as *M. edulis*, then differences between Pacific and Atlantic *M. trossulus* should provide novel

insights into the evolutionary history of *Mytilus*. Other cytogenetic techniques such as C-banding, in situ hybridization to localize the 18s, 5.8s and 28s ribosomal genes and silver staining to localize nucleolar organizer regions may also enhance our knowledge of *Mytilus* species and clarify the evolutionary history and systematic status of several populations (González-Tizón et al., 2000).

The objective of the present study is to provide a better understanding of the available genetic markers for *Mytilus* in order to optimize studies of hybridization. The combined use of natural populations and laboratory-produced families allows a wider comprehension of hybridization and the direct observation of inheritance patterns. The transmission of mtDNA to the progeny is analyzed in laboratory-produced families, so that the precise mode of inheritance and the disruption of DUI can be related to observations from natural populations. Finally, preliminary cytogenetic observations are presented that may provide important insights into the evolutionary history of the genus *Mytilus*.

3.2. Materials and methods

A total of 250 individuals was genotyped with 2 allozyme (MPI and EST-D) and 3 nuclear DNA markers (ME, ITS and MAL-I) to study the diagnostic power of each marker and patterns of introgression (see below for details on markers). Mussels were collected from Reach Run (RR, figure 2.1) in 2001 and only individuals ranging from 26.5 to 63.1 mm (mean length 41.1mm) were used to ensure successful allozyme electrophoresis. After genotyping with the 5 markers, mussels were classified according to a hybrid index. A value of one point was assigned for each *M. trossulus* allele, e.g. individuals homozygous for *M. trossulus* for all loci were classified as 10 and individuals homozygous for all *M. edulis* alleles were scored as 0. Scores from 1 to 4 characterize *M. edulis*-like hybrids, scores from 6 to 9 represent *M. trossulus*-like hybrids and a score of 5 represents putative F1 hybrids or backcrosses homozygous for different species in two or more loci. As both allozyme markers possess multiple diagnostic alleles within each species, species-specific alleles for each enzyme system were combined to form a

synthetic allele for each species (E or T), following the approaches of Skibinski et al. (1980), Rawson et al. (1996b) and Comesaña et al. (1999). The number of hybrids identified by each marker alone and in combination with each other was used to infer the diagnostic power of the markers. Mussels that produced unclear results for any marker were genotyped twice more with that marker, and if the data remained ambiguous the samples were excluded from the analysis. From the total of 250 mussels a subsample of 68 was also genotyped with the mtDNA marker COIII, as were another 112 individuals used as parents to produce laboratory families in 2000, 2001 and 2002.

A total of 523 individuals from 16 families produced in the laboratory was used to investigate inheritance patterns. Two different groups of families were used for the analysis. The first contained families produced in July/August 2000 from wild mussels collected at Chance Cove (2000 cohort). Parents were genotyped with 2 allozyme (Mpi and Est-D) and 5 DNA markers (ME, ITS, MAL-I, COIII and PLII). From a total of 32 families produced in that summer, only 7 families in which parents were heterozygous for at least one marker were selected for the analysis. The second group was produced in August 2002, from which 9 families were genotyped for the study (2002 cohort). Parents used to form these families were F1 hybrids produced in the laboratory in 2000 in addition to cultured mussels collect from Goose Arm (GA, figure 2.1). Of these crosses, 5 were F1 hybrids crossed to F1 hybrids and the other 4 were F1 hybrids crossed either to one of the parent species (F1 backcrosses) or to other backcrosses (advanced backcrosses). Hatchery techniques for spawning and larval rearing are described in chapter 4. After 12 months approximately 30 individuals from each of the 18 families were genotyped with the ME marker. In addition, mussels from three of these families were also genotyped with ITS, five families with MAL-I and four families with COIII. Expected mendelian ratios were compared with observed ratios using a G-test followed by a sequential Bonferroni procedure to minimize the chance of making a type I error.

3.2.1. Allozyme electrophoresis

The digestive gland of each mussel sampled was homogenized in approximately 500 µl 0.01 M dithiothreitol solution and centrifuged for 30 seconds at 13 000 rpm. The

supernatants were then used to genotype individuals with Mpi (mannose-6-phosphate isomerase; E.C. 5.3.1.8) and Est-D (esterase-D, E.C 3.1.1.1). Horizontal starch-gel (Sigma starch) electrophoresis was carried out on 11% gels at 4°C following the procedures of Skibinski et al. (1980) and McDonald and Koehn (1988), except for the Mpi electrode buffer (150mM tris, 45 mM citric acid, pH 7.5). Allele nomenclature follows previous studies (e. g. McDonald and Koehn, 1988; McDonald et al., 1991; Comesaña et al., 1999). The Mpi locus is considered diagnostic for *Mytilus edulis* and *M. trossulus* (Stewart et al., 1995; Comesaña et al., 1999; Penney and Hart, 1999), although Varvio et al. (1988) referred to it as virtually diagnostic. The Est-D locus is also highly diagnostic, with only one allele (which was rare) common to the two species (reviewed in Gosling, 1992).

3.2.2. DNA extraction

Approximately 100 mg of mantle edge tissue from each mussel was used for DNA extraction. Samples were incubated with 200 µg proteinase K (Sigma) in 500 µl lysis buffer containing 50 mM Tris HCl (pH 8.0), 1.0% SDS and 25 mM EDTA at 37°C for 12 to 18 hours. Alternatively, some samples (62) were incubated at 55°C for 2 to 4 hours with no difference in DNA quality. Samples were then extracted with an equal volume (500µl) of phenol: chloroform: isoamyl alcohol (24: 24: 1), the aqueous phase removed and equal volume of 95% ethanol at -20°C added to precipitate DNA. The DNA recovered was dissolved in 200 µl sterile distilled water, although some DNA samples from the 2000 cohort were preserved in 100 µl TE (10mM Tris-HCl, 1mM EDTA) for long-term storage.

3.2.3. Markers

Markers were selected from the literature and only those known to differentiate between *M. edulis* and *M. trossulus* were used. All molecular markers were therefore expected to be diagnostic for the two species.

3.2.3.1. GLU and ME

GLU and ME are two nuclear DNA markers that amplify different parts of the gene that encodes the mussel polyphenolic adhesive protein produced by the pedal gland. The marker GLU (Rawson et al., 1996a) is diagnostic for *M. edulis*, *M. trossulus* and *M. galloprovincialis* and the primer sequences are JH-5 GTAGGAACAAAGCATGAACCA and JH-54 GGGGGGATAAGTTTTCTTAGG. The PCR amplification protocol was adapted from Rawson et al. (1996a) and the reaction mixture contained approximately 100 ng DNA, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 50 pmol of each primer and one unit of Taq polymerase (Promega). Reactions were performed in a programmable thermocycler (MJ Research Inc.) with the amplification buffer supplied by the manufacturer plus sterile distilled water (total volume 25 µl). An initial denaturation at 94°C for 3 min preceded 35 cycles of 94°C for 20 s, 53°C for 20 s and 72°C for 45 s . Approximately 12 µl PCR products were mixed with 1.5µl loading dye (bromophenol blue and xylene cyanol 1:1) and separated on 2.5 % agarose gels (Sigma) stained with ethidium bromide. Electrophoresis was carried out in 0.5X Tris-borate-EDTA (TBE) buffer for 50 min at approximately 100V. All gels were run with a positive control (sample of known genotype), a negative control (no DNA template) and a 1 Kb ladder (GibcoBRL). Digital images of the gels were obtained to confirm genetic identification afterwards. Reaction mixtures and electrophoresis of PCR products followed the same protocol for all DNA markers, although different amplification conditions were used for each marker.

The ME marker (Inoue et al., 1995) is expected to produce DNA fragments of 180 bp for *M. edulis* and 168 bp for *M. trossulus*. The primer sequence is ME-15, CCAGTATACAAACCTGTGAAGA and the reverse sequence is ME-16, TGTTGTCTTAATAGGTTTGTAAGA. For approximately 30% of the samples PCR amplification followed the protocol of Inoue et al. (1995) i.e. 30 cycles of 94°C for 30 s, 56°C for 30 s and 70°C for 90 s . For the remaining 70% of the samples, the protocol was 35 cycles at 94°C for 30 s, 50°C for 60 s and 72°C for 30 s followed by a final extension period of 20 min. at 72°C. To avoid repetition of results by using GLU and ME, only 54

mussels were genotyped with both markers to allow a comparison between them. The remaining samples from Reach Run were genotyped only with ME.

3.2.3.2. ITS

The internal transcribed spacer (ITS) regions that lie between the 18S and 28S nuclear rDNA coding regions form the target sequence for the *Mytilus*-specific primer developed by Heath et al. (1995). The primer sequences are ITS 1, GTTTCCGTAGGTGAACCTG and ITS 2, CTCGTCTGATCTGAGGTCG. Amplification conditions consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 20 s, 50°C for 20 s and an original extension phase at 72°C for 45s which was modified to 2 min. The expected fragment size of the PCR product is 1250 bp. and a digestion step is required to produce species-specific patterns. Digestions were performed for 12 hours at 37°C in a reaction volume of 15 µl. For each sample, the restriction enzyme HhaI (from *Haemophilus haemolyticus*; 0.5 units) was mixed with 3 µl of the buffer supplied by the manufacturer (Pharmacia), 6.5 µl sterile distilled water and 5 µl PCR product. The restriction enzyme was then inactivated at 65°C for 20 min before electrophoresis. The ITS marker is expected to behave as a single gene locus in *Mytilus*, showing mendelian-like inheritance (Heath et al., 1995; Rawson et al., 1996b)

3.2.3.3. MAL-I

The *Mytilus* Anonymous Locus (MAL-I) primer targets a coding region of the DNA and was developed by Rawson et al. (1996b) and redesigned by Rawson et al. (2001). The primer sequences JH-2, GAAGCGTATTTGGTCACTGGCAC and PR-9, GTCATAAAATGGAACATCTGAGTC are expected to amplify a 650 bp DNA fragment for *M. edulis* and *M. trossulus* that also contains part of a non-coding sequence from an adjacent intron. Amplification conditions consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 20 s, 52°C for 20 s and 72°C for 45 s . The resulting fragment was then digested with the restriction enzyme SpeI to produce smaller species-specific fragments using the same protocol as for ITS.

3.2.3.4. PLIIa

The PLIIa primer targets part of the coding region for the protamine-like sperm protein PLII and was developed by Heath et al. (1995) based on the published cDNA sequence of Carlos et al. (1993). The primer sequences PLIIa, GAGCCCAAGTAGGAAATCCCG and CCTTCGCATTGTTAGATTTATT produce a DNA fragment of 475 bp for both species. Amplification was performed with a denaturation phase at 94°C for 3 min, followed by 35 cycles of 94°C for 20 s, 50°C for 20 s and 72°C for 45 s. Species-specific fragments were produced after digesting the PCR product with the restriction enzyme HinfI. The resulting banding pattern does not completely discriminate between genotypes since the marker is dominant and the hybrids show the same pattern as *M. trossulus* homozygotes.

3.2.3.5. COIII

The COIII marker amplifies a fragment of the cytochrome oxidase c subunit III gene of the mitochondrial DNA. The primer sequences of the marker developed by Zouros et al. (1994) are FOR1, TATGTACCAGGTCCAAGTCCGTG and REV1, ATGCTCTTCTTGAATATAAGCGTACC. The primer amplifies a fragment of same size (860 bp) for both female and male mtDNA types for *M. edulis* (F-ed and M-ed) and also for *M. trossulus* (F-tr and M-tr). Amplification conditions were modified from Zouros et al. (1994) and consisted of a denaturation phase at 94°C for 2 min, followed by 35 cycles of 94°C for 60 s, 54°C for 20 s and 72°C for 60 s . A final extension period of 5 min. at 72°C after the cycles was added to the original protocol. Because digestion with only the EcoRI restriction enzyme (Zouros et al., 1994; Toro, 1999; Comesaña et al., 1999) may produce ambiguous results as the F-tr and M-ed types cannot be differentiated from the undigested fragment, the restriction enzyme Acc1 was also added to produce the four possible mitotypes following the approach of Saavedra et al. (1996). The expected fragments for EcoRI are approximately 450, 340 and 70 bp for M-tr and 520 and 340 for F-ed, while F-tr and M-ed should show the same pattern as the undigested fragment of

860 bp. On the other hand, the Acc1 enzyme should produce 740 and 120 bp fragments for M-ed and 500, 230 and 130 bp for F-tr, while F-ed and M-tr are not digested and should show the 860 bp fragment. Thus, the two restriction enzymes together provide accurate identification for all possible combinations of mtDNA in one individual. As not all the mussels from Reach Run were genotyped with both restriction enzymes, only 68 mussels from that population were used for the analysis. In addition, another 112 mussels used to produce laboratory families in 2000, 2001 and 2002 were also genotyped with COIII, allozymes and the other molecular markers to support the analysis of mtDNA inheritance.

3.2.4. Karyotype and location of ribosomal DNA loci

Samples used for karyotype analysis were collected from Salmonier Cove and Cape Broyle, Newfoundland, in May and August 2002. Once in the laboratory, animals were placed in tanks with filtered sea water and fed continuously on a suspension of *Isochrysis* sp. and *Tetraselmis* sp. microalgae for 10 days. Metaphases were obtained by treatment with colchicine (0.005%) for 8 h. Gills were then dissected, treated twice with 0.56% KCl (15 min) and fixed in ethanol – glacial acetic acid (3:1). Fixed gills were used to prepare slides for karyotype analysis. Chromosome measurements and classification followed Levan et al. (1964). The centromeric index (100 x length of short arm x total chromosome length $^{-1}$) was the main feature used to compare species.

For the fluorescence *in situ* hybridizations the DNA probe used was pDm 238 from *Drosophila melanogaster* containing the repeat unit 18S-5.8S-28S rDNA (Roiha et al. 1981) labeled with digoxigenin-11-dUTP using a Nick Translation Kit (Boehringer Mannheim). Chromosomal DNA was denatured by immersing the slides in DNAase-free RNAase (100 μg L^{-1} in 2X SSC) for 1 h at 37°C, then incubated in pepsin (10% in 100 mM HCl), post-fixed in formaldehyde (1% in PBS 50 mM) for 10 min, washed in 2X SSC for 10 min and finally dehydrated in a graded ethanol series and air-dried. The hybridization solution (50% formamide in 2X SSC, 10% dextran sulphate, 0.33% SDS, 10 μg salmon sperm DNA, and 100 ng labeled DNA probe) was denatured for 15 min at 75°C. Then, 30 μL of this hybridization solution was applied to each slide under a sealed

coverslip. Hybridization was performed in a slide-PCR (MJ Research, MJ 100) as follows: 7 min at 75°C, 2 min at 55°C, 30 s at 50°C, 1 min at 45°C, 2 min at 42°C, 5 min at 40°C, 5 min at 38°C, 5 min at 37°C, and, finally, overnight at 37°C in a moist chamber. Post-hybridization washes were carried out for 5 min at 42°C in 2X SSC, 10 min in 20% formamide and 0.2X SSC, 5 min in 0.1X SSC, 5 min in 2X SSC and, finally, 5 min in 0.1 M Tris, 0.15 M NaCl, 0.05% Tween–20. After blocking the slides for 30 min in 0.1 M Tris–HCl, 0.15 M NaCl, and 0.5% Boehringer Mannheim blocking reagent, probe hybridization sites were detected by immunocytochemical incubations in mouse anti-digoxigenin, rabbit anti-mouse-FITC (fluorescein isothiocyanate) and goat anti-rabbit-FITC. Chromosomes were counterstained with propidium iodide (50 ng mL^{-1} anti-fade) and visualized and photographed using a Nikon Microphot AFX microscope.

3.3. Results

Of the 250 individuals genotyped from Reach Run, 214 produced positive results for all five markers and were used for the analysis. Among the failures, five samples did not produce bands for any of the DNA markers, suggesting that the DNA extraction was not effective. Eight samples failed to produce reliable bands for ITS, seven samples for MAL-I and two for ME. However, the major source of possible scoring error was the allozyme markers, for which there were 19 samples that produced unreliable or faint bands and were excluded from the analysis.

A marked increase in the number of hybrids detected was observed with the increase in the number of markers used in the analysis (figure 3.1). Animals homozygous for the five markers are considered "pure" species hereafter, keeping in mind that the status of pure may be equivocal for a few samples as five markers represent only a small part of the genome. Considering the five markers together, the sample consisted of 41.2 % pure *M. edulis* and 21.0 % pure *M. trossulus*. Among backcrosses, 20.1 % were *M. edulis*-like and 17.7 % were *M. trossulus*-like (figure 3.2). The frequencies of mussels in the different backcross categories are given in figure 3.3. Only two individuals showed the hybrid index of 5, being putative F1 hybrids. One of them was homozygous for

different species in two loci, being reclassified as a backcross. Therefore only one individual was heterozygous for all markers, representing a frequency of 0.47 % of F1 hybrids in the population. Only 8 individuals showed signs of introgressed genes, being homozygous at one locus for one of the species but having the main genetic background (hybrid index) from the other species. These cases were distributed equally between the two species. Among these mussels, three were homozygous for the other four markers of the main genetic background, demonstrating a greater level of introgression. ITS was the only marker in which signs of introgression were not observed and MPI was the marker with most individuals (three mussels) homozygous for the locus in the opposite genetic background. The results for inheritance in the laboratory families were analyzed for each marker separately and are reported in the following sections.

3.3.1. GLU and ME

As expected, since they target the same gene, the GLU and ME markers produced exactly the same results for most samples, although five individuals showed *M. trossulus* bands for ME but no bands for the GLU marker. In addition, three other samples showed the hybrid pattern for ME, but only the *M. edulis* band was visible with GLU. This would have resulted in a misclassification of these animals if only GLU had been used. Bands for *M. trossulus* were consistently fainter than *M. edulis* bands with the GLU marker and, in general, the ME marker showed clearer, stronger and more unambiguous bands than the GLU marker. The banding pattern for ME was exactly as reported by Inoue et al. (1995) with a 180 bp fragment for *M. edulis*, a 168 bp for *M. trossulus* and hybrids showing both bands. Although both of the amplification conditions used for ME produced good results, the alternative cycling condition with an annealing temperature of 50°C produced clearer bands than the original protocol from Inoue et al. (1995). For the GLU marker the rarer 380 bp fragment for *M. edulis* was not observed in the present study and, in addition to the 350 bp band expected for *M. edulis* and the 240 bp band for *M. trossulus* (Rawson et al., 1996a), several smaller bands (<200 bp) were occasionally produced.

Results for the Reach Run population showed that ME was able to identify 10.3 % of the sample as hybrids, close to the average of the five markers (11.3 %) and, in combination with other markers, ME also showed an average resolving power for identifying hybrids (table 3.1). The 10.3 % of hybrids detected in the population by the ME marker represents only 26.5 % of all the possible hybrids identified with the 5 markers together.

For the laboratory crosses, at least one of the parents was heterozygous for ME in 16 of the families. In general, the offspring of these crosses followed a Mendelian inheritance, although some exceptions occurred (table 3.2). Of 11 crosses between heterozygotes and homozygotes, 3 did not show the expected Mendelian ratio of 1:1 (G test, $p< 0.05$), but after the sequential Bonferroni correction all crosses conformed to the expected Mendelian ratio (table 3.2). If the offspring of the families are combined ($N = 313$), the observed frequency is then close to the expected value, i.e. 46.1 % homozygotes and 53.9 % heterozygotes. The five crosses involving two F1 hybrids showed a ratio similar to the expected 1:2:1, although one of the crosses showed a marked deficit of *M. edulis* homozygotes (3.4 %). In addition, in another family only 14 % *M. edulis* were homozygous and the remaining families all showed less than the expected 25 % *M. edulis*, but not significantly different from the expected. Although the G test was significant for one cross ($p = 0.002$), after the sequential Bonferroni correction no significant difference was observed in any of the crosses.

3.3.2. ITS

The digested fragments of the 1250 bp ITS-PCR product showed a very similar pattern to that described by Heath et al. (1995). *M. edulis* also produced a 450 bp fragment, a 180 bp fragment and several smaller fragments (<100 bp). For *M. trossulus*, most of the samples showed the expected 280 and 180 bp fragments, but 7 individuals (9.1 %) exhibited a fragment of approximately 260 bp instead of the 280 bp and 12.9 % possessed both fragments. Hybrids showed the fragments of both species and 11.1% of the hybrids showed the 260 bp for *M. trossulus*. The ITS marker was able to identify 18 hybrid individuals that represented 8.4 % of the natural population or 21.7 % of the total

of hybrids identified with the 5 markers together (table 3.1). Surprisingly, for the laboratory produced cohorts ITS did not show the Mendelian-like inheritance suggested by Heath et al. (1995) and Rawson et al. (1996b). The three families formed by F1 hybrids crossed to F1 hybrids showed similar results and a markedly higher frequency of hybrids (mean 87.0 %) than the expected 50 % (table 3.2). Mussels from a second sample from one of these families (family 12, N = 30) were genotyped and results were confirmed, showing 90% hybrids.

3.3.3. MAL-I

Although the original studies (Rawson et al., 1996b; 2001) do not mention the expected fragment size for both species, a clear differentiation between species was observed in the present study. *M. edulis* showed a single ~ 640 bp fragment while *M. trossulus* showed a ~ 360 bp and a few samples (7.47 %) also showed a ~210 bp fragment. The MAL-I marker identified the lowest frequency of hybrids (5.1 %) of all markers used in the present study. In combination with the other markers, MAL-I also identified fewer hybrids than the average (table 3.1). The laboratory produced crosses showed frequencies concordant with a Mendelian inheritance for MAL-I (table 3.2).

3.3.4. COIII

The banding pattern observed for the COIII marker was exactly as expected based on previous studies, although other bands of variable size were occasionally observed. Restriction fragments cut with EcoRI produced bands for F-ed and M-tr mtDNA and Acc1 produced bands for F-tr and M-ed mtDNA, thus EcoRI and Acc1 complemented each other. Only mussels genotyped with the two restriction enzymes are discussed below. Of the 68 individuals from Reach Run that were genotyped with COIII and the other markers, approximately half showed *M. edulis* mtDNA (54.4 %) and the remainder showed *M. trossulus* mtDNA. There was a high concordance between mtDNA and the genetic background of the individuals. All individuals that were considered pure *M. edulis* (25 mussels) or pure *M. trossulus* (17 mussels) for the allozyme and molecular

markers also possessed mtDNA from the same species. Although the advanced backcrosses showed the same pattern, there were a few exceptions. Among 12 *M. edulis*-like backcrosses 2 had *M. trossulus* female mtDNA (F-tr). One *M. trossulus*-like backcross showed the expected F-tr mtDNA, but also the *M. edulis* male mtDNA (M-ed), being the only individual with a hybrid genotype for mtDNA. One other *M. trossulus*-like backcross possessed *M. edulis* mtDNA. As animals were not sexed before genotyping, it was not possible to correlate sex and mtDNA, however there was a higher frequency of individuals homoplasmic for female mtDNA (63.2 %) than heteroplasmic individuals. However, according to Saavedra et al. (1996), some *M. trossulus* males can carry a variant M-tr mitotype (M-trO) that is not distinguishable with EcoRI digestion from the standard F-tr mitotype and therefore would be mistaken for a female (if not previously sexed) or a homoplasmic male.

The pattern of mtDNA variation described above was also observed in the parents that were genotyped with the 6 markers and used to produce laboratory families in 2000 and 2001 (65 mussels). Of a total of 12 putative homoplasmic males for only female mtDNA, two were pure *M. edulis*, one was an *M. edulis*-like backcross, 6 were pure *M. trossulus* and 3 were *M. trossulus*-like backcrosses. Among individuals with mtDNA from both species, 2 showed F-ed and M-tr mtDNA and one showed F-tr and M-ed mtDNA. Among backcrosses, two females with a predominantly *M. trossulus* background possessed *M. edulis* mtDNA. Another two *M. edulis* females and one *M. trossulus* female had both M and F molecules from the same species as their main genetic background.

Parents used in the 2002 crosses (42 mussels) should be analysed separately as they included 14 F1 hybrids produced in the laboratory in 2000 and other mussels from a natural population (Goose Arm). Among males from the natural population, one *M. edulis*-like, one pure *M. trossulus* and three *M. trossulus* individuals were homoplasmic, showing only female mtDNA. One individual showed a hybrid pattern with F-ed and M-tr mtDNA and one *M. trossulus* female also showed male mtDNA (M-tr mtDNA). Among the F1 hybrids produced in the laboratory, all females (5 mussels) had only the expected F molecules. Two F1 males fathered by an *M. edulis* were used in the crosses and both lacked male mtDNA. Of the other 6 F1 hybrid individuals that were fathered by *M.*

trossulus, three possessed the expected mtDNA from both species, two had only female mtDNA from *M. trossulus*, and surprisingly, one mussel contained female mtDNA from *M. edulis* and also female mtDNA from *M. trossulus*.

Of the four laboratory-produced families genotyped with COIII, three showed the expected distribution of F molecules in the offspring. One family (family 10) was the product of a female F1 hybrid with the F-tr mitotype crossed to an apparently homoplasmic *M. edulis* male (with only the F-ed mtDNA), and all the offspring showed only the F-tr mitotype (N = 25). The other cross was between an *M. trossulus*-like female and an *M. trossulus* male that was also apparently homoplasmic, and the offspring (family 1) showed only F-tr mtDNA (N = 60).Furthermore, the offspring (family 14) of a cross between two F1 hybrids (F-tr female and F-tr/M-ed male) also possessed only female mtDNA (N = 18). The most surprising result was obtained from a cross between an *M. edulis* female and an *M. trossulus*-like male that contained F-tr mtDNA and also M-tr mtDNA (family 8). Of 30 offspring genotyped, 21 showed the expected F-ed mtDNA, whereas the remaining 9 had only F-tr mtDNA. As these results suggesting transmission of female mtDNA by a heteroplasmic male were unexpected, a second set of 28 mussels from the same family was genotyped with essentially the same result, five individuals carrying only *M. trossulus* female mtDNA. Since the male involved in this cross was a backcross, the offspring had a genotype pattern for the other markers that was different from that of any other family produced in the laboratory. The possibility of genetic contamination could therefore be eliminated. Although the number of these aberrant individuals was small, a correlation was observed between these individuals and their genotype at the ME locus. The offspring showed the expected 50% *M. edulis* and 50% hybrids for the ME marker (father was heterozygote for ME), but all individuals that inherited the female mtDNA from the father (14 mussels) were hybrids for ME.

The results for the laboratory crosses described above are considered preliminary as no male mtDNA was detected in any individual, probably because the genotyped mussels were young and reproductive tissue had not yet been developed. A small number of mussels from two other families of the same 2000 cohort (N = 12) was genotyped after 24 months when their gonads were developed, and male mtDNA was observed in 5

individuals, whereas in the previous year no male mtDNA had been observed in these families. In addition, 6 individuals from the family that showed the unusual transmission of F molecules by a male (family 8) were genotyped with COIII after 24 months. All showed the F-ed molecules from the mother, as expected, and two mussels also had the M-tr molecules inherited from their *M. trossulus* father.

3.3.5. Karyotype and location of ribosomal DNA loci

Of a total of 126 mussels treated with colchicine, only 6 showed chromosomes during metaphase that were suitable for karyotype analysis and *in situ* hybridizations. As the screening process is time consuming, higher priority was given to *M. trossulus* individuals since it has been reported that Pacific populations differ in chromosome arrangements from other *Mytilus* populations studied so far (Martínez-Lage et al., 1997b). Five *M. trossulus* individuals yielded cells in metaphase suitable for chromosome measurements. Only one *M. edulis* individual had the chromosomes measured since the main focus of the study was to determine karyotype differences among *M. trossulus* populations. The diploid complement of 28 chromosomes was confirmed for both species. According to the centromeric classification of Levan et al. (1964) all *M. trossulus* individuals showed 6 metacentric chromosomes and 8 submetacentric or subtelocentric chromosomes (table 3.3). All *M. trossulus* chromosomes showed an unequivocal classification, with the exception of chromosome 5, which varied between submetacentric and subtelocentric (centromeric index 24.58 ± 0.83). The lowest centromeric mean for metacentric chromosomes was 43.32, far above the limit of 37.5 determined by Levan et al. (1964). The greatest centromeric mean for submetacentric chromosomes was 31.87, therefore no chromosome lay close to the border between metacentric and submetacentric. The only *M. edulis* sample examined also had 6 metacentric chromosomes and the centromeric index was similar to that recorded by Insua et al. (1994).

Two in situ hybridizations were performed totaling 12 slides containing cells with chromosomes in metaphase. Both were successful and produced fluorescent bands of medium to strong intensity, but overlapping of chromosomes produced unclear results in

7 samples. The number of 18s-5.8s-28s ribosomal DNA regions stained by the technique in the remaining samples varied from 2 to 5 areas. No clear conclusion could be drawn from the samples analyzed, although at least two 18s-5.8s-28s ribosomal DNA regions are present in *M. trossulus*.

3.4. Discussion

The number of markers necessary to answer specific questions concerning a hybrid zone depends primarily on the goals of the research. A small number of markers, say 2 or 3, will only provide a coarse classification of pure species with hybrids as a general group. Invariably, a small number of markers will underestimate the frequency of hybrids in a sample and limit the categories of different kinds of hybrids (Boecklen and Howard, 1997). Many advanced backcrosses will probably be mistaken for the parent species, giving a false indication of limited hybridization and introgression. On the other hand, logistical difficulties restrict the allocation of advanced backcrosses into finely divided categories, as the number of markers necessary is too high to be practical and the immense possibilities for genetic combinations would obscure the analysis (Floate et al., 1994). As the use of several markers to describe hybridization in detail involves high cost and effort, one should carefully consider the optimal number of markers to be used. The present study confirms for a *Mytilus* hybrid zone that an increment in the number of markers also increases the number of hybrids that are identified (figure 3.1) and allows a better understanding of the nature of these hybrids (figure 3.3). Previous studies based exclusively on allozyme markers suggested that very limited, if any, hybridization occurs in the northwest Atlantic (Koehn et al., 1984; Varvio et al. 1988; Bates and Innes, 1995). The use of Mpi alone identified 5% hybrids in Nova Scotia (Mallet and Carver, 1995) and less than 10 % in Newfoundland (Penney and Hart, 1999). On the other hand, a multilocus approach identified 22.8% hybrids in Nova Scotia with two nuclear markers and one mtDNA marker (Saavedra et al., 1996). Comesaña et al. (1999) identified 26 % hybrids in Newfoundland with 4 markers and concluded that half would have been mistakenly classified as parent species if only Mpi and Est-D had been used. These values

are very close to those presented in figure 3.1 for a single site in Newfoundland, in which 38 % of mussels were identified with 5 markers as hybrids. The use of several markers also permits the subclassification of hybrids and demonstrates that most hybrids are actually advanced backcrosses and that very few F1 hybrids occur in the area. Obviously, the frequency of hybrids identified at a particular site also depends on the species composition and the actual frequency of hybrids at that site. As the *Mytilus* hybrid zone in the northwest Atlantic is complex, with a patchy distribution of genotypes, the relative frequencies of both species and their hybrids may vary on a microgeographic scale. Reach Run was chosen because it showed the highest frequency of hybrids in the static cohort analysis in chapter 2 (size class below 40 mm). This explains the slightly higher frequency of hybrids than has been observed in previous studies.

Another important question to be considered is the effort necessary to work with each marker in relation to the answers it can provide (Silva and Russo, 2000). Starch gel electrophoresis is in general time consuming and requires that tissue samples are fresh or properly preserved (frozen at $-70^{\circ}C$ or lyophilized). Although cellulose acetate has been a viable alternative to starch gels, results for Est-D are not consistent for *Mytilus* (Penney and Hart, 1999) and Mpi produced more reliable results on starch gels (present study). In addition, both allozyme markers used in the present study produced more ambiguous and unclear bands than did the molecular markers. On the other hand, the cost and difficulty of allozyme electrophoresis are lesser than molecular techniques that require primers, chemicals, specific equipment and training. The greater polymorphism of allozyme markers observed in *Mytilus* may obscure the analysis, but may also reveal differences at a population level and allows observations on the interactions between several alleles from both species. However, molecular markers can also exhibit polymorphism. For example, Bierne et al. (2003b) observed 32 different alleles for the molecular mac-1 primer in *Mytilus* populations from Europe and pointed out the advantage of this marker, which can detect variation within each species while being virtually diagnostic between species.

The resolving power, cost and practicality of the PCR-based markers available for *Mytilus* should also be considered. Markers like ME and GLU in which the PCR

product can be resolved directly on gels have an advantage over those such as ITS and MAL that require digesting the PCR product with restriction enzymes. Only one of ME and GLU is necessary, as they target the same gene. In the present study ME consistently produced more unambiguous and clearer bands than GLU. A few reservations should be mentioned about PLIIa and ITS, nuclear markers that require restriction enzymes to produce species-specific fragments. The protamine-like sperm protein marker (PLIIa) is a dominant marker, therefore the heterozygote shows the same pattern as one of the homozygotes and the assumption that the population is in Hardy-Weinberg equilibrium has to be made in order to calculate allele frequencies. Hardy-Weinberg equilibrium tends to be violated in hybrid zones (due to non-random fertilization, differential selection and immigration), and the use of PLIIa is therefore limited. Nevertheless, it may provide important insights into reproductive isolation since the marker targets a sperm protein that may have species-specific properties during fertilization (chapter 4). Although some authors have claimed that the ITS marker exhibits Mendelian-like inheritance (Heath et al., 1995; Rawson et al., 1996b), the present results from three crosses involving F1 hybrids showed an excess of hybrids in the offspring of all families. DNA contamination or genotyping error may be possible explanations for the discrepancy, although unlikely, as results in the present study were consistent among families and repeated with a second set of mussels from one of the families. The internal transcribed spacer (ITS) regions lie between the 18S and 28S nuclear rDNA coding regions and for higher eukaryotes several copies of the ribosomal gene have been commonly reported (Hillis and Dixon, 1991). González-Tizón et al. (2000) observed that 3 different regions for 18s-5.8s-28s ribosomal genes located in different chromosomes are present in *Mytilus,* and the present study confirmed at least two different regions for *M. trossulus*. Insua et al. (2001) also observed a variable number (more than 2) of 18-28S rDNA regions in *M. edulis*. This may partly explain the present results for the ITS marker, since multiple copies of the gene would produce a higher frequency of hybrid bands in F2 hybrids. However, the reason that the marker seems to show Mendelian-like inheritance in some mussels remains unknown. Further research is

necessary for an improved understanding of ITS inheritance and caution should be exercised when analyzing laboratory produced cohorts with ITS.

Although not all markers are completely diagnostic and the necessary effort and resolving power associated with each one varies considerably, the restricted number of markers currently available for *Mytilus* makes them all extremely valuable for revealing biological patterns. Although a single diagnostic marker should be able to provide important information about hybrid zones (e.g. Penney and Hart, 1999), a combination of several makers increases the definition of genotype categories observed in the hybrid zone. It is only after classifying backcrosses according to a hybrid index with the aid of several markers that important aspects such as bimodality can be revealed in hybrid zones. The distinction between unimodal and bimodal hybrid zones has important implications for their maintenance. In an analysis of several hybrid zones, Jiggins and Mallet (2000) concluded that bimodal hybrid zones are invariably associated with strong assortative mating and represent an advanced stage of speciation, while unimodal hybrid zones show little assortative mating. The clear bimodal pattern observed in figure 3.3 confirms the observations of Bates and Innes (1995) and supports the conclusion from chapter 4 that assortative fertilization may play an important role in maintaining the *Mytilus* hybrid zone in the northwest Atlantic. In addition, the study of hybrid fitness coupled to a hybrid index can reveal different patterns of fitness in the various hybrid genotypes, which is a central question in understanding hybrid zones (Arnold and Hodges, 1995). Hybrid vigour in the F1 generation and hybrid breakdown in subsequent generations are probably two important characteristics of hybridization that can explain the balance of the hybrid zone. Hybrid vigour is also potentially useful to improve aquaculture production of mussels (discussed in chapters 5 and 6). Finally, of particular relevance is the use of several markers to study introgression and to produce a genetic map that can relate specific parts of the genome to traits of interest (Rieseberg, 1998).

The question why the different markers produced distinct results requires an understanding of how different parts of the genomes of each species interact with each other and with the environment. Differential introgression could allow some genes, or groups of genes, to invade the other species genome preferentially if adaptive advantage

is conferred to this gene in the opposite genetic background and environment. Although the role of hybridization and introgression in the evolutionary process has been generally neglected and underemphasized (Rieseberg, 1998), many cases of introgression have been documented in animal hybrid zones, suggesting that it is a relatively common phenomenon (Dowling and Seccor, 1997). Transfer of adaptations from one species to another may be an important consequence of hybridization that promotes adaptive evolution (Rieseberg and Wendel, 1993). Recombination between divergent genomes can also create new adaptations if advantageous epistatic interactions are formed. However, this action should be limited, since original quantitative trait loci (QTL) and epistatic relations would also be broken up due to recombination, reducing the fitness of hybrids. The principle of introgression is based on the fact that although hybrids are in general less fit than the parent species, some of the hybrids may be fitter than one or both parent species and these new genetic combinations are passed on to future generations. This is particularly true for fecund species like *Mytilus* in which a single backcross can produce millions of different recombinant genotypes. Fit hybrid genotypes can then allow the invasion of the parent species' genome if better adapted genes or a group of genes are favored by selection (Barton, 2001). Arnold (1997) has compiled some substantial evidence showing that hybridization may have critical importance in adaptive evolution, either through introgression or through the formation of new hybrid lineages.

The rate of introgression of a particular gene depends on the selective pressure and the linkage relationship to other genes. If introgression occurs at different rates in different parts of the genome, then different markers are likely to reflect the selective forces acting on this specific gene or part of the genome. Genes responsible for reproductive isolation that reduce hybrid fitness are expected to show lower introgression rates, while neutral or positively selected genes may introgress at a higher rate. Harrison (1990) suggested that the genetic architecture of a species barrier could be inferred from patterns of differential introgression, and that if a genetic map is created then the genes responsible for isolation can be located. Although most markers used in inheritance studies are presumably neutral, this is not true for the *Mytilus* markers. Only ITS is presumably neutral as it targets non-coding regions of the DNA, although these regions

could always be linked to adjacent genes under selection. The ME marker codes for byssal proteins that may play a role in fitness and may show some adaptive significance. Finally, PLIIa, a sperm protein, may be involved directly in reproductive isolation, and selection on the gene has been observed (Riginos and McDonald, 2003). Two other markers developed to distinguish *M. edulis* and *M. galloprovincialis* in Europe (mac-1; Daguin et al., 2001 and Efbis; Bierne et al., 2002a) are presumably neutral, but have not been tested in *M. edulis* -*M. trossulus* hybrid zones. Among the molecular markers used in the present study, only MAL-I has an unknown function. However, this marker may be under selective pressure or close to a gene that contributes to reduced hybrid fitness, explaining the lower frequency of hybrids observed for MAL-I in the present study. Linkage to each other or equivalent selection pressures may explain the similar results among the other markers. Beaumont (1994) observed linkage between several allozyme markers in *M. edulis* and suggested that Mpi and Est-D are part of the same linkage group.

In the present study only 8 individuals out of 214 showed signs of introgression, being homozygous for one species at one locus and showing the genetic background of the other species. Of these 8 mussels, only 3 were homozygous for the other species at all other loci analyzed. Although only small numbers were observed, these mussels represent introgressed genotypes which can only be produced by matings in which both parents are hybrids for that particular locus. The low number of these genotypes suggests that barriers to hybridization that maintain reproductive isolation are strong enough to prevent or reduce gene flow across the species barrier, but it also demonstrates that introgression is possible. Introgression of nuclear markers has been observed in the European hybrid zone (Bierne et al., 2003b) and in the Baltic Sea hybrid zone (Borsa et al, 1999; Riginos et al., 2002). Introgression of mtDNA has also been recorded in both hybrid zones (Rawson and Hilbish, 1995b; 1998; Quesada et al., 1999; Zbawicka et al., 2003; Quesada et al., 2003) but the phenomenon may be related to the mode of mtDNA inheritance present in *Mytilus*.

The unusual doubly uniparental inheritance (DUI) in *Mytilus* probably predates the speciation of *M. edulis*, *M. trossulus* and *M. galloprovincialis* (Rawson and Hilbish,

1995b; Stewart et al., 1995). Although the pattern of DUI is similar in the three species in the *Mytilus edulis* complex, disruption of DUI has been commonly observed and shows marked geographical variation. In North America, disruption of DUI seems to be more frequent and is probably related to the blocking of mtDNA introgression. Zouros et al. (1994) used laboratory crosses to show that DUI breaks down in interspecific crosses between *M. edulis* and *M. trossulus* (40% of males were homoplasmic) but not in homospecific crosses. Rawson et al. (1996b) also suggested that hybridization is responsible for the high level of DUI disruption observed in natural populations from the hybrid zone between *M. trossulus* and *M. galloprovincialis* on the west coast of North America. On the other hand, Rawson and Hilbish (1998) and Quesada et al. (1998) observed that DUI is not disrupted and introgression occurs in the *M. edulis – M. galloprovincialis* hybrid zone, but Wood et al. (2003) recorded high levels of DUI disruption in laboratory crosses. Moreover, extensive introgression of *M. edulis* mtDNA into *M. trossulus* likely caused replacement of mtDNA in populations inside the Baltic Sea (Quesada et al., 1999; Zbawicka et al., 2003; Quesada et al. 2003). It is not known if disruption of DUI is coupled to introgression.

In the present study no pure individual of either species was observed to carry the mtDNA of the other species, suggesting that mtDNA introgression is blocked in early generations of hybridization in the northwest Atlantic, and confirming the observations of Saavedra et al. (1996) and Comesaña et al. (1999). However, the occurrence of some advanced backcross individuals with mtDNA from the other species and the presence of hybrids containing mtDNA from both species suggest that mtDNA introgression may be possible. One of the most striking cases of introgression comes from the *Mytilus* hybrid zone at the entrance to the Baltic Sea. Possibly due to adaptation to lower salinity, *M. trossulus* has preferentially colonized the Baltic Sea, but the mitochondrial DNA of *M. trossulus* has been totally replaced by introgressed *M. edulis* mtDNA (Quesada et al., 1999; Zbawicka et al., 2003; Quesada et al., 2003). This phenomenon is probably coupled with a masculinization event, in which *M. edulis* F molecules would have introgressed into *M. trossulus* and then invaded the paternal mtDNA lineage, assuming the role of M molecules (Quesada et al., 2003). Introgression of other nuclear genes from *M. edulis* into

M. trossulus has also been observed in this hybrid zone (Borsa et al., 1999; Riginos et al., 2002). It is unknown whether mtDNA introgression has adaptive significance or is merely a consequence of DUI disruption due to hybridization. Although the same two species hybridize in the northwest Atlantic, mtDNA introgression has not been observed (Saavedra et al., 1996; Comesaña et al. 1999). Two factors should be considered when comparing the two hybrid zones. First, the specific environmental conditions and the ecological interactions where the hybrid zones are located may influence selection patterns and hybridization level. Secondly, genetic differences between *M. edulis* and *M. trossulus* from both sides of the Atlantic (Gosling, 1992b) due to isolation and differential selection patterns may also alter the manner in which the two species interact in different areas.

Several studies on *Mytilus* around the world have reported the disruption of DUI. Males homoplasmic for only female mtDNA (Fisher and Skibinski, 1990; Rawson and Hilbish, 1995a; Wenne and Skibinski, 1995; Saavedra et al., 1997; Wood et al., 2003) and heteroplasmic females (Fisher and Skibinski, 1990; Zouros et al. 1994; Stewart et al., 1995; Quesada et al., 1996; Garrido-Ramos et al., 1998; Wood et al., 2003) have been described in natural populations and laboratory crosses. Although more frequently observed in hybrid zones, the disruption of DUI is not exclusively coupled to hybridization. The present observations of individuals classified as putative pure species that violated DUI and of many F1 and F2 hybrid individuals that exhibited DUI confirm the variability of DUI disruption. According to Rawson et al. (1996b), disruption of DUI should occur more frequently in F1 hybrids and then decrease in frequency in later generations of backcrosses. The observation of males that lack M molecules may have implications for the masculinization process, which occurs when F molecules invade the M lineage and assume the role of M molecules (Hoeh et al., 1997; Saavedra et al., 1997). If some homoplasmic males contain only F mtDNA and mitochondria are invariably present in the sperm and passed to the egg (Sutherland et al., 1998), then we should expect these F molecules to be incorporated into the egg. Dalziel and Stewart (2002) observed small amounts of female mtDNA in male gonads and Zbawicka et al. (2003) were able to detect F molecules in sperm. The mode of sex determination in *Mytilus* is

probably under the control of the female nuclear genome, which allows preferential replication of M molecules in embryos that will become males, but not in female embryos (Zouros, 2000; Kenchington et al, 2002). The inheritance of F molecules from a male may allow the preferential replication of these F molecules in male embryos, as if they were M molecules, and presumably start the masculinization process. Although two of the crosses in the present study involved homoplasmic males, determination of the fate of the mtDNA inherited from the father was hampered by the low sensitivity of the technique to detect small amounts of mtDNA and by the fact that the offspring were genotyped when young. Some of the families produced in 2000 from presumably homoplasmic males are now known to have some male offspring, therefore analysis of mtDNA in the gonad of these males should provide further evidence of the masculinization process. However, this can only be observed in hybrid males, otherwise these "masculinized" F molecules would show no difference from the usual F molecules inherited from the mother in homospecific crosses. An entirely monospecific population of *M. galloprovincialis* in the Black Sea showed males that were presumably homoplasmic, but Laudokakis et al. (2002) were able to demonstrate that these males actually reflected a recent masculinization process that had spread to the whole population. Therefore, males that have been considered homoplasmic in many studies may simply represent masculinization phenomena that the mtDNA markers are unable to detect due to the low divergence between the F molecule and the new masculinized molecule. The masculinization process may start with a heteroplasmic male, in which for some reason F molecules are incorporated into the sperm. In addition to several other DUI disruptions previously reported, the present detection of the inheritance of F molecules from a heteroplasmic male is a novel observation of DUI disruption that may also be involved in the masculinization process. Other unusual exceptions to DUI have been reported such as individuals heteroplasmic for three or more genomes (Hoeh et al., 1991) and the co-transmissions of multiple molecules by males (Quesada et al., 2003). Analysis of mitochondrial molecules in the sperm and their transmission, replication and presence in the adult offspring from hybrid crosses should provide further evidence on DUI

disruption and possibly establish a clearer relationship between DUI disruption and introgression or/and the masculinization process.

Another potentially useful approach for understanding hybridization between *Mytilus* species is cytogenetics. Although the sample size in the present study was relatively small for karyotype analyses (N = 5), the preliminary results were consistent and suggested that *M. trossulus* from the northwest Atlantic has 6 metacentric chromosomes. The number of metacentric chromosomes seems to be the most conspicuous difference between *Mytilus* species since other chromosome variations have shown very little agreement among several cytogenetic studies. Gosling (1992b) suggested that the inconsistency among karyotype studies might reflect differences in methodology, but it could also demonstrate real structural plasticity that is independent from species differences. The presence of 6 metacentric chromosomes in *M. edulis* is probably the most consistent observation for several European populations (Moynihan and Mahon, 1983; Dixon and Flavell, 1986; Thiriot-Quiévreux, 1984; Insua et al., 1994; Martínez-Lage et al., 1995; Martínez-Lage et al., 1997a). For *M. trossulus*, Insua et al. (1994) and Martínez-Lage et al. (1995) observed 6 metacentric chromosomes in Baltic Sea populations, but Martínez-Lage et al. (1997a) observed 7 metacentric chromosomes in Pacific populations. The authors viewed the results as significant differences between Pacific and Baltic populations and suggested that chromosome changes may have taken place after a *M. trossulus*-like ancestral form invaded the Atlantic. These changes would have been retained in the divergent species that presumably originated in the Atlantic (*M. edulis, M. galloprovincialis* and a possible *M. trossulus* variant). This hypothesis is consistent with a *Mytilus* invasion into the Atlantic that probably took place when the Bering Strait first opened around 3.2 million years ago (Vermeij, 1991; Rawson and Hilbish, 1995b). However, the idea that *M. trossulus* in the Atlantic diverged from Pacific populations before the origin of *M. edulis* is inconsistent with allozyme data comparing *Mytilus* populations from different oceans (Varvio et al., 1988; McDonald et al., 1991; Väinölä and Hvilsom, 1991).

The present observation that *M. trossulus* from the northwest Atlantic differs in karyotype from Pacific populations challenges the hypothesis that the present hybrid zone

has been formed by secondary contact after the last glacial maximum, when *M. trossulus* would have re-invaded the Atlantic through the Arctic route (chapter 2). The presence of the same chromosomal arrangement in *M. trossulus* from the Baltic Sea and northwest Atlantic suggests that the species may have re-invaded the Atlantic before the last glacial maximum (during previous interglacial periods) and that the hybrid zone may have already existed somewhere in the Atlantic before that time. However, the hypothesis of a rapid fixation of a new chromosomal arrangement, following a founder event for example, cannot be ruled out as an explanation for a recent hybrid zone (formed after the last glacial maximum). Väinölä (2003) used allozyme differentiation to suggest that *Macoma balthica* have re-invaded the Atlantic repeatedly during the Pleistocene and that genetically different populations persisted in the Atlantic during the last glacial period. In addition, there is strong evidence that the Baltic Sea was first colonized by marine species around 9000 years ago (Varvio et al., 1988, Andrén et al., 2000), therefore Baltic mussels should have originated from an *M. trossulus* population that also exhibited 6 metacentric chromosomes. Environmental fluctuations may have altered the species distribution (and the presumed Atlantic hybrid zone location) during the last interglacial maximum (around 9000 years ago) and *M. trossulus* may then have colonized the Baltic Sea, showing subsequent divergence from northwest Atlantic populations due to strong selective pressure in the Baltic Sea. Further studies are necessary to understand the differences in the karyotype of *M. trossulus* from the Baltic (Insua et al., 1994; Martínez-Lage et al., 1995) and northwest Atlantic (present study) and that described by Martínez-Lage et al. (1997a) for Pacific populations. Based on chromosome differences, Martínez-Lage et al. (1997a) suggested that *M. trossulus* from the Baltic should be considered a different taxon than Pacific *M. trossulus*. Karyotype analysis from Hudson Bay and northern Labrador should determine if any dispersal of *M. trossulus* from the Pacific to the Atlantic is currently taking place. The degree of molecular divergence between *M. trossulus* populations from different areas should also provide important insights into the evolutionary history of *Mytilus*.

A further understanding of *Mytilus* hybrid zones depends on the appropriate use of the available genetic tools and the development of new molecular markers that

differentiate the species forming the complex. The combined use of several markers in the present study demonstrates that an increment in the number of markers is able to identify more hybrids and distribute them into several hybrid classes, demonstrating the clear bimodal character of the northwest Atlantic hybrid zone. Very low levels of introgression were observed, although some individuals showed genes homozygous for the species opposite to the main genetic background. Introgression of mtDNA also seems limited in the northwest Atlantic (although possible), and the several cases of DUI disruption observed may influence introgression rate and the origin of masculinization events. The observation that the karyotype from Atlantic *M. trossulus* populations differ from those of Pacific populations may provide an important insight into the evolutionary history of *Mytilus*.

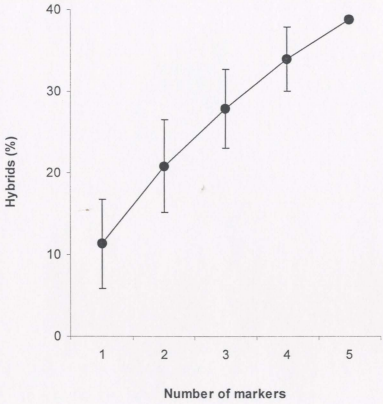

Figure 3.1. Percentage hybrids identified in a sample from Reach Run relative to the number of markers used to genotype individuals. Error bars represent standard deviation. N = 214 mussels.

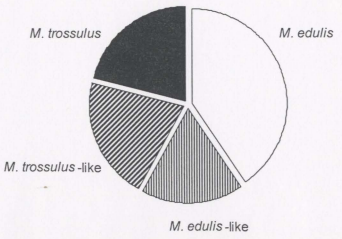

Figure 3.2. Relative frequency of putative pure parent species (*M. edulis* and *M. trossulus*) and backcrosses (grouped according to the main genetic background) at Reach Run. N = 214 mussels.

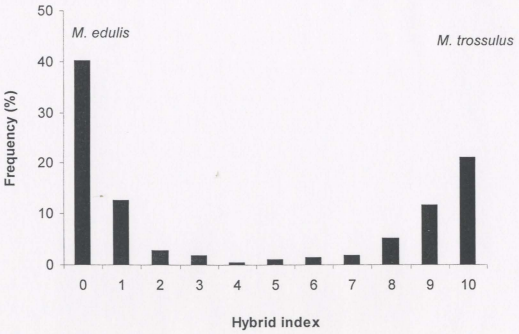

Figure 3.3. Relative frequency of putative pure *M. edulis*, *M. trossulus*, F1 hybrids and backcrosses from Reach Run. Hybrid index based on 5 codominant genetic markers (ME, ITS, MAL, MPI and EST-D) in which 1 score was attributed for each *M. trossulus* allele. 0 = *M. edulis*, 5 = putative F1 hybrid and 10 = *M. trossulus*. N = 214.

Table 3.1. Relative frequency of hybrids, *M. edulis* and *M. trossulus* identified with each marker and the various combinations of the 5 markers. N = 214.

Marker	Frequency (%)		
	Hybrids	*M. edulis*	*M. trossulus*
ME	10.3	57.0	32.7
MPI	13.1	51.9	35.0
EST-D	19.6	47.7	32.7
MAL	5.1	55.6	39.3
ITS	8.4	55.6	36.0
ME / MPI	21.5	49.1	29.4
ME / EST	25.7	47.2	27.1
ME / MAL	13.6	55.1	31.3
ME / ITS	17.3	54.7	28.0
MPI / EST	29.0	42.5	28.5
MPI / MAL	18.7	49.1	32.2
MPI / ITS	21.0	48.6	30.4
EST / MAL	23.4	45.8	30.8
EST / ITS	26.6	45.3	28.0
MAL / ITS	11.7	54.7	33.6
ME/MPI/EST	33.2	41.6	25.2
ME/MPI/MAL	23.4	48.6	28.0
ME/MPI/ITS	26.6	47.7	25.7
ME/EST/MAL	27.6	45.3	27.1
ME/EST/ITS	31.3	44.9	23.8
ME/MAL/ITS	19.2	54.2	26.6
MPI/EST/MAL	31.3	41.6	27.1
MPI/EST/ITS	34.1	40.7	25.2
MPI/MAL/ITS	23.4	48.1	28.5
EST/MAL/ITS	28.5	44.9	26.6
ME/MPI/EST/MAL	34.6	41.1	24.3
ME/MPI/EST/ITS	38.8	40.2	21.0
ME/EST/MAL/ITS	32.7	44.4	22.9
ME/MPI/MAL/ITS	28.0	47.7	24.3
MPI/EST/MAL/ITS	35.5	40.7	23.8
ME/MPI/EST/MAL/ITS	38.8	40.2	21.0

Table 3.2. Relative frequency of *M. edulis*, *M. trossulus* and hybrids in the offspring of laboratory produced families. F = female, M = male and N = sample size, * = 2002 crosses involving F1 hybrids, h = homoplasmic male for only female mtDNA, c = second set of samples for results confirmation, a = EcoRI restriction enzyme, b = AccI restriction enzyme.

Cross	F x M	Marker	Frequency (%)			N
			M. edulis	Hybrids	*M. trossulus*	
1	H x T	Me	0.0	50.0	50.0	30
1[c]	H x T	Me	0.0	64.1	35.9	39
2	H x T	Me	0.0	73.3	26.7	30
3	T x H	Me	0.0	54.5	45.5	22
4	T x H	Me	0.0	57.7	42.3	26
5	T x H*	Me	0.0	27.6	72.4	29
6	H* x E	Me	30.0	70.0	0.0	30
7	H* x E	Me	36.7	63.3	0.0	30
8	E x H	Me	46.7	53.3	0.0	30
8[c]	E x H	Me	45.7	54.3	0.0	35
9	T x E	Me	0.0	100	0.0	30
10	H* x E	Me	48.0	52.0	0.0	25
11	H x E	Me	65.4	34.6	0.0	26
12	H x H*	Me	3.4	51.7	44.8	29
13	H x H*	Me	13.6	59.1	27.3	22
14	H x H*	Me	20.0	43.3	36.7	30
15	H x H*	Me	20.0	56.7	23.3	30
16	H x H*	Me	23.3	53.3	23.3	30
12	H x H*	ITS	3.3	93.3	3.3	30
14	H x H*	ITS	3.3	86.7	10.0	30
15	H x H*	ITS	13.3	80.0	6.7	30
2	H x T	MAL	0.0	52.2	47.8	23
9	T x E	MAL	0.0	100	0.0	15
11	H x E	MAL	56.3	43.8	0.0	16
12	H x H*	MAL	22.2	33.3	44.4	18
14	H x H*	MAL	26.7	40.0	33.3	30
15	H x H*	MAL	13.8	37.9	48.3	29
1	H xT (FT x MT[h])	COIII [a]	0.0	0.0	100.0	30
1	H xT (FT x MT[h])	COIII [b]	0.0	0.0	100.0	24
1[c]	H xT (FT x MT[h])	COIII [a]	0.0	0.0	100.0	25
8	E x H (FE x MT)	COIII [a]	70.0	0.0	30.0	30
8	E x H (FE x MT)	COIII [b]	71.4	0.0	28.6	21
8[c]	E x H (FE x MT)	COIII [b]	82.1	0.0	17.9	28
10	H x E (FT x ME[h])*	COIII [a]	0.0	0.0	100.0	25
14	H x H (FT x MT)*	COIII [a]	0.0	0.0	100.0	15
14	H x H (FT x MT)*	COIII [b]	0.0	0.0	100.0	18

Table 3.3. Chromosome measurements and centromeric classification of *M. trossulus*. RL= relative length, SE = standard error, CI = centromeric index, CC = centromeric classification according to Levans et al. (1964), m = metacentric, sm = submetacentric, st = subtelocentic. N = 5 metaphase.

Chrom.	RL	SE	CI	SE	CC
1	8.76	0.49	43.32	0.93	m
2	8.45	0.51	29.09	0.87	sm
3	7.93	0.43	27.59	0.85	sm
4	7.80	0.34	48.22	0.26	m
5	7.58	0.37	24.58	0.83	sm/st
6	7.30	0.30	43.53	0.78	m
7	7.15	0.31	27.20	0.95	sm
8	6.97	0.25	43.63	0.88	m
9	6.95	0.37	31.87	0.90	sm
10	6.62	0.29	44.80	0.70	m
11	6.62	0.23	30.74	0.81	sm
12	6.24	0.24	29.70	0.75	sm
13	5.93	0.61	45.34	0.54	m
14	5.74	0.50	31.75	0.57	sm

Chapter 4

Incomplete reproductive isolation in the blue mussel (*Mytilus edulis* and *M. trossulus*) hybrid zone in the northwest Atlantic: gamete recognition and larval viability

4.1. Introduction

Evolutionary forces driving reproductive isolation of marine animals with external fertilization are poorly understood, and very little is known about the specific mechanisms responsible for maintaining species identity when two divergent species make secondary contact in the ocean. The classic scenario for allopatric speciation is the accumulation of incidental genetic differences when two populations are geographically isolated, resulting in by a barrier to gene flow (e.g. Dobzhanski, 1937; Orr and Pesgraves, 2000). This genetic divergence, caused by natural selection and/or random genetic drift, may eventually result in intrinsic reproductive isolation unless the two populations become reconnected and genetic exchange occurs. In the case of secondary contact between diverging populations, a hybrid zone may be formed which provides excellent opportunities to study populations undergoing different stages of the speciation process and the specific mechanisms involved in it. For some broadcast spawning marine invertebrates the relative importance of these mechanisms may differ, as gene flow is greatly facilitated by high fertility, free mixing of gametes and the potential for long distance dispersal during the larval stage. Genetic differences resulting in reproductive isolation are initially expressed as partial pre- or postzygotic barriers to gene flow and most commonly as a combination of both, since most hybrid zones contain multiple barriers (Arnold, 1997). Prezygotic barriers prevent or reduce the formation of hybrid zygotes, and may include spatial (habitat preference) or temporal (variation in reproductive periods) factors, behavioural differences and gamete incompatibility. For terrestrial animals, sexual behaviour is probably one of the most important barriers to

gene flow among sympatric species, and genetically complex mate recognition systems (MRS) are well developed (Futuyma, 1998). For externally fertilizing marine species from temperate waters, such as mussels, the reproductive season is very short and closely coupled with environmental cycles. In addition, species distributions overlap greatly and behavioural isolating mechanisms are almost absent. In this case, gamete recognition, either morphological or chemical, should be the primary prezygotic isolating mechanism. Postzygotic barriers to gene flow involve reduced fitness of hybrid offspring. Hybrid non-viability or sterility has been extensively studied in *Drosophila* and the genetic basis of reduced hybrid fitness is relatively well understood (Orr and Pesgraves, 2000; Orr and Turelli, 2001). Hybrid inferiority can sometimes be expressed only in F2 or backcross generations and in many cases the degree of hybrid inferiority is closely related to environmental conditions (Harrison, 1990).

The rates at which pre- and postzygotic processes evolve should be similar if natural selection is not involved (Coyne and Orr, 1997), but there is considerable variation among taxa. Extrinsic postzygotic incompatibilities mediated by the environment may be driven by natural selection to evolve rapidly (Hendry, 2000), while intrinsic incompatibilities that are neutral within populations may be expected to accumulate at a more constant rate (Edmands, 2002). Prezygotic isolation could also be accelerated by reinforcement if hybrid offspring have reduced fitness and natural selection promotes the evolution of prezygotic mechanisms that prevent the "wastage" of gametes in mal-adapted hybrids (Dobzhanski, 1940; Butlin, 1989). Speciation after a founder effect could also produce accelerated prezygotic isolation according to the model of Gavrilets and Boake (1998), in which prezygotic mechanisms would be more efficient in preserving species identity than postzygotic mechanisms. Finally, there is recent evidence of accelerated prezygotic evolution, even in totally allopatric populations, as a consequence of rapid changes in only a few loci (Lessios and Cunninghan, 1990; Knowlton, 1993; Metz et al., 1998; Hellberg et al., 2000). Several examples of accelerated prezygotic evolution come from marine species, and sometimes a change in a small number of genes, such as those associated with gamete recognition proteins, may produce reproductive isolation independently of the presence of other postzygotic

mechanisms. The relative importance of pre- and postzygotic mechanisms may be different in marine broadcasters and copulating species because behavioural recognition systems are very limited in free-spawning animals. Therefore, reproductive isolation may depend strongly on sperm morphology or on a few proteins expressed on the gamete surface.

In the last few years two of these proteins and their functions have been extensively studied. Lysin in the abalone (*Haliotis* sp.) and other gastropods and bindin in sea urchins are proteins involved in sperm binding and the acrosome reaction, a critical species-specific reaction chain that occurs when sperms adhere to the egg surface (Vacquier, 1998). These molecules represent just a small part of our limited understanding of the genes involved in the fertilization process, but the fact that they provide species recognition is of great relevance for marine species. Takagi et al. (1994) isolated an analogous protein in *Mytilus* (M7 lysin), and Riginos and McDonald (2003) suggested positive selection for this molecule in the Baltic Sea *M. trossulus* - *M. edulis* hybrid zone.

The "*Mytilus edulis* complex" is basically composed of three species (*M. edulis*, *M. trossulus* and *M. galloprovincialis*), and whenever any two of them meet hybridization occurs (Gosling, 1992b). The most studied of these hybrid zones is that between *M. edulis* and *M. galloprovincialis* in Europe (reviewed by Gardner, 1994a), but increasing attention has been paid in the last few years to the *M. edulis – M. trossulus* hybrid zone in the northwest Atlantic. Natural hybridization in this area has been documented by Mallet and Carver (1995), Saavedra et al. (1996), Comesaña et al. (1999), Penney and Hart (1999) and Toro et al. (2002). Successful production of F1 hybrids in the laboratory has been achieved by Zouros et al (1992), Freeman et al. (1994) and Saavedra et al. (1996), but interspecific fertilization success or early viability have not been quantified. In a preliminary experiment, Toro (1999) found no differences in fertilization success between intra and interspecific crosses but suggested that the higher early mortality observed in hybrids was due to differences in the time of sexual maturity (and consequently gamete quality) or to genetic incompatibilities between the two species. Recently, Rawson et al. (2003) provided good evidence for gamete incompatibility between the two species from

pair-mating experiments and by assessing fertilization success at the embryo (8 to 16 cells) stage, and proposed that this is likely to be the principal barrier to gene flow between *M. edulis* and *M. trossulus*. The relative importance of different barriers to gene flow obviously merits more research, as the role of postzygotic mechanisms in the reproductive isolation of these species has never been examined empirically in post-larval stages. Furthermore, gamete competition, either as assortative fertilization or sperm precedence, is generally neglected in pair-mating experiments, although it could be instrumental in preventing hybridization. If species distributions and spawning periods overlap, it is likely that gametes of both species are present at the same time in the water column, and gamete interactions may play an important role in determining hybridization rate. Bierne et al. (2002) found evidence for preferential intraspecific fertilization when gametes of *M. edulis* and *M. galloprovincialis* were in competition, but no difference between intra- and interspecific fertilization was observed without competition. Pair-mating experiments performed by Beaumont et al. (1993) also did not show any significant difference in fertilization rate between intraspecific and hybrid crosses of these two species. Although conspecific sperm precedence may be more evident in marine species that fertilize externally, the phenomenon has also gained increasing attention in terrestrial species, in which post-copulatory (or post-insemination) prezygotic barriers have become clearer in the last few years (Howard, 1999).

The present study focuses on possible mechanisms operating as genetic barriers at the fertilization stage in the blue mussel (*Mytilus edulis* and *M. trossulus*) hybrid zone in the northwest Atlantic. The findings of Rawson et al. (2003) are extended by using a larger number of pair-matings, allowing gamete competition and analyzing offspring viability at the early larval stage. To investigate a possible mechanism preventing cross fertilization, sperm morphology of both species was also compared, since Hodgson & Bernard (1986) found significant morphological differences between the spermatozoa of *Mytilus edulis* and *M. galloprovincialis* in England. Surprisingly, *M. trossulus* sperm morphology has never been described for northwest Atlantic populations. Considering that *M. trossulus* is the most genetically distinct species of the complex (Rawson and Hilbish, 1995b; Hilbish et al., 2000), we hypothesize that the sperm of *M. trossulus*

differs morphologically from that of *M. edulis* and that gamete incompatibilities are greater in the northwest Atlantic hybrid zone than in the *M. edulis – M. galloprovincialis* hybrid zone in Europe.

4.2. Materials and Methods

4.2.1. Fertilization success and larval abnormality (pair-matings)

Ripe mussels were collected in July and August 2001 from 5 different aquaculture sites in Newfoundland: Reach Run, Charles Arm, Trinity Bay, Drac Bay and Salmonier Cove (see chapter 2 for details), and transported to the laboratory in refrigerated containers. For each trial a small piece of mantle tissue (ca. 20 mg) was taken from each of 30 mussels for preliminary genotyping with one nuclear DNA marker (ME: Inoue et al., 1996). Mussels were then held in air at 4°C overnight before experimental procedures. Heterozygotes for the ME marker were excluded from the spawning trials. Mussels were carefully rinsed in sea water before induction of spawning and all instruments that had been in contact with gametes were washed with hot fresh water and sterilized with 90% ethanol to avoid cross contamination. Spawning was induced by placing animals in separate containers with 2 liters of filtered (1 μm), UV sterilized sea water at 15°C (FSW). Spawned oocytes were sieved on a 37 μm nylon screen, rinsed with FSW and resuspended in 300 ml FSW. Sperm were separated from debris by passing the suspension through a 12 μm nylon screen. Sperm and egg concentrations were determined with a Coulter Multisizer and gametes mixed at a 100:1 sperm to egg ratio. Approximately 50 000 eggs were placed in each of several 1.5L containers, yielding a density of 33 eggs per ml. All gametes were utilized within 2 hours of spawning to ensure a similar quality of gametes in all crosses. Spawned mussels were frozen at –70°C for further allozyme and DNA analysis. Two allozymes (MPI and Est-D), 4 nuclear DNA markers (ME, ITS, MAL and PLIIa) and one mitochondrial DNA marker (COIII) were used to genotype parents (see chapter 3). Mussels from each site were used in separate trials, so that only crosses within sites were performed. A total of 10 trials and 174

different crosses was conducted, but only 6 trials provided factorial designs and therefore only 108 crosses were included in the analysis. In the previous summer (August, 2000) 9 preliminary trials were performed to help establish experimental design. All crosses were pair-matings of only one male and one female. Individual crosses were duplicated, with the exception of trial 10 and some crosses in trial 14, in which half-sib crosses of the same genotype class were used as replicates. Full factorial designs were used when possible (ExE, ExT, TxE, TxT) and in most cases there were three levels of replication: true replicates of the same cross, paternal or maternal half-sib crosses with the same genotypes and other unrelated crosses of the same genotype class (ExE, ExT, TxE or TxT). For all heterospecific crosses the female was also crossed to one or more homospecific males as a control.

To evaluate fertilization success the numbers of normal larvae, abnormal larvae and unfertilized eggs were recorded from digital images taken under an Olympus light microscope (400 X) 72 hours after fertilization. In all crosses at least 3 images of the offspring were used as pseudoreplicates. To confirm that genetic contamination had not occurred, 116 mussels were genotyped after 45 days. In addition, a further 448 (20 days old) larvae were genotyped for a sperm competition experiment which used the same parents (see below). Only 3 of the 564 mussels genotyped were found to be contaminants. A nested Anova was used to compare fertilization success and larval abnormality among genotypes. As the homogeneity of variance requirement was violated in some comparisons, a non-parametric Kruskal-Wallis test was also performed.

4.2.2. Sperm competition

For sperm competition experiments the same spawning procedures were followed as were used in the pair-matings, since the same mussels were used. The only difference was that males were transferred simultaneously to separate containers with clean seawater to ensure similar age and quality of gametes. Sperm were counted with a Coulter Multizer and suspensions were prepared with approximately equal numbers of sperm from one *M. edulis* and one *M. trossulus*. One hour after the males were moved to new containers, sperm suspensions were added to the egg suspensions of single females

(50 eggs ml^{-1}) at a final ratio of 50 sperm of each species per egg. A total of 15 different sperm competition trials was carried out in duplicate containers. Replicates were pooled after 20 days and offspring were genotyped with one nuclear DNA marker (ME).

4.2.3. Controlled mass mating

One mass mating experiment was conducted in July 2003 following the procedures of Bierne et al. (2002) to determine assortative fertilization when several adults are present. Spawning methodology was as described for the pair-mating experiments, but gametes were pooled before fertilization. Five males and five females of each species that spawned copiously were chosen for the trial. Animals used in the experiment were produced in the laboratory in 2000 and genotypes were known because their parents had been genotyped with the 7 available markers that distinguish the *Mytilus* species. Genotypes of spawned mussels were subsequently confirmed with the ME marker to rule out the possibility of contamination. Males and females were selected from unrelated families to avoid the effect of inbreeding depression. The concentration of gametes spawned was measured for each individual male with a Coulter Multisizer, and approximately $2x10^7$ sperm from each male were pooled. Eggs from each female were counted separately and each female contributed $2x10^5$ eggs to the pool. Fertilization occurred in two separate containers with 10L of seawater filtered (1 μm) and sterilized by UV irradiation, giving a final concentration of 50 eggs L^{-1} and a 100:1 sperm to egg ratio. Embryos were allowed to develop undisturbed for 72 hours, when cultures were sieved, a sample of each replicate preserved in 90% ethanol and the remaining larvae returned to clean water. Larvae were initially fed $15x10^3$ cells ml^{-1} *Isochrysis galbana* (T-ISO) daily and water was changed 3 times a week. At day 10 larvae from both replicates were pooled, another sample taken and culture densities reduced to 5 larvae ml^{-1}. Larvae were grown to metamorphosis with an increasing amount of food up to a daily concentration of 10^5 cells ml^{-1} *I. galbana* (T-ISO), *Chaetoceros muelleri* and *Tetraselmis suecica* (5:3:2 ratio, respectively). A final sample was taken 45 days after fertilization, and metamorphosed larvae discarded.

Progeny from random mating were tested for deviation from Hardy-Weinberg equilibrium using only one molecular marker (ME), as parents were known to be homozygous for all markers available. As PCR amplification of DNA from 72 hours-old embryos was inconsistent, DNA from 10 days old (50 larvae) and 45 days old spat (50 spat) was used to test for deviation from the Hardy-Weinberg equilibrium with Genepop software.

4.2.4. Sperm morphology

Sperm morphology of *M. edulis* and *M. trossulus* was compared by three different methods. Measurements of acrosome length, nucleus width and total sperm length (without tail) were made from transmission electron micrographs from gonad tissue of 12 males. These measurements are considered to be the most variable and diagnostic between *Mytilus* species (Hodgson & Bernard, 1986). Animals were genotyped with EST-D and MPI at the time gonad tissue was fixed. To avoid the problem that the gonad may contain immature, smaller gametes, samples of sperm from some males that spawned copiously in the fertilization trials in 2001 were preserved in Karnovsky EM fixative (5% glutaraldehyde). Samples were subsequently washed in 0.1M sodium cacodylate buffer, dehydrated and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and photographed in a Zeiss-109 transmission electron microscope. For scanning electron microscopy samples were dehydrated with acetone, critical point dried and coated with gold. Photographs were taken in a Hitachi S-570 scanning electron microscope. These individuals were genotyped with the same 7 markers used in the pair-mating experiment. Finally, the availability of a Coulter Multisizer facilitated the volume determination of thousands of sperm from several individuals (16 *M. edulis* and 11 *M. trossulus*). However, for non-spherical particles the Coulter Multisizer can only calculate volume in spherical equivalents, so data cannot be compared with other morphological measurements, but the technique allows a comparison between a large number of sperm from both species and their hybrids. A one-way Anova was used to compare sperm dimensions.

4.3. Results

4.3.1. Fertilization success (pair-matings)

There was considerable variation between females in fertilization success for both intra- and interspecific crosses (Figure 4.1). Of the 22 females crossed, 11 showed a significantly greater fertilization success by males from the same species while the remaining 11 females had a similar fertilization success by males from both species, although in these cases intraspecific crosses almost always showed a greater mean fertilization percentage (Fig. 4.1). Three females completely failed to produce hybrid larvae, yet their eggs were successfully fertilized by males of the same species.

The several trials probably yielded variable results because they were performed at different times in the reproductive season and with animals from several sites (Figure 4.2A). For example, in trials 10 and 14 the great majority of females that spawned were *M. edulis* (18 altogether) from a total of 20 females that spawned in both trials. On the other hand, in trials 16 and 17 the total number of females that spawned was 12 *M. trossulus* compared with 2 *M. edulis*. As a result of the different number of females from each species in the trials, of the 10 trials performed, 4 did not produce factorial designs and were not included in the analysis. Trials 12 and 15 used mussels from Charles Arms, a site with almost only *M. edulis*, so no *M. trossulus* individuals were available for crosses. Trial 17 also produced only *M. edulis* offspring (parents from Reach Run) and trial 18 produced only *M. trossulus* offspring (parents from Drac Bay). Details on spawning dates, origin of parents and number of families produced are presented in figure 4.2.

Despite the large variability among females and trials, intraspecific crosses showed an overall significantly greater fertilization success when all crosses were analyzed together in a nested Anova and the non-parametric Kruskal-Wallis test (Figure 4.3, $p < 0.01$). Intraspecific crosses showed a mean fertilization success of 85.8 %, while interspecific crosses showed 51.3 % fertilization success. No significant difference was

observed between intraspecific crosses (E x E and T x T) or between interspecific crosses (E x T and T x E). When trials were analyzed separately, again more eggs were fertilized in intraspecific crosses than in interspecific crosses, with the exception of trial 13 where fertilization success was over 95% in all crosses (Figure 4.2A). Overall, only half the females showed clear incompatibilities against sperm of the other species, while the other half showed equal receptivity to heterospecific sperm.

4.3.2. Abnormal larvae (pair-mating)

The percentage of normal larvae at 72 hours also varied considerably among females and among trials (Figure 4.4). All females produced a greater or equal mean percentage of normal larvae in intraspecific crosses than in hybrid crosses. Only 5 out of 18 females had a significantly greater percentage of normal larvae in intraspecific crosses than in interspecific crosses (Anova followed by sequential Bonferroni correction, $p < 0.05$). *M. edulis* females, when crossed with *M. edulis* males, produced an average of 68% normal larvae, whereas the same females produced an average of only 48% normal hybrid larvae. *M. trossulus* females produced an average of 62% normal larvae in intraspecific crosses and 52% in interspecific crosses. Although there was a general trend for intraspecific crosses to produce greater mean percentages of normal larvae, there was no statistical difference in the percentage of normal larvae produced by the four types of cross (Figure 4.5, $p > 0.05$ for nested Anova and Kruskal-Wallis). However, when intraspecific crosses (E x E and T x T) were pooled, the difference between intraspecific and interspecific (E x T and T x E) crosses was significant ($p < 0.01$). When trials were analyzed separately, intraspecific crosses produced a significantly greater number of normal larvae than did interspecific crosses in 4 out of 6 trials (Figure 4.2B). In most trials one or the other species produced better larvae, probably as a result of the difference in optimum timing of spawning for each species. Combined results for fertilization success and abnormal larvae for each intra and interspecific cross are shown in figure 4.6. Typical offspring from an interspecific cross showing unfertilized eggs and abnormal larvae are illustrated in figure 4.7.

4.3.3. Sperm competition (gamete choice)

The clearest result in the sperm competition experiments (15 trials) was that in most cases the sperm of one male were evidently more successful than those of the other (Table 4.1). Four trials (3, 4, 11 and 15) did not show deviations from the expected 1:1 ratio associated with random fertilization. Curiously, in two of the trials the sperm from a male of one species were able to fertilize almost all the eggs of a particular female of the other species (trials 13 and 14). In the remaining 10 trials there was a clear preference for eggs to be fertilized by the sperm of the same species. The percentage fertilization that each male achieved separately with a particular female without competition (pair-mating experiment) is also given in Table 4.1. Note that the relative percentage of each genotype in the offspring is not related to the fertilization success of that particular female in the single pair-matings. For example, in some crosses that were not successful in the pair-matings (0% fertilization for both males in cross 2) a few eggs could have been fertilized in the sperm competition experiment and a small number of larvae may have survived in this experiment but not in the pair-mating. The results for cross 11 were surprising as the heterospecific male was not able to fertilize any egg in the pair mating but was able to father a relatively high percentage of the offspring (40%), despite competing with sperm of a successful homospecific male (90% fertilization in pair-mating).

4.3.4. Controlled mass mating

A clear deviation from the expected ratio of parental and hybrid genotypes was observed for 10 days old larvae ($G = 13.39$, $df = 1$, $p < 0.001$). Considering that ME is inherited as a Mendelian marker (chapter 3), the expected proportion of genotypes in the offspring would be 25% for each parental genotype and 50% for hybrids. The observed proportion of 40% *M. edulis*, 32.5% *M. trossulus* and 22.5% hybrids at day 10 shows a clear deficiency of heterozygotes, although there were more *M. edulis* than expected in relation to *M. trossulus* (Table 4.2). At 45 days the percentage of hybrids observed was

even lower (12.5%) and significantly different from the expected ($G = 25.60$, $df = 1$, $p < 0.0001$).

4.3.5. Sperm morphology

One possible explanation for the lower fertilization success observed in interspecific crosses could be differences in sperm morphology that prevent gamete recognition before fertilization. However, sperm head length, acrosome length and nucleus width in *M. edulis*, *M. trossulus* and their hybrids were almost identical when compared by transmission and scanning electron microscopy (Table 4.3, figure 4.8). There was no significant difference between the two species in the equivalent spherical volume of sperm determined with the Coulter Multisizer (Table 4.3), although there was considerable variation within species and individuals, and the overall equivalent spherical volume of sperm ranged from 1.6 to 4.2μm. Sperm for only two hybrids were analyzed with the Coulter Multisizer and no difference from the parent species was observed. Most sperm examined by electron microscopy contained 5 mitochondria for both species, although some possessed 6 mitochondria.

4.4. Discussion

Reproductive isolation between *Mytilus edulis* and *M. trossulus* in the northwest Atlantic is not complete, but the very low frequency of F1 hybrids and a strong bimodal distribution of genotypes suggest that one or more mechanisms operate to prevent or reduce hybridization. Toro et al. (2002) observed that in general the reproductive season coincides, but some discrete differences in timing of spawning between the two species exist in Newfoundland. In Maine, Maloy et al. (2003) found a considerable overlap in spawning time for both species. Both studies concluded that temporal displacement in spawning is unlikely to be the only mechanism preventing hybridization.

Spatial distributions of both species overlap considerably from Newfoundland to Maine (Comesaña et al., 1999; Penney and Hart, 1999; Rawson et al., 2001). Despite the

patchy distribution characteristic of a mosaic hybrid zone, most sites in Newfoundland contain mussels of both species and their hybrids (chapter 2). Penney and Hart (1999) found only one site in Newfoundland to be monospecific from 40 sites sampled and almost all sites contained some hybrid genotypes. As mussels form dense aggregations, the likelihood of gamete encounters between the two species is great, and therefore the potential exists for extensive hybridization and erosion of species identity if no isolating mechanisms are present.

The results of the present study suggest that gamete recognition and incompatibility are likely to be important isolating mechanisms between *M. edulis* and *M. trossulus*. The decreased fertilization success observed in interspecific crosses is consistent with the observations of Rawson et al. (2003). Although other factors such as age, readiness to spawn, nutritional condition and gamete competence may affect fertilization success, the consistency within females for greater homospecific fertilization success observed in the present study suggests some species-specific interactions at this stage. Moreover, both studies demonstrate that although there is a general tendency for homospecific crosses to be more successful, not all females are incompatible with heterospecific males and some females are as receptive to heterospecific males as they are to homospecific males. The use of a large number of pair-mating crosses and sperm competition trials in the present study suggests that these are not incidental or isolated cases and that a lack of specificity may be relatively common between the two species, at least under some circumstances. The nature of this specificity and the reason for the occasional failure in fertilization is unknown but a comparison with other better-studied systems can provide some possible explanations.

Differences in sperm morphology can produce major reproductive barriers between species, and sperm morphology has been a good indicator of species divergence in many taxa, including the Mytilidae (Guidi and Rebecchi, 1996; Garrido and Gallardo, 1996). However, this is not the case for *M. edulis* and *M. trossulus* as no difference was observed in their sperm morphology. The fact that the sperm of *M. galloprovincialis* is twice the length of that of *M. edulis* and *M. trossulus* (Hodgson and Bernard, 1986; Komaru et al., 1995) does not support the hypothesis that *M. edulis* is more closely

related to *M. galloprovincialis* than it is to *M. trossulus* (Varvio, 1988; Gosling, 1992b, Rawson and Hilbish, 1995b). However, over the period of their speciation, *M. edulis* and *M. galloprovincialis* probably came into contact several times during glaciation events when the Mediterranean was repeatedly isolated and reconnected to the Atlantic during the Pleistocene due to sea level fluctuations. On the other hand, *M. trossulus* and *M. edulis* were probably completely isolated during most of the Pleistocene in the Pacific and the Atlantic respectively. The idea of reinforcement is very appealing to explain the difference in the morphology of the *M. galloprovincialis* sperm, once prezygotic mechanisms may have been strengthened to prevent hybridization during repeated secondary contact. However, several other alternatives are possible and sperm divergence in this case would be fairly ineffective or secondary, as there is more hybridization between *M. edulis* and *M. galloprovincialis* in the European hybrid zone than between *M. edulis* and *M. trossulus* in the northwest Atlantic (Gardner, 1994a; Comesaña et al., 1999).

It is more likely that the gamete incompatibilities seen in *Mytilus* are due to recognition proteins expressed on the gamete surface in a similar way to those observed for sea urchin, abalone and other gastropod species (Metz and Palumbi, 1996; Hellberg et al., 2000). Bindin proteins in echinoderms interact with the egg membrane during sperm attachment and egg-sperm fusion, and display amino acid divergence between and within several orders, genera and species (Zigler and Lessios, 2003a, b). Lysin proteins in *Haliotis* are responsible for sperm penetration of the chorion layer and are species-specific (Galindo et al., 2003). The M7 lysin in *Mytilus* is probably analogous, but not homologous, to gastropod lysin, but there is no evidence that this protein is responsible for species recognition during fertilization (Riginos and McDonald, 2003). Further research is necessary to identify the precise factors involved in gamete recognition between *Mytilus* species and to understand why the mechanism sometimes allows interspecific fertilization. The reason that this specificity occasionally fails is a much more complex subject, and the present data only allows conjectural, although relevant, argument. Many other studies have found a similar pattern in which some females are receptive to heterospecific sperm but others are not (Palumbi, 1998, 1999; MacCartey and

Lessios, 2002). Sperm-egg recognition proteins in marine invertebrates show adaptive evolution due to positive selection (Lee et al. 1995; Metz and Palumbi, 1996; Metz et al. 1998; Hellberg and Vacquier, 1999; Hellberg et al. 2000; Swanson and Vacquier, 2002; Riginos and MacDonald, 2003, Galindo et al. 2003). Although the cause of this accelerated evolution is not understood and may not be the same in different taxa, positive selection suggests some adaptive value in altering the amino acid sequence of these proteins. The highly polymorphic nature of *Strongylocentrotus* bindin within species may be a result of positive selection on new amino acid changes but may also represent intraspecific assortative mating (Metz and Palumbi 1996; Palumbi, 1999; Geyer and Palumbi 2003). Recognition of polymorphic alleles within species implies compatibility variation at these loci in females that co-evolved to match the corresponding protein in the male, therefore assortative mating should be expected. Gaffney et al. (1993) also observed assortative mating between some oyster individuals that came from the same population. The *Mytilus* M7 lysin shows lower levels of polymorphism than the echinoderm bindin (Riginos and McDonald, 2003), yet some of the polymorphisms could explain species recognition failure if they predate the divergence of *M. edulis* and *M. trossulus* and both species have retained some shared alleles. Alternatively, female alleles could be neutral to reproductive success and would then have relaxed selection for compatibility with conspecific males, as proposed in the models of Nei at al. (1983) and Wu (1985), and therefore occasional interspecific fertilization may occur. Riginos and MacDonald (2003) also observed recombination among these *Mytilus* alleles, suggesting that long-term hybridization can potentially break down the recognition system developed in allopatry.

This study, together with those of Vacquier (1998), Palumbi (1999), Rawson et al. (2003) and many others, implies that drifting eggs in the ocean are able to differentiate, to a certain extent, among sperm from genetically divergent males. Hamel and Mercier (1994) observed that diffusible chemical compounds from the eggs of sea urchins were able to immobilize heterospecific sperm in the vicinity of the egg, but not homospecific sperm. Miller et al. (1994) demonstrated that sperm from the zebra mussel are chemically attracted to drifting eggs and suggested that the mechanism is species-

specific. Conspecific sperm precedence, long overlooked as a mechanism reducing hybridization, has been receiving increasing attention, since many examples of post-insemination prezygotic barriers have been discovered in the last few years (Howard, 1999). The coevolution of sperm and the female tract in many copulating species is instrumental in enhancing conspecific fertilization success (Pesgraves et al., 1999). Although empirical evidence is limited for marine species, eggs of at least some taxa may be able to distinguish chemically between sperm from different males, even conspecific males. Sexual selection is probably one of the strongest forces driving evolution, and there is extensive documentation of female preferences in mating animals (see Anderson, 1994 for comprehensive review). Basically, the penalty for a female that chooses an inappropriate mate is greater than for a male, since the female produces fewer but more energetically costly gametes, and is therefore likely to be more selective in choosing a mate (Bateman's principle; Bateman, 1948). If this principle also applies to marine broadcast spawners, these indicators for 'good genes' must be expressed on the sperm itself, as individuals do not meet. A situation in which several sperm are attached to one egg is probably common in dense aggregations of mussels. The evolution of at least three different mechanisms to block polyspermy in *Mytilus* supports the idea that several sperm may compete for the same egg (Togo et al. 1995, Togo and Morisawa, 1997). Furthermore, Lyu and Allen (1999) observed greater fertilization success in oysters when more than one sperm was attached to the egg, suggesting that, to a certain extent, competition may also be beneficial to some marine species. Many species, including humans, increase production of sperm when risk of competition is imminent (Gage, 1991; Baker and Bellis, 1993; Gage and Bernard, 1996). As they grow, mussels produce more gametes (Thompson, 1984), thereby increasing the chance of fertilization and offspring number. It is unlikely that in 200 million years of mytilid evolution there have been changes only in the number of gametes produced and not in their qualitative composition. LaMunyon and Ward (1998, 1999, 2002) observed that sperm from mating individuals were larger and faster than sperm from self-fertilizing individuals of the nematode *Caenorhabditis elegans*, and suggested that competition was responsible for the evolution of these larger and faster sperm, which are able to physically displace and outcompete

smaller ones. The authors managed to increase sperm size in competing lines by 20% over 60 generations compared with non-competing controls. Furthermore, Hosken et al. (2003) observed that male dung flies that were more successful in sperm competition also produced higher quality offspring, suggesting that multiple mating improves offspring success by allowing 'fitter' sperm to fertilize the egg. Likewise, marine invertebrates may also improve fertilization and offspring success by keeping several sperm attached to the egg, allowing time for a superior sperm to penetrate and fertilize it. Moderate gamete incompatibility, non-random fertilization and a sire-dam interaction effect on oyster larval viability suggest that the interaction of male and female genetic components may also play an important role in fertilization success (Gaffney et al., 1993). Variability in sperm-egg binding has also been observed in oysters, indicating variation in gamete affinity among conspecific matings (Luy and Allen, 1999). Competition may also allow intraspecific assortative mating that favours specific beneficial genetic combinations. The results of the sperm competition and the controlled mass mating experiments in the present study, showing clear assortative mating, suggest that competition may enhance conspecific fertilization. However, in both experiments offspring were only genotyped after several days (20 and 10 days, respectively), so postzygotic mortality could also explain the results, and it is likely that both pre- and postzygotic mechanisms prevent hybrid formation in *Mytilus*.

Another hypothetical mechanism that could allow some sperm to outcompete others is the production of larger amounts of a particular protein that eggs can detect and recognize as a trait of good sperm or 'good genes'. This would work in concordance with, or secondarily to, species-specific recognition proteins, but in some cases it could overexpress them, resulting in heterospecific fertilization even in competition.

The results of this study on gamete competition suggest that in *Mytilus* there is not only recognition of conspecific gametes but also preferential fertilization when choice is available, although this preference is not always for the homospecific male. However, other physiological aspects of gamete competence and early mortality could also explain the observations. A different approach would take into consideration life-history, ecological adaptation and fertilization kinetics to explain the variability in receptivity for

heterospecific gametes. Levitan (1998; 2002) observed that characteristics of the gametes of the three distinct species of *Strongylocentrotus* vary according to population density. Individuals from dense populations have fast sperm (advantageous in competition) and more selective eggs while scattered populations have slower but long-living sperm and less selective eggs that allow more hybridization. Although mussels live in dense aggregations where sperm competition is expected, they are also well known for long distance dispersal during larval stage and the ability to colonize new sites. Colonization of uninhabited areas may have been evolutionarily critical during glacial and interglacial periods, when species distribution shifted regularly to match environmental fluctuations. This idea implies the possibility of a natural variation in egg receptivity among individuals in the population in order to maximize fertilization efficiency under different conditions. Furthermore, egg receptivity may vary according to other endogenous factors if gamete competition is not intense. Eggs could relax their specificity with time if few sperm are available (as encounter is reduced by dispersal) in the same way that many plants allow more hybridization as the stigma ages. Older eggs may be more receptive to any kind of sperm as their capacity for fertilization diminishes.

One common theme among all the alternative hypotheses presented above is that gametes are not all created equal. Although extensive care was taken to synchronize gamete age in the present experiments, their 'physiological age' depends also on the nutritional condition of the parents, readiness to spawn, life-history and other inherited traits. The present results, showing high levels of abnormal larvae even in some homospecific crosses, suggest that gamete quality may also be a critical variable in fertilization trials. Nonetheless, the factorial design of the present study allowed the distinction between genetic and gamete quality factors that affect interspecific crosses. Benzie and Dixon (1994) observed that starfish gametes obtained at the end of the breeding season showed lower fertilization success and larval viability than those from earlier in the season. Furthermore, gametes fertilized 4 or 5 hours after being spawned were qualitatively poorer than recently spawned gametes, and high densities during fertilization often produced a high percentage of abnormal larvae. Fertilization success and embryonic development are also affected by environmental factors such as salinity,

temperature and pH (Christen et al., 1986). Any of these reasons may have contributed to the high levels of abnormality observed in some crosses in the present study, but polyspermia due to high density of gametes is a common factor producing abnormal larvae (Loosanoff and Davis, 1963). An experimental design that included sequential fertilization with the same parents would add important knowledge on interspecific gamete interaction over time.

More important than the discussion of why some females exhibit more heterospecific fertilization than others is the fact that most of them did not do so in the laboratory crosses performed, which implies that the fertilization stage is probably critical in reducing hybridization, and therefore in maintaining reproductive isolation. The question whether laboratory experiments reflect the natural situations remains to be answered. Conditions like those in the present study (100 sperm per egg) are probably not common in nature. Results such as those of Hamel and Mercier (1994), who observed that *M. edulis* is able to fertilize and be fertilized (over 20% in some cases) by taxa as distant as echinoids or asteroids also may not represent the natural reality. Nonetheless, considering the logistical difficulties in studying fertilization in the ocean, this kind of experimental approach should still provide important insights into how prezygotic isolation is maintained. Advances in PCR techniques and the development of new markers should provide individual genetic signatures that allow tracking of parentage. This would greatly benefit experiments that investigate assortative mating between and within species when several adults spawn simultaneously.

The *Mytilus* hybrid zone in the northwest Atlantic appears to be relatively young, possibly no older than 13,000 years, when the last glacial period ended and the Pacific and Atlantic oceans were reconnected through the Arctic Ocean. However, the two species probably diverged around 3 million years ago (Vermeij, 1991). The consequences of hybridization and the fate of the hybrid zone are unknown, but the combination of several pre- and postzygotic mechanisms seems to maintain reproductive isolation and species identity, indicated by the very low frequency of F1 hybrids and the strong bimodal distribution of genotypes (chapter 3). At this time it would be premature to suggest that gamete incompatibility is the principal barrier to gene flow between the two

species, since post-zygotic mechanisms have never been studied empirically. Nevertheless, the present study provides clear evidence that barriers to hybridization at the fertilization stage are likely to be important mechanisms contributing to partial reproductive isolation. The higher level of abnormality in hybrids from some crosses suggests that postzygotic mechanisms may also play an important role in maintaining reproductive isolation. This and other postzygotic mechanisms in the northwest Atlantic mussel hybrid zone are more fully discussed in chapter 5.

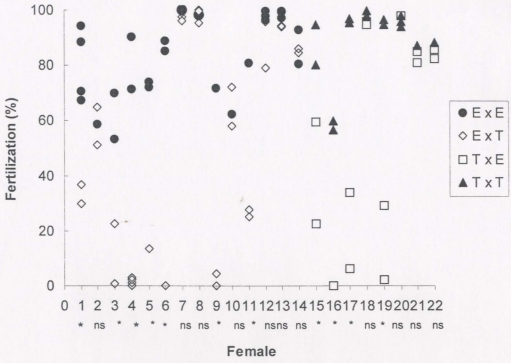

Figure 4.1. Mean fertilization success of individual *Mytilus* spp. females crossed to homospecific and heterospecific males.

E x E = *M. edulis* female x *M. edulis* male

E x T = *M. edulis* female x *M. trossulus* male

T x E = *M. trossulus* female x *M. edulis* male

T x T = *M. trossulus* female x *M. trossulus* male

ns = non significant; * = $p < 0.05$ (One-way Anova followed by a sequential Bonferroni correction comparing homospecific and heterospecific fertilization success).

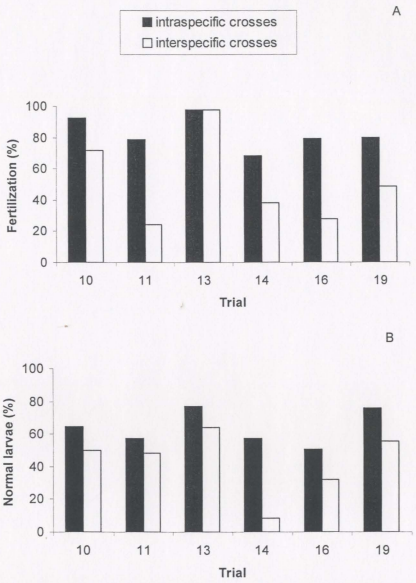

Figure 4.2. Percentage fertilization success and percentage normal larvae for each trial performed in 2001. Sites where parents originated, spawning dates and the number (N) of intraspecific and interspecific crosses (respectively) are listed below. Trial 10: Reach Run, June 20, N = 12 and 6. Trial 11: Salmonier Cove, July 11, N = 8 and 9. Trail 13: Cap Cove, July 14, N = 5 and 7. Trial 14: Salmonier Cove, July 21, N = 4 and 4. Trial 16: Drac Bay, July 27, N = 8 and 8. Trial 19: Salmonier Cove, August 3, N = 4 and 5. Trials 1 to 9 were preliminary experiments performed in 2000, trials 12 and 15 used mussels from Charles Arm that were all *M. edulis* and trials 17 and 18 did not produce a factorial design.

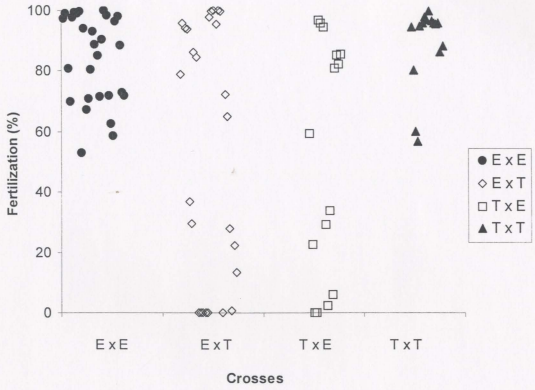

Figure 4.3. Fertilization success of the different possible crosses involving *Mytilus* spp. individuals (same crosses as in figure 4.1) grouped by genotype.
E x E = *M. edulis* female x *M. edulis* male
E x T = *M. edulis* female x *M. trossulus* male
T x E = *M. trossulus* female x *M. edulis* male
T x T = *M. trossulus* female x *M. trossulus* male

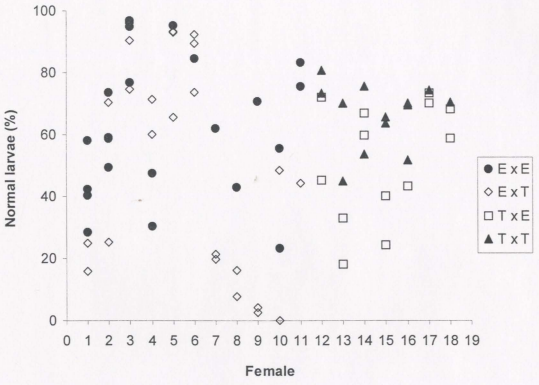

Figure 4.4. Percentage of normal larvae produced by each individual *Mytilus* spp. female crossed to homospecific and heterospecific males.

E x E = *M. edulis* female x *M. edulis* male

E x T = *M. edulis* female x *M. trossulus* male

T x E = *M. trossulus* female x *M. edulis* male

T x T = *M. trossulus* female x *M. trossulus* male

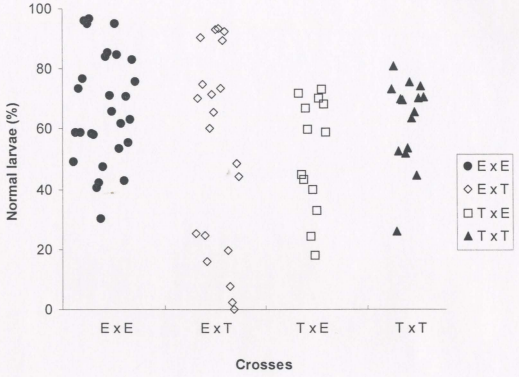

Figure 4.5. Percentage of normal larvae in the different possible crosses involving *Mytilus* spp. individuals (same crosses as in figure 4.4) grouped by genotype.

E x E = *M. edulis* female x *M. edulis* male

E x T = *M. edulis* female x *M. trossulus* male

T x E = *M. trossulus* female x *M. edulis* male

T x T = *M. trossulus* female x *M. trossulus* male

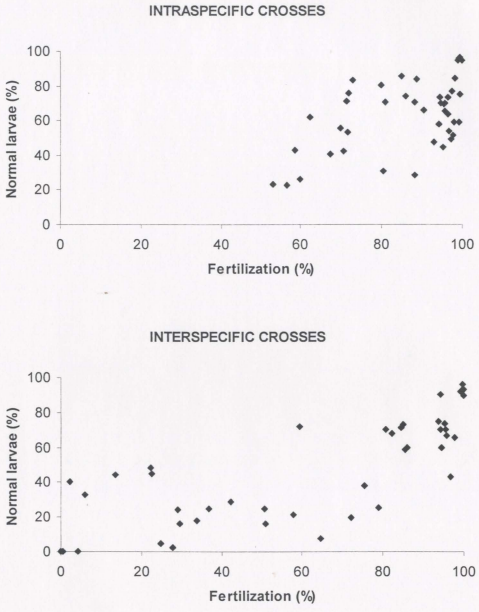

Figure 4.6. Correlation between fertilization success and percentage of normal larvae for intraspecific (A) and interspecific crosses (B).

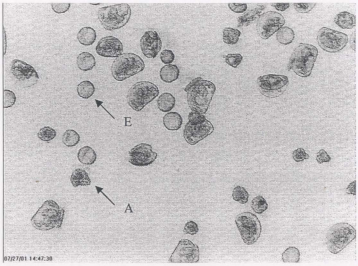

Figure 4.7. Fertilization success and abnormality in a cross between a *M. edulis* female and a *M. trossulus* male pictured after 72 hours. Note the unfertilized eggs (E) and abnormal larvae (A). Normal larvae are D shaped.

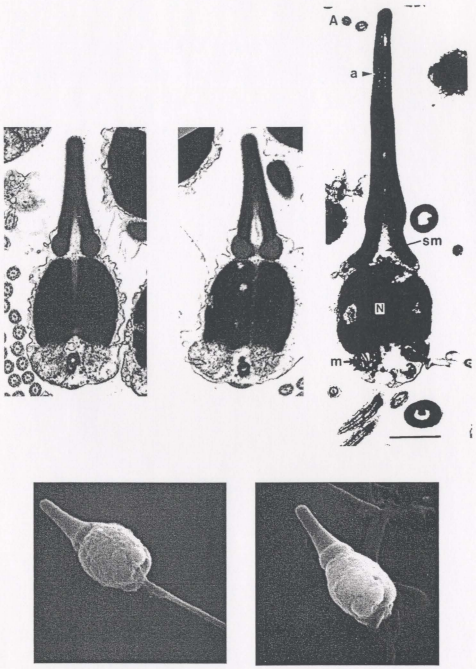

Figure 4.8. Transmission electron micrographs of the spermatozoon from *M. edulis* (top left), *M. trossulus* (center) and *M. galloprovincialis* (top right, from Hodgson and Bernard, 1986) and scanning electron micrographs of the spermatozoon from *M. edulis* (bottom left) and *M. trossulus* (bottom right). Bar = 1 μm.

Table 4.1. Frequency distribution of offspring genotyped after 20 days in crosses where sperm from one male of each species competed for eggs of a single female. E = *M. edulis*, T = *M. trossulus*, n/a = data not available, F = female, M = male and N = number of larvae genotyped.

Cross	Genotype	Offspring (%)				Pair-mating fert.(%)	
	F x M + M	*M. edulis*	Hybrid	*M. trossulus*	N	F x E	F x T
1	T x E+T		3.33	96.7	30	0.00	12.2
2	T x E+T		0.00	100	20	0.00	0.00
3	T x E+T		66.7	33.3	30	n/a	n/a
4	T x E+T		66.7	33.3	30	n/a	n/a
5	T x E+T		4.00	96.0	25	85.4	88.1
6	T x E+T		21.8	78.1	32	95.7	100
7	T x E+T		27.3	72.7	33	33.7	95.7
8	T x E+T		2.78	97.2	36	89.4	92.7
9	T x E+T		0.00	100	35	80.8	86.1
10	T x E+T		7.41	92.6	27	84.9	86.1
11	E x E+T	60.0	40.0		30	90.3	0.00
12	E x E+T	93.3	6.67		30	90.3	0.00
13	E x E+T	0.00	100		30	92.92	85.9
14	E x E+T	13.3	86.7		30	92.92	n/a
15	E x E+T	66.7	33.3		30	86.33	78.2

Table 4.2. Observed and expected numbers of both parental and hybrid individuals in the offspring of a controlled mass mating laboratory experiment. Expected results refer to a ratio of 1: 1: 2 for both parental species and hybrids, respectively, assuming that random mating occurred.

	M. edulis	Hybrids	*M. trossulus*	Total (N)
Day 10	18	9	13	40
Day 45	20	5	15	40
Expected	10	20	10	40

Table 4.3. Acrosome length, total length of sperm and nucleus width of the spermatozoon of *M. edulis* and *M. trossulus*.
N= number of sperm measured and number of mussels between parentheses. No significant difference between *M. edulis*, *M. trossulus* and hybrids was observed on any measured character (one-way Anova, $p > 0.05$).

	N	Acrosome length (µm ± SD)	Total length (µm ± SD)	Nucleus width (µm ± SD)	Acrosome/nucl. (µm ± SD)	Diameter equivalent (µm ± SD) **
M. edulis	82 (6)	2.14 ± 0.16	4.23 ± 0.52	1.65 ± 0.21	1.98 ± 0.24	2.55 ± 0.39
M. trossulus	57 (5)	2.18 ± 0.16	4.35 ± 0.26	1.60 ± 0.23	2.00 ± 0.09	2.57 ± 0.44
Hybrids	10 (1)	2.20 ± 0.11	4.18 ± 0.15	1.58 ± 0.06	1.90 ± 0.06	2.60 ± 0.46
M. gallo. *	18 (5)	5.08 ± 0.13	n/a	1.76 ± 0.13	n/a	n/a

* *M. galloprovincialis* data from Hodgson and Bernard (1986).

** Data collected from Coulter Multisizer. Represents the spherical equivalent for a non-spherical particle. N = ca. 9.6×10^4 sperm (14 mussels) for *M. edulis*, 7.1×10^4 sperm (11 mussels) for *M. trossulus* and 1.7×10^4 sperm (3 mussels) for hybrids.

Chapter 5

Hybrid fitness in a mussel mosaic hybrid zone in the northwest Atlantic

5.1. Introduction

One of the most challenging tasks in studies of marine speciation is to identify the various mechanisms responsible for reproductive isolation and their relative importance. Upon secondary contact and hybridization, diverging species face the paradox of maintaining genetic identity despite the great potential for gene flow facilitated by the high fertility and planktonic larval dispersal of many invertebrate species. In some marine taxa, forces responsible for reproductive isolation have to be particularly strong to counteract the high gene flow, otherwise species would merge following secondary contact. Alternatively, several different mechanisms could work together to maintain reproductive isolation despite their minor individual effects. The relative importance of isolating mechanisms in broadcast spawning marine invertebrates is probably different from that of terrestrial animals as a result of distinct reproductive modes. Physical contact and behavioural mate recognition cues are usually instrumental in preserving the genetic identity of most terrestrial species, but are almost absent in marine broadcast spawners (Palumbi, 1994). Due to logistical difficulties in assessing fertilization and tracking parentage in the ocean, speciation and hybridization are far better understood in terrestrial species than in marine species. Thus, model organisms such as the fruit fly *Drosophila*, the mouse *Mus* or the toad *Bombina* have provided evolutionary biologists with a good understanding of the mechanisms involved in hybridization and speciation of terrestrial species (e. g. Coyne and Orr, 1997, Orr and Turelli, 2001, Vines et al., 2003).

Although often overlooked and less well studied, hybridization in marine organisms probably occurs at the same rate as in terrestrial species. For example, Gardner (1997) was able to identify 108 cases of natural hybridization among several marine taxa,

suggesting that hybridization may also be common in marine species. However, in only a few of these cases has sufficient information been collected to allow the identification of possible mechanisms responsible for reproductive isolation. Among these, the *Mytilus edulis* complex is one of the most studied examples of hybridization in marine species. Although there have been great advances in understanding pre-zygotic isolating mechanisms in several groups (reviewed in chapter 4), post-zygotic isolation of marine invertebrate species is seldom studied because of difficulties in raising offspring through complex life cycles and long generation times (Palumbi, 1994).

The blue mussels *Mytilus edulis* and *M. trossulus* interbreed in the northwest Atlantic, forming a wide and patchy hybrid zone that provides excellent opportunities to study marine speciation and specific barriers to gene flow. In contrast to the well-studied *M. edulis* - *M. galloprovincialis* hybrid zone in Europe, very little is known about how the *M. edulis* – *M. trossulus* hybrid zone is maintained in the northwest Atlantic. Toro et al. (2002) suggested that temporal displacement in spawning could reduce hybrid formation in Newfoundland, but Maloy et al. (2003) found no differences in spawning time in Maine, concluding that other factors must be involved in reproductive isolation. Rawson et al. (2003) and the present work (chapter 4) have provided clear evidence of pre-zygotic mechanisms preventing hybridization at the fertilization stage. Gamete recognition and fertilization success are likely to be important barriers preventing gene flow between *M. edulis* and *M. trossulus*. However, the high percentage of advanced backcross individuals (25%) in natural populations in Newfoundland (Comesaña et al., 1999) suggests that pre-zygotic barriers alone are probably not strong enough to maintain reproductive isolation. Post-zygotic mechanisms have rarely been documented in hybrid zones between *Mytilus* species and other marine organisms.

Post-zygotic reproductive isolating mechanisms are those that result in reduced fitness of individuals of mixed ancestry expressed as a decrease in either fertility, viability or reproductive success of F1, F2 or backcross individuals relative to the parental species. The reduced fitness of hybrids may have an entirely genetic basis, being the result of problems in development of heterozygous and recombinant genotypes (often in embryogenesis) or irregularities in meiosis leading to lower fertility or complete sterility

(Harrison, 1990). Individuals of mixed ancestry may also have lower fitness which is expressed only under specific environmental conditions (Kruuk et al., 1999).

Postzygotic isolation is commonly a consequence of the accumulation of between-locus incompatibilities, as Dobzhansky (1937) and Muller (1942) first proposed. The Dobzhansky-Muller model postulates that postzygotic isolation arises in allopatry as a side effect of ordinary evolutionary divergence, and perfectly functional co-adapted genes from one species may lose their function or produce deleterious epistatic interactions if brought together in a different genetic background when hybridization and recombination occur. While the Dobzhanski-Muller model only explains intrinsic genetic mechanisms reducing hybrid fitness, extrinsic environmental mechanisms may also play an important role in the success of hybrids. In fact, the models explaining hybrid zones differ exactly on the importance given to genetic or environmental factors (discussed in chapter 2).

Jiggins & Mallet (2000) suggested that postzygotic incompatibilities are not only isolating mechanisms themselves but are also necessary for stabilizing incomplete assortative mating in bimodal hybrid zones, otherwise recombination will eventually destroy associations between species-specific recognition loci. In this situation, linkage disequilibrium between recognition loci must be maintained through selection against hybrids in order to preserve species identity. For broadcast spawning marine species, in which behavioural and other mate recognition cues are mostly absent, the evolution of very few loci related to gamete recognition proteins may be able to produce strong reproductive isolation (Palumbi, 1994). For these species, postzygotic selection against hybrids could play a minor role in preserving co-evolved mate recognition systems responsible for prezygotic isolation. Nonetheless, genetic incompatibilities among other gene systems not related to gamete recognition could still cause reproductive isolation through deleterious epistatic combinations that reduce hybrid fitness.

Another important consequence of postzygotic barriers to hybridization is that they may be able to accelerate prezygotic mechanisms through a process known as reinforcement. This controversial model, first proposed by Dobzhansky (1940), implies that selection against hybrids should increase prezygotic isolation because individuals that

mate with their own species are favoured. An interaction between partial pre- and postzygotic isolation would accelerate the evolution of stronger reproductive barriers between diverging species.

The *Mytilus* hybrid zone in the northwest Atlantic shows a clear bimodal distribution with very few F1 and F2 hybrids but several advanced backcross individuals that resemble one or other parent species (Toro et al., 2004). This implies that at least some hybrids are viable and reproductively successful. In fact, several other hybrid zone studies support the hypothesis that not all hybrids are uniformly unfit and that some hybrid groups can be as fit or even fitter than the parent species (Arnold and Hodges, 1995). Hybrid vigour is a well-known phenomenon and has been extensively used to improve agricultural and animal production. It has been suggested that hybrid vigour occurs at the larval stage in *M. edulis – M. trossulus* laboratory hybridization (Freeman et al., 1994; Toro, 1999). Beaumont et al. (1993) also suggested hybrid vigour in *M. edulis* and *M. galloprovincialis* hybrid larvae, but Bierne et al. (2002) did not observe any increase in fitness of the same kind of hybrids, nor did Matson et al. (2003) in *M. trossulus* and *M. galloprovincialis* hybrids. In most studies where hybrid vigour has been observed in mussels, the results are not consistent and may have been biased by density-dependent growth rate and the analysis of only a few crosses. Surprisingly, apart from larval studies, no other information is available for *Mytilus* hybrid fitness in other life-history stages. Here, laboratory and field data on the fitness of F1 hybrids between *M. edulis* and *M. trossulus* from fertilization to maturity are presented. Hybrid reproductive success and F2 viability are also discussed in the light of recent studies of adaptive evolution through hybridization and introgression.

5.2. Materials and Methods

5.2.1. Survival to day 10

The percentage of larvae that survived to day 10 was estimated using offspring from 38 families produced in the pair-mating experiment in Chapter 4 (2001 cohort), in addition to 27 other families produced in the previous summer (2000 cohort). Parents

were genotyped with ME, ITS, MAL, PLII, COIII, MPI and EST-D (see chapter 3 for details). For both cohorts, spawning and fertilization procedures were as described in chapter 4. Larvae were fed 15 $x10^3$ cells ml^{-1} *Isochrysis galbana* (T-iso) daily from day 3 to day 10 and were kept in static, filtered, UV sterilized seawater (15°C), which was changed 3 times a week. At day 10, larvae were sieved and resuspended in 100 ml filtered seawater. Two samples of 1 ml were taken from each family to estimate total number of larvae. As replicates of crosses were pooled at 72 hours, results for day 10 represent pseudo-replications, but only families in which the replicates did not statistically differ from each other for fertilization success and larva abnormality at 72 hours were used in the analysis. The several crosses with the same genotype (e.g. E x E or E x T) were used as replicates. For the analyses, values for percentage survival were transformed (arcsine square root of proportion surviving) and used in a one-way analysis of variance. Backcrosses are hereafter considered to be all animals of mixed genotypes with the exception of F1 and F2 hybrids, which are referred to separately. A hybrid index incorporating 5 diagnostic markers was used to determine the genetic composition of backcrossed individuals (table 5.1). Although this group includes animals with large variability in their genetic composition (almost all backcrosses were unique), they were pooled to facilitate the analyses, although specific cases are discussed separately when appropriate.

5.2.2. Larval stage

One experiment was conducted with the 2000 cohort to assess larval growth and survival until metamorphosis. Five families with different genotypes were grown in duplicate 1.5 liter buckets with standard hatchery techniques described in chapter 4. A preliminary experiment conducted four weeks before suggested increased growth rate for hybrid larvae, but results could have been misleading as there was higher mortality of hybrid larvae (mainly in the first week of life) and culture densities were not carefully adjusted. This probably led to more food, less stress and consequently a better growth rate in some cultures than in others. Freeman et al. (1994) and Toro (1999) also identified the possibility of similar bias in their experiments. To avoid the influence of higher hybrid

mortality in the first week of life, the main experiment was started with 10 day-old larvae, and to prevent density-dependent growth rate culture density was adjusted every week. Densities at the beginning of the experiment were approximately 10 larvae/ml and the total number of larvae was $15x10^3$ with the exception of one family (T x E) that had approximately half the number, but the same density, as the others. Densities were reduced to 5 larvae ml^{-1} at day 15 of the experiment and the last samples were taken when approximately 50% of larvae were undergoing metamorphosis in most cultures (day 30 of experiment). A subsample of known volume was used to determine survival and the shell lengths of 30 larvae from each replicate were measured with an Olympus compound microscope. Cultures continued for another 60 days but only data on the number of spat that survived was collected as differential mortality during metamorphosis probably affected subsequent growth rate, since densities were not adjusted.

5.2.3. Laboratory cohorts

Two similar experiments were conducted to evaluate hybrid fitness at the juvenile stage. On November 2000 approximately 700 individuals from 23 families produced in the laboratory were isolated in individual containers (35 x 5 cm cylindrical tubes with nylon mesh on both ends). Families started with 2 replicate containers, all in one tank (800L) provided with a continuous flow of unfiltered seawater. Genotypes were determined by scoring the parents for ME, ITS, MAL, PLII, COIII, MPI and EST-D. Parents originated from a wild population in Chance Cove, Newfoundland. After 6 months, survival was evaluated and families were moved to a continuous flow-through system at a density of 100 mussels per container. All families were isolated in 1.8L buckets and placed in 2 communal tanks (one for each set of replicates). Unfiltered seawater from the same source was supplied to each container for 18 hours a day at a flow rate of 20 ml min^{-1}. During the remaining 6 hours, water was recirculated with approximately 2.3 x 10^5 cells ml^{-1} live *Isochrysis galbana* (T-Iso), *Chaetoceros muelleri* and *Tetraselmis suecica* per day (5:5:2 ratio respectively). In December 2001 another 2 tanks were added to the system to accommodate 30 new families (one replicate in each tank) produced in the summer of 2001. Parents came from 5 different aquaculture sites

(see chapter 2) and were genotyped with the same 7 markers as the 2000 cohort. To compensate for the larger number of animals in the system, in addition to the live algae, concentrated algae paste (Instant Algae©, Six Species Shellfish Blend, Reed Mariculture Inc.) was added 3 to 5 times a week. After 6 months, mortality and size class distribution were determined for the 2001 cohort and densities were adjusted from the initial 500 mussels to 100 mussels per replicate. Both cohorts were monitored periodically for growth and survival. Data for the 2000 cohort was obtained for months 3 to 27 and for the 2001 cohort months 4 to 22.

5.2.4. Field

In October 2001 several families produced the previous year in the laboratory were pooled to form four groups of known genotype: pure *M. edulis* (E - 5 families), pure *M. trossulus* (T - 4 families), F1 hybrid offspring from *M. edulis* females crossed with *M. trossulus* males (ET – 5 families) and F1 hybrids from *M. trossulus* females crossed with *M. edulis* males (TE – 4 families). Each family contributed a similar number of individuals to the pooled groups. Each of the four groups was then divided into subgroups of 100 mussels (replicates) and placed in pearl nets for grow-out in different environments. Three replicates of each genotype were placed in each of the three mussel farms chosen for their environmental characteristics and mussel species composition. In summary, Charles Arm is a semi-closed inlet where almost only *M. edulis* is found, Salmonier Cove is a deep inlet where *M. trossulus* is abundant in smaller size classes and Long Harbour is a shallow, productive environment where both species coexist (see chapter 2 for details).

Temperature was monitored daily with a thermograph. Salinity and chlorophyll *a* data was provided by the Shellfish Environmental and Biological Monitoring Program from CTD (Seabird Inc.) data collected seasonally. Mussels were sampled 3 times per year for measurements of growth and survival rates, and moved to clean pearl nets at every sampling. The grow-out experiment lasted 24 months with the exception of Charles Arm, where the farm owner required that the mussels were taken out after 12 months.

5.2.5. Reproduction and F2 success

To test the hypothesis that hybrids are sterile or less reproductively active than "pure" genotypes, results for reproductive development from chapter 2 are discussed from the hybridization point of view. In addition to the gonad maturation analysis described in chapter 2, the number of eggs spawned per female was estimated in July 2003. Mussels from the genotype groups growing in pearl nets in Long Harbour were brought to the laboratory and induced to spawn (methods described in chapter 4). Approximately 30 mussels of each genotype were isolated and allowed to spawn for 6 hours starting at the moment the first gametes were observed. Eggs were then sieved, resuspended in 300 ml filtered seawater and 2 samples taken to determine with a Coulter Mutiziser the total number of eggs spawned during that period. No attempt was made to quantify fertilization success, but 16 F1 hybrids were inter-crossed to produce 12 families of F2 hybrids and also backcrossed to both parent species to produce another 18 families. In addition, F1 hybrids from the same 2000 cohort that were kept in the laboratory throughout their lives were crossed on August 2002 to produce 21 families of F2 hybrids and backcrosses that were grown in the laboratory for a period of 1 year. Although fertilization success and culture conditions were not carefully controlled, results are also presented due to their relevance in determining hybrid fitness.

5.3. Results

5.3.1. Survival to day 10

In general, more larvae survived to day 10 in intraspecific crosses than in interspecific crosses, but there were some exceptions (table 5.1). Percentage survival to day 10 represents exclusively mortality after fertilization (endogenous post-zygotic mechanisms). The percentage of larvae remaining in relation to the number of eggs available for fertilization (5×10^3 eggs) represents pre- and postzygotic mechanisms acting together at the fertilization stage. There was no significant difference (Anova, p =

0.75) between the percentage of normal larvae observed at 72 hours (76.4 %) and the percentage of survival at day 10 (74.8 %) in intraspecific crosses, but in interspecific crosses percentage of normal larvae at 72 hours (59.5 %) was significantly higher than survival at day 10 (48.3 %) (Anova, $p = 0.03$). This difference suggested that more 'apparently normal' larvae died in interspecific crosses than in intraspecific crosses. Survival to day 10 was significantly greater in intraspecific than in interspecific crosses (Anova, $p < 0.001$). No statistical difference was observed between hybrids from the 2000 cohort and 2001 cohort, but intraspecific crosses had a better survival in the 2000 cohort (figure 5.1). There was also no difference between pure *M. edulis* and pure *M. trossulus* crosses or between the two kinds of hybrids in any of the cohorts (figure 5.1). Hybrids and backcrosses showed the lowest survival and highest variance.

5.3.2. Larval stage

Data for final shell length of larvae (day 40) fell into two subsets of families. Pure *M. edulis* and F1 hybrid TE formed one subset that showed a significantly smaller shell length than the other subset, which was composed of the remaining 3 families (Anova and Duncan multiple comparison for homogeneity of subsets, $p < 0.05$, figure 5.2). Results for mortality were inconsistent and may have been affected by protozoan contamination in some replicates. Nonetheless, one of the families produced from an advanced backcross individual did not show any sign of contamination and still exhibited greater mortality than the other families during the larval stage (Anova, $p < 0.05$), almost no spat surviving to day 90 (figure 5.3). There was no significant difference in survival rate among the other families. In general, hybrid larvae performed as well as pure larvae when growth and survival are considered only from day 10 to metamorphosis, with the exception of one backcross family in which survival was compromised during the larval stage.

5.3.3. Laboratory cohorts

After 6 months survival was significantly greater in *M. edulis* families than in most of the other groups in the 2000 cohort, except the F1 hybrid (ET), in which mean survival values were intermediate between those of the parent species but not significantly different from either of them (one-way Anova, figure 5.4). Survival was significantly lower in the other F1 hybrid (TE) than in all groups but the backcrosses, the group with the lowest mean survival. The proportion of large spat (> 3 mm) was greater in both F1 hybrids than in either pure species. *M. trossulus* had the lowest proportion of animals in the larger size class (Fig. 5.4). Although *M. edulis* showed the greatest mean values for survival and backcrosses showed the lowest values after densities were readjusted to 100 mussels per replicate in October 2001, there was no significant difference in survival among any of the groups (Anova, $p = 0.23$, figure 5.5).

There was a significant difference in final shell length among the groups in the 2000 cohort (Anova, $p < 0.01$, figure 5.6). After 27 months, shell length was greater in *M. edulis* than in the other groups, except the F1 hybrid ET. Although no significant difference was observed between the two kinds of F1 hybrids, they were both intermediate to the parent species and not significantly different from the species with which they share the same maternal genotype. F1 hybrids ET were significantly different from *M. trossulus*, but not different from *M. edulis*. Conversely, F1 hybrids TE were significantly different from *M. edulis* but not from *M. trossulus*. This tendency to show a maternal effect was clearly observed in the field experiments, where carrying capacity was not a limiting factor and more time was allowed for growth. Backcrosses also showed mean values for shell length intermediate to the parent species, but were only significantly different from *M. edulis* (fig. 5.6). The deviation in the growth curve in figure 5.6 just after densities were reduced to 100 mussels per container shows that density was a limiting factor on growth in all groups. The later decrease in growth rate, even during the summer, also suggests that density affected growth rate in the laboratory, but all groups were equally affected. For the 2001 cohort the same trends were observed as the 2000 cohort, although data were obtained for a shorter period. From months 4 to

22, *M. edulis* showed significantly greater survival than F1 hybrids TE (Anova, $p< 0.05$), but no difference from *M. trossulus* and F1 hybrid ET (figure 5.7). The other groups did not differ from each other. No differences were observed among groups for growth rate over the same period (Anova, figure 5.8).

5.3.4. Field

Results from grow-out experiments at mussel farms followed the same trend as the laboratory experiments, but growth was faster, as conditions were more favourable. Although grow out in Charles Arm had to be terminated earlier than at the other sites, growth rate showed a very consistent pattern among all sites. *M. edulis* and F1 hybrids from *M. edulis* females (ET) had a faster growth rate than the other groups and were not statistically different from each other at any site (figure 5.9). Growth rate for all the genotypes was greatest at Long Harbour, probably due to the favourable environmental conditions (productive and warmer waters). At this site differences between genotypes were more apparent and, surprisingly, F1 hybrids from *M. edulis* females (ET) were significantly larger than the other groups before the last summer, but equal to *M. edulis* after the summer. At all sites *M. trossulus* and F1 hybrids from *M. trossulus* females (TE) showed very similar growth rates, both being significantly lower than the two other groups (Anova, $p < 0.05$). A maternal effect on hybrid growth was clear at all sites, hybrids ET being similar to *M. edulis* and hybrids TE being similar to *M. trossulus*.

Survival rates varied among sites, although mortality was very low at all sites before the last summer (average of 16%) when the highest mortality occurred for most groups (figure 5.10). The most consistent pattern observed was a clearly better performance of *M. edulis* at both sites for which the experiment lasted 2 years. At Long Harbour and Salmonier Cove, although a greater mortality was observed for all groups during the second summer, *M. edulis* survived better than the other three groups, which did not differ from each other in survival rate (fig 5.10). No significant difference in survival was observed among any groups at Charles Arm, but the experiment at this site was terminated earlier than at the others and mussels did not experience a complete summer in which they were reproductively active.

5.3.5. Reproduction and F2 success

Although *M. trossulus* developed more gonad tissue than *M. edulis* in the first year of maturation, probably reflecting earlier reproduction, hybrids also developed gonads and once again showed a strong maternal effect (chapter 2, fig. 2.8). The number of eggs that hybrids spawned also showed a maternal effect (Fig. 5.11). However, mussels in this experiment were much larger than those used in chapter 2 (mean lengths 37.64 mm and 17.75 mm, respectively) and may have been in their second reproductive season. *M. edulis* and F1 hybrids ET spawned more eggs than *M. trossulus* and F1 hybrids TE (Fig. 11). Nonetheless, hybrids spawned a reasonable number of eggs (mean 5.5 x 10^5 eggs in 6 hours) and at least some of them were viable. Of the 30 crosses performed with F1 hybrids (crossed to other F1 hybrids or the parent species), 26 produced normal "D-larvae" larvae after 72 hours. Larval cultures were terminated after 10 days, and although no attempt was made to quantify fertilization and larval success, more than half the families contained large numbers of healthy larvae, comparable with other intraspecific crosses performed in previous years. Families produced by crossing F1 hybrids in 2002 also showed a similar pattern. A total of 21 families (F2 hybrids and backcrosses) was produced from 26 crosses performed with F1 hybrids. After 10 days, all larvae in 4 of these families had died and after metamorphosis (40 days) another 3 families showed zero survival. During the following 11 months, 100% mortality occurred in only one other family. Of the 13 families that survived to 12 months, 8 were backcrosses of F1 hybrids to the parent species and 5 were F2 hybrids. Seven of these remaining families ended with very few individuals (less than 50), 3 with moderately high numbers (over 500) and the other 3 (including two F2 families) produced more than 4 x 10^3 mussels after 12 months.

5.4. Discussion

Hybridization between two divergent species normally results in reduced fitness of hybrid progeny, but sometimes hybrid vigour is observed (Barton, 2001). The

increased fitness of F1 hybrids is usually attributed to overdominance (heterozygote advantage), but it could also result from the masking of deleterious recessive alleles or favourable epistasis (Edmands, 2002). Frequently, however, hybrid fitness declines after the F1 generation (hybrid breakdown) due to recombination and deleterious epistatic interactions, as the Dobzhansky-Muller model predicts (Dobzhansky, 1937, Muller, 1942). In addition, reduction of fitness in hybrids is commonly expressed in the F1 generation as a result of incompatibilities between diverging genomes and/or the disruption of local adaptation to a specific environment (e.g. Howard et al. 1993; Gaffney et al. 1993; Gaffney and Allen, 1993; Orr and Irving, 2001).

Although some F1 hybrids may be as viable as the parent species or even superior in some cases it is often expected that hybrid breakdown will emerge in later generations (Endler, 1977). The present findings, that some backcrosses have lower fitness than parent species and F1 hybrids, are in complete accordance with the Dobzhansky-Muller model, which predicts that epistatic incompatibilities are expressed when homozygous alleles that are neutral or beneficial in one genetic background encounter conflict if combined with alleles from a different genetic background. In this situation, F1 heterozygotes and some backcrosses would not necessarily express the deficiency. There is strong evidence that hybrid sterility and non-viability in many animal taxa evolved according to the Dobzhansky-Muller model (see Orr, 1995 for review). Results from the present study not only fit the model but also suggest that hybrid breakdown plays a role in reproductive isolation in *Mytilus*. Backcrosses consistently performed more poorly than the other groups. Survival of backcrosses was lower than in the parental genotypes at day 10 but not different from that of F1 hybrids. A conspicuously lower survival in one backcross family during the larval stage and through metamorphosis suggests that hybrid breakdown occurs at several different stages of development. In addition, survival to six months and two years in the laboratory showed that some backcross families were clearly selected against, supporting the hypothesis that genetic incompatibility in later generations of hybrids reduces gene flow and helps to maintain reproductive isolation. Lower fitness of hybrids is usually attributed to the breakdown of co-adapted gene complexes, although other explanations are possible,

particularly in the genus *Mytilus*. The unusual pattern of mitochondrial DNA inheritance in *Mytilus* in which males also possess a lineage that is passed from fathers to sons in addition to the usual female lineage (Zouros et al., 1992; 1994) adds an additional obstacle to genetic compatibility. This 'doubly uniparental inheritance' (DUI, Zouros et al., 1992) allows the possibility of hybrids with one nuclear genetic background and a different mitochondrial DNA, potentially increasing the likelihood of incompatibility. The facts that DUI frequently breaks down upon hybridization and many male hybrids in nature do not carry the paternal lineage (Rawson et al., 1996) suggest that cytoplasm-nuclear incompatibilities may reduce hybrid fitness. Cytonuclear epistatic interactions often cause genomic incompatibility between plant species and between some animal species (Liu et al., 1995; Ulloa et al., 1995; Cruzan and Arnold, 1999). There is also a possibility that the two *Mytilus* species differ in chromosomal arrangements. Data suggest that *M. trossulus* from the Pacific has 7 metacentric chromosomes while *M. edulis* from Europe has only 6 (Martinez-Lage et al., 1995; Gonzalez-Tizon et al., 2000). The cytogenetics of northwest Atlantic populations of *Mytilus* has never been investigated (but see chapter 3). Hybridization between species that differ in chromosome composition is possible (although not frequent) and a few hybrid zones are known (Reed and Sites, 1995; Rieseberg, 2001). The most common problem for such hybrids is that they are unable to complete meiosis properly and produce viable gametes, resulting in postzygotic isolation (Navarro and Barton, 2003).

Although hybrids are usually observed to have lower fitness than parental genotypes, this does not preclude the formation of later generations and novel hybrid recombinants. Natural selection will eventually eliminate negative gene combinations, but it may also maintain positive interactions that may appear, allowing these novel beneficial combinations to spread into the population (Rieseberg et al., 1996). The common observation that a small fraction of hybrid genotypes is as fit or fitter than the parental species (Grant, 1966; Reed and Sites; 1995, Burke et al., 1998) has usually been overlooked because in general the majority of hybrids have lower fitness. The large variance in backcross fitness for growth rate and survival in the present study suggests that some specific combinations may be neutral or even advantageous in *Mytilus*. The

success of some specific backcrosses may allow introgression of beneficial genes from one species to another, promoting adaptive evolution through hybridization. The role of hybridization and introgression in the evolutionary process has generally been neglected and under-emphasized (Rieseberg, 1998), but recently a full body of theory has been developed to explain the importance of introgressive genes in the evolutionary process (reviewed in Wu, 2001). Beneficial introgression may occur in the blue mussel hybrid zone in the Baltic Sea, where mitochondrial DNA from *M. edulis* has completely replaced *M. trossulus* mtDNA (Quesada et al., 1999), possibly conferring more adaptability to the environment. If these same species are adapted to different environmental conditions in the northwest Atlantic (different temperature regimes, for example) but overlap in distribution and hybridize considerably, then recombination could eventually permit some genes to introgress from one species into the other. Assuming that *M. edulis* is warm adapted and *M. trossulus* more cold adapted, *M. trossulus* individuals could benefit from *M. edulis* introgressive genes that improve fitness in warmer water, and conversely, *M. edulis* could benefit from introgression of *M. trossulus* genes for adaptation to cold waters. The effects of long-term hybridization and introgression through a semi-permeable hybrid zone have yet to be demonstrated, but both species may exchange beneficial alleles that increase fitness in particular environments. Although temperature is probably one of the most important factors determining species distribution, other adaptations that differentiate the two species (e. g. salinity, wave exposure, feeding regime) could potentially increase or decrease hybrid fitness, depending on the environment. Salinity tolerance, in particular, is believed to play a role in *M. edulis* and *M. trossulus* distribution in the Baltic Sea and the northwest Atlantic due to differential physiological adaptation (Johannesson et al. 1990; Varvio et al. 1988; Väinölä and Hvilsom, 1991; Gardner and Thompson, 2001; Qiu et al, 2002). The observation that hybrids are often less fit than the parental species in both parental habitats but are particularly good at colonizing novel or disturbed habitats suggests that the environment probably plays an important role in maintaining some hybrid zones (Harrison, 1993). In addition, many hybrid zones form a narrow cline exactly on a biogeographic boundary, suggesting that hybrids are usually less fit in both parental habitats but sometimes equal

or superior in intermediary habitats (Endler, 1977). This could allow high levels of recombination in the hybrid zone, and introgression of novel co-adapted gene systems may then occur. Marine hybrid zones are particularly susceptible to recombination as the long larval dispersal period extends the area of overlap between species and the high fecundity allows more hybridization and variability in recombinant types. The patchy distribution of both *M. edulis* and *M. trossulus* in Newfoundland suggests that the environment is probably a key factor determining the distribution of both species. Other mosaic hybrid zones have been closely correlated with adaptation to specific environments (Howard, 1986; Harrison and Rand, 1989; Sites et al., 1995; Harrison and Bogdanowicz 1997, Vines et al., 2003), but not much is known about what factors determine *Mytilus* distribution in the northwest Atlantic. A combination of factors, such as temperature and salinity, may be responsible for the mosaic distribution. On a macrogeographic scale, temperature is likely to limit the distribution of *M. trossulus* to southern Maine (Rawson et al., 2001), while *M. edulis* occurs as far south as Cape Hatteras (Koehn et al., 1984; Koehn, 1991; Seed, 1992). In Europe, tolerance to low salinity probably maintains allopatric populations of *M. trossulus* in the Baltic Sea (Johannesson et al. 1990; Väinöla and Hvilsom, 1991). Adaptation to specific ranges of temperature and other environmental factors are also likely to maintain partial reproductive isolation between *M. edulis* and *M. galloprovincialis* (Gardner et al. 1993; Wilhelm and Hilbish, 1998), which also form a hybrid zone in which the mosaic pattern is probably correlated with environmental adaptations (Gardner, 1994a; Bierne et al., 2002b; Fuentes et al., 2002). The patchy distribution over a broad area in this and the northwest Atlantic hybrid zones provides a large number of microgeographic realms for hybridization to occur, in which some hybrids may have an advantage in transitional or disrupted areas.

The few studies that have investigated *Mytilus* hybrid fitness focused on the larval stage of F1 hybrids, and some of the results suggested the occurrence of hybrid vigour (expressed as increased growth rate) at this stage of development (Beaumont et al, 1993; Freeman et al., 1994; Toro, 1999). However, all authors identified the possibility that higher initial mortality in hybrids resulted in density-dependent growth rate at the

larval stage, and results were not consistent within and between trials. Similarly, the present study also observed high mortality of hybrid mussels during the first week in some crosses, but not in all. As expected, the higher mortality of hybrids observed at day 10 was closely related to the higher percentage of abnormal larvae at 72 hours (table 1). Abnormal larvae were probably not able to acquire food in the first days of life as a consequence of shell malformation and inability to swim. Abnormal development, often during embryogenesis, is known to be one of the major causes of hybrid non-viability (Harrison, 1990). In addition, the higher proportion of hybrid larvae that possessed an apparently normal "D" shell at 72 hours but died before day 10 also supports the hypothesis that post-zygotic mechanisms at early stages of development reduce hybrid viability. Lu and Bernatchez (1998) observed daily mortality rates 2.4 to 4.7 times higher in hybrid embryos of lake whitefish than in pure species. It is important to note that some hybrid crosses in the present study showed very low percentages of abnormality and mortality in larvae. Maternal effects, gamete quality and genetic compatibility are particularly important to larval development, therefore a large variance in offspring performance is usually observed in bivalve larvae from laboratory crosses (Bayne, 1983; Utting & Millican, 1997). The present results suggest that hybrid superiority at the larval stage can occasionally occur (but not frequently), although this may be due to factors other than genetic overdominance. A larger number of crosses and more genetic markers would provide a better understanding of the interactions among divergent genomes. Studies of later stages of development should also provide a better understanding of the genetic components of hybrid fitness. The factorial crossing design of the present study allowed the observation that several heterospecific crosses produced a high percentage of abnormal larvae while their homospecific half-siblings were mostly normal, ruling out the influence of gamete quality in many cases. This clearly indicates that the reduced fitness observed in some F1 hybrids and backcrosses has a genetic basis. On the other hand, some other hybrid combinations could benefit from heterosis. If the genetic architecture of parents from divergent species does not produce any deleterious effects when combined, then hybrids could be normal or even express heterozygosity advantage. The present field experiments revealed that hybrid individuals that survived the larval stage

showed equal or intermediate fitness, and in some occasions even superior fitness (growth rate), to one or both parent species.

The advantage conferred by heterozygosity in bivalves has been a controversial subject but there is evidence that individuals with higher levels of heterozygosity exhibit increased fitness in monospecific populations (Zouros and Pogson, 1994; Hedgecock et al., 1995, 1996). In contrast to Beaumont et al. (1993) and Toro (1999), larval growth data in the present study do not support the hypothesis of hybrid vigour at this stage, although a larger number of families should be analyzed to assess differences between and within genotypes. Hybrid vigour, defined as the increased fitness of hybrids in relation to both parent species, was observed on one occasion, when F1 hybrids ET showed a faster growth rate and higher survival in Long Harbour than other groups until the first reproductive season. The fact that hybrid superiority was observed at the warmer and more productive site suggests that heterosis may benefit some hybrid individuals under favourable environmental conditions. However, high mortality was observed in this group during the following summer and the final shell length was not different from that of *M. edulis*. High temperatures during the second summer were probably detrimental to mussels, coinciding exactly with the period of significant investment of energy reserves in reproduction. Summer mortality of mussels, particularly *M. trossulus*, has often been described in the Pacific and Atlantic (Dickie et al., 1984; Mallet et al., 1990; Myrand and Gaudreault, 1995; Mallet and Carver, 1995; Tremblay et al., 1998 a, b). The higher mortality of hybrids is probably related to the fact that *M. edulis* is better adapted to warmer temperatures than *M. trossulus* (chapter 2, figure 2.5). Proteins produced by epistatic loci may be less efficient or costlier to produce if hybridization breaks down the coadapted gene system of diverging populations. The metabolic efficiency of enzymes is strongly affected by temperature, so enzymes are designed to work in a specific temperature range (Freeman and Herron, 2001). A decrease in metabolic efficiency in different environmental conditions has been demonstrated in hybrids of copepods and crabs (Burton, 1990; Combs et al., 1997). If *M. trossulus* is better adapted to lower temperatures, the metabolic cost in warmer waters should increase as enzyme efficiency decreases, therefore hybrid fitness would also decrease owing to the presence of *M.*

trossulus alleles that results in 'mixed', maladapted enzymes. The strong maternal effect also makes F1 hybrids from *M. edulis* females (ET) fitter than TE hybrids and *M. trossulus* in the study area (Newfoundland), as *M. edulis* itself is presumably better adapted to these environmental conditions. Fuentes et al. (2002) observed that selection against hybrids of *M. edulis* and *M. galloprovincialis* in Spain is highly associated with lower levels of the stress protein calreticulin and heat shock protein 70. Hilbish et al. (1994) also observed a correlation between temperature and physiological adaptation of these two species. Although mortality was in general low after metamorphosis in most of the present experiments, there was a tendency for *M. trossulus*, both kinds of F1 hybrids and backcrosses to suffer greater mortality than *M. edulis*. F1 hybrids were inferior to *M. edulis* in survival at 6 months and 2 years in laboratory experiments, but ET hybrids were equal to *M. trossulus* at 6 months (2000 cohort) and 18 months (2001 cohort). The same pattern was observed in the field, but the maternal effect on hybrids was more conspicuous. Although high mortality was observed in all groups during the first summer of reproduction, *M. edulis* had a better survival rate than *M. trossulus* and hybrids. Mortality in the field over the summer showed a very similar pattern to mortality of laboratory mussels exposed to high temperatures after the spawning season (figure 2.6).

A comparison with the data from the static cohort analysis at 5 aquaculture sites (chapter 2) suggests that *M. edulis* has a clear advantage over *M. trossulus* and hybrids in most parts of Newfoundland. The significant reduction in frequency of *M. trossulus* and hybrids in the larger size classes in the static cohort analysis was not as evident in the present controlled growth experiment, suggesting that other factors than environmental adaptation may be involved. Predation, mainly by sea stars, is a major source of mussel mortality in Newfoundland farms. In the present experimental conditions, predators were prevented from reaching the mussels. Differential resistance of *Mytilus spp.* to parasites has never been evaluated in the northwest Atlantic, but Fuentes et al. (2002) observed that hybrids from *M. edulis* and *M. galloprovincialis* were more susceptible than the pure species to mortality caused by the protist *Marteilia refringens*. Strength of attachment to the substrate and competition for space are also important factors excluded from the present experimental design as the genotypes were kept separately in cages or buckets

and drop-off due to weak byssal attachment was prevented. Some mussel farmers have suggested that *M. trossulus* individuals take longer to produce byssal threads than *M. edulis* , and also produce weaker threads (Sean Macneill, personal communication). Variation in strength of attachment is probably one of the causes of differential mortality in the European *M. edulis – M. galloprovincialis* hybrid zone (Gardner and Skibinski, 1991; Willis and Skibinski, 1992). In addition to metabolic malfunction associated with reduced adaptation to a particular environment, lower fitness could also be the result of some intrinsic characteristic of the species that is originally adapted to different predators, competitors and life-history strategies.

M. edulis was also superior to *M. trossulus* in terms of growth rate, and a strong maternal effect was observed in the hybrids. Penney et al. (2002) obtained similar results in Newfoundland, hybrids being typically intermediate to the parent species, but often statistically similar to one or other of them. Although growth rate is an important component of fitness, it is probably not the ideal fitness correlate in this particular *Mytilus* hybrid zone since there is evidence that the two species differ in their life history. Results from chapter 2 suggest that *M. trossulus* invests earlier than *M. edulis* in reproduction, whereas *M. edulis* commits more resources to shell deposition in the first year, thus the trade off must be balanced in order to estimate fitness. From the evolutionary point of view early reproduction is a clear advantage, but on the other hand early mortality could nullify the advantage. High fecundity and post-spawning mortality of young *M. trossulus* individuals could be counterbalanced by longevity and many spawning seasons in *M. edulis*. A better understanding of life-history strategies of both species would provide a clearer estimate of fitness. It is particularly important to understand if those characteristics of *M. trossulus* that are commonly associated with invasive species (e. g. small eggs, early reproduction, see chapter 2 for details) occur only in the hybrid zone and reflect some character displacement to avoid competition with *M. edulis* or if these are intrinsic characteristics of *M. trossulus* even outside the hybrid zone.

The present study suggests that intrinsic postzygotic mortality represents an additional reproductive isolating mechanism between *M. edulis* and *M. trossulus*. In addition to the prezygotic mechanisms described in chapter 4, reduced hybrid viability,

mainly in the early stages of development, may be an important factor reducing hybridization and preserving species identity. Adaptation to different environmental conditions may also play an important role in regulating hybrid fitness. As in other hybrid zones, the *Mytilus* hybrid zone in the northwest Atlantic is probably maintained by a combination of several factors and partial post-zygotic isolation is probably critical in preserving in each species the co-adapted genetic interactions responsible for prezygotic isolation.

The consistently higher variance for some fitness components (growth and survival) in hybrids, particularly backcrosses, implies that hybrid breakdown is likely to occur in later generations of *M. edulis* x *M. trossulus* hybrids. However, it also suggests that some backcrosses can occasionally perform well. The fact that F1 hybrids and backcrosses are viable and probably as fertile as pure individuals (figures 2.8 and 5.11) implies that many different kinds of hybrid progeny can be produced and that natural selection plays an important role in determining their success. Although several F1 hybrids from the present work were successfully crossed with each other and backcrossed to both parent species, producing thousands of individuals of a second generation of hybrid mussels, the overall fitness of these progeny has yet to be determined. The expected result is that in most cases hybridization will break down co-adapted gene complexes, leading to combinations of inferior viability that are eliminated by selection (Mayr, 1954), but hybridization could occasionally produce better adaptations that would enhance the evolutionary process (Wu, 2001). Future research is necessary to understand the relative importance of post-zygotic mechanisms in preserving genetic integrity in *Mytilus* species and to understand the role that hybridization plays in the evolutionary process.

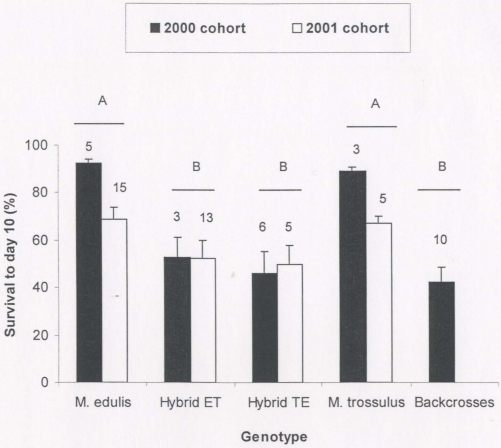

Figure 5.1. Percentage larval survival of parent species, F1 hybrids and backcrosses 10 days after fertilization. Error bars represent standard error and the number above is the number of crosses for each genotype. Different letters above the columns represent significant differences between genotypes (Anova on pooled cohorts and Duncan multiple comparison test for homogeneity of subsets, $p < 0.05$). Genetic composition of backcrosses is detailed in table 5.1 (crosses 56 to 65).

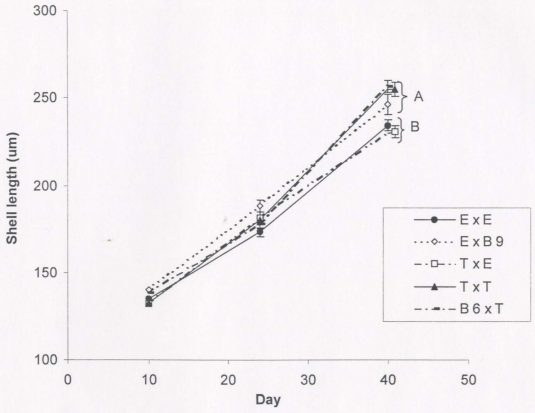

Figure 5.2. Larval growth in *Mytilus* spp. families from day 10 to metamorphosis (day 40). N = 100 larvae per family per sample date (50 per replicate). Error bars represent standard deviation. Different letters represent significant differences between genotypes (Anova and Duncan multiple comparison test for homogeneity of subsets, $p < 0.05$). Number after backcrosses represents hybrid index based on 5 genetic markers (see table 5.1 and figure 3.3).

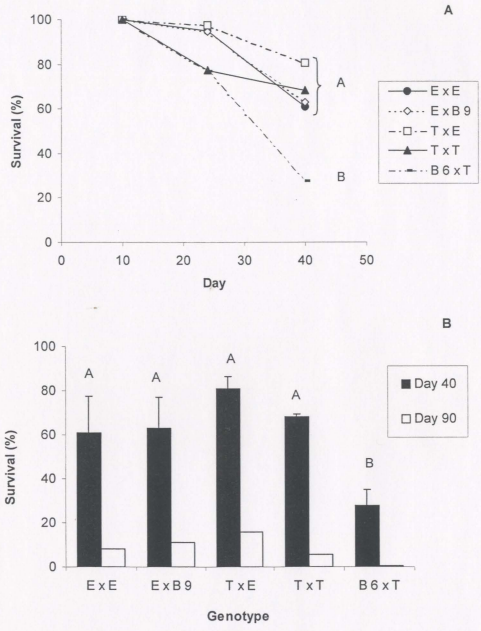

Figure 5.3. Survival of *Mytilus* families during the larval stage (A) and after metamorphosis (B). Error bars represent standard error. Different letters above the columns represent significant differences between genotypes (Anova and Duncan multiple comparison test for homogeneity of subsets, $p < 0.05$).

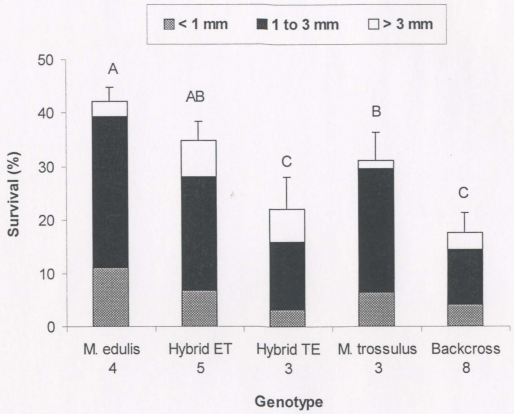

Figure 5.4. Survival and size class distribution of *Mytilus* spp. juveniles grown in the laboratory from months 5 to 12. Error bars represent standard deviation. N = number of families (below genotypes) x 2 replicates. Different letters above the columns represent significant differences between genotypes (Anova and Duncan multiple comparison test for homogeneity of subsets, $p < 0.05$).

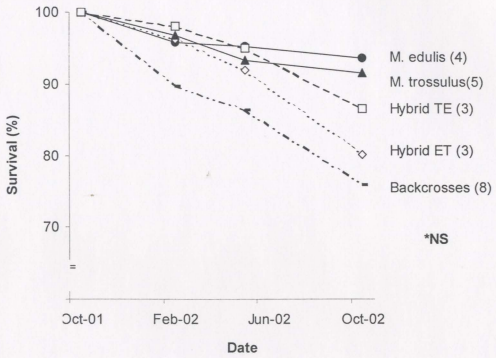

Figure 5.5. Mean survival of *Mytilus* families in the laboratory from months 15 to 27. Number after genotype represents number of families. N = number of families x 2 replicates. * No significant difference was observed among the groups (Anova, $p>0.05$).

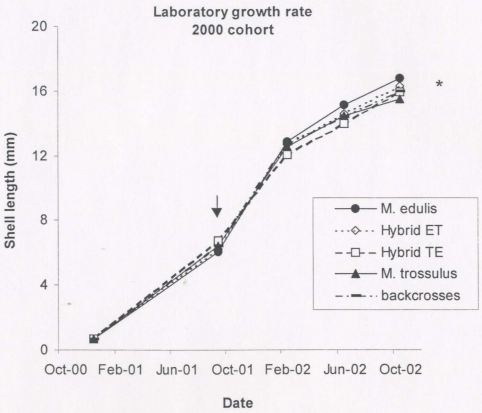

Figure 5.6. Mean growth rates of *Mytilus* spp. families in the laboratory from months 5 to 27. Arrow points to when densities were reduced to 100 mussels per replicate and mussels were moved to a flow-through system. Sample size (N), final mean shell length (SL), standard error (SE), Anova and Duncan multiple comparison test for homogeneity of substes are presented below (* $p < 0.05$).

	N (families)	N	SL ± SE	* Duncan
M. edulis	4	400	16.79 ± 0.32	A
Hybrid ET	5	482	16.26 ± 0.14	AB
Hybrid TE	3	300	15.95 ± 0.23	BC
M. trossulus	3	300	14.48 ± 0.13	C
Backcrosses	8	632	15.72 ± 0.12	BC

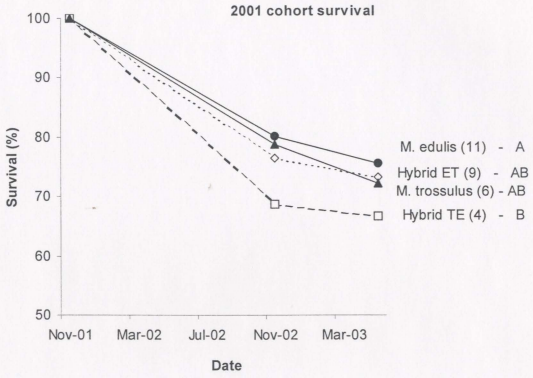

Figure 5.7. Survival of *Mytilus* families from the 2001 laboratory cohort from months 4 to 22. Numbers after genotypes represent number of families. Different letters represent significant differences between genotypes (Anova and Duncan multiple comparison test for homogeneity of subsets, $p < 0.05$).

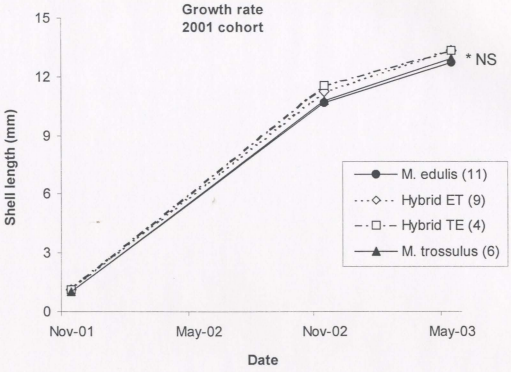

Figure 5.8. Growth rate for 2001 laboratory cohort families from months 4 to 22. N = 50 to 100 larvae per family per sample date x number of families (between parentheses). * No significant difference was observed among any groups (Anova, $p > 0.05$).

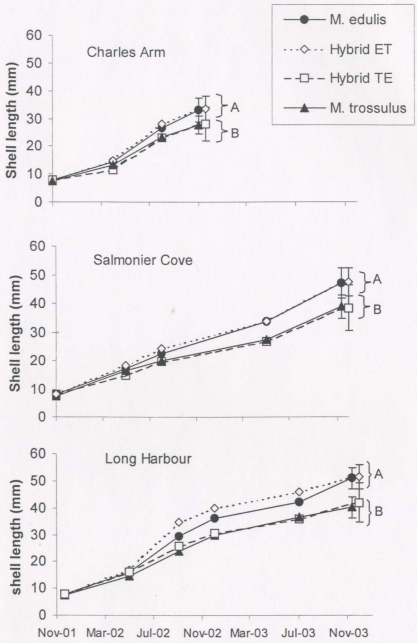

Figure 5.9. Growth rate of laboratory produced mussels at three aquaculture sites in Newfoundland. Groups with different letters are significantly different (Anova and Duncan multiple comparison test for homogeneity of subsets, $p<0.05$).

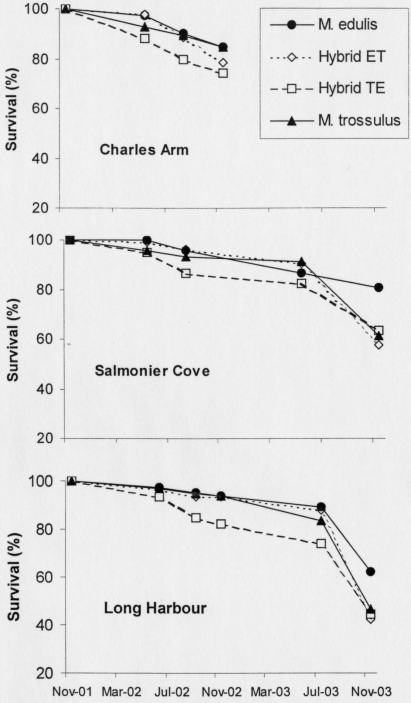

Figure 5.10. Survival of laboratory produced mussels for a period of 24 months at three aquaculture sites in Newfoundland .

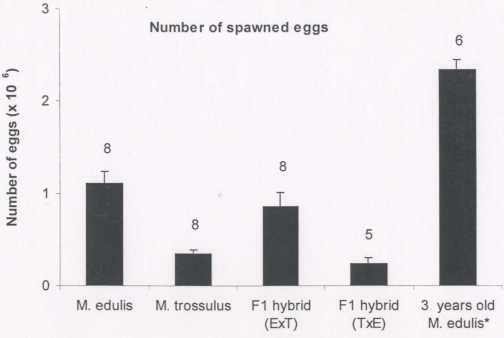

Figure 5.11. Number of eggs spawned in the laboratory during a period of 6 hours. Error bars represent standard error. Numbers above bars represent number of mussels. Average mussel length is 37.64 mm. Control 3 years old *M. edulis* are cultured mussels from Charles Arm with average length of 54.25 mm.

Table 5.1. Percentage of fertilized eggs, normal larvae and survival to day 10 of *Mytilus* families produced in the laboratory in 2001.

Cross	Genotype	Fertilized (%)	Normal / Fertilized (%)	Survival / Fertilized (%)	Remaining / Eggs (%)
1	E x E	97.32	49.27	47.50	46.23
2	E x E	99.14	58.70	59.63	59.12
3	E x E	96.50	73.49	68.73	66.32
4	E x E	97.94	58.91	54.58	53.45
5	E x E	97.53	76.65	71.76	69.98
6	E x E	99.33	96.03	89.29	88.70
7	E x E	99.11	94.91	94.00	93.16
8	E x E	99.60	96.71	86.92	86.58
9	E x E	94.20	57.98	48.51	45.70
10	E x E	70.70	42.31	41.44	29.30
11	E x E	80.36	30.36	37.40	30.05
12	E x E	100.0	95.02	94.00	94.00
13	E x E	98.27	84.64	80.86	79.46
14	E x E	72.88	83.11	77.82	56.72
15	E x E	71.92	75.61	79.84	57.43
16	E x E	98.12	92.07	92.99	91.24
17	E x E	100.0	94.21	96.02	96.02
18	E x E	97.66	90.89	89.95	87.85
19	E x E	88.12	87.19	87.93	77.49
20	E x E	97.50	92.99	95.01	92.63
21	E x T	78.88	25.30	22.23	17.54
22	E x T	95.55	70.24	57.61	55.05
23	E x T	94.13	90.27	82.88	78.01
24	E x T	93.67	74.60	61.65	57.75
25	E x T	36.76	24.71	14.15	5.20
26	E x T	29.62	15.96	11.11	3.29
27	E x T	97.80	65.52	40.43	39.54
28	E x T	99.81	92.95	74.79	74.65
29	E x T	100.0	93.25	85.91	85.91
30	E x T	95.37	73.45	56.60	53.98
31	E x T	100.0	89.51	84.90	84.90
32	E x T	99.61	92.22	68.73	68.46

Table 5.1. Cont

Cross	Genotype	Fertilized (%)	Normal / Fertilized (%)	Survival / Fertilized (%)	Remaining / Eggs (%)
33	E x T	13.38	44.25	18.19	2.43
34	E x T	71.96	70.26	56.60	40.73
35	E x T	86.13	72.66	64.69	55.71
36	E x T	87.33	67.93	37.40	32.66
37	T x E	96.80	43.34	35.37	34.24
38	T x E	80.78	69.98	57.61	46.54
39	T x E	84.95	73.20	63.67	54.09
40	T x E	82.28	68.05	65.70	54.05
41	T x E	85.42	58.67	26.28	22.45
42	T x E	83.17	58.33	49.53	41.19
43	T x E	55.73	31.17	16.17	9.01
44	T x E	73.06	37.66	28.30	20.68
45	T x E	68.97	44.52	41.44	28.58
46	T x E	67.75	65.51	63.68	43.14
47	T x E	89.04	86.17	77.82	69.29
48	T x T	94.80	69.91	71.76	68.03
49	T x T	95.54	69.32	66.71	63.73
50	T x T	97.70	51.69	54.58	53.32
51	T x T	86.08	74.12	72.77	62.64
52	T x T	88.12	70.47	68.73	60.57
53	T x T	93.33	96.26	91.97	85.84
54	T x T	94.79	87.33	88.94	84.31
55	T x T	86.17	89.12	85.91	74.03
56	E x B9	73.44	65.86	54.58	40.08
57	T x B9	69.05	38.70	25.27	17.45
58	T x B9	87.33	77.17	67.72	59.14
59	B4 x E	82.96	66.89	58.62	48.63
60	B6 x E	77.66	37.26	31.33	24.33
61	B9 x E	62.45	15.43	12.13	7.57
62	B6 x B9	80.37	20.33	17.18	13.81
63	B4 x T	81.54	64.72	54.58	44.50
64	B6 x T	85.06	41.58	37.39	31.81
65	B9 x T	86.79	73.66	63.67	55.26

Chapter 6

Aquaculture production of blue mussels (*Mytilus edulis* and *M. trossulus*) in an Atlantic Canada hybrid zone

6.1. Introduction

Mussel culture has proved to be a sustainable and profitable activity in Atlantic Canada for the last two decades. The finding that two different species (*Mytilus edulis* and *M. trossulus*) coexist and hybridize in the northwest Atlantic has important implications for the mussel culture industry. Difficulties in distinguishing between the species using morphological characters have been overcome with the discovery of diagnostic enzyme genetic markers (e. g. Gartner-Kepkay et al., 1980; Koehn et al., 1984; Varvio et al., 1988; McDonald et al., 1991) and the development of species-specific DNA markers (Zouros et al., 1994; Heath et al., 1995; Inoue et al., 1995; Rawson et al., 1996a). Accurate genetic identification has allowed comparison of the distribution, ecology, and fitness of parent species and their hybrids and the interactions between them (Mallet and Carver, 1995; Comesaña et al, 1999; Penney and Hart, 1999; Gardner and Thompson, 2001; Toro et al., 2002; Rawson et al., 2003). Applied aquaculture research has focused on the relative performance of *M. edulis* and *M. trossulus* at different sites, which is a major concern to the mussel industry (Mallet and Carver, 1995; Penney et al., 2002). However, other aspects such as physiological adaptation, genetic background, competition and life-history traits have a direct impact on species fitness and overall mussel production. Therefore, a basic biological perspective on both species and how they interact is also critical for successful aquaculture production.

The blue mussel industry in eastern North America dates from the beginning of the century (Lutz, 1992), and Newfoundland has long had a prominent role in supplying wild mussels for the North American market (Sutterlin et al., 1981). The development of

the mussel aquaculture industry and competition with other countries in the 1970s led to a shift in production to Prince Edward Island, leaving Newfoundland with a secondary role. Currently Prince Edward Island accounts for 81.7% (16 875 tonnes) of Canadian mussel production, but Newfoundland has shown a steady increase, reaching 1700 tonnes in 2002 (DFO, 2003). After the cod moratorium in 1992, aquaculture has been seen as an important alternative to support former fishermen and the Canadian government has funded basic and applied research to enhance the mussel industry. The rapid expansion of mussel culture has resulted in a need for improved knowledge of the species' biology (Heggberget, 1997). Currently, *Mytilus edulis* is the most important mussel species for aquaculture production and has been extensively studied. On the other hand, very little is known about the biology of *M. trossulus* in the northwest Atlantic, nor about the interactions between the two species and between them and their hybrids.

Despite the successful production of mixed stocks of both species in Atlantic Canada, there is a perception that *M. trossulus* is less desirable for aquaculture. This assumption is primarily based on the work of Mallet and Carver (1995), which showed greater production of *M. edulis* than in *M. trossulus* at some of their study sites in Nova Scotia. Penney et al. (2002) also observed that *M. edulis* in Newfoundland was superior to *M. trossulus* in some production traits and suggested that the substitution of mixed stocks for pure *M. edulis* stocks would improve production. No studies have directly compared monospecific stocks of *M. edulis* with mixed stocks nor pure stocks of both species under aquaculture conditions. Recent awareness of the potential risks of species transfer has led to a requirement for careful consideration before moving animals or plants to other areas. Transplantation of stocks or the introduction of exotic species should be balanced against ecological risks such as the concomitant introduction of diseases, parasites and predators to new areas and potential competitive interactions with local species (Shatkin et al., 1997). The legal requirements and the economics involved in the production of the alternative stock in relation to the local stock must also be carefully examined, since the consequences of transplantation may be irreversible. Alternative solutions that improve the performance of the local stock should also be considered before introducing alien stocks to new areas.

Mussel culture in areas where two species hybridize must take into consideration the ecological interactions between the species and the performance of hybrids. The overall production in the hybrid zone is greatly influenced by the level of hybridization, since hybrids represent up to 38% of the mussel numbers at some sites (chapters 2 and 3). "Hybrid vigour", the increased fitness of hybrids in relation to both parent species, is a well-known biological phenomenon that has been extensively exploited to improve agricultural and animal production. Beaumont et al. (1993), Freeman et al. (1994) and Toro (1999) suggested that hybrid vigour occurs in *Mytilus* at the larval stage (but see Bierne et al., 2002a; Matson et al., 2003 and chapter 4). The considerable genetic variability observed in hybrids (chapter 3) probably results in concomitant wide variability in hybrid fitness regardless of the stage of development (chapter 5), therefore some hybrid genotypes may potentially be superior to both parent species while other hybrid genotypes may be inferior. The genetic and environmental context of each area should also be established to assess the relative performance of each species and their hybrids exposed to a wide range of environmental conditions. Furthermore, mussel culture is strongly based on the principle of thinning, in which intra- and interspecific competition and specific environmental conditions play an important role in determining the genetic background of the mussels that reach commercial size. Mixed species stocks or stocks containing hybrids may be favoured if unequal competitive interactions during the thinning process allow the better adapted and more productive individuals to survive preferentially to stages at which high densities are no longer necessary.

As the mussel market in Atlantic Canada is largely based on whole fresh product, some shell characteristics that differ between species may also have commercial implications. Morphometric studies of mussel shells have focused on a variety of characteristics that can be used to differentiate the species comprising the *Mytilus edulis* complex (Beaumont et al., 1989; McDonald et al., 1991; Sarver and Foltz, 1993; Innes and Bates, 1999; Gardner, 1996). However, other specific shell traits that could have direct influence on aquaculture production may differ between *M. edulis* and *M. trossulus* (Mallet and Carver, 1995). The present study focuses on some of these species-specific differences that are not commonly studied but may have important implications for the

mussel industry. Shell weight, fragility, colour and internal volume are compared in mussels from several aquaculture sites in Newfoundland with different environmental conditions. In addition, the growth and survival of both species and their hybrids in culture conditions (chapter 5) are compared with mixed stocks and discussed from an aquaculture perspective. Finally, differential settlement behaviour was investigated with the objective of providing alternative solutions to improve local stock production by preferentially collecting the spat of whichever genotype shows the greatest productivity.

6.2. Materials and Methods

To study differences in shell morphology between *M. edulis*, *M. trossulus* and their hybrids the length, width, height and weight were measured in both valves of 450 mussels (figure 6.1). Internal volume was also determined by weighing the volume of water required to fill each valve. Shells were from mussels used in the static cohort analysis in chapter 2, i.e. collected in 2001 from 5 different mussel farms (Cap Cove, Charles Arm, Drac Bay, Reach Run and Salmonier Cove), and were genotyped with the nuclear DNA marker ME (Inoue et al., 1995). To test differences between genotypes an analysis of covariance was performed using shell length as the covariate. The same shells were also used to investigate the relationship between shell colour and genotype. Shells were classified subjectively into four groups; light brown, brown, blue and dark blue. Groups were then compared by genotype in a one-way Anova.

A subsample (185 shells) was used to determine breakage resistance. A device designed to test bone strength in the Atlantic Veterinary College (AVC, University of Prince Edward Island) was adapted to measure the pressure a mussel shell can tolerate before breaking. A single valve was placed horizontally on a bench with the inner side facing down and a metal plate was mechanically lowered to contact the shell. Pressure was gradually increased until the valve fractured. Software developed by Dr. Ireland (AVC) was then used to calculate the strain energy and the force required to rupture the shell. An analysis of covariance was performed using shell length as the covariate to assess differences in shell breaking force among *M. edulis*, *M. trossulus* and hybrids.

Growth rate and mortality were used as correlates of the production performance of pure species and mixed stocks. In addition to the pearl nets containing pure *M. edulis*, pure *M. trossulus*, F1 hybrids and backcrosses produced in the laboratory (chapter 4), another set of three replicates containing mixtures of equal numbers of *M. edulis* and *M. trossulus* was deployed in Long Harbour. The three replicates were randomly distributed among the other 15 pearl nets, each replicate containing 50 individuals of each species. *M. edulis* individuals were marked before deployment by immersing the mussels in a solution of calcein in seawater (100 mg L^{-1}) for 12 hours. The calcein should be incorporated into the shell, forming a ring which can be subsequently visualized since it fluoresces under UV light. Mussels were deployed in November 2001 and monitored periodically until July 2003. Hybrids were not used in the mixed stock groups.

An experiment was conducted in the summer of 2003 to determine whether there were genotype specific differences in settlement behaviour that could be exploited for aquaculture purposes by preferentially collecting spat from one or the other species. At the beginning of the reproductive season (early June) two ropes 9 meters long were deployed vertically at two mussel farms (Long Harbour and Salmonier Cove), each rope extending from 3 to 12 meters. Ropes were previously dried and cleaned before deployment. After the spawning season (September) ropes were retrieved from the mussel farms and checked for the presence of newly settled mussels. In the laboratory, mussels from 3 different sections of each rope, corresponding to depths of 3 to 4 meters (top), 7 to 8 meters (middle) and 11 to 12 meters (bottom), were collected separately. As ropes were submerged at an initial depth of 3 meters, surface samples were taken separately from the head ropes. A subsample of 30 mussels ranging from 5 to 20 mm length was taken from each depth and each mussel genotyped with the ME marker.

6.3. Results

Shells from the parent species differed significantly in height, weight, internal volume and width. Shell height in *M. edulis* was greater than in *M. trossulus*, regardless

of shell length (fig. 6.2, $p < 0.01$). The greater length/height ratio for *M. trossulus* (2.24) in relation to *M. edulis* (1.97) accounted for the more elongate shape that characterizes *M. trossulus*. Shells from *M. edulis* were also significantly heavier than those of *M. trossulus* (figure 6.3, $p<0.001$), wider (fig. 6.4, $p < 0.05$) and possessed a greater internal volume (fig. 6.5, $p< 0.001$). Hybrids showed intermediate values for all shell variables. Shell height for hybrids was intermediate to values for the parent species, being significantly lower than *M. edulis* and greater than *M. trossulus* ($p < 0.02$). Hybrids did not differ significantly from either parent species in shell width or volume ($p > 0.5$). Shells of hybrids were significantly lighter than those of *M. edulis* ($p< 0.001$) but not significantly different in weight from *M. trossulus* shells ($p > 0.5$).

A correlation between shell colour and genotype was also observed. Although the great majority of shells were dark blue or blue (85.6 %), a significantly higher proportion of the brown (59.1%) and light brown (76.2%) shells observed were identified as *M. trossulus* (fig. 6.6 A) ($G = 63.8$, $df = 6$, $p < 0.001$), whereas 70.1% of dark blue shells were *M. edulis*. When shells from each species were classified separately by colour, most shells (95%) of *M. edulis* were dark blue or blue, whereas 26.3% of *M. trossulus* shells were lighter in colour (blue or light brown) (fig. 6.6 B). However, the classification did not unequivocally differentiate each species as some *M. trossulus* individuals with a dark shell and some *M. edulis* individuals with a light colour shell were also observed. In addition, 10.04 % of the mussels were also classified as striped, a shell morph previously described for *M. edulis* (Innes and Haley, 1977b; Mitton, 1977; Newkirk, 1980). The relative frequency of striped mussels was very similar for *M. edulis* (10.6%), *M. trossulus* (9.37%) and hybrids (9.09%).

M. edulis shells were significantly more resistant to breakage than those of *M. trossulus* (fig 6.7). Mean strain energy corrected for a standard shell length (50 mm) was 17056.1 kilodynes cm^{-1} for *M. edulis* and 9629.6 kilodynes cm^{-1} for *M. trossulus*. Breaking strength was 194.6 and 97.8 kilodynes cm^{-1} for *M. edulis* and *M. trossulus*, respectively. Exactly the same pattern was observed if only mussels of commercial size (shell length 50-70 mm) were considered (fig. 6.7 B). Although sample size for hybrids

was much smaller, shells from hybrids were more fragile than *M. edulis* shells, but not significantly different from those of *M. trossulus*.

Growth rates for mixed stock containing equal numbers of pure *M. edulis* and pure *M. trossulus* in the same pearl net were similar to those of *M. edulis* ($p = 0.094$) but significantly higher than the growth rate of *M. trossulus* ($p < 0.001$) (figure 6.8). Because the calcein mark was not retained throughout the experiment, species differences within the pearl nets containing mixed stocks could not be determined. Mortality was relatively low after 20 months in Long Harbour and not significantly different among the genotypes. *M. edulis* showed 89.0% survival, *M. trossulus* 83.5 % and the mixed stock 80.6 %.

At both study sites there was a tendency for *M. edulis* larvae to settle at deeper parts of the experimental ropes while *M. trossulus* settled preferentially on the upper parts of the ropes (figure 6.9; Long Harbour, $G = 47.60$, $df = 6$, $p<0.0001$; Salmonier Cove, $G = 20.67$, $df = 6$, $p<0.002$).

6.4. Discussion

In areas where the two mussel species coexist and hybridize, growers must consider several biological aspects of both species and the way they interact with each other and with the environment. Although reproductive competence is the main component of fitness from an evolutionary perspective, survival and growth rates are the most important traits from an aquaculture point of view. The difference in growth rate between the two species was not great in the present study, although *M. edulis* showed significantly greater shell length after 24 months at all study sites (chapter 4). Penney et al. (2002) only observed a greater growth rate (shell length) for *M. edulis* on one occasion, but live weight of mussels and shell weight were significantly greater for *M. edulis* than for *M. trossulus* at all sites. Similarly, Mallet and Carver (1995) could only find differences in shell length for some of their study sites in Nova Scotia, although *M. edulis* showed consistently greater shell weight and tissue weight than *M. trossulus*. Since the mussel market is predominantly based on product weight, shell weight is an important

characteristic that partially determines the value of the product. Furthermore, the greater fragility of *M. trossulus* shells observed in the present study probably results in losses during harvesting and process phases, as observed by Mallet and Carver (1995). In this case, alternative markets that require secondary processing could be explored for this species. Different methodologies could be adapted for mussel culture depending on species frequencies and alternative marketing strategies should allow profitable operations for *M. trossulus* even if the species is less productive than *M. edulis*. In British Columbia, where the hatchery production of spat allows genetic selection programs, a colour morph (golden mussel) has been exploited to make the product more appealing and valuable. Although the species produced on the west coast of North America are predominantly *M. galloprovincialis* and *M. edulis*, a similar approach could be taken to add value to *M. trossulus* in the northwest Atlantic.

Shell colour is probably an inherited trait and intraspecific variation has long been known. Innes and Haley (1977b) observed that *M. edulis* has brown-striped or black shells and suggested that a single locus mechanism is responsible for shell colour. Newkirk (1980) also used pair-mating families to reach a similar conclusion, but raised the possibility that additional loci may influence mussel colour. Adaptation to the environment may play a part in determining shell colour since heat absorption is directly correlated with colour. A latitudinal cline from Virginia to Maine, in which the frequency of dark shells increases to the north, may reflect adaptation to maintain an energetic balance (Mitton, 1977). Light coloured shells in southern (warmer) areas would prevent overheating during the summer and dark shells in colder areas would prevent freezing and support the maintenance of a positive energetic balance. However, Newkirk (1980) found the opposite relationship, i.e., darker shells further south, but his study area is now known to contain a mixture of both species and *M. edulis* is the predominant species at the site where most dark shells were observed (Ostrea Lake). The genotype composition of the other site, where a higher frequency of light colour shells was observed (Spanish Ship Bay), has not yet been examined, although several nearby sites showed high frequencies of *M. trossulus* (Mallet and Carver, 1999). The observation that the light coloured mussels were significantly smaller than the dark mussels (Newkirk, 1980) is compatible

with species-specific differences in growth rate and shell colour, since *M. trossulus* shows slower growth and a higher frequency of light coloured shells. The mixture of species in the hybrid zone may therefore obscure the relationship between colour and temperature since the two species are probably adapted to different environmental conditions and show marked macrogeographic displacement (chapter 2). The higher frequency of light coloured shells observed for *M. trossulus* in the present study may reflect the southern limit of the species, where some individuals could benefit from lower energy absorption that prevents overheating and summer mortality. On the other hand, *M. edulis* is probably more adapted to warmer waters, so individuals with a dark shell would be have an advantage in the northern (colder) areas where high energy absorption may raise the body temperature.

Shell colour is probably one of the most noticeable traits that mussel farmers use to classify mussels intuitively. Anecdotally, the "brown and weak" shells are often assumed to be *M. trossulus* in Newfoundland, an assumption that may hold true in many cases since the species has a more fragile and less heavy shell which is often light in colour (figs. 6.7, 6.3 and 6.6). However, none of these traits is completely diagnostic, and most hybrids have intermediate characteristics. Apart from enzyme and DNA markers, shell morphology has been the primary means of differentiating species of the *Mytilus* complex. Canonical discriminant analysis of morphometric variation has used as many as 18 different shell measurements (McDonald et al., 1991; Mallet and Carver, 1995; Toro, 1999; Innes and Bates, 1999) to differentiate the species, but hybridization usually confounds the analysis and the method is too complex and time consuming to be routinely used at mussel farms. However, the relationship between shell length and shell height differs significantly between species and may be used as a simple tool to classify individuals. Freeman et al. (1992) and Mallet and Carver (1995) also observed that shell height is an important discriminating factor between *M. edulis* and *M. trossulus* that showed consistent results among several sites. Although hybrids still exhibit intermediate values and cultured mussels tend to be uniform in shape, the more elongated shell shape is the most conspicuous morphological trait associated with *M. trossulus*.

The relatively heavier shells of *M. edulis* suggest that they are also thicker, a trait that confers an advantage to mussels by protecting them from predation (Beadmam et al., 2003). Oystercatchers exhibit a strong preference for mussels with thinner shells (Meire and Ervynck, 1986; Sutherland and Ens, 1987). In addition, Nagarajan et al. (2002) observed that these birds also discriminate between shell colours to maximize mussel predation, showing a preference for brown-shelled mussels over the commoner black-shelled morph that occurs in Britain. If the same situation occurs in Newfoundland, predation should be greater on *M. trossulus*, since this species has a higher frequency of brown-shelled individuals. However, predation by other organisms such as starfish, sea urchins and crabs may be more influenced by the size of the adductor muscle or the strength of the byssus (Côté, 1995; Reimer and Tedengren, 1996; Leonard et al., 1999). As the mussels in the present experiment were inside pearl nets and therefore protected from predators, the mortality observed was probably caused primarily by endogenous or environmental factors rather than predation. Several other aspects of mussel predation deserve further research, particularly now that there is evidence that the two species differ in many aspects that may have important implications for aquaculture production.

Although most of the mortality observed in the present study may not have been caused by predation, results from the static cohort analysis at five aquaculture sites (chapter 2) suggest that many more *M. edulis* individuals than *M. trossulus* reach commercial size. Despite the higher frequency of *M. trossulus* in the smaller size classes, the shift in species frequency with size class shows that differential mortality or different life-history strategies may be important factors contributing to overall production. Under culture conditions, high initial stock densities usually compensate for the high mortality observed during the growing period. Compensatory overstocking is a common practice, particularly for species in which competition-independent mortality is important during the self-thinning period (Fréchette et al., 1996). Although initial high densities can potentially decrease predation and compensate for natural mortality, the practice may also increase intra and interspecific competition-dependent mortality and retard growth rate. Therefore, optimal stock density is critical for aquaculture production, which aims at maximizing yield without inhibiting growth rate. Food availability and mortality should

also be considered when estimating the optimum stock density for a particular area. Initial stock densities of 100 to 200 mussels per 30.5 cm of sleeve are recommended for suspended culture in Atlantic Canada (Mallet, 1989). These values should generate competition-independent and competition-dependent mortality, resulting in as much as 63% mortality, depending on initial stock density (Fréchette et al., 1996). In areas where both species are cultured together, the competitiveness of each species may play an important role during the thinning process. Assuming that *M. edulis* is better adapted to the environmental conditions of the Canadian Maritimes, this species could be at an advantage if competing with less well adapted *M. trossulus* individuals. Differences in byssal strength may also influence the numbers of each mussel species that reach commercial size (Willis and Skibinski, 1992). Therefore mixed stocks may improve production by allowing the more productive *M. edulis* individuals to survive the thinning period preferentially and grow faster than pure stocks that would face greater intraspecific competition and exhibit slower growth. In the present study pure *M. edulis* and pure *M. trossulus* individuals held in the same pearl net did not show any significant difference in growth rate from pure *M. edulis*. However, these results for mixed stocks may not represent aquaculture conditions, since the low density used did not allow competitive interactions and very low mortality was observed. Furthermore, hybrids were not included in the experimental design, whereas naturally occurring stocks may contain up to 38% hybrids (chapter 3). Because the calcein mark was not retained throughout the experiment, species-specific mortality could not be determined. Nevertheless, the fact that growth rate for the mixed stock was much more similar to that of *M. edulis* than that of *M. trossulus* suggests that higher mortality of *M. trossulus* may have occurred. Survival estimates for both species and hybrids cultured together are critical for a further understanding of competitive interactions that may affect growth rate. Although Penney et al. (2002) concluded that pure *M. edulis* stocks should perform better than mixed stocks, their approach did not allow measurements of survival for both species, nor did they directly compare pure stocks with mixed stocks. Further research is required to investigate the comparative performance of pure stocks and mixed stocks under aquaculture conditions in order to improve production techniques. The fact that *M. edulis*

shows an overall greater production than *M. trossulus* at most culture areas in Atlantic Canada does not necessarily imply that monospecific stocks are more productive than mixed stocks for all culture conditions.

The relative performance of each species depends on the specific environmental conditions under which the mussels are growing. Areas where *M. trossulus* dominates or enjoys a competitive advantage may favour that species for culture purposes. The northerly distribution of *M. trossulus* suggests that this species is better adapted to colder waters (chapter 2), therefore in areas such as the Northern Peninsula of Newfoundland and the coast of Labrador *M. trossulus* may show superior performance to *M. edulis*. On the other hand, the work of Mallet and Carver (1995) may only reflect specific environmental conditions that favour *M. edulis* in Nova Scotia, and the results may not be general since the patchy distribution of both species suggests some ecological differentiation at a macro- and microgeographic scale. Stocks transplanted to a different area may not show the same performance because the different environmental conditions at the new location may alter metabolism and consequently survival and growth rate. Yanick et al. (2003) observed that transplanted mussels (*M. trossulus*) exhibited higher mortality and lower growth rates than the local stock, suggesting that differential adaptation may be important even for relatively close populations. Similarly, Johannesson et al. (1990) observed almost total mortality in reciprocal transplantation experiments between the North Sea and the Baltic Sea. Seed origin also influences the growth rate of *M. galloprovincialis* in Europe (Babarro et al., 2000). Furthermore, considerable intraspecific variation among *M. edulis* stocks has been observed in the northwest Atlantic (Tremblay et al., 1998d) and stocks that are predominantly *M. trossulus* sometimes perform better than some *M. edulis* stocks but more poorly than others (Mallet and Carver, 1999).

The concern of growers in Newfoundland to avoid collection of *M. trossulus* spat can only be justified if in the future pure stocks of *M. edulis* prove to be more profitable than mixed stocks or pure *M. trossulus* stocks. In this case, alternative solutions such as preferential collection of *M. edulis* spat from the original site should be considered before introducing alien stocks to new areas. The differences in settlement

patterns between species may allow farmers to target *M. edulis* spat if necessary. The preference of *M. trossulus* to settle closer to the surface than *M. edulis* may reflect an intrinsic behavioural trait. Kenchington et al. (2001) observed exactly the same pattern of *M. trossulus* preferentially settling in shallower waters in Nova Scotia, although Freeman and MacQuarrie (1999) did not observe any difference in depth preference between the two species for pre-settling larvae in a mesocosm experiment. Depth may not be the determining factor for larval settlement, and the present results may also reflect species-specific aggregation behaviour or habitat specialization. Preference for specific sediments and interactions with adults and other settling larvae may influence settling behaviour of mussels (Bierne et al., 2003a) and other bivalves (Snelgrove et al., 1999). An alternative hypothesis is that temporal displacement in spawning between the two species allows the first competent larvae to settle in prime areas.

Finally, hybridization should be taken into consideration when productivity is being estimated, since hybrid vigour may influence production in areas of hybridization. The present study observed that hybrids were generally inferior to or intermediate between the parent species for most studied traits, although in some cases hybrids were superior to both parent species (chapter 4). Although hybridization between two divergent species usually results in reduced fitness of hybrid progeny, hybrid vigour can occasionally be expressed in some individuals (Barton, 2001). This superiority frequently declines after the F1 generation (hybrid breakdown) due to recombination and deleterious epistatic interactions, as the Dobzhansky-Muller model predicts (Dobzhansky, 1937; Muller, 1942). However, greater hybrid fitness may persist in later generations as a consequence of overdominance (heterozygote advantage) or favourable epistasis (Edmands, 2002). Heterozygote advantage has been controversial in bivalve studies (Gaffney, 1990; 1994), but a relationship between the degree of heterozygosity and heterosis has been suggested for *Mytilus edulis* (Zouros and Pogson, 1994; Hedgecock et al., 1995; 1996). The potential to improve mussel production through a genetic program that includes hybridization exists mainly for areas such as British Columbia where most production depends on hatchery produced spat. In areas where spat is collected directly from the environment, hybridization may enhance production if fitter hybrids survive

preferentially to market size or grow faster than pure individuals. However, growers have little control of the genetic composition of the local stocks, and the relative frequency of hybrids could vary from site to site. Nonetheless, mussel culture in areas of hybridization may benefit from heterosis and the overall production may be similar to that of monospecific cultures. Again, monospecific stocks must be compared with mixed or hybrid stocks before a final conclusion can be reached. Further research on settlement time and preference for each species may clarify whether selective collection of spat can be a useful tool to improve mussel production.

The competitive world market for mussels demands that the industry continually pursue new alternatives to maximize production. Basic research can support industry development, mainly when novel information leads to a new situation that can potentially alter production. The recent discovery that a large part of the mussel production in the Canadian Maritimes occurs in areas where two different species coexist and hybridize merits further research in order to advise mussel growers on how to improve production. Several aspects of the biology of both species must be examined, especially for *M. trossulus*, which has received much less attention than *M. edulis*. The present study contributes to a better understanding of the biology of both species and reopens the discussion on how mussel production could be improved in areas of hybridization. Further studies are necessary to identify the more productive stocks and determine how they perform under different environmental conditions. The great complexity of this hybrid zone in the northwest Atlantic requires each area to be considered independently. A cautionary approach should be taken to mussel introduction and transfers since the consequences may sometimes be adverse and severe. Alternative solutions must always be examined to minimize damage to the environment and the aquaculture industry.

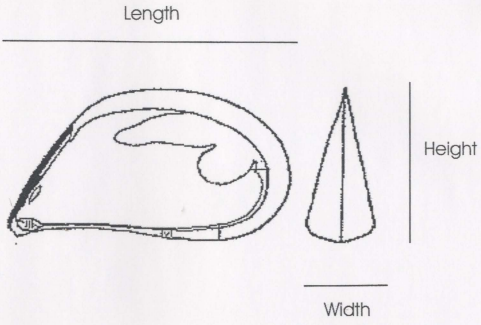

Figure 6.1. Shell morphometric characters measured for species identification: shell length, height and width. Modified from McDonald et al. (1991).

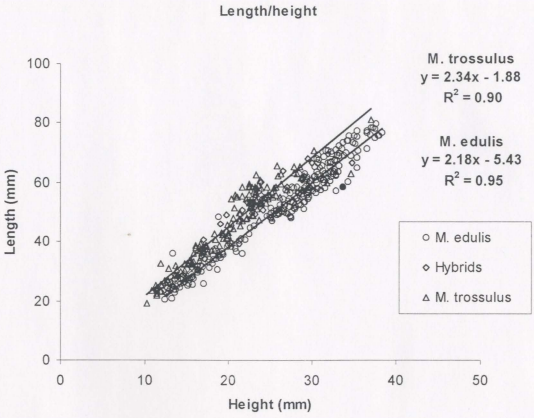

Figure 6.2. Correlation between shell height and shell length for *M. edulis*, *M. trossulus* and their hybrids. N = 450.

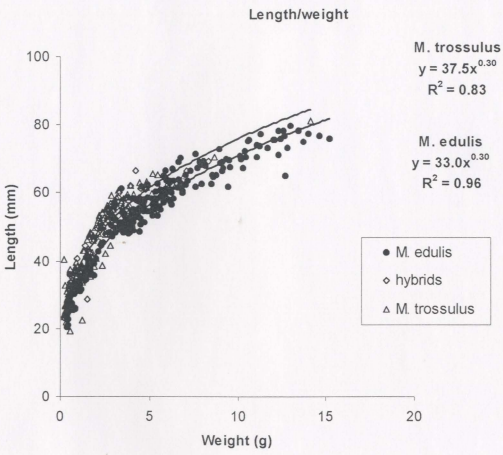

Figure 6.3. Correlation between shell weight and shell length for *M. edulis*, *M. trossulus* and hybrids. N = 450.

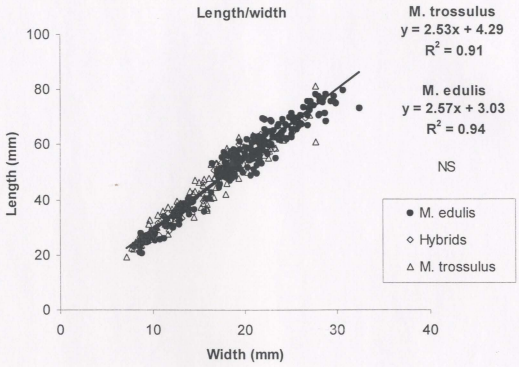

Figure 6.4. Correlation between shell width and shell length for *M. edulis*, *M. trossulus* and their hybrids. N = 450.

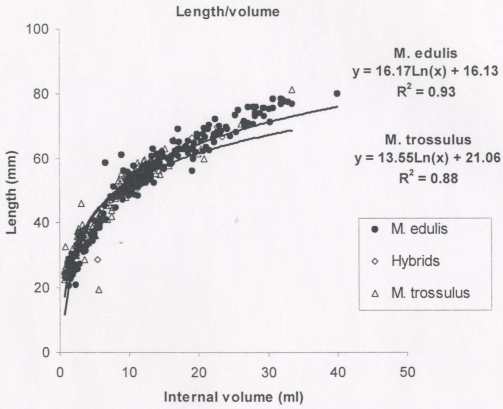

Figure 6.5. Correlation between internal volume of shell and shell length for *M. edulis*, *M. trossulus* and their hybrids. N = 450.

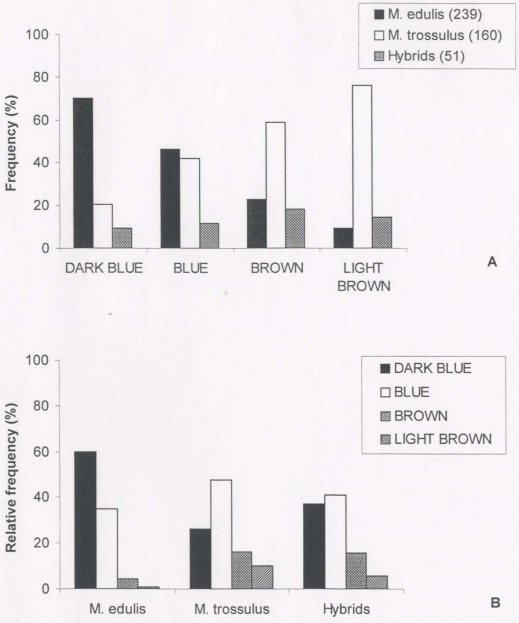

Figure 6.6. Frequency of colour classes by genotype (A) and species frequency by shell colour (B). Numbers between parentheses represent sample size.

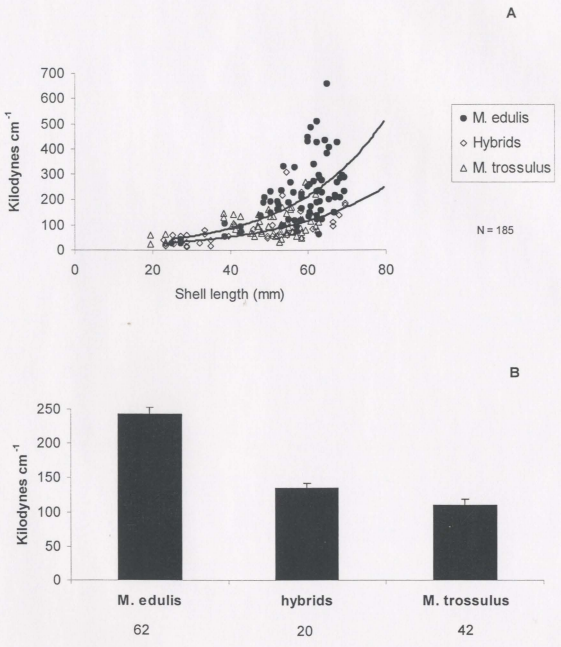

Figure 6.7. Energy necessary to rupture cultured mussel shells (A) and mean values for breaking strength by species for commercial size mussels (50 to 70mm) standardized for 60 mm mussels (B). Numbers below columns represent sample size.

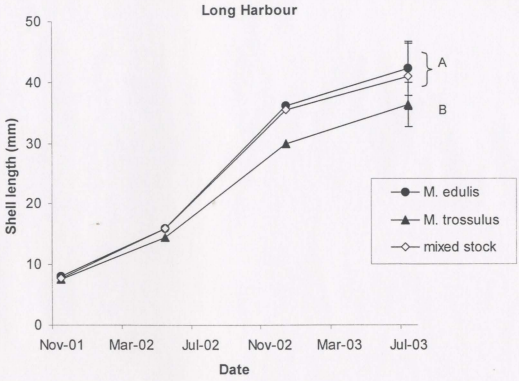

Figure 6.8. Growth rate of laboratory produced pure stocks of *M. edulis*, *M. trossulus* and mixed stocks containing 50% of *M. edulis* and 50% of *M. trossulus*. N = 150 mussels for each genotype.

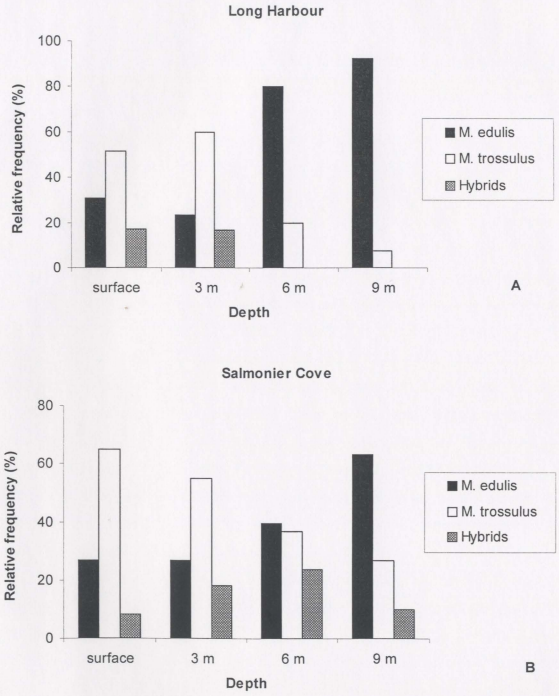

Figure 6.9. Species frequencies of recently settled mussels by depth in Long Harbour (A) and Salmonier Cove (B). N = 30 mussels per depth for each site.

Chapter 7

General discussion and conclusions

The study of *Mytilus* hybrid zones can reveal important aspects of marine evolution and speciation. The present study provides evidence that temperature tolerance is an important factor affecting physiological activity and consequently determining the distributions of both species. The observation that *M. trossulus* is less tolerant of high temperatures than *M. edulis* suggests that physiological differentiation may contribute to reproductive isolation since discrete biogeographic distributions help to maintain the genetic identity of the species. The establishment of *M. trossulus* populations south of Maine is probably limited due to lower tolerance of the species for high temperatures. The northern limit of *M. edulis* and the position of the hybrid zone may reflect migrational stochasticity. The secondary contact between the species probably occurred during an interglacial period or after the last glacial maximum, when *M. edulis* presumably expanded to the north from a temperate refuge in North America and *M. trossulus* expanded to the south from the Arctic Ocean when the Arctic route was reopened. It is also possible that both species are still expanding their ranges, but differential adaptation and competition prevent or slow down the establishment of permanent populations in areas where the other species is dominant. The constant southward direction of the Labrador Current may also limit the dispersal of *M. edulis* to the north and facilitate dispersal of *M. trossulus* from Labrador into the hybrid zone. The presence of only *M. trossulus* in the five sites sampled in Labrador suggests that this area may provide large numbers of larvae to the hybrid zone through the Labrador Current. Poorer adaptation to the environmental conditions of Newfoundland would then result in a higher mortality of *M. trossulus* in the area. Differential survival was suggested by the decrease in the relative frequency of *M. trossulus* in the larger size classes in all study sites in Newfoundland and by the greater mortality observed in the field and in temperature experiments. Differences in life-history strategies may also influence the

observed pattern since *M. trossulus* reproduces earlier, spawns smaller eggs and possibly has a shorter generation time than *M. edulis*. Finally, the occurrence of *M. edulis* populations inside Hudson Bay suggests that an undescribed hybrid zone may occur between *M. edulis* and *M. trossulus* in the Hudson Strait or somewhere inside Hudson Bay.

The multi-marker approach used in the present study allowed a better understanding of the available markers for *Mytilus* species and demonstrated that the number of hybrids identified in a given sample depends on the number of markers utilized. The use of several markers followed by a hybrid index classification also provided evidence of a clear bimodal distribution of mixed genotypes in the hybrid zone, showing very few F1 hybrids, but approximately 38% advanced backcrosses. Although low levels of introgression were observed, the presence of a few individuals homozygous for alleles from the opposite species suggests that introgression can be a potential means of adaptive evolution in *Mytilus*. No individual of either parent species contained mtDNA from the other species, implying that introgression of mtDNA is limited in the northwest Atlantic hybrid zone. However, many exceptions to doubly uniparental inheritance (DUI) were observed. The presence of females containing the male mtDNA haplotype and males lacking the male mtDNA in hybrid and pure individuals suggests that the disruption of DUI is not only a result of hybridization. In addition, the inheritance of female molecules from a heteroplasmic male is reported for the first time, and this may be associated with the masculinization process that occurs in mtDNA molecules of *Mytilus*. Cyto-nuclear incompatibilities may also be an important factor responsible for the partial reproductive isolation observed in this hybrid zone.

The results on fertilization success from the present study suggest that gamete recognition and/or gamete incompatibility are likely to be important isolating mechanisms between *M. edulis* and *M. trossulus*. The decreased fertilization success observed in several interspecific crosses is consistent with pre-zygotic isolation that helps to maintain species identity. As no difference between the spermatozoon of the species has been observed, recognition of species-specific proteins may be the determining factor responsible for reducing interspecific fertilization. The higher proportion of abnormal

larvae in some hybrid crosses than in intraspecific crosses suggests that postzygotic mechanisms may also play an important role in maintaining reproductive isolation. F1 hybrids and backcrosses evaluated from larval stage to maturity were in general less fit than pure *M. edulis* and usually intermediate between the parent species or equal to *M. trossulus*. Selection against *M. trossulus* and *M. trossulus* –like hybrids due to differential adaptation to the environmental conditions of Newfoundland probably influenced the observed pattern. Although selection against hybrids was stronger during the first week of life (probably due to genetic incompatibilities), a higher mortality of hybrids and *M. trossulus* was also observed in the field experiments, mainly after the reproductive season. Nonetheless, many hybrids showed fitness similar to that of the parent species and a marked maternal effect was evident in growth rate and other reproductive traits. *M. edulis*-like hybrids behaved similarly to *M. edulis* and *M. trossulus*-like hybrids similarly to *M. trossulus*. The large variance observed in hybrid growth rate suggests that different hybrid classes show different fitness and that some hybrid individuals may be as fit as, or even fitter than, the parent species. Reproductive success was similar in hybrids and the parent species and many laboratory-produced F2 hybrids survived to 2 years, implying that post-zygotic isolation is only partial in *Mytilus*, but probably critical in preserving co-adapted genetic interactions in each species. Despite the overall greater mortality of hybrids, some hybrid individuals can express hybrid vigour (as growth rate) and hybridization could be used to improve aquaculture production. Other intrinsic differences observed between the species such as settlement behaviour, shell morphology and breakage resistance may also be relevant to aquaculture production in the future.

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