# LIFE HISTORY OF GENETIC VARIATION IN *Mytilus edulis* (LINNAEUS, 1758) AND *M. trossulus* (GOULD, 1850) IN A HYBRID ZONE ON THE EAST COAST OF NEWFOUNDLAND



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# LIFE HISTORY AND GENETIC VARIATION IN *Mytilus edulis* (Linnaeus, 1758) AND *M. trossulus* (Gould, 1850) IN A HYBRID ZONE ON THE EAST COAST OF NEWFOUNDLAND

by

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## ABSTRACT

Two mussel species (*Mytilus edulis* L. and *Mytilus trossulus* Gould) were found sympatrically in two locations (Bellevue and Chance Cove) in eastern Newfoundland. There was genetic evidence for naturally occurring hybrids between *M. edulis* and *M. trossulus*. Mussels were classified as pure forms or hybrids (F1, F2 and from backcrosses) based on four diagnostic markers, two allozyme loci (*Mpi* and *Est-D*) and two nuclear PCR-based DNA markers (*ITS* and *Glu-5*). In addition, a PCR-based mtDNA marker (*COIII*) was used to characterize the distribution of mtDNA mitotypes among pure and hybrid individuals. All females and males from pure species were found to be homoplasmic and heteroplasmic, respectively, for the mtDNA genomic combination. Two individuals showed heterospecific combination of the F *edulis* genome and the M *trossulus* genome (both classified as F1 hybrids using four nuclear diagnostic markers). There were differences in the proportions of pure *M. edulis* and *M. trossulus* and hybrids between sites within each location. *M. edulis* and hybrids were associated with protected sites, *M. trossulus* with exposed sites. Life history variation between species was also found, whereby *M. trossulus* was the predominant form among small individuals (larvae, spat and juveniles) and *M. edulis* among the large individuals, suggesting a strong viability selection against pure *M. trossulus* and its hybrid backcrosses. The overall low frequency of hybrids at the adult stage (7.28 %) and the results of artificial hybridization in the laboratory, which showed an increased proportion of abnormal larvae among interspecific crosses, indicate strong selection against hybrids, and clearly showed them to be at selective disadvantage, supporting the tension-zone model for the early stages of the mussel life-history. *M. trossulus* and hybrids spawned over a prolonged period of time (from late spring to early autumn), while most *M. edulis* individuals spawned simultaneously in late July. Hybrid mussels exhibited spawning activity intermediate between that of the parental species. *M. trossulus* showed a higher reproductive output than *M. edulis* of similar shell length, while hybrids showed intermediate fecundity values between *M. edulis* and *M. trossulus*. Post-spawning mortality in *M. trossulus* seems to be associated with reproductive stress, but further studies should be carried out to establish the cause of the summer mortality which affects mainly *M. trossulus*.

*Solo subiendo a la colina podrás contemplar el llano....*

*A ti, Rodrigo.... querido hermano.*

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Finally, loving thanks to my parents who gave me what all children should have.

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| | |
|---|---|
| **ANOVA** | **= Analysis of variance** |
| **BE** | **= Bellevue exposed** |
| **BP** | **= Bellevue protected** |
| **BSC** | **= Biological species concept** |
| **CE** | **= Chance Cove exposed** |
| **CSC** | **= Cohesion species concept** |
| **CP** | **= Chance Cove protected** |
| **ESC** | **= Evolutionary species concept** |
| **DUI** | **= Double uniparental inheritance** |
| **F1** | **= First filial generation** |
| **MT** | **= Metric tons** |
| **MYA** | **= Million years ago** |
| **NORs** | **= Nucleolar organizer regions** |
| **PCR** | **= Polymerase chain reaction** |
| **PSC** | **= Phylogenetic species concept** |
| **RFLPs** | **= Random fragment length polymorphisms** |

# I. INTRODUCTION

## I.1. GENERAL

### I.1.1. GENUS *Mytilus* Linné 1758

The genus *Mytilus* belongs to the family Mytilidae, which according to Soot-Ryen (1969) originated in the Devonian era (about 400 mya). Mussels belonging to the genus *Mytilus* are semi-sessile epibenthic bivalves which are attached to a hard substrate, or anchored to other mussels, with byssal threads secreted by the pedal glands in the foot (Seed and Suchanek, 1992). These byssal threads allow the mussel to accomplish some degree of movement in order to migrate towards the outer edge of the clump (Harger, 1968). Mussels present separate sexes, though rare instances of hermaphroditism have been reported (Seed, 1976; Beaumont and Abdul-Matin, 1994; see Chapter 3). The reproductive cycle of any mussel population is the result of a complex balance between exogenous factors such as food availability, temperature, salinity, and position in the intertidal zone, and endogenous factors such as nutrient reserves and genotype (Seed, 1976; Sastry, 1979; Rodhouse et al., 1986; Hilbish and Zimmerman, 1988; Seed and Suchanek, 1992). Interaction between these factors requires the synchrony of gamete development within the population. Such synchrony is important for an oviparous species and ensures that larvae are in the water at the optimum time for their growth and survival (Sastry, 1979). For environments in which variations in physical factors are not large (especially in those factors that influence patterns of phytoplankton production), the reproductive cycle of the mussel is less variable (Newell et al., 1982). In estuaries and open bays where annual variations in environmental factors (salinity, temperature, inorganic nutrients, etc.) are large, the reproductive cycle of mussels can be expected to vary (Thompson, 1979, 1984b; Lowe et al., 1982). Spawning occurs when the eggs and sperm are released directly into the water column, where fertilization occurs. In *Mytilus edulis*, males usually spawn first, and the presence of sperm in the water

stimulates the females to stop filtering (Newell and Thompson, 1984) and start spawning. This synchronous spawning ensures that the sperm and eggs are in the water column at the same time. Spawning may take the form of mass spawning, in which an individual mussel liberates most of its gametes over a short period (Thompson, 1984a), but another type of spawning has been described in which gametes are continually ripening and are liberated in a "dribble" spawn (Seed and Suchanek, 1992).

The meroplanktonic larval stage in mussels may last from 2 to 10 weeks, depending on the environmental conditions in the water column (Seed and Suchanek, 1992). During this stage mussel larvae are passively carried by water currents, sometimes over large distances (Scheltema, 1978). The large number ($7\text{-}40 \times 10^6$) (Thompson, 1979) and small size (60-90 μm) of eggs (Seed and Suchanek, 1992) produced by mussels are typical of the planktotrophic reproductive strategy in which output is maximized but nutrient investment per egg is small (Bayne, 1976b; Strathmann, 1985). This strategy may enhance dispersal, although it produces an egg with very low nutrient reserves, which may reduce the survival of the mussel larvae under certain conditions (e.g., high temperature, insufficient food or lack of a suitable substrate) (Bayne, 1965, 1976a; Bayne et al., 1982).

Once the larva reaches the pediveliger stage it uses its foot to make contact with the substrate and starts searching for a filamentous material (Bayne, 1976b). If the substrate is suitable, the larva metamorphoses and becomes a juvenile form (plantigrade), which attaches with byssal threads (=primary settlement). It has been suggested that this primary settlement enables the juvenile mussel to grow in an environment free from the competition for food and space which can occur in large mussel beds (Thorson, 1957). After the plantigrade reaches around 1.5 mm shell length on the filamentous substrate, it detaches itself and becomes planktonic (Bayne, 1976b). When this drifting plantigrade meets a mussel bed, it is stimulated to generate new byssal threads and attach itself to the substrate or directly onto the shell of another mussel (=secondary settlement) (Bayne, 1976b).

Growth rate in *Mytilus* is highly variable and is influenced by exogenous (temperature, salinity, food supply and tidal exposure) and endogenous (genotype,

reproduction) factors. Individual mussels of similar size from a single cohort may show widely different growth rates under apparently identical conditions, indicating that genotype may also play a role (Innes and Haley, 1977; Singh and Zouros, 1978; Koehn and Gaffney, 1984; Gentili and Beaumont, 1988; Beaumont, 1991; Hawkins et al., 1994; Hawkins and Day, 1996; Toro and Paredes, 1996a, b). In some subtidal environments *M. edulis* can reach lengths of 60-80 mm within two years (Winter et al., 1984), whereas in the high intertidal zone growth is substantially reduced, and mussels may attain lengths of only 20-30 mm after 15-30 years (Seed, 1976). In studies involving populations of *M. edulis* from different latitudes in the northern hemisphere, it has been found that the southern population has a much faster growth rate for the first two years and the maximum length attained is 55 mm after 4-5 years (Rodhouse et al., 1986), while mussels from the northern population reach an asymptotic length of over 95 mm after 11-12 years (Thompson, 1984a).This differential growth rate with latitude may be a consequence of the shorter feeding season for the northern population, and the increased longevity in high latitude populations may be a result of reduced metabolism (Seed, 1976).

Mussels in temperate waters of the northern and southern hemispheres, growing continuously submerged in areas of high food availability such as those in raft-based mariculture operations, can grow as much as 50 mm per year (Incze and Lutz, 1980; Winter et al., 1984). Therefore, several environmental factors influence growth rate in *Mytilus* in temperate waters. Shell growth is usually rapid between the spring and autumn, and slow or absent in winter months (Seed and Suchanek, 1992). Flesh weight, by contrast, has more variable seasonal peaks associated with the annual reproductive cycle and food availability events such as the spring bloom (Kautsky, 1982; Hilbish, 1986). Thompson (1984a,b) reported that mussels in Newfoundland initially invest most of the available energy in shell and somatic growth, but as the animal grows older, more energy is diverted to reproductive tissue. Higher fecundity values in mussels have also been associated with more heterozygous individuals within a population (Rodhouse et al., 1986).

### I.1.2. WORLDWIDE DISTRIBUTION OF *Mytilus*

Mussels in the genus *Mytilus* are abundant and widely distributed marine bivalves which occur in temperate and boreal waters of all oceans and major seas of both northern and southern hemispheres (Soot-Ryen, 1955). They represent an important component of the intertidal and subtidal communities and estuaries, in terms of the number of individuals and of biomass and production, and are also of considerable economic importance to aquaculture in many regions of the world ( Hickman, 1992; Seed, 1992; Seed and Suchanek, 1992; Aiken, 1993). Gosling (1992a) and Seed (1992) give a detailed description of the world distribution of the most studied species within the genus *Mytilus*, based on electrophoretic and morphological evidence. Since then, more evidence on the macro-and micro-distribution of these species has become available, most of which is based on electrophoretic surveys and the use of new molecular tools such as mtDNA and nuclear-DNA markers, combined in some cases with analysis of shell morphology (Sarver and Foltz, 1993; Sanjuan et al., 1994, 1996, 1997; Bates and Innes, 1995; Heath et al., 1995, 1996; Inoue et al., 1995, 1997; Mallet and Carver, 1995; Suchanek et al., 1997; Hunt and Scheibling, 1998; Toro, 1998). These studies have confirmed, extended or reduced the previously reported geographic ranges of some of the species within the genus ***Mytilus*** (Gosling, 1992a). For instance, the presence of *M. trossulus* in southeastern Nova Scotia (Mallet and Carver, 1995; Hunt and Scheibling, 1998) and eastern Newfoundland (Bates and Innes, 1995) confirms the study of Koehn et al. (1984), which describes mussel populations as belonging to what they called "genetically distinct Group III" (Koehn et al., 1984). Furthermore, *M. trossulus* has now been described from northern Japan (Suchanek et al., 1997; Inoue et al., 1997), extending its range south from that previously reported (McDonald et al., 1991). However, so far no occurrences of *M. trossulus* have been reported below latitude 35° N or in the southern hemisphere. *M. edulis* occurs at similar latitudes as *M. trossulus*, although *M. edulis* is also found in the southern hemisphere (McDonald et al.,

1991; Toro, 1998). *M. galloprovincialis*, on the other hand, is restricted to warmer waters and the species has not been reported above 51° latitude in either hemisphere.

### I.1.3 ECONOMIC IMPORTANCE OF *Mytilus*

Mussels have a number of characteristics that make them ideal for intensive commercial mariculture. The high fecundity and recruitment of natural mussel populations allow small mussels to be collected from natural seed beds (Incze and Lutz, 1980; Hickman, 1992). The plantigrades can also be collected directly on fibrous spat collectors which provide the filamentous substrate needed for primary settlement (Mason, 1976). The ability of mussels to attach to surfaces such as filamentous ropes makes it possible to cultivate them easily in off-bottom systems such as rafts or long-lines (Lutz, 1980; Winter et al., 1984). This trait is very important to commercial mariculture because it reduces the amount of fouling from feces and pseudofeces, lowers the incidence of pearls and, more importantly, enhances growth and reduces mortality from benthic predators (Winter et al., 1984; Hickman, 1992). According to Lutz (1980) mussels grow faster than most traditional shellfish species, generate a higher ratio of meat to total weight, and are nutritionally superior.

Unlike that of most other aquatic species, wild mussel production is much lower than cultured mussel production. In 1995, total mussel production -both capture and culture- was 1.2 million MT, up from 950,000 MT in 1985 (New, 1997). The increase came almost entirely from enhanced culture production. The mussel capture fisheries have declined to 21% of the total mussel harvest in recent years. Mussel capture fisheries peaked in 1992 and 1994 at 277,000 MT and then declined by 13% in 1995, as a result of lower production in Denmark and Thailand, the two major producing countries. *Mytilus edulis* (blue mussel) is the main mussel taken in the wild. In the North Sea, it accounts for over one half of the total mussel capture fishery. *Mytilus galloprovincialis* (Mediterranean mussel) is second in importance, with an annual average catch of 40,000 MT between 1985 and 1995.

Among the leading countries in cultured mussel production, China produced about 500,000 MT (including various unidentified species) from 1991-1993, decreasing to 415,000 MT in 1995. Italy replaced Spain as the second major producer of cultured mussels in 1995 with 95,000 MT. Other important producing countries are The Netherlands, Korea, France, New Zealand and Thailand. In Canada, *Mytilus edulis* is the predominant mussel species cultivated. Production of mussels is concentrated in the Atlantic Provinces (Newfoundland, Prince Edward Island, Nova Scotia and New Brunswick). Mussel production first became established in Prince Edward Island in the 1970's, and this province has become the leader in North America (approximately 8,000 MT in 1996, estimated to be 10,000 MT in 1997 and projected to be over 12,000 MT in 1998).

Mussel culture in Newfoundland, Nova Scotia and New Brunswick did not begin until the mid 1980's. After a developmental period, Newfoundland appears poised for rapid growth. In 1996 the eastern provinces produced 8,700 MT of mussels with value of $20,000,000 Canadian (PEI 86%, NS 5.8%, NF 5%, NB 2%, PQ 1.2%) (Kielley, 1997).

While the physical geography varies in the Atlantic provinces, the environment of Newfoundland is particularly well suited for mussel culture. The shoreline topography presents many large, well protected, sheltered coves and inlets which provide ideal growing conditions. Most sites are located in sparsely populated areas and are therefore little affected by industrial, municipal or domestic pollutants (Thompson, 1984b; Brown). However, a major concern for growers in Newfoundland is to avoid collection of *M. trossulus* seed which may have inferior growth characteristics (Mallet and Carver, 1995). Further information on the distribution of the two species and their ecology is required.

### 1.1.4. TAXONOMY OF *Mytilus*

Despite the worldwide distribution of the genus *Mytilus*, as well as its scientific and commercial importance, the taxonomy and systematics of this genus are still uncertain (McDonald and Koehn, 1988; Varvio et al., 1988; Johannesson et al., 1990; Väinölä, 1990;

Koehn, 1991; McDonald et al., 1991; Gardner, 1992; Gosling, 1992a; Seed, 1992; Toro, 1998). Early classifications based on external shell morphology tended to be complex and confusing (Soot-Ryen, 1955; Seed, 1976). Shell shape is highly influenced by local environment, making identification very difficult when based only on shell morphology (Seed, 1969, 1978, 1980, 1992; Innes and Bates, 1999). Moreover, the hybridization which occurs in areas where two species of *Mytilus* are found sympatrically (Skibinski et al., 1978; McDonald and Koehn, 1988; Coustau et al., 1991; Väinölä and Hvilsom, 1991; Sarver and Foltz, 1993; Bates and Innes, 1995; Mallet and Carver, 1995; Comesaña and Sanjuan, 1997; Inoue et al., 1997) further confuses the taxonomy. Although *M. californianus* and *M. trossulus* on the west coast of North America can be easily identified by shell morphology when larger than 20 mm shell length, they are indistinguishable when smaller than 10 mm (Suchanek, 1978).

Mussel populations have been defined using allozyme characters instead of morphological characters (Koehn et al., 1984; McDonald and Koehn, 1988; Varvio et al., 1988; Coustau et al., 1991; McDonald et al., 1991; Sanjuan et al., 1994; Viard et al., 1994; Bates and Innes, 1995; Sanjuan et al., 1997), or, more recently, by using mitochondrial or nuclear DNA sequences (Edwards and Skibinski, 1987; Blot et al., 1990; Côrte-Real et al., 1994a, 1994b; Heath et al., 1995; Geller et al., 1994; Heath et al., 1996; Inoue et al., 1997; Comesaña et al., 1998; Toro, 1998). These studies have shown that the genus *Mytilus* is composed of at least three morphologically similar but genetically distinct species which are distributed world-wide (McDonald et al., 1991; Gardner, 1992; Seed, 1992; Beynon and Skibiski, 1996), *M. edulis* Linnaeus, 1758 (in eastern USA and Canada, northern Europe, Argentina, Chile, the Falkland Islands, and Kerguelen Island), *M. galloprovincialis* Lamarck, 1819 (in the Mediterranean, western Australia, Tasmania, New Zealand, sympatrically with *M. edulis* in parts of Great Britain, Ireland and France, and introduced into Japan, Hong Kong, South Africa, and southern California), and *M. trossulus* Gould, 1850 (in the northern Pacific from Siberia to central California, the Canadian Atlantic provinces, and the Baltic Sea). *M. californianus* Conrad (1837), another species within the genus *Mytilus*, is found

only on the Pacific coast of North America (Seed, 1976). *M. californianus* has long been recognized as a distinct species (Soot-Ryen, 1955), although its range overlaps those of *M. trossulus* and *M. galloprovincialis*.

Other methodologies such as cytology, inmuno-electrophoresis and gamete ultrastructure have also been applied to inter- and intraspecific studies of *Mytilus* taxa. The genus *Mytilus* is a complex of closely-related species which share the same basic karyotype in terms of chromosome morphology (diploid number 2n=28) (Ahmed and Sparks, 1970; Thiriot-Quiévreux and Ayraud, 1982; Moynihan and Mahon, 1983; Thiriot-Quiévreux, 1984 Dixon and Flavell, 1986; Insua et al., 1994), and the taxa cannot be differentiated by a single karyological character (Insua et al., 1994). The presence of five metacentric chromosome pairs in *M. galloprovincialis* can be used to differentiate this species from *M. trossulus* and from *M. edulis*, which both have six metacentrics. The location of Ag-NORs (silver stained chromosomal nucleolar organizer regions) is similar in *M. edulis* and *M. galloprovincialis*, but not in *M. trossulus*, which also presents NORs on metacentric pair 4 (Martinez-Lage et al., 1995), although this character seems not to be fully diagnostic because it depends on NOR activity (Insua et al., 1994). The immuno-electrophoretic study of Brock (1985) indicated that *M. edulis* and *M. galloprovincialis* were conspecific, although the author included *M. trossulus* from the Baltic Sea as *M. edulis* in her analysis. Gamete ultrastructure has also been used for solving various systematic and phylogenetic problems in the Metazoa. However, Hodgson and Bernard (1986) concluded that within the genus *Mytilus*, spermatozoon morphology alone is insufficient to designate *M. edulis* and *M. galloprovincialis* as separate species. Other studies have shown great intra-specific variation that precludes differentiation between species in the genus *Mytilus* (Healy, 1996; Kafanov and Drozdov, 1998).

The taxonomic status of these genetically and/or morphologically distinguishable forms of *Mytilus* has been the subject of considerable debate for a number of years. Some authors, such as Gosling (1984) and Johanneson et al. (1990), have suggested that the members of the "*M. edulis* complex" (*M. edulis*, *M. galloprovincialis* and *M. trossulus*) are

ecotypes or varieties, while other authors, such as McDonald and Koehn (1988), Koehn (1991), MacDonald et al. (1991) and Seed (1992) recognize them as separate species. *M. edulis* and *M. trossulus* coexist in the Baltic Sea, where hybridization and introgression are common, leading Väinölä and Hvilson (1991) to suggest that these two taxa be considered as semispecies (defined as taxa intermediate between subspecies and species (Mayr and Ashlock, 1991)). The systematic position of *M. galloprovincialis* has been controversial (Bayne, 1976a; Beaumont et al., 1989; Gardner, 1992). In some studies *M. galloprovincialis* is described as a species distinct from *M. edulis* based on a set of traits (Koehn et al., 1984; McDonald and Koehn, 1988; Koehn, 1991; McDonald et al., 1991), while in other studies it is regarded as a subspecies (a replacement of the term "variety" in its meaning of "geographic race" (Mayr and Ashlock, 1991)) based on the same set of traits (Gosling, 1984; Väinölä and Hvilson, 1991; Gardner, 1992).

Any debate about species-level taxonomy is largely dependent on the species definition which is used. However, most studies on the taxonomical status of mussels within the genus *Mytilus* have not mentioned or defined which species concept they have employed. Seed (1978, 1992) pointed out, in the context of the taxonomic status of *M. galloprovincialis*, that "the systematics of these mytilids emphasizes the problems inherent in obtaining a satisfactory and practical species definition, especially when the concept is extended geographically". Most of the work has been done on the systematic status of *M. edulis* L. and the Mediterranean mussel *M. galloprovincialis* Lmk. (review in Gardner, 1994). It is thought that *M. edulis* is the ancestral form which may have evolved from the Modiomorphidae, and that *M. galloprovincialis* evolved in the Mediterranean Sea during the Pleistocene (Barsotti and Meluzzi, 1968; Skibinski et al., 1980; Fisher and Skibinski, 1990; Seed, 1992; Gosling, 1994). Recently a genetically distinct mussel type from the Baltic Sea, the Pacific coast of North America and the Atlantic provinces of Canada has been described. Koehn et al. (1984) believed that this mussel may constitute a separate species (as no evidence for hybridization was detected at that time) and they suggested the name *M. trossulus* Gould, 1850 for this taxon (McDonald and Koehn, 1988). According to Koehn

(1991) *M. trossulus*, which inhabits colder waters in the northen hemisphere, may have evolved from some cold tolerant genotype during the Pleistocene glacial period. However, there is some evidence that its presence in the Baltic Sea is recent (Varvio et al., 1988; Väinölä and Hvilson, 1991). *M. trossulus* is widely accepted as a distinct species, based on both genetic and biogeographic criteria (McDonald et al., 1991; Geller et al., 1994), while the status of *M. galloprovincialis* as a species separate from *M. edulis* is debatable (Gosling, 1984; Koehn, 1991; McDonald et al., 1991; Gardner, 1992).

In areas where two members of the "*Mytilus edulis* complex" are found sympatrically, there is always hybridization between them. This fact, and the results of artificial hybridization studies between *M. galloprovincialis* and *M. edulis* (Beaumont et al., 1993) and between *M. edulis* and *M. trossulus* (Zouros et al., 1992), indicate that there is little evidence of genetic incompatibility. Unfortunately, it is difficult to agree on how much hybridization two taxa are allowed to exhibit and still be considered separated species, or conversely, how much hybridization is permissible between taxa before they are considered conspecific. This is again a problem when the biological or isolation species concept is applied to hybridization between allopatric taxa in a contact zone, as occurs in *Mytilus*.

Gardner (1992) has evaluated various species concepts with respect to the taxonomic status of *M. galloprovincialis*. According to the definition of the biological species concept (BSC), species are "groups of actually or potentially interbreeding populations, which are reproductively isolated from other such groups" (Mayr, 1970). For this species concept reproductive isolation (the two species do not interbreed in nature) is more important than any morphological differences that they may have. Therefore under the BSC hybridizing species such as those in the "*Mytilus edulis* complex" are not true species. Although this concept is one of the most widely used, there are others which are perhaps more useful to describe the "*Mytilus edulis* complex". The evolutionary species concept (ESC), for example, defines species as "a single lineage of ancestor-descendent populations which maintains its identity from other such lineages and which has its own evolutionary and historical fate" (Wiley, 1978, 1981). The important point for the ESC is that hybridization

is permitted, but at the same time both species must maintain their separate identities. A number of authors (Woodruff et al., 1988; Liu et al., 1991; Sarver et al., 1992), however, have used the ESC when two species are found sympatrically at a site (such as *M. edulis* and *M. trossulus*), and it is very difficult to separate them based on morphological traits alone. Moreover, until recently there was no single genetic marker (allozyme or DNA) that allowed differentiation between these "species".

In the phylogenetic species concept (PSC), a species is considered as "the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent" (Cracraft, 1989). The same author notes that "even if two sister-taxa hybridize, both can still be considered to be species if each is diagnosable as a discrete taxon". Under this concept "reproductive isolation" is not important, but the presence of "diagnostic characters" is required. Recently, numerous new powerful genetic markers have become available which allow us to distinguish among taxa within the genus *Mytilus* (Heath et al., 1995; 1996; Rawson et al., 1996a; Inoue et al., 1997; Comesaña et al., 1998). However, a difficulty with this concept is that it is based mainly on the resolving power of the molecular tools available today or in the future.

Finally, the cohesion species concept (CSC) concentrates on processes that cause groups of organisms to be similar to one another (Templeton, 1989). This concept considers that "the species is the most inclusive population of individuals having the potential for phenotypic cohesion through intrinsic cohesion mechanisms" (sharing a common developmental genetic system, physiology and ecology). The focus of this concept is on cohesion mechanisms such as gene flow, stabilizing selection, and common ecological factors that keep species homogeneous. There is little evidence that taxa within the genus *Mytilus* are cohesive, so according to the CSC criteria these are not good species.

In conclusion, it can be stated that the different forms of *Mytilus* may not at this stage merit full species status. According to the present evidence, these taxa could be in the process of early sympatric speciation or experiencing intergradation after a period of allopatry. According to Gardner (1992), we may treat each of these taxa as a semispecies

(using the trinomial nomenclature), as component members of the *Mytilus edulis* superspecies (note that we do not even agree on a species definition), or we may retain the binomial nomenclature and eventually accept the phylogenetic species concept.

## I.2. HYBRID ZONES

### I.2.1. EVOLUTIONARY IMPORTANCE

Hybrid zones are regions in which species that are genetically distinct meet and interbreed, producing offspring of mixed ancestry (Harrison, 1993). Recently, Harrison (1990) pointed out that hybrid zones serve as "windows on evolutionary process", providing opportunities for studying the effects of gene flow, linkage, and the strength and forms of selection on genetic systems. The study of hybrid zones has been invigorated by new theory (Harrison, 1993) and new techniques, especially the use of molecular markers, so that hybrid zones are now seen to be much more complex than was previously believed.

Hybrid zones may be stable or unstable with respect to position and genetic dynamics. In recent decades, several authors have focused on the development of models to explain the stability and maintenance of hybrid zones. The literature cites two kinds of models, one which proposes that the fitness differences between hybrid and pure species genotypes are environmentally mediated (exogenous selection) (Slatkin, 1973, 1975; Endler, 1977) and the other which proposes that they are genetically mediated (endogenous selection) (Key, 1968, Barton and Hewitt, 1985). In the "environmental gradient models" the hybrids could be more or less fit as a consequence of their interactions with their environment. In the "tension zone models" selection occurs against hybrids, which are intrinsically less fit than "pure" individuals owning to genetic incompatibilities, without an ecological component to selection (=endogenous selection (Moore and Price, 1993)). However, Wilhelm and Hilbish (1998) pointed out that "in practice it is very difficult to

distinguish between environmental gradient and tension zone models for the maintenance of any given hybrid zone, since they yield similar predictions".

In recent years, most efforts to explain the apparent stability of hybrid zones have focused on models based on endogenous hybrid inferiority (Barton and Hewitt, 1985, 1989; Barton and Gale, 1993; Harrison, 1993; Shaw et al., 1993). Exogenous selection may be more difficult to demonstrate, but has been convincingly shown by Harrison and Rand (1989), Cruzan and Arnold (1993), Moore and Price (1993) and more recently by Wilhelm and Hilbish (1998).

### I.2.2. *Mytilus* HYBRID ZONES

When two species of the "*Mytilus edulis* complex" are found sympatrically, there is always hybridization between them (Gosling, 1992a). The size of a hybrid zone depends on its location. In Europe the width of the hybrid zone between *M. edulis* and *M. galloprovincialis* is about 1400 km, while that between North Sea *M. edulis* and Baltic *M. trossulus* is about 150 km (Väiniölä and Hvilson, 1991). In Atlantic Canada, there is limited information for the size and geographic location of the hybrid zone between *M. edulis* and *M. trossulus*, although recent studies (Bates and Innes, 1995; Saavedra et al., 1996; Comesaña et al., 1998) have made some progress in characterizing it. The size of the hybrid zone of *M. trossulus* and *M. galloprovincialis* on the Pacific coast of North America has been the subject of several studies (McDonald and Koehn, 1988; Sarver and Foltz, 1993; Geller et al., 1994; Rawson and Hilbish, 1995), and its range appears to extend from Vancouver Island (Heath et al., 1995) to San Diego Bay (Suchanek et al., 1997). The recent discovery of a hybrid zone in northern Japan (Innoue et al., 1997) is not well documented, but the data available suggest that it is a very narrow zone in south-west Hokkaido.

Most studies on mussel hybrid zones have focused on the exogenous and endogenous factors that maintain the genetic integrity of the species, despite the potential for hybridization and long-range larval dispersal. Results from electrophoretic, nuclear and

mitochondrial DNA analyses have indicated that such zones are spatially complex, containing a mixture of pure, hybrid and introgressed individuals.

#### I.2.2.1. Europe

Europe is the most extensively studied region of mussel hybridization (Gosling, 1984; Gosling, 1992a; Seed, 1992; Comesaña and Sanjuan, 1997; Comesaña et al., 1998). *Mytilus galloprovincialis* and *M. edulis* coexist and hybridize in different proportions in large areas of the British and Atlantic French coasts (Skibinski and Beardmore, 1979; Gosling and Wilkins, 1981; Skibinski, 1983; Skibinski et al., 1978, 1980, 1983; Coustau et al., 1991). The patchy distribution pattern observed for the two mussel species and their hybrids in these regions of contact and hybridization suggests that there are ecological differences between the species (Skibinski and Beardmore, 1979; McDonald and Koehn, 1988; Hilbish et al., 1994). There is some evidence from laboratory crosses of *M. edulis* and *M. galloprovincialis* that hybrid larvae have higher mortality rates than those of pure crosses (Seed, 1992; Beaumont et al., 1993). Furthermore, there is evidence that fecundity and timing of spawning of *M. edulis* and *M. galloprovincialis* populations can differ at certain localities (Gardner and Skibinski, 1990a; Gardner, 1992; Seed, 1992), which can result in partial reproductive isolation.

The distribution of the two species in most studied sites within the hybrid zone seems to be correlated with some environmental factors, whereby *M. edulis*-like mussels occur at highest frequency in sheltered, less saline waters, and *M. galloprovincialis*-like mussels occur at highest frequency in more exposed waters of high salinity (Comesaña and Sanjuan, 1997). There is a strong genotype-dependent viability within the hybrid zone, in which *M. galloprovincialis* has a significantly higher survival rate than *M. edulis*, while hybrids are intermediate in fitness (Skibinski, 1983; Gardner and Skibinski, 1988; Skibinski and Roderick, 1991; Wilhelm and Hilbish, 1998). Several hypotheses have been advanced to explain the strong viability selection which occurs in this hybrid zone. Physiological

differences like differential susceptibility to thermal stress (Hilbish et al., 1994) and differential resistance to parasitism (Coustau et al., 1991) have been found between the two species (Hilbish et al., 1994). It also seems that the heterogeneity of the marine environment could be playing a role in the maintenance of the genetic integrity of the species. Studies on this hybrid zone have shown that in *M. galloprovincialis*-like genotypes a greater byssal attachment strength seems to be responsible for a relatively higher frequency of this species in more exposed environments (Gardner and Skibinski, 1991; Willis and Skibinski, 1992). A recent study by Wilhelm and Hilbish (1998) indicated that the genetic structure of the hybrid population in south-west England is maintained by a balance between selection against *M. edulis*-like genotypes and their replacement through larval immigration.

#### I.2.2.2. Pacific coast of North America

Recent studies have shown that mussels on the Pacific coast of North America are not *M. edulis*, as previously reported (Seed, 1976). McDonald and Koehn (1988) identified *M. galloprovincialis* in southern California, *M. trossulus* in northern California, and the presence of hybrids around San Francisco Bay. This finding was later confirmed by Rawson et al. (1996a) and Suchanek et al (1997). The region of contact and hybridization between *M. trossulus* and *M. galloprovincialis* in California is not as well documented as the European hybridization zone, although hybridization has been reported along the Pacific coast of the U.S.A. (McDonald and Koehn, 1988; Koehn, 1991; Sarver and Foltz, 1993; Geller, 1994; Geller et al., 1994; Rawson and Hilbish, 1995) and Canada (Heath et al., 1995). According to Sarver and Foltz (1993), only 71 out of 1250 individuals (5.7%) from their collections of mussels along the Pacific coast of the U.S.A. could be classified as possible hybrids on the basis of 15 enzyme loci. Heath et al. (1995) used PCR-based nuclear markers to study mussels on the west coast of Canada and reported only 5.4% hybrids. Rawson and Hilbish (1995), using an mtDNA (PCR/RFLP) assay, concluded that hybridization of *M. trossulus* and *M. galloprovincialis* was rare in southern California. In

general, these are low percentages of hybrids compared with those reported for the hybrid zone in Europe, which are higher and may vary from site to site, for instance 25-50% reported by Sanjuan et al. (1994), 80% by Hilbish et al. (1994) and 27-49% by Comesaña and Sanjuan (1997). Rawson et al. (1996a) reported 20.7% hybrids in San Francisco Bay, of which 66% were putative F1 genotypes. However, in a more recent study along the Pacific coast of North America, Suchanek et al. (1997) reported 55% hybrids, 34% *M. galloprovincialis* and 11% *M. trossulus* in mussel samples taken in San Francisco Bay, near the Golden Gate Bridge. These studies may imply that the distribution of the two species and their hybrids is patchy. It also seems that the relationship between *M. trossulus* and *M. galloprovincialis* in California is different from that between *M. edulis* and *M. galloprovincialis* in the contact zone in Europe, where *M. edulis* predominates in sheltered bays and estuaries and *M. galloprovincialis* is more common on wave-exposed shores (Comesaña and Sanjuan, 1997). In California the degree of exposure does not appear to be a significant factor influencing the distributions of *M. trossulus* and *M. galloprovincialis* (Sarver and Foltz, 1993), a situation which could be related to the presence of *M. californianus*, which out-competes other mytilids on exposed rocky shores (Harger, 1970a, b).

#### 1.2.2.3. Atlantic coast of North America

The micro- and macrogeographic distribution of the genus *Mytilus* on the Atlantic coast of North America has been studied by examining genetic variation at several polymorphic enzyme loci (Gartner-Kepkay et al., 1980; Koehn et al., 1984; Varvio et al., 1988; McDonald et al., 1991; Bates and Innes, 1995). Early genetic studies of *Mytilus* populations on the east coast of North America suggested that *Mytilus edulis* was the only species present (Koehn et al., 1976; Gartner-Kepkay et al., 1980), but later Koehn et al. (1984) showed that populations of *Mytilus* spp. in Atlantic Canada were composed of two genetically distinct forms found sympatrically at some locations. These two genetically

distinct forms of *Mytilus* (groups II and III of Koehn et al., 1984) were later confirmed to belong to the species *M. edulis* and *M. trossulus*, respectively (Varvio et al., 1988; McDonald et al., 1991; Bates and Innes, 1995; Mallet and Carver, 1995; Saavedra et al., 1996; Comesaña et al., 1998; Hunt and Scheibling, 1998). In Atlantic Canada, there is limited evidence for interbreeding between *M. edulis* and *M. trossulus* in nature (Koehn et al., 1984; Varvio et al., 1988), despite the sympatric occurrence of both species and the successful production of viable hybrids in laboratory crosses (Zouros et al., 1992; 1994a, Saavedra et al., 1996). The lack of hybrids detected may in part be due to the failure to use highly diagnostic allozyme markers and/or an insufficient sample size. A recent study on the genetic variation of these two species of mussels in Newfoundland showed that the distribution of a hybrid index based on three partially diagnostic loci (*Est-D*, *Pgm*, *Lap*) provided no evidence for hybridization (Bates and Innes, 1995). Two studies in Lunenburg Bay, Nova Scotia, estimated the degree of natural hybridization to be < 5% (Mallet and Carver, 1995) using the *Mpi* locus and 22.8 % (Saavedra et al., 1996) using *Mpi* and *Est-D* loci.

#### 1.2.2.4. Japan

Inoue et al. (1997) used a PCR-based DNA marker (Inoue et al., 1995) to show that a hybrid zone of *M. galloprovincialis* and *M. trossulus* may be located in the region of Hiura and Hakodate, northern Japan. These authors also confirmed the study carried out by Wilkins et al. (1983) reporting the presence of *M. galloprovincialis* along the entire coast of Japan (rather than *M. edulis* populations as previously reported (Seed, 1976)), including Hokkaido and the northern part of Honshu, indicating a more cold water distribution for this species. According to Wilkins et al. (1983) *M. galloprovincialis* was accidentally introduced to Japan from Europe before 1935. The other species, *M. trossulus*, is present in Hokkaido (although it was originally identified as *M. edulis* (Wilkins et al., 1983)), although Inoue et al. (1997) observed that "pure" *M. trossulus* was not found at the northern tip of Honshu

(which is only 20 km south of Hokkaido), despite the enormous marine traffic between the two islands, and suggested that Hokkaido may be the southern limit of the North Pacific *M. trossulus*. At Hiura and Hakodate, mussels having both *M. galloprovincialis* and *M. trossulus* type sequences were found, which were presumed by the authors to be caused by hybridization and introgression between the two species (Inoue et al., 1997). Because this is a recent finding, more studies are required to establish a detailed distribution for the two species and their hybrids in northern Japan.

## I.3. OBJECTIVES

The main hypothesis tested in this study is that there is no differences in the life history of *Mytilus edulis-M. trossulus*.

The following are the specific objectives to be addressed:

- Determine whether there is micro-geographic genetic differentiation of *Mytilus edulis* and *M. trossulus* and detect the presence of naturally occurring hybrids.

- Estimation of the degree of natural hybridization at two different locations and, at each location, for two different environments (one exposed and other more sheltered and protected from wave action).

- Determine if both *Mytilus* species and their hybrids are distributed differentially according to size.

- Examine the possible intrinsic incompatibilities between *edulis* and *trossulus* nuclear and mitochondrial genomes in mussels of different size from different environments.

- Estimation of the frequency distribution of both *Mytilus* species and their hybrids at the larval and spat stages of the life-history.

- Determine the timing of spawning of *M. edulis* and *M. trossulus* and their hybrids in their natural environment.

- Determine spawning frequency and fecundity under natural conditions for *Mytilus edulis*, *M. trossulus* and their hybrids, using gonado-somatic index, qualitative histological staging, and quantitative stereology.

- Evaluation of fertilization, viability and growth success of pure lines and hybrids of *M. edulis* and *M. trossulus* during the crucial early stages of their development, based on laboratory crosses using a factorial design.

- Evaluation of shell morphometrics as an approach to distinguish among *M. edulis*, *M. trossulus* and their hybrids using sympatric populations and a combination of individuals from three different sites.

# II. CHAPTER 1.

## Applications of PCR-based nuclear markers to discriminate among *Mytilus edulis*, *M. trossulus* and their natural hybrids, and the analysis of their frequency distributions among geographic locations and stages of their life cycles

### II.1. INTRODUCTION

Natural hybrid zones provide an opportunity to study the genetics of speciation and the origin of the ecological and genomic interactions which maintain the integrity of species (Arnold, 1992; Harrison, 1993). The hybrid zones are spatially complex, and in some areas their location is determined by specific environmental factors (Skibinski and Roderick, 1991). In areas where species of the "*Mytilus* complex" are found sympatrically, there is always hybridization between them (Gosling, 1994). Some of these contact zones are located on the Pacific coast of North America, between *M. galloprovincialis* and *M. trossulus* (McDonald and Kohen, 1988; Heath et al., 1995), in southwest England, between *M. edulis* and *M. galloprovincialis* (Skibinski et al., 1978; Edwards and Skibinski, 1987; Gardner et al., 1993; Gardner, 1994), and in the Baltic Sea, between *M. edulis* and *M. trossulus* (Väinölä and Hvilsom, 1991). In Atlantic Canada, there is limited evidence for interbreeding between *M. edulis* and *M. trossulus* in nature (Koehn et al., 1984; Varvio et al., 1988), despite their sympatric occurrence and the successful production of viable hybrids in laboratory crosses (Zouros et al., 1992; 1994a; Saavedra et al., 1996). Undetected hybrids may in part be due to the failure to use highly diagnostic allozyme markers and/or an insufficient sample size. Several studies have suggested a potential hybrid zone between two species of mussels in Atlantic Canada (McDonald et al., 1991; Freeman et al., 1994; Mallet and Carver, 1995). A recent study based on allozyme analysis has established the presence of two species of blue mussels, *Mytilus edulis* and *M. trossulus*, in eastern Newfoundland, although the distribution

of hybrid index scores, based on three partially diagnostic loci (*Est, Pgm, Lap*), provided no clear evidence for hybridization (Bates and Innes, 1995).

Most marine benthic invertebrates show spatial genetic heterogeneity, despite the long-lived planktonic stage which enhances dispersal and tend to homogenize the genetic structure of populations. Selection or immigration of genetically different larvae (Koehn et al., 1980; Gartner-Kepkay et al., 1983; Hilbish, 1985; Wilhelm and Hilbish, 1998) may counteract the dispersal capabilities of the larvae, resulting in spatial or temporal genetic heterogeneity. Several studies report that selection seems to be most intense at larval and spat stages (Hilbish, 1985; Hilbish and Koehn, 1985; Gosling and McGrath, 1990), because predation during the pelagic stage and larval metamorphosis may increase the mortality rate (Wendell and Garton, 1995).

For organisms with complex life histories such as mussels (a prolonged, mobile, planktonic larval stage, two distinct phases of larval settlement and a semi-sessile adult stage), it is particularly important that early life stages (such as the planktonic larval stage and spat) can be investigated, especially within hybrid zones. Until recently, studies carried out on the relationship between benthic mussel communities and the planktonic distribution of mussel larvae have been based on detailed morphological analysis of plankton samples (Ramorino and Campos, 1983; Pulfrish, 1997). When these investigations are carried out in areas where more than one mussel species is sympatrically or parapatrically present, some difficulties in identifying larvae are often encountered (Olson et al., 1991; Cragg, 1996), especially in temperate waters, where reproduction is highly seasonal (Bayne, 1976a, b) and most invertebrate species have overlapping spawning times (Olive, 1992; Minchin, 1993). Moreover, the size and shape of prodissoconch II shells of larval bivalves vary considerably (Loosanoff and Davis, 1963; Bayne, 1965; Pulfrish, 1997), making identification more difficult. Allozyme electrophoresis techniques, in general, have been difficult to apply to larvae or spat of bivalves (Skibinski et al., 1983;Gosling and McGrath, 1990) (but see Hu et al., 1992; Wendell and Garton, 1995). The polymerase chain reaction (PCR) (Mullis and Faloona, 1987; Buffery, 1993) is potentially a sufficiently sensitive method for characterizing

invertebrate larvae at very early stages (Olson et al., 1991), e.g., those of *Mytilus edulis* (Côrte-Real et al., 1994b; Sutherland et al., 1998). This technique, using specific genetic markers, could be very useful in planktonic surveys of bivalve larvae. In the present study, a static cohort analysis of *Mytilus* spp. and two diagnostic PCR-based nuclear-DNA markers were used to investigate the micro-geographic distribution pattern of *M. edulis* and *M. trossulus*, and to detect the presence and frequencies of their natural hybrids. Different stages of the life cycles of the species and hybrids, including planktonic larvae, newly settled post larvae and spat were also examined, in order to investigate the existence and timing of any change in their frequencies that may suggest that selection affects one or more of the species.

## II. 2. MATERIAL AND METHODS

### II. 2.1. STUDY SITES AND SAMPLING

Adult mussels (*M. edulis* Linnaeus, 1758; *M. trossulus* Gould, 1850) were collected subtidally by SCUBA at a depth of approximately 1.5 m below mean low tide at two locations, Chance Cove and Bellevue, located in Trinity Bay, east coast of Newfoundland, in October 1995, June 1996, October 1996 and October 1997. At each location, two sites were sampled, one exposed to wave action, (Chance Cove exposed, CE; Bellevue exposed, BE) and another protected and sheltered (Chance Cove protected, CP; Bellevue protected, BP) (Figure 1). Representative samples (Hap hazzard) of approximately 200-300 mussels were taken from each mussel bed at each site, including a range of sizes from 15 mm to 98.6 mm shell length. During June 1996 three random samples, each composed of 200-300 mussels, were taken along the longest axis of the mussel bed at each of the four sampling sites. This allowed for the collection of a representative sample from each mussel bed, which can contain several thousands of mussels. The mussels were brought alive to the laboratory, shell length was measured with an electronic caliper (0.01 mm), and the animals dissected.

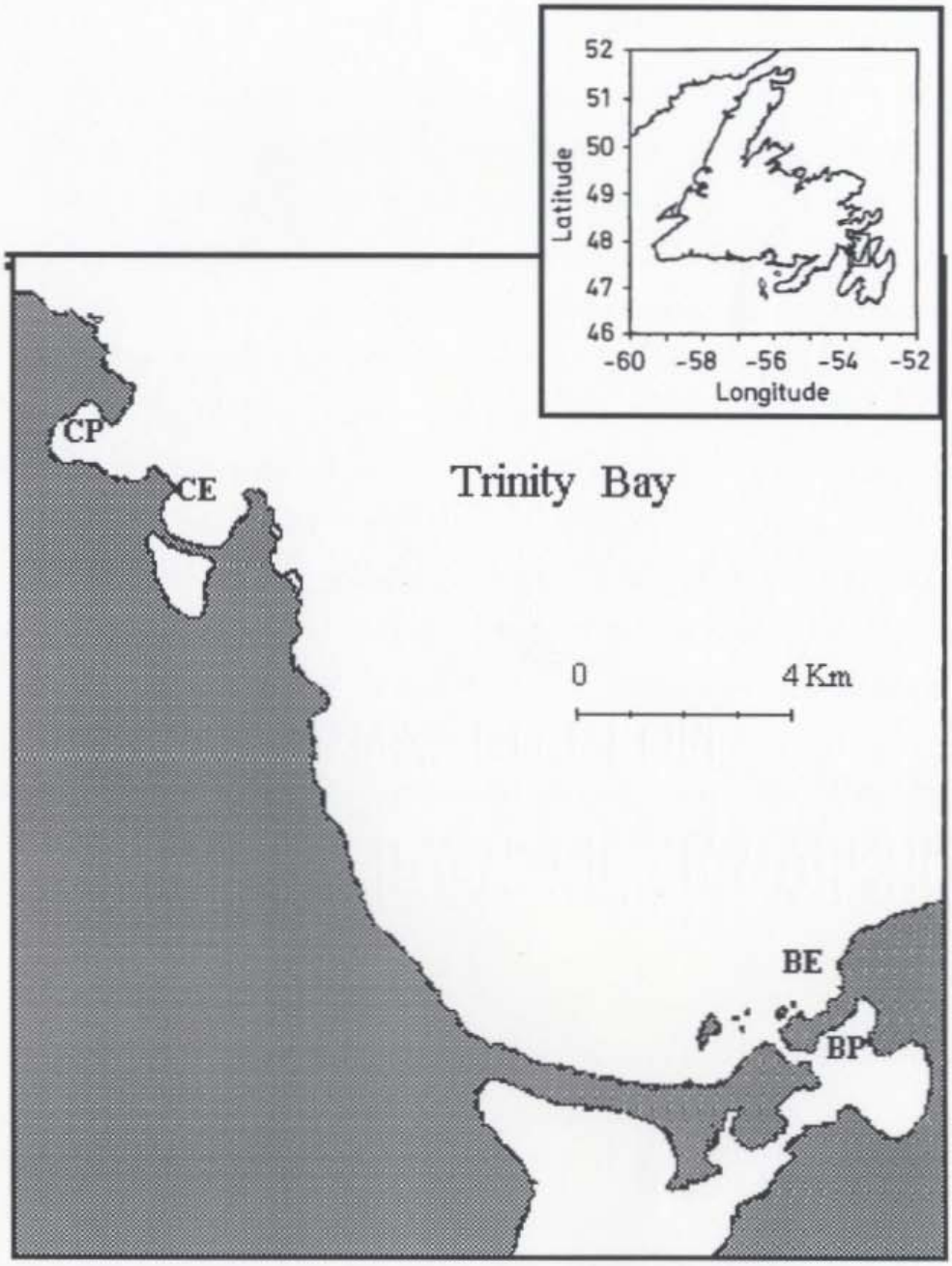



**Fig. 1.** Map of sample sites in eastern Newfoundland: Bellevue Protected (BP), Bellevue Exposed (BE), Chance Cove Protected (CP), Chance Cove Exposed (CE).

A small piece of mantle edge tissue (approximately 200 mg) was removed, placed in a 1.5 ml Eppendorf tube, fixed with 95% ethanol and stored at -20°C to await analysis.

Mussel spat were collected from brown filamentous algae in three consecutive years (0.6-1.9 mm, primary settlement) and from within mussel clumps (2.0-14 mm, secondary settlement) at each of the sites described in the above paragraph during the months of October and December 1995, September, October, and December 1996, and September and December 1997. The samples were placed in 95% ethanol and refrigerated until they could be analysed. Approximately 250 mussels from each site were collected on each sampling date.

Planktonic larvae were qualitatively sampled in standard 10 minutes plankton tows (20 µm mesh) at the same sites sampled for adult mussels at Bellevue and Chance Cove, Newfoundland, during June, July, August, September, October 1996, June and September 1997, and August, 1998. Also, D-shape larvae obtained from pure crosses of *M. edulis*, *M. trossulus* and their reciprocal hybrid crosses (see Chapter 4) were obtained by rearing larvae from laboratory crosses of the two species (Scarpa et al., 1994; Toro and Sastre, 1995; Toro and Paredes 1996b).

### II.2.2. DNA EXTRACTION

#### II.2.2.1 Juvenile and adult mussels

Approximately 50-100 mg mantle-edge tissue was removed from each mussel, coarsely chopped and digested in 500 µl lysis buffer (50-mM Tris-HCl , pH 8.0, 1.0 % SDS; 25 mM EDTA) with 200 µg proteinase K (Sigma) at 37 °C overnight. The solution was then extracted twice with 500 µl of an equal volume of phenol-chloroform-isoamyl alcohol (24:24:1) followed by 95% ethanol precipitation at -20°C. The extracted DNA was resuspended in 200 µl ultra-pure sterile distilled water.

#### II.2.2.2 Spat

For smaller mussels (< 5 mm in length), the whole flesh was dissected out of the shell and used. In recently settled spat and very small individuals (< 2 mm in length), the whole animal was used. Total DNA extraction followed as described above.

#### II.2.2.3 Larvae

Total DNA was extracted from around 200 individual larvae from each location at every sampling date. Furthermore, 60 D-shape larvae from each pure and hybrid larval culture (see Chapter 4), were analysed in order to see if there was any variation in the RFLPs among individual larvae of the same species. No variation among larvae from "pure" crosses was detected with either DNA-marker. The DNA extraction procedure was similar to that described for the mantle tissue, except that each larva was first measured with a graduated eyepiece fitted to a Wild stereomicroscope at 40X magnification, and isolated using a Pasteur pipette. Each individual larva was then placed in a separate 1.5 ml Eppendorf tube and washed twice in 0.5 ml distilled water before DNA extraction. Individual larvae were scored for genotype at each marker locus on the basis of diagnostic RFLPs.

### II.2.3 SPECIES MARKERS

Two polymerase chain reaction (PCR) based nuclear-DNA species markers were used in the present study. *Glu-5* and *ITS* are co-dominant DNA markers producing two specific *M. edulis* and *M. trossulus* patterns and distinct patterns for hybrids.

### II.2.3.1 *ITS*

This marker, developed by Heath et al. (1995), is based on the internal transcribed spacer (*ITS*) regions between the 18S and 28S nuclear rDNA coding region. The primers used were ITS 1 5'-GTTTCCGTAGGTGAACCTG-3' and ITS2 5'-CTCGTCTGATCTGAGGTCG-3', with an expected PCR gene fragment size of 1250 bp. Primers were synthetised at the Core facility for Protein/DNA chemistry at Queen's University and shipped as the ammonium salt, dry, in a sterile tube. Dissolution was carried out in ultra-pure distilled water, according to the manufacturer's instructions and the concentration needed. Working solutions of primers were kept at -20 °C (-70°C for long-term storage).

#### II.2.3.1.1. PCR cycling

Standard PCR amplifications were carried out in 25-µl reaction mixtures (500 µl thin walled PCR microtubes, Gordon Technologies) containing 2 µl DNA template (1:10 dilution), 0.2 mM each of the four deoxyribonucleotide triphosphates (dNTPs) (Sigma), 2.0 mM $MgCl_2$, primers at 0.4 mM, 1 unit of Taq (*Thermus aquaticus* strain YT1) DNA polymerase (Promega), the manufacturer-supplied PCR buffer and sterile distilled water. The reaction mixtures were overlaid with a drop of mineral oil (Sigma) to prevent evaporation, and were then placed in a programmable thermocycler (MJ Research Inc.). The thermal cycler protocol 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 72°C for 2 min. Rigorous precautions were taken to prevent template contamination during the PCR procedure: positive displacement pipettes (Eppendorf) and autoclaved, sterile tips, tubes and reagents were used, and a negative control without the DNA template was run with every batch of samples.

#### II.2.3.1.2. Restriction analysis

Five µl of each amplified PCR-product was digested for 12 h at 37°C with 0.5 unit of the restriction enzyme *Hha* I from *Haemophilus haemolyticus* in a total volume of 15 µl, including 3 µl of buffer supplied by the manufacturer (Pharmacia) and 6.5 µl of ultra-pure distilled water. Enzyme inactivation was accomplished at 65 °C for 20 min in a water bath and the sample cooled for 20 min at room temperature. The digested products were electrophoretically fractionated in 3 % agarose gels (2% Sigma and 1% NuSieve GTG agarose) with 0.5X Tris-borate-EDTA (TBE) buffer for 30 min at 112 V. A negative control with no PCR-product was run with every batch of samples. A 1Kb DNA ladder (GibcoBRL) was run on each gel for sizing the DNA fragments. The random fragment length polymorphisms (RFLPs) were visualized by placing the gels in a solution of ethidium bromide (0.5 µg $ml^{-1}$) and photographing them under ultraviolet illumination. Individual mussels were scored for genotype at each marker locus on the basis of diagnostic RFLPs.

### II.2.3.2. *Glu-5*

A second nuclear-DNA marker, *Glu-5*, developed by Rawson et al. (1996a), targets the gene encoding the polyphenolic adhesive protein produced by the pedal gland. The primer used was JH-5 5' -GTAGGAACAAAGCATGAACCA- 3' and the reverse primer JH54 5' -GGGGGGATAAGTTTTCTTAGG- 3'.

#### II.2.3.2.1 PCR cycling

Standard PCR amplifications were carried out in 25-µl reaction mixtures containing approximately 50 ng of DNA template, 2.5 nmol dNTPs, 2.0 mM $MgCl_2$, 50 pmol of each primer, 1 U of Tfl DNA polymerase (Promega), the manufacturer-supplied PCR buffer, and sterile distilled water. The reaction mixtures were overlaid with a drop of mineral oil (Sigma)

to prevent evaporation, and were then placed in the thermocycler. The thermal cycler protocol consisted of an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94°C for 20 s, 53 °C for 20 s and 72 °C for 45 s. PCR products were then directly resolved on 3 % agarose gels stained with ethidium bromide and scored for species using Polaroid photos taken under UV light. A negative control without DNA template was run with every batch of samples.

### II.2.4 SIZE FREQUENCY ANALYSIS

Shell lengths of all sampled mussels were measured to the nearest 0.01 mm with digital calipers, and each mussel assigned to the appropriate size class (5 mm intervals) (Comesaña et al., 1998).

### II.2.5 STATISTICAL ANALYSES

Comparisons of genotype frequency distributions were made with R X C G-tests of independence (Sokal and Rohlf, 1981) using the Systat 5.1 (Wilkinson, 1991) and Zaykin and Pudovkin (1993) computer programs. The probability estimates of null hypothesis (homogeneity) were performed using Monte Carlo simulations as suggested by Roff and Bentzen (1989). Bonferroni-adjusted probabilities for multiple comparisons were applied (i.e., divide the critical level, $\alpha$=0.05, by the number of comparisons) (Sokal and Rohlf, 1987; Wilkinson, 1991). Two way analysis of variance and nested analysis of variance (ANOVA) were performed on log transformed values for shell length (Mallet and Carver, 1995), to test for differences among sites, among species within sites and for the genotype x site interaction term, using the Systat 5.1 (Wilkinson, 1991) statistical package.

## II.3. RESULTS

### II.3.1 SPECIES MARKERS

#### II.3.1.1 *ITS* nuclear-DNA marker

The restriction digestion of the 1250-bp *ITS* PCR amplified product with HhaI produced three specific RFLPs. In *M. edulis*, the 1250-bp fragment was cut into two 450-bp fragments and two 180-bp fragments. In *M. trossulus* the ITS-PCR product was cut into two 280-bp fragments, two 180-bp fragments and several <100-bp fragments (Figure 2). This assay, using a co-dominant marker, was also able to separate the hybrids, in which patterns from both species were present on the gel (Figure 2).

#### II.3.1.2. *Glu-5* nuclear-DNA marker

This PCR assay also produced species-specific patterns. In *M. edulis*, two different banding patterns were found, 92 per cent of *M. edulis* mussels producing a single 350-bp band and 8 per cent producing one 350-bp band and one 380-bp band. In *M. trossulus* this PCR assay produced only a single primary band of 240-bp. Hybrid mussels presented RFLPs patterns from both species (Figure 3).

### II.3.2 MICRO-DISTRIBUTION PATTERNS

#### II.3.2.1 Larvae

The data show that PCR is a sufficiently sensitive technique to permit genotyping of individual mussel larvae. Figure 4 (A to D) shows that every site sampled contained individuals from each of the three mussel types (*M. edulis*, *M. trossulus*, hybrids). A higher

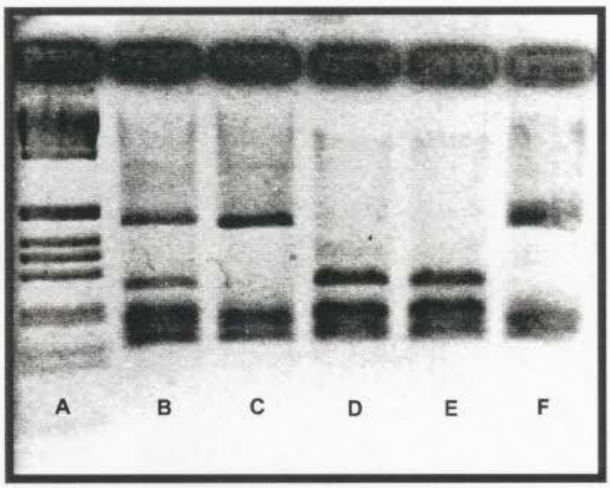



**Fig. 2.** Photo negative of ethidium bromide stained 3% agarose gel transilluminated with ultraviolet light showing the RFLPs patterns produced by the ITS nuclear marker for ***Mytilus edulis*** (lines C and F), ***Mytilus trossulus*** (lines D and E) and the hybrid (line B). Molecular weight marker (Gibco BRL 1Kb ladder (line A).

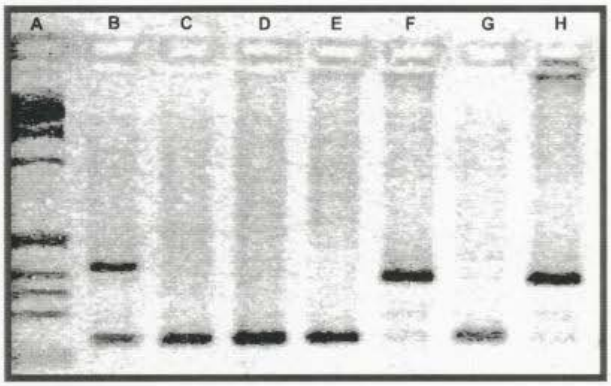



**Fig. 3.** Photo-negative of ethidium bromide stained 3% agarose gel transilluminated with ultraviolet light showing the RFLPs patterns produced by the *Glu-5* nuclear marker for ***Mytilus edulis*** (lines F and H), ***Mytilus trossulus*** (lines C, D, E and G) and the hybrid (line B). Molecular weight marker (Gibco BRL 1Kb ladder) (line A).

frequency of *M. trossulus*, lower frequency of *M. edulis* and intermediate frequencies of hybrids were found in all samples (Figure 5). There was no difference in genotype frequency among sites or between locations (pooling sites at each location) for the August 9 and September 3, 1996 samples (Table 1). Also, no significant differences in genotype frequencies for larvae were found between sites (protected vs exposed) within locations (Table 1). The size range of the larvae analysed by this method ranged between 113 (s.d = 8.57) μm and 298 (s.d. = 6.54) μm (Figure 4, E-H). However, no attempt was made to analyse size differences among species, due to the mixture of cohorts in each sample.

No significant differences ($P>0.01$) in the species frequencies (*M. edulis, M. trossulus* and hybrids) for larvae were found among years at Bellevue protected, Chance Cove protected and Chance Cove exposed sites (Table 2). At Bellevue exposed a significant G value was obtained ($P<0.01$, $G=16.31$, $df=4$). However, after excluding the hybrids from the analysis, there was no difference among years ($P>0.01$, $G=5.70$, $df=2$), indicating that the difference among years at this site was the result of small fluctuations in the frequency of hybrids.

#### II.3.2.1 Spat

The mussel spat collected during the autumn of 1995, 1996 and 1997 were arbitrarily divided into three size groups, one group including the spat collected from filamentous algae (0.6-1.9 mm), the other two groups including spat with size ranges between 2.0-9.9 mm and 10.0-14.9 mm respectively. Figures 6, 7, 8 and 9 show the species frequencies of the spat for each size range in three consecutive years. A higher frequency of *M. trossulus*, lower frequencies of hybrids and intermediate frequencies for *M. edulis* are evident, especially at the exposed sites at both locations, while at the more protected sites the frequency of *M. trossulus* is lower than at the exposed sites and the frequency of *M. edulis* and hybrids shows no clear pattern (Table 3, Figures 6 to 9). There were no significant differences in the species frequencies for the smallest size range of spat among sites, between sites within locations

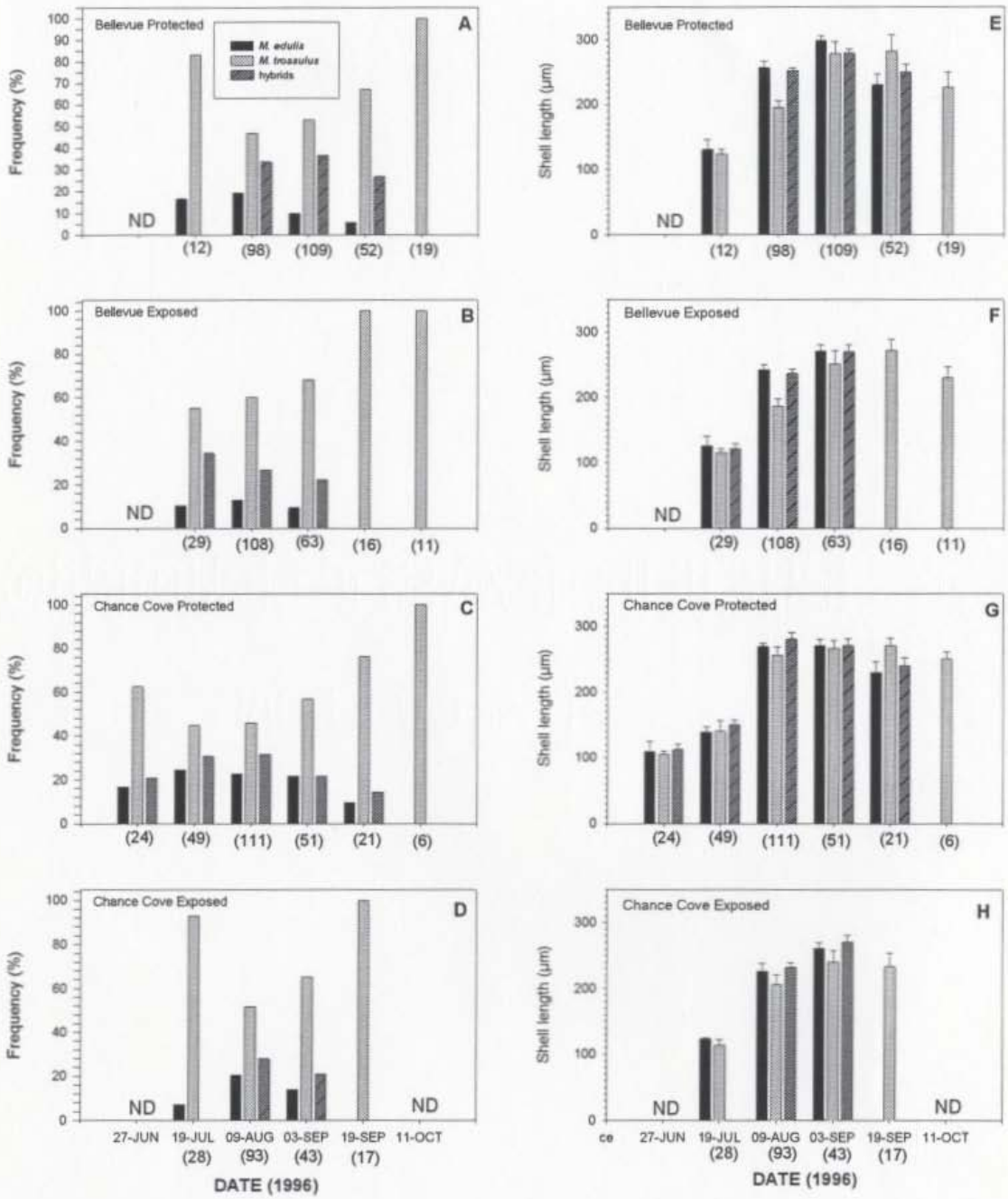



**Fig. 4.** Frequency distribution of *Mytilus edulis*, *Mytilus trossulus* and hybrids larvae (A to D) and mean (±S.E.) shell length (E to H) for each location (BP, BE, CP and CE) from plankton tows taken regularly during the summer-autumn 1996. ND=no data. Number of larvae in parentheses.

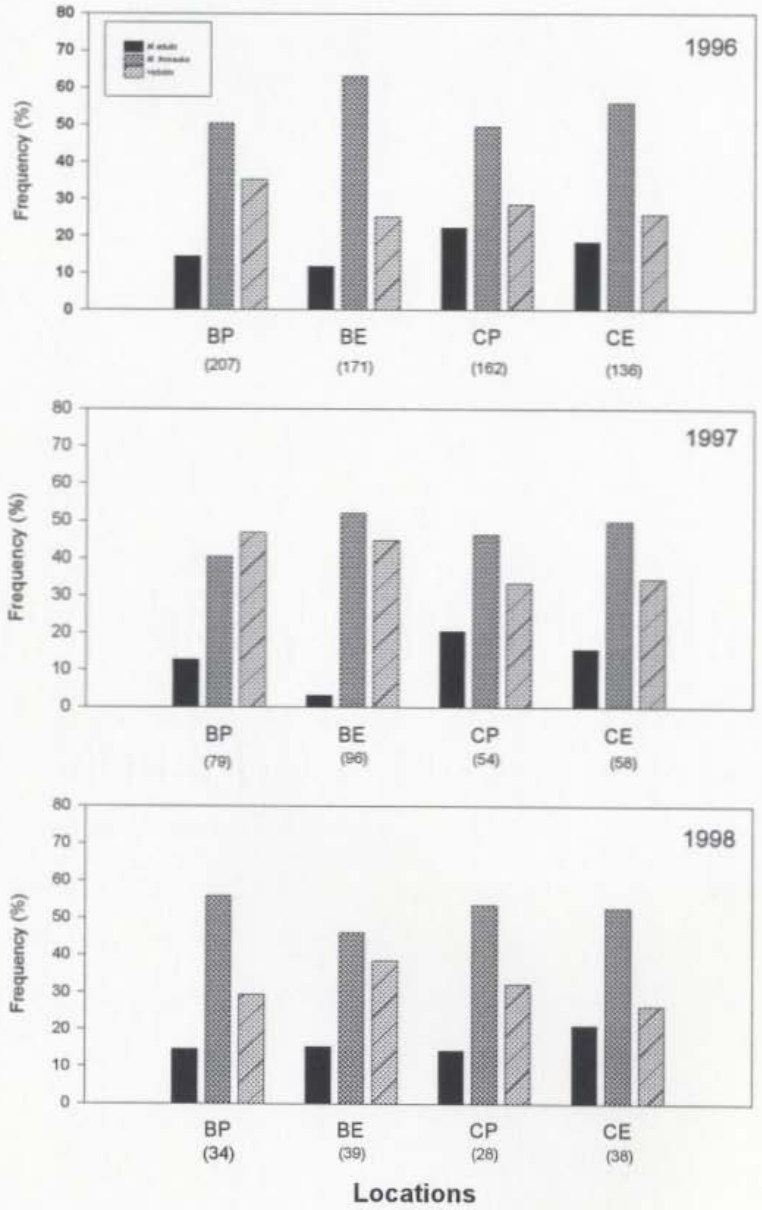



**Fig. 5.** Frequency distribution of *Mytilus edulis*, *Mytilus trossulus* and hybrids larvae for each location (BP, BE, CP and BE) for samples taken during August 9/September 3, (pooled) 1996 (A), September 2, 1997 (B), and July 31, 1998 (C). Number of individuals in parentheses.

**Table 1:** Results of R X C G-tests of independence among genotype frequencies (***M. edulis***, ***M. trossulus*** and hybrids) at the larval stage, comparing 1) locations and 2) sites within each location for samples taken on August 09 and September 03 1996. The critical values of the type I error were adjusted using Bonferroni method. Significance level is indicated (ns = non significant, * = significant)

| | | August 09 | P | September 03 | P |
|---|---|---|---|---|---|
| Among sites (BP,BE,CP and CE) $\chi^2_{(6)} = 12.59$ | | G= 6.59 | ns | G=11.34 | ns |
| Between sites $\chi^2_{(2)} = 5.99$ | BP vs BE | G= 3.79 | ns | G= 4.21 | ns |
| | CP vs CE | G= 0.65 | ns | G= 4.21 | ns |
| | BP+CP vs BE+CE | G= 6.90 | ns | G= 4.37 | ns |
| Between locations $\chi^2_{(2)} = 5.99$ | BP+BE vs CP+CE | G= 2.25 | ns | G= 5.47 | ns |

**Table 2:** Results of R X C G-tests of independence among genotype frequencies (***M. edulis***, ***M. trossulus*** and hybrids) at the larval stage comparing among three years 1) at the four locations and 2) sites within locations for samples taken in August 09, September 03 1996 (pooled), September 02 1997 and July 31 1998. The critical values of the type I error $\alpha$ were adjusted using the Bonferroni method. Significance level is indicated (ns = non significant; * = significant).

| | | G | *P* |
|---|---|---|---|
| Among years for BP<br>$\chi^2_{(4)} = 9.488$ | | 4.40 | ns |
| Among years for BE<br>$\chi^2_{(4)} = 9.488$ | | 16.31 | * |
| Among years for CP<br>$\chi^2_{(4)} = 9.488$ | | 1.31 | ns |
| Among years for CE<br>$\chi^2_{(4)} = 9.488$ | | 1.83 | ns |
| Among years for:<br>$\chi^2_{(4)} = 9.488$ | BP+CP | 4.64 | ns |
| | BE+CE | 15.48 | * |
| | BP+BE | 13.81 | ns |
| | CP+CE | 1.94 | ns |

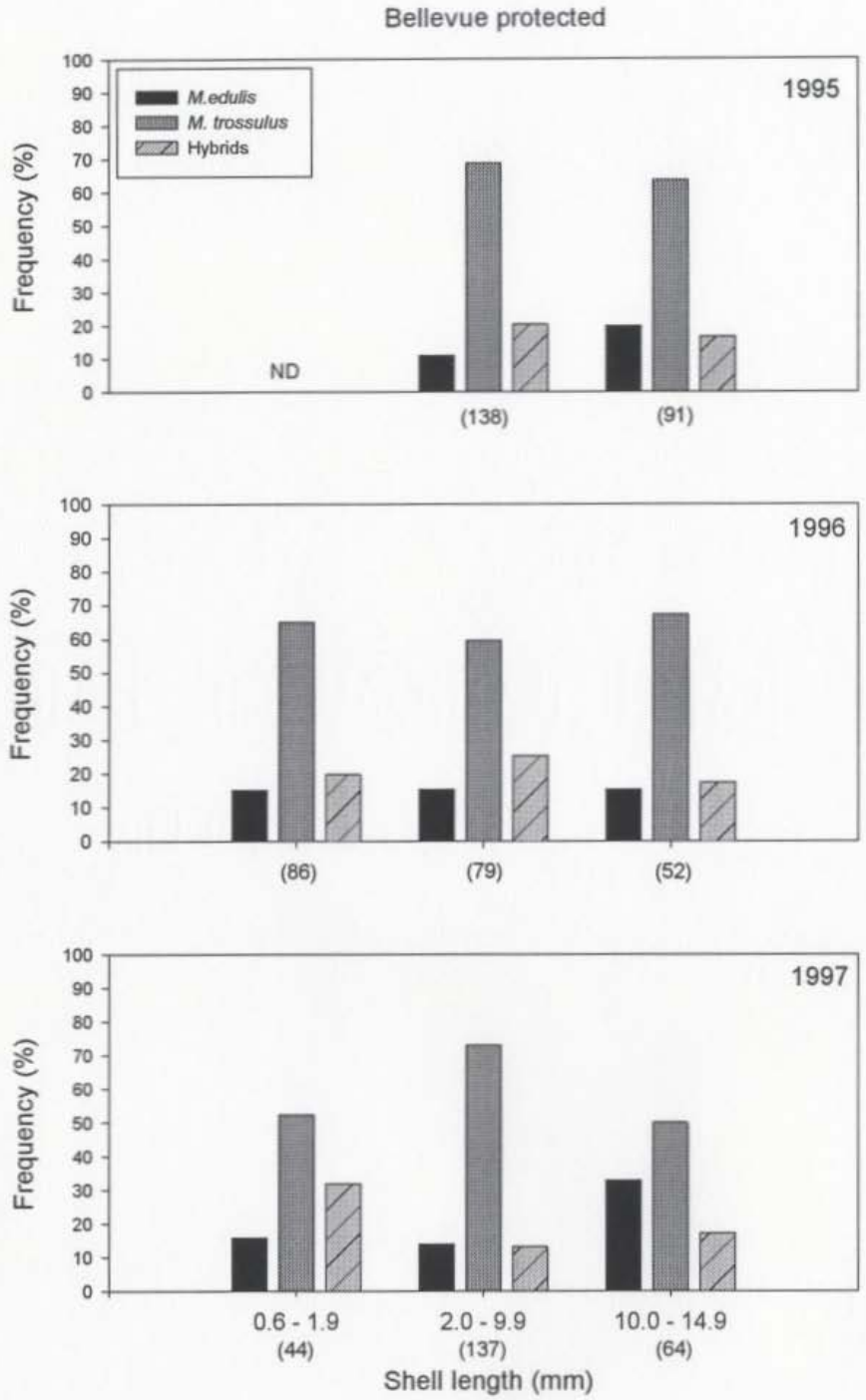



**Fig. 6.** Frequency distribution of *Mytilus edulis*, *Mytilus trossulus* and hybrids at the spat stage, divided into three shell length ranges in Bellevue protected (BP) for samples taken during three consecutive years (1995, 1996 and 1997). Number of individuals in each length class in parentheses.

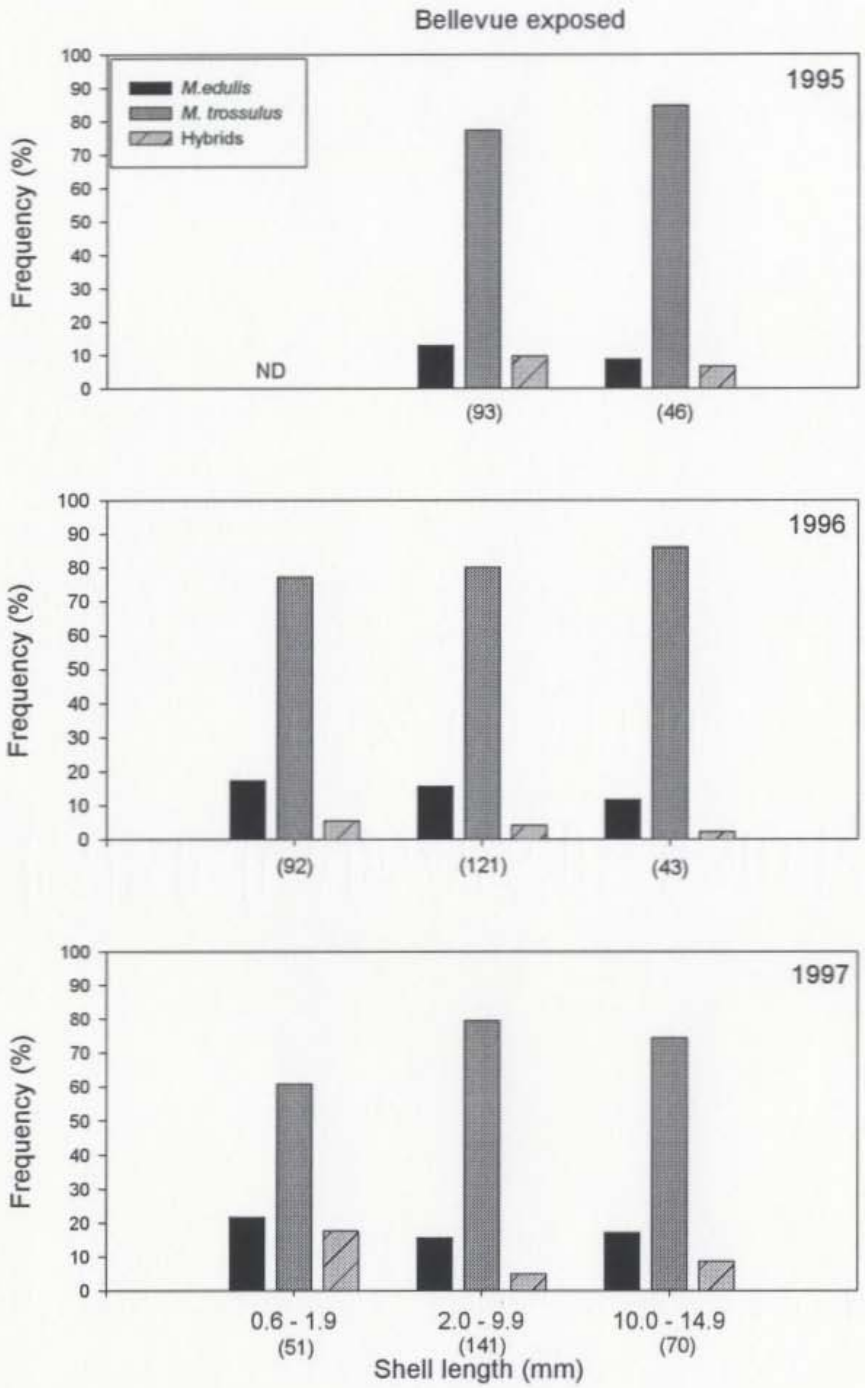



**Fig. 7.** Frequency distribution of *Mytilus edulis*, *Mytilus trossulus* and hybrids at the spat stage, divided into three shell length ranges in Bellevue exposed (BE) for samples taken during three consecutive years (1995, 1996 and 1997). Number of individuals in each length class in parentheses.

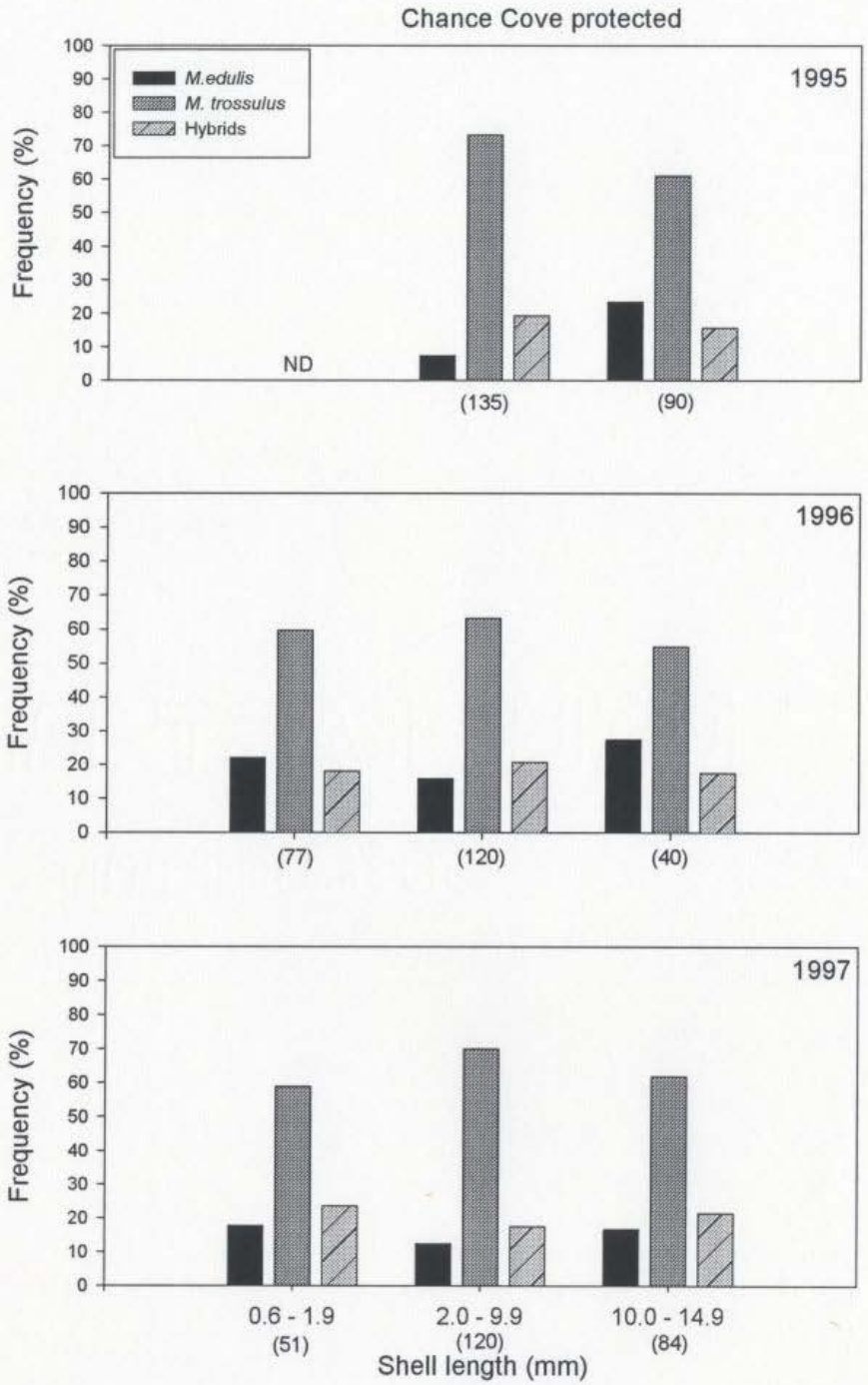



**Fig. 8.** Frequency distribution of *Mytilus edulis*, *Mytilus trossulus* and hybrids at the spat stage, divided into three shell length ranges in Chance Cove protected (CP) for samples taken during three consecutive years (1995, 1996 and 1997). Number of individuals in each length class in parentheses.

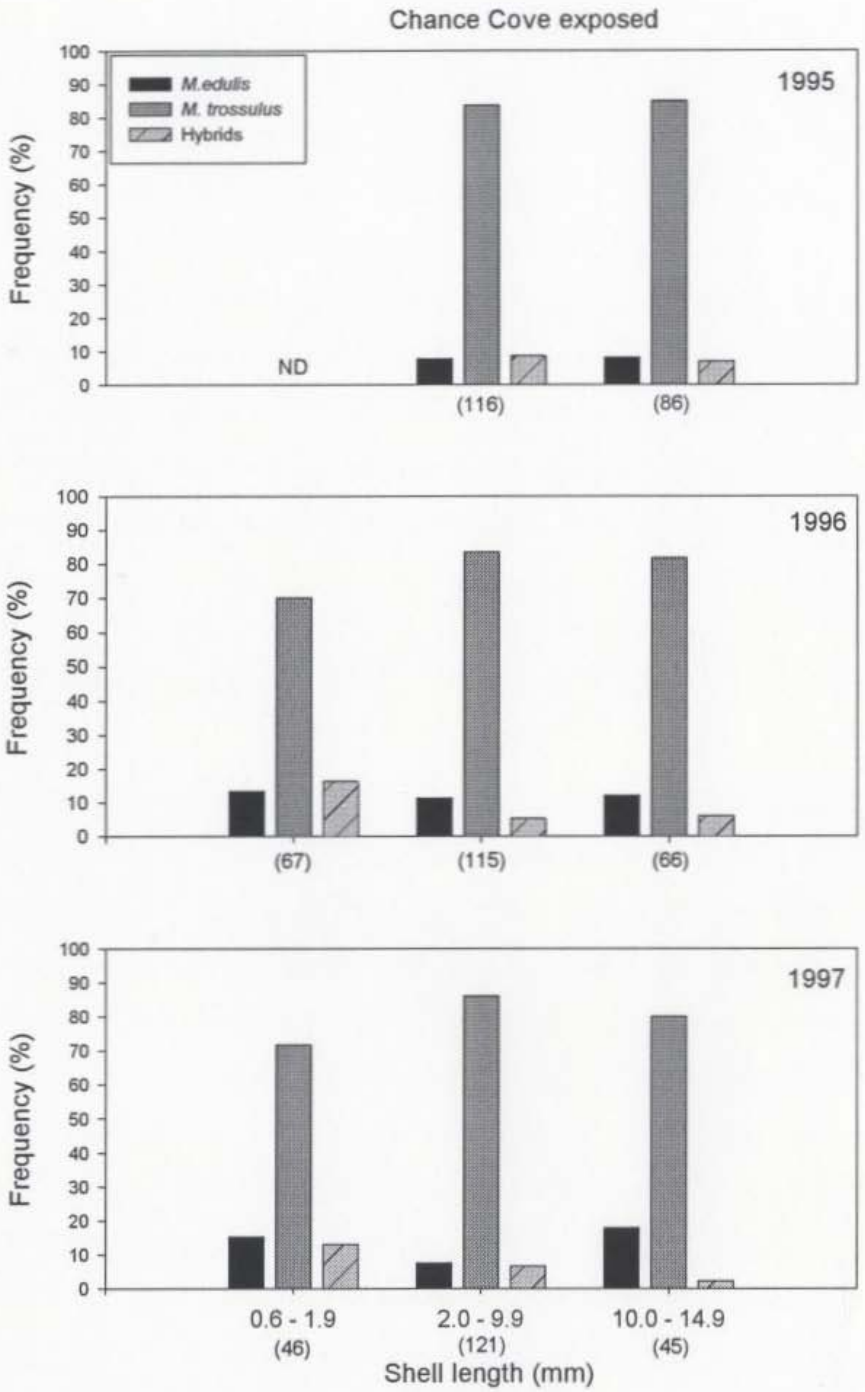



**Fig. 9.** Frequency distribution of *Mytilus edulis*, *Mytilus trossulus* and hybrids at the spat stage, divided into three shell length ranges in Chance Cove exposed (CE) for samples taken during 1995, 1996 and 1997. Number of individuals in each length class in parentheses.

and between locations (Table 3). However, genotype frequencies showed significant site differences in most G-tests for spat in the 2.0-9.9 and 10.0-14.9 mm size ranges (Table 3), with the exception of the comparison between locations (BP+BE vs CP+CE), in which no significant differences in genotype frequencies were found.

There were no significant overall differences in species frequencies among years for any of the sites studied, with the exception of Bellevue exposed in the smallest size range ($P<0.01$) (Table 4). In general, the genotype frequencies (*M. edulis*, *M. trossulus* and hybrids) showed no differences among the years studied, indicating that the genetic composition of the mussels that recruit each year at each of these sites does not vary significantly.

#### II.3.2.1 Adults

The relationships between the frequencies of *M. edulis*, *M. trossulus*, and hybrids and shell length are shown in figure 10 for a sample taken in October 1995, figure 11 for a sample taken in June 1996, figures 12, 13, 14 and 15 for a sample taken in October 1996 and in figure 16 for a sample taken in October 1997. Mussels were considered to be adults when gonad development and gamete storage in the mantle tissue were first observed (age at first reproduction), which generally occurs at about 15-20 mm shell length in *M. trossulus* (Suchanek, 1981) and 20-25 mm shell length in *M. edulis* (Seed, 1969; see also Chapter 3).

The relative frequency of each genotype was strongly dependent upon size class. *M. trossulus* was the predominant species in the smallest size classes for all samples (Figures 10 to 16). An increase in the frequency of *M. edulis* individuals and a decrease in frequency of *M. trossulus* individuals with shell length was observed at all sites and locations (Figures 10 to 16). The change in frequency of both species with increasing size was gradual, and the hybrid frequencies tended to decrease in the larger size classes, although no clear pattern was observed.

**Table 3:** Results of R X C G-tests of independence among genotype frequencies (***M. edulis***, ***M. trossulus*** and hybrids) at the spat stage, comparing 1) locations and 2) sites within each location for three size ranges ( 0.6 - 1.9 , 2.0-9.9 and 10.0-14.9 mm), for samples taken during 1995, 1996 and 1997. The critical values of the type I error $\alpha$ were adjusted using the Bonferroni method. Significance level is indicated ( * = significant).

| | | 1995 | 1996 | 1997 |
|---|---|---|---|---|
| Among sites (BP,BE,CP and CE) $\chi^2_{(6)} = 12.59$ | | | | |
| | 0.6 - 1.9 mm | | G=11.59 | G= 6.31 |
| | 2.0 - 9.9 mm | G=13.35 | G=35.04 * | G=18.65 * |
| | 10.0 - 14.9 mm | G=19.44 * | G=16.32 | G=20.44 * |
| Between sites $\chi^2_{(2)} = 5.99$ | BP vs BE | | | |
| | 0.6 - 1.9 mm | | G= 2.59 | G= 2.65 |
| | 2.0 - 9.9 mm | G= 4.66 | G=20.00 * | G= 5.68 |
| | 10.0 - 14.9 mm | G= 6.55 | G= 6.35 | G= 8.43 |
| | CP vs CE | | | |
| | 0.6 - 1.9 mm | | G= 2.14 | G= 2.14 |
| | 2.0 - 9.9 mm | G= 5.77 | G=14.99 | G= 9.45 |
| | 10.0 - 14.9 mm | G=12.64 | G= 8.92 | G= 8.76 |
| | BP+CP vs BE+CE | | | |
| | 0.6 - 1.9 mm | | G= 6.35 | G= 4.08 |
| | 2.0 - 9.9 mm | G=10.53 | G=33.25 * | G=13.31 |
| | 10.0 - 14.9 mm | G=19.01 * | G=13.35 | G=13.70 |
| Between locations BP+BE vs CP+CE $\chi^2_{(2)} = 5.99$ | | | | |
| | 0.6 - 1.9 mm | | G= 2.04 | G= 1.39 |
| | 2.0 - 9.9 mm | G= 2.89 | G= 0.33 | G= 3.56 |
| | 10.0 - 14.9 mm | G= 0.24 | G= 0.68 | G= 2.31 |

**Table 4:** Results of R X C G-tests of independence among genotype frequencies (***M. edulis, M. trossulus*** and hybrids) at the spat stage for three different size ranges (0.6-1.9, 2.0-9.9 and 10.0-14.9 mm) among three different years (1995, 1996 and 1997), at the four locations and sites within locations. $\chi^2_{(4)} = 9.488$. The critical values of the type I error $\alpha$ were adjusted using the Bonferroni method. Significance level is indicated (ns = non significant).

| | Size range (mm) | G | *P* |
|---|---|---|---|
| Among years for BP | 0.6- 1.9 | 2.57 | ns |
| | 2.0- 9.9 | 6.51 | ns |
| | 10.0-14.9 | 6.19 | ns |
| Among years for BE | 0.6- 1.9 | 6.54 | ns |
| | 2.0- 9.9 | 3.49 | ns |
| | 10.0-14.9 | 3.87 | ns |
| Among years for CP | 0.6- 1.9 | 0.73 | ns |
| | 2.0- 9.9 | 5.22 | ns |
| | 10.0-14.9 | 2.79 | ns |
| Among years for CE | 0.6- 1.9 | 0.27 | ns |
| | 2.0- 9.9 | 2.26 | ns |
| | 10.0-14.9 | 3.69 | ns |
| Among years for: BP+CP | 0.6- 1.9 | 2.43 | ns |
| | 2.0- 9.9 | 9.44 | ns |
| | 10.0-14.9 | 1.35 | ns |
| Among years for: BE+CE | 0.6- 1.9 | 2.13 | ns |
| | 2.0- 9.9 | 4.87 | ns |
| | 10.0-14.9 | 5.19 | ns |
| Among years for: BP+BE | 0.6- 1.9 | 7.49 | ns |
| | 2.0- 9.9 | 6.78 | ns |
| | 10.0-14.9 | 6.13 | ns |
| Among years for: CP+CE | 0.6- 1.9 | 0.12 | ns |
| | 2.0- 9.9 | 5.33 | ns |
| | 10.0-14.9 | 1.45 | ns |

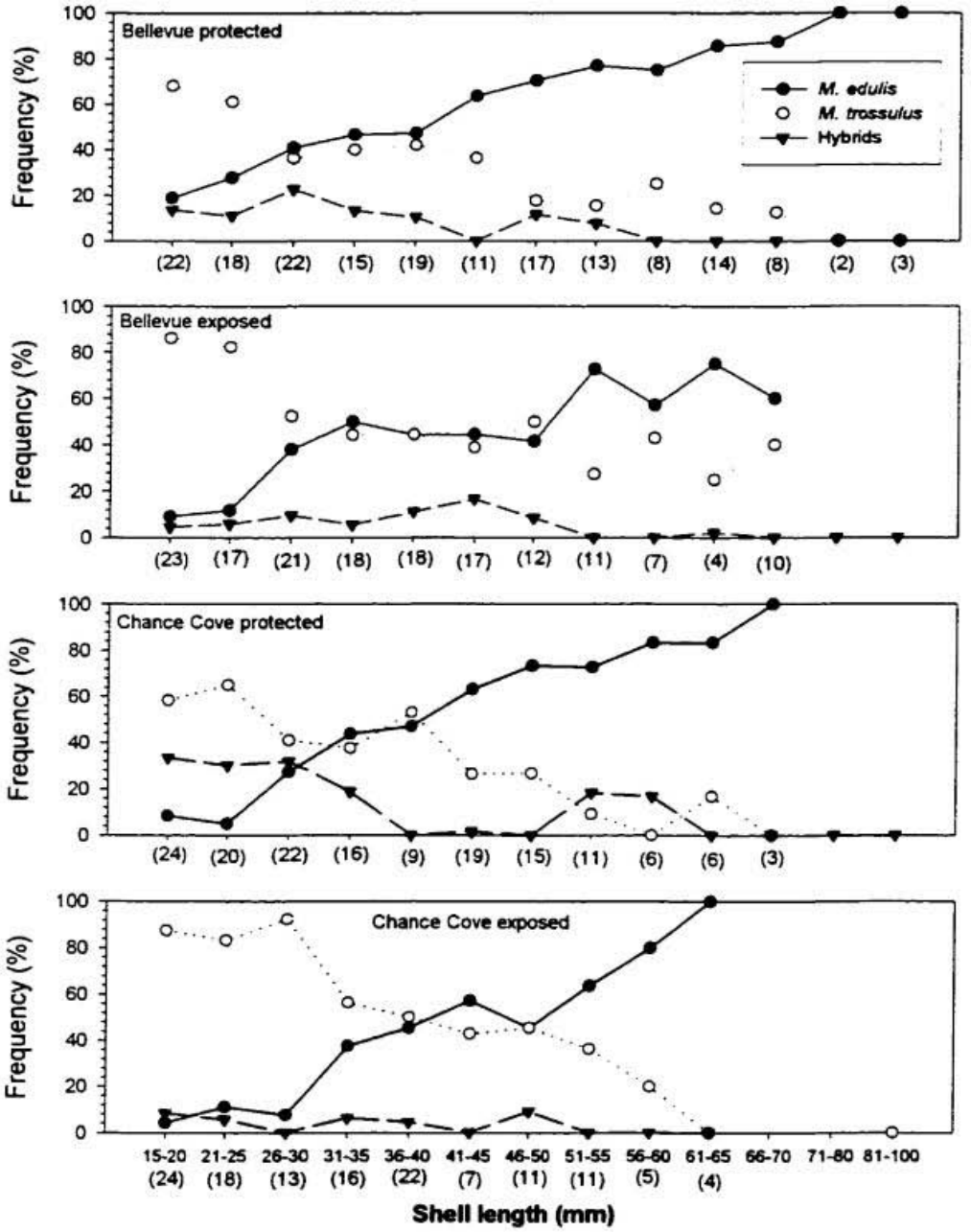



**Fig. 10.** Frequencies of *M. edulis* (black circles), *M. trossulus* (open circles) and hybrids (inverted black diamonds) plotted against shell length classes for four populations, from a sample taken in October 1995, in eastern Newfoundland. Number of individuals in each length class in parentheses.

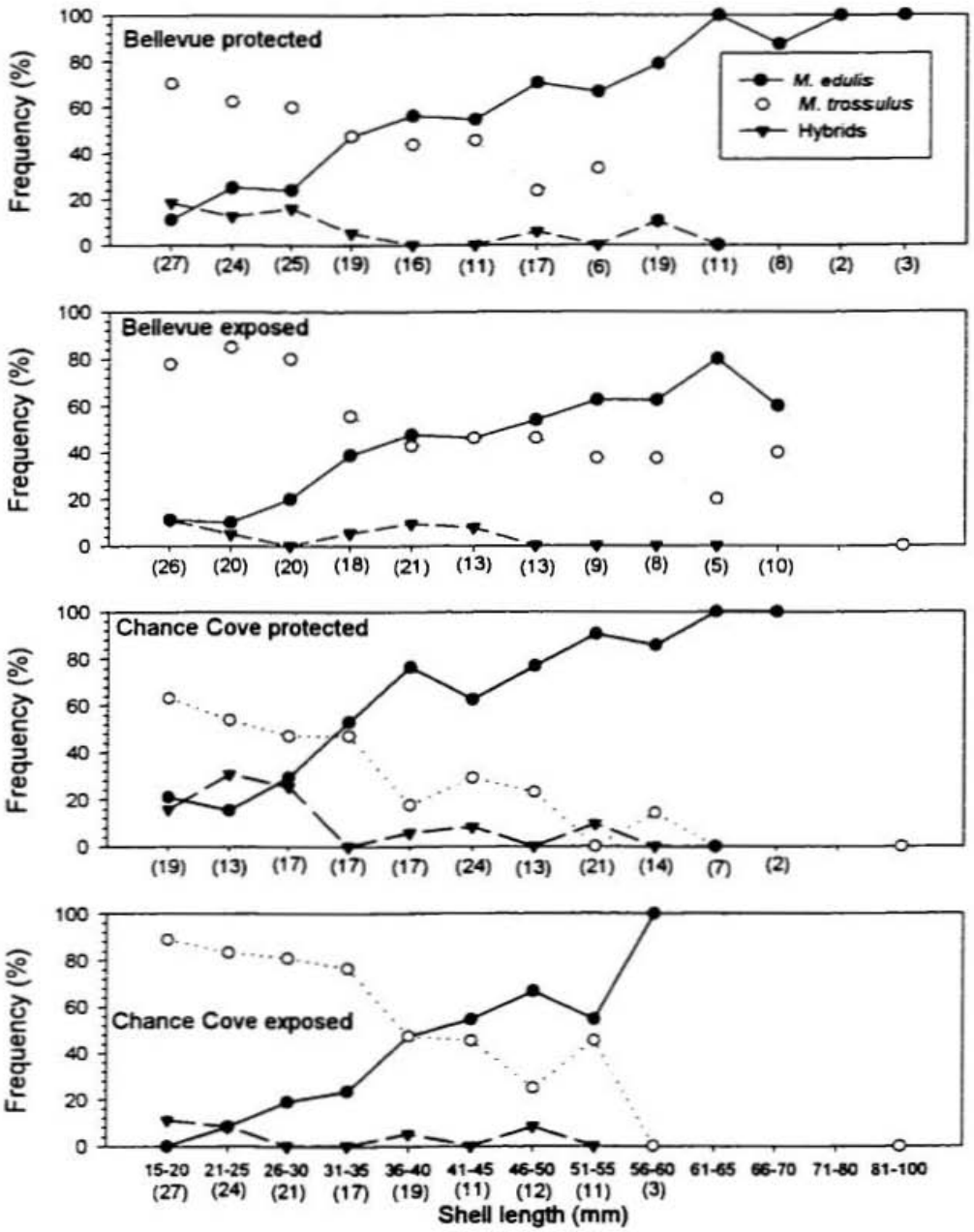



**Fig. 11.** Frequencies of *M. edulis* (black circles), *M. trossulus* (open circles) and hybrids (inverted black diamonds) plotted against shell length classes for four populations, from a sample taken in June 1996, in eastern Newfoundland. Number of individuals in each length class in parentheses.

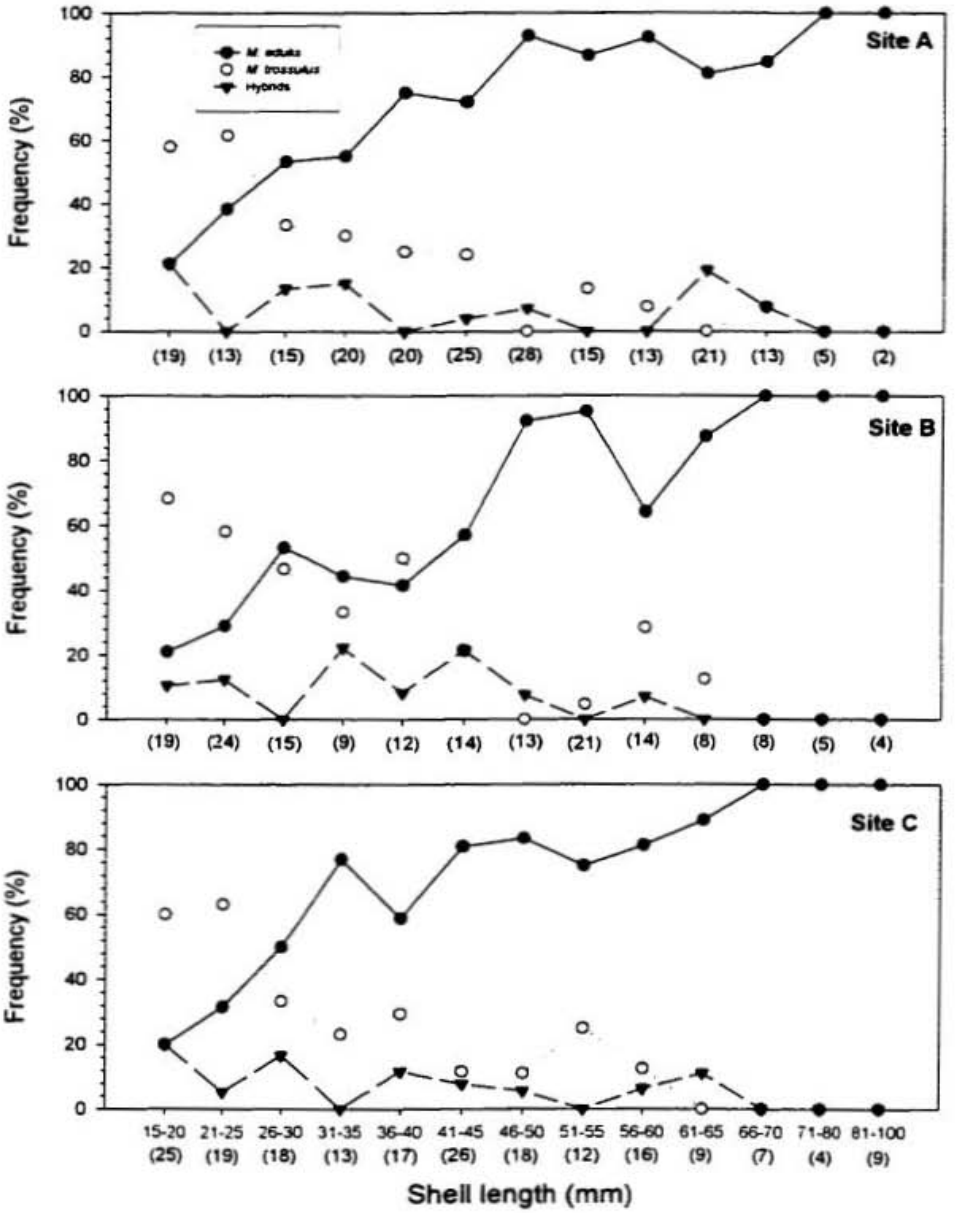



**Fig. 12.** Frequencies of *Mytilus edulis* (black circles), *Mytilus trossulus* (open circles) and hybrids (inverted black diamonds) plotted against shell length classes for random samples taken along three sites of the mussel bed at Bellevue protected location (BP) in October 1996. Number of individuals in each length class in parentheses.

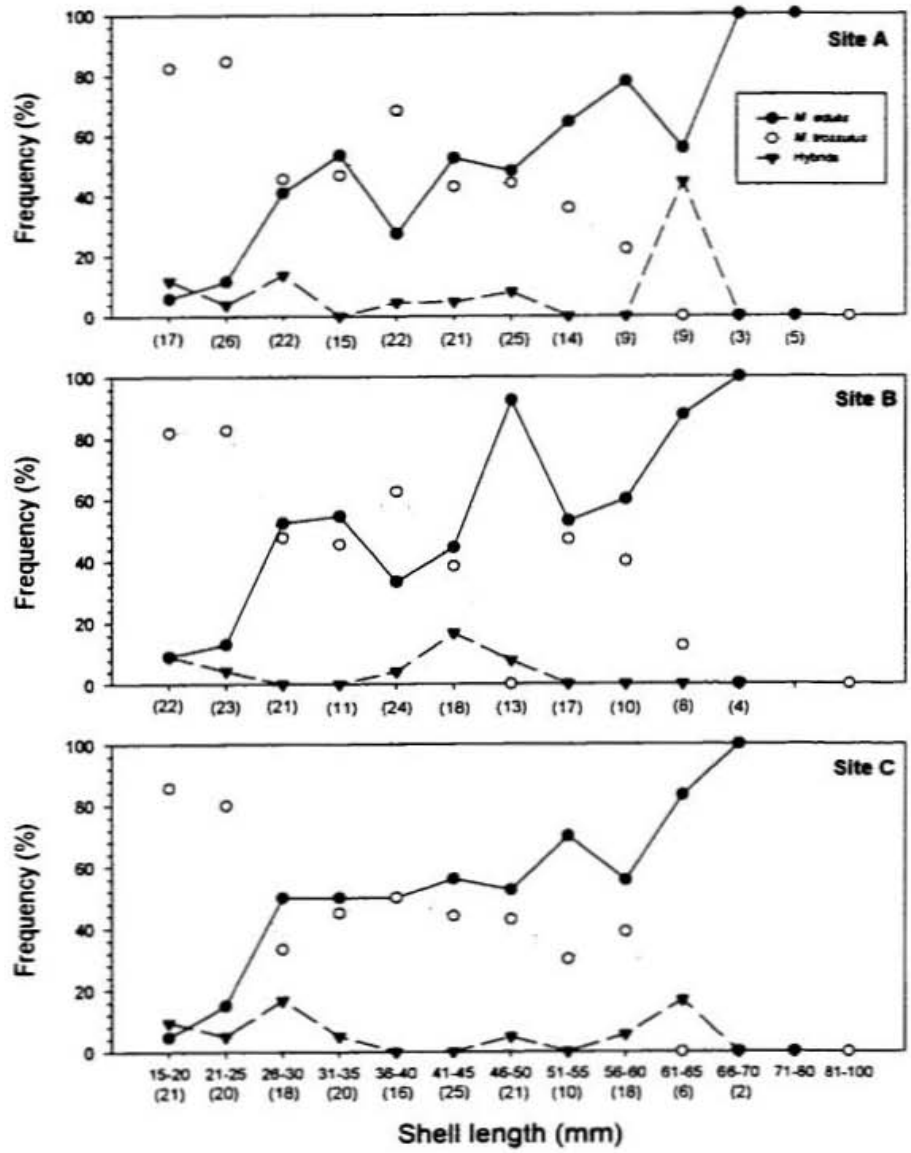



**Fig. 13.** Frequencies of *Mytilus edulis* (black circles), *Mytilus trossulus* (open circles) and hybrids (inverted black diamonds) plotted against shell length classes for random samples taken along three sites of the mussel bed at Bellevue exposed location (BE) in October 1996. Number of individuals in each length class in parentheses.

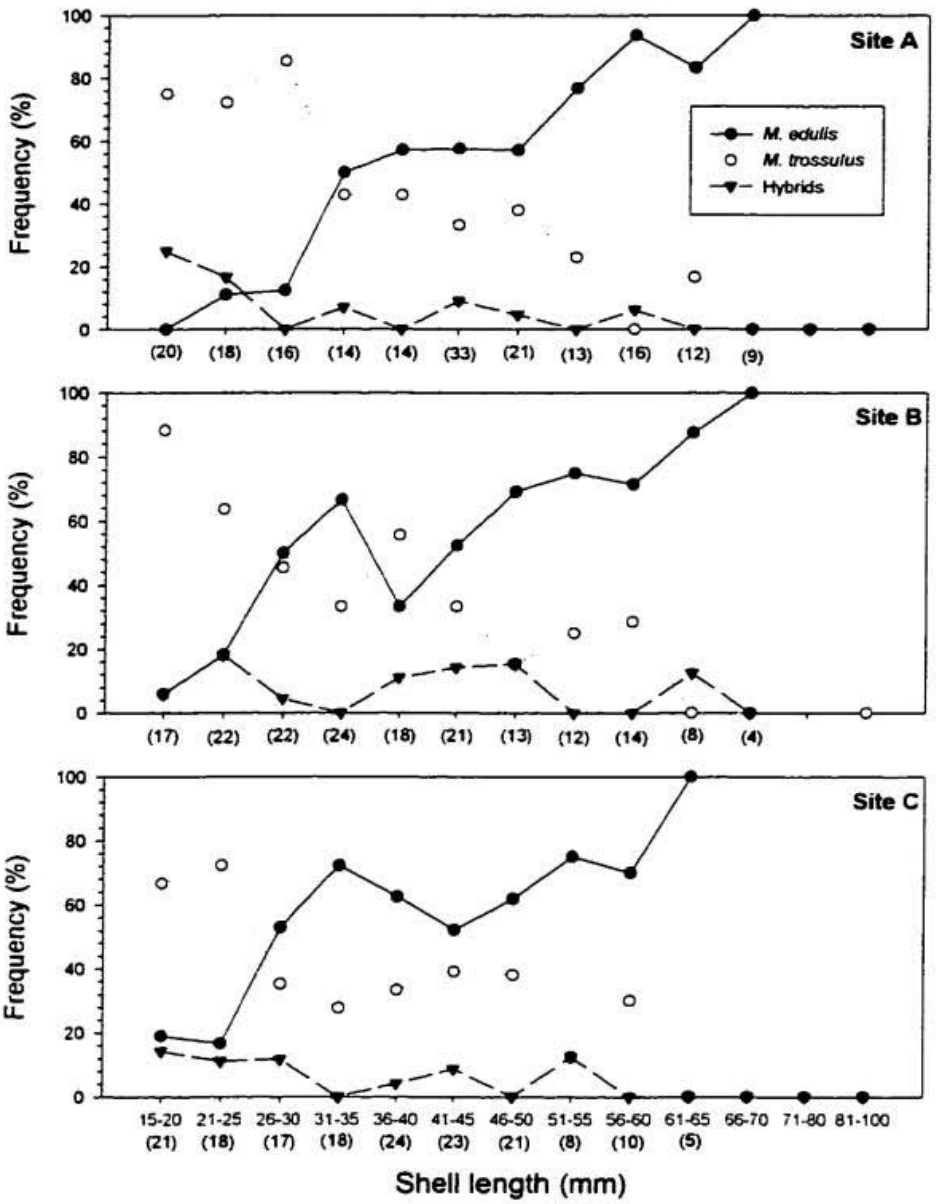



**Fig. 14.** Frequencies of *Mytilus edulis* (black circles), *Mytilus trossulus* (open circles) and hybrids (inverted black diamonds) plotted against shell length classes for random samples taken along three sites of the mussel bed at Chance Cove protected location (CP) in October 1996. Number of individuals in each length class in parentheses.

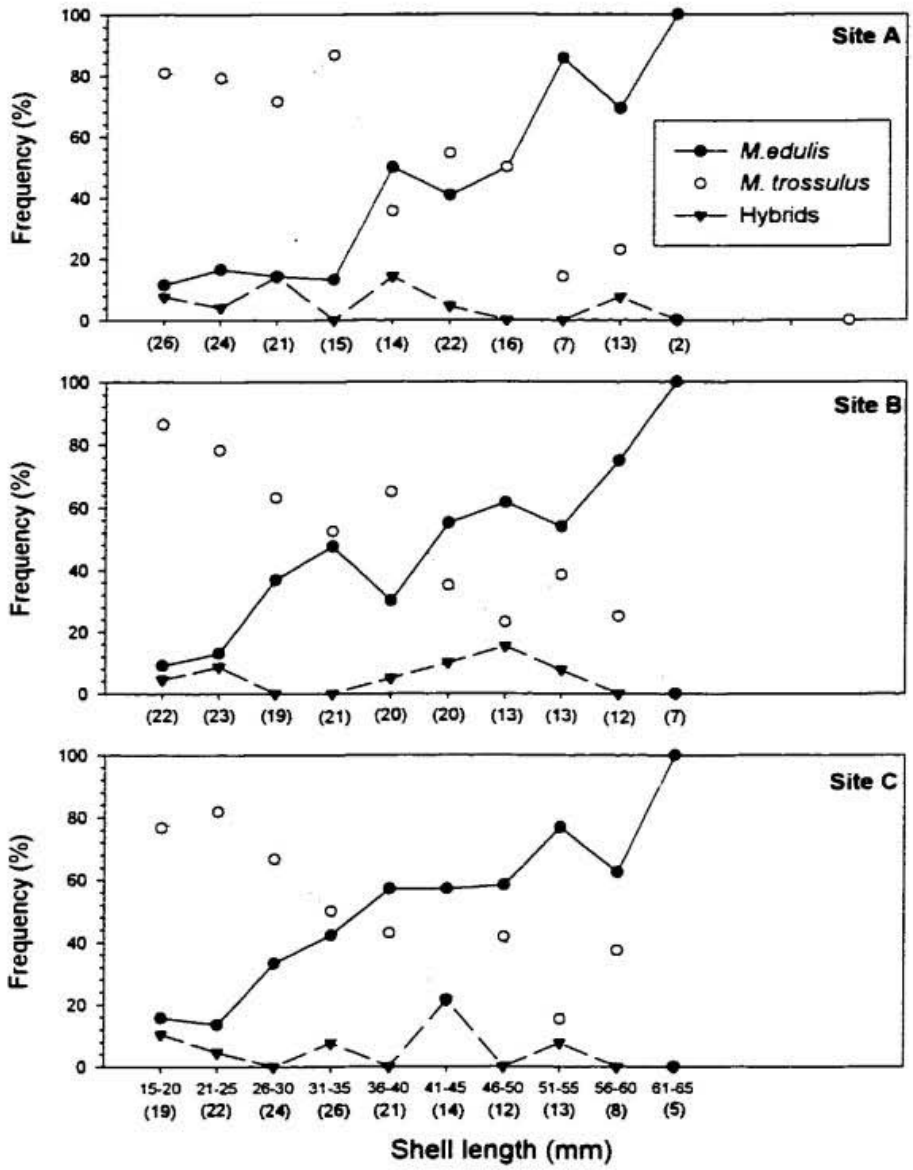



**Fig. 15.** Frequencies of *Mytilus edulis* (black circles), *Mytilus trossulus* (open circles) and hybrids (inverted black diamonds) plotted against shell length classes for random samples taken along three sites of the mussel bed at Chance Cove exposed location (CE) in October 1996. Number of individuals in each length class in parentheses.

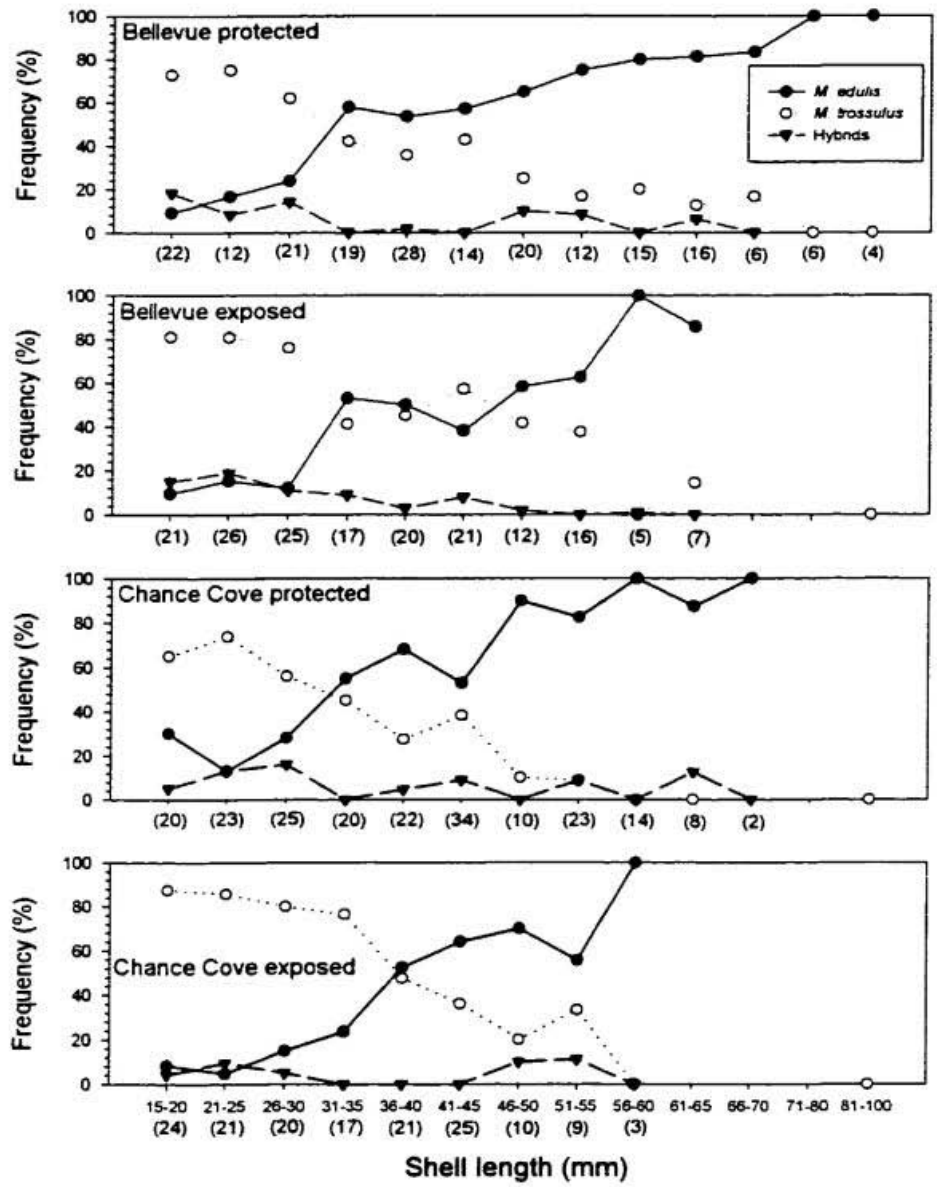



**Fig. 16.** Frequencies of *Mytilus edulis* (black circles), *M. trossulus* (open circles) and hybrids (inverted black diamonds) plotted against shell length classes for four populations, from a sample taken in October 1997, in eastern Newfoundland. Number of individuals in each length class in parentheses.

Extensive sampling at both locations at each site was carried out in October 1996, in order to evaluate the possibility of a patchy distribution of genotype frequencies (*M. edulis*, *M. trossulus* and hybrids) within each site. Three representative (hap hazzard) samples along each of the mussel beds at these sites showed that there were no significant differences in genotype frequencies among samples taken near the edge of the mussel bed and at the centre of the same mussel bed ($P>0.05$; $G= 4.72$ for BP, $G=1.48$ for BE, $G=0.37$ for CP and $G=4.29$ for CE, $df=4$). Significant differences were found among all four sites ($P<0.05$, $G=107$, $df=6$). The significant difference in species frequencies between more exposed sites and more sheltered and protected sites is particularly interesting (Table 5). *M. edulis* showed a higher frequency at the more protected sites (65.4% and 51.2% for BP and CP, respectively), while *M. trossulus* was predominant at the more exposed sites (50.2% and 54.9% for BE and CE, respectively). The same significant difference in genotype frequency was obtained by pooling data from both exposed sites (BE and CE) and both protected sites (BP and CP) ($P<0.01$, $G= 78.15$, $df=2$). A significant difference was found in the genotype frequencies between the locations (Bellevue and Chance Cove), pooling both sites at each location ($P<0.01$, $G=22.38$, $df=2$). *Mytilus edulis* was predominant (55%) at Bellevue, while *M. trossulus* was more frequent at Chance Cove (47%). However, after pooling data (1995 + 1997) from both sites at each location (BP+BE vs CP+CE), there were no significant differences in genotype frequencies between locations. There was a significant difference in genotype frequencies among years (1995, 1996 and 1997) for the Bellevue protected site only (Table 6).

A two way ANOVA of shell length (log transformed) of mussels at the spat (>2.0 mm) and adult stages showed significant effects of Site ($F_{(3,2)}=97.21$, $P<0.01$), Species ($F_{(2,6354)} = 1012.24$, $P<0.001$) and Site x Species interaction ($F_{(6,6354)} = 26.12$, $P < 0.01$). This significant interaction indicated that the differences in shell length among the three groups of mussels varied among sites. However, the mean shell length of *M. trossulus* was lower than that of *M. edulis* at every site, while the mean shell length of hybrids showed intermediate values between the two "pure" *Mytilus* species (Figure 17).

**Table 5:** Results of R X C G-tests of independence among species frequencies (***M. edulis***, ***M. trossulus*** and hybrids) at the adult stage (>15 mm), comparing samples within sites, among sites, between locations and sites within each location for samples taken in October 1995, October 1996 and October 1997. The critical values of the type I error $\alpha$ were adjusted using the Bonferroni method. Significance level is indicated ( * = significant).

| | | 1995 | 1996 | 1997 |
|---|---|---|---|---|
| Among sites (BP,BE,CP and CE) $\chi^2_{(6)} = 12.59$ | | G= 40.36 * | G=107.51 * | G=32.64 * |
| Between sites $\chi^2_{(2)} = 5.99$ | BP vs BE | G= 23.55 * | G= 68.47 * | G=12.60 * |
| | CP vs CE | G= 8.24 | G= 18.36 * | G= 19.95 * |
| | BP+CP vs BE+CE | G= 28.69 * | G= 78.15 * | G= 31.89 * |
| Between locations $\chi^2_{(2)} = 5.99$ | BP+BE vs CP+CE | G= 5.81 | G= 22.86 * | G= 0.11 |

**Table 6:** Results of R X C G-tests of independence for genotypes frequencies (***M. edulis***, ***M. trossulus*** and hybrids) at the adult stage (>15 mm) among three different years (1995, 1996 and 1997), at the four sites, between locations and between sites within locations. $\chi^2_{(4)} = 9.488$. The critical values of the type I error $\alpha$ were adjusted using the Bonferroni method. Significance level is indicated (ns = non significant; * = significant).

| | G | P |
|---|---|---|
| Among years for BP | 36.25 | * |
| Among years for BE | 6.88 | ns |
| Among years for CP | 2.87 | ns |
| Among years for CE | 3.55 | ns |
| Among years for: BP+CP | 18.59 | * |
| Among years for: BE+CE | 4.94 | ns |
| Among years for: BP+BE | 31.55 | * |
| Among years for: CP+CE | 3.04 | ns |

A nested ANOVA showed that the differences among mean lengths of the three species at each site were statistically significant ( $F_{(8,6354)} = 286.89$, $P < 0.01$).

### II.3.3 LIFE HISTORY VARIATION

There were significant differences in genotype frequencies among all life-history stages at all four sites sampled during 1996 and 1997 (Table 7). In order to identify the life-history stage(s) responsible for this high G value, genotypes for the eight life-history stages were pairwise tested on ontogenetic sequence (Table 8). Larvae were significantly different from the adult IV (over 61 mm) stage ($P < 0.01$) and the genotype frequencies from adults I (15.0-30.9 mm) to adults II (31.0-45.9 mm) were also statistically different ($P < 0.01$) (Table 8). The difference between adults and larvae was the result of fluctuations in the frequency of *trossulus* and hybrid genotypes, these two genotypes being more frequent at the larval stage. The increasing frequency of *M. edulis* and decreasing frequency of *M. trossulus* and hybrids among adult I, adult II and adult III stages (Table 8) can clearly be seen in figures 10 to 16.

Surprisingly, only two of eight G-tests comparing larvae with spat I (0.6-1.9 mm) resulted in a significant difference in the genotype frequencies (Table 8), which indicates that in general there is no strong selection against any particular genotype (*M. edulis*, *M. trossulus* or hybrids) at this very delicate stage of larval metamorphosis. However, when *M. edulis* and *M. trossulus* genotypes were pooled and tested against hybrids, there was a significant difference between the frequencies of larvae versus spat I (0.6-1.9 mm) ($P<0.001$) for samples taken in 1996 ($G=16.08$) and 1997 ($G=20.25$). The overall frequency of hybrids for both locations at the larval stage was 29.14 % for 1996, 41.11 % for 1997 and 31.65 % for 1998. There was no significant difference in the frequency of *M. edulis*, *M. trossulus* (pooled) and hybrid larvae among all four sites ($P>0.01$) for any of the three years analysed.

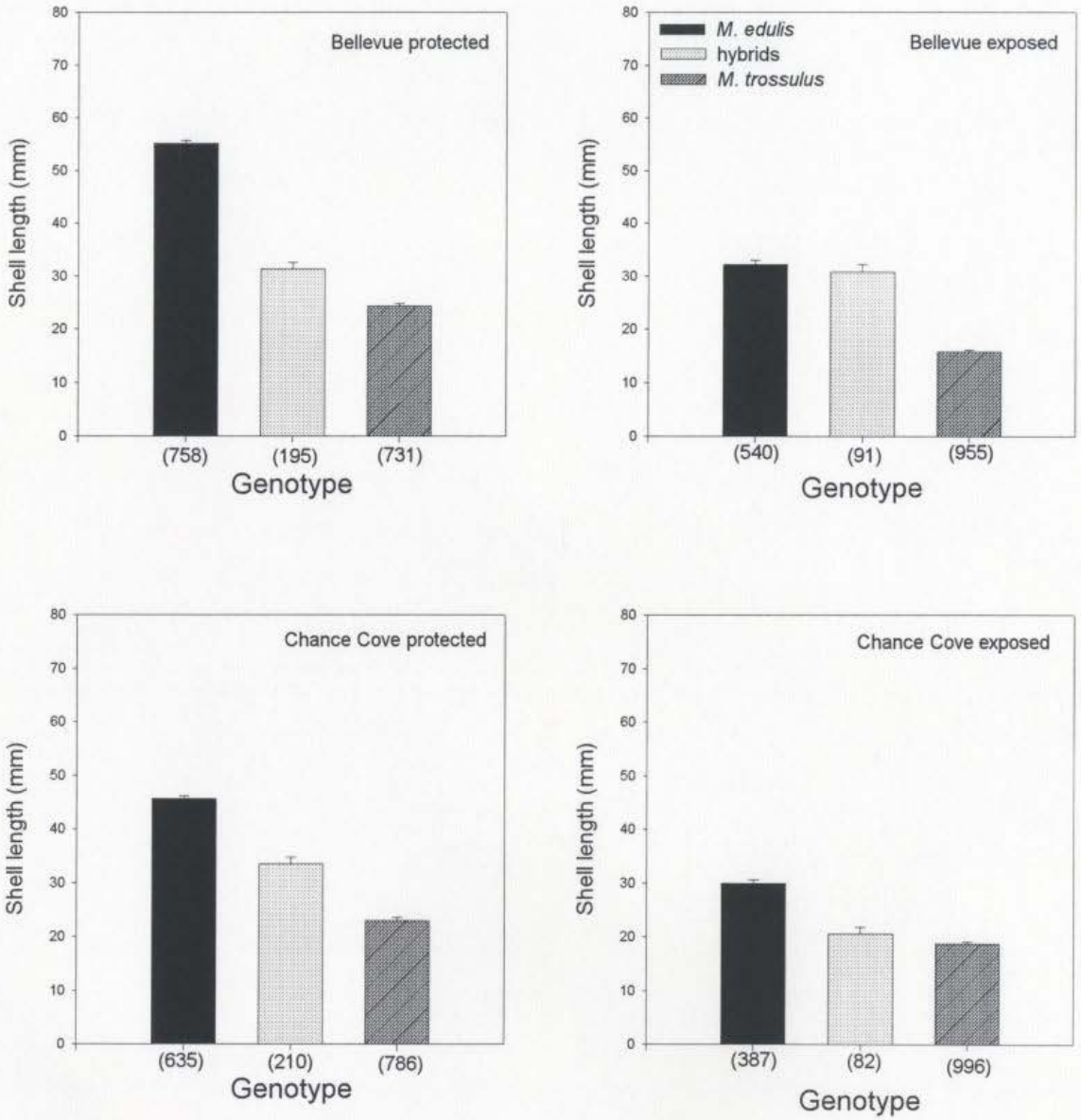



**Fig. 17.** Mean shell length (±SE) of *Mytilus edulis*, hybrids and *M. trossulus* individuals (size range 2.0 - 109.5 mm), scored using one or two PCR-based nuclear markers (*ITS, Glu-5*). Pooled from samples taken during 1995, 1996 and 1997 for each site (Bellevue protected, Bellevue exposed, Chance Cove protected, Chance Cove exposed). Number in parentheses show the number of individuals scored.

**Table 7:** Results of R X C G-tests of independence among species frequencies (***M. edulis***, ***M. trossulus*** and hybrids) among all (8) size (life-history) stages during two consecutive years (1996, 1997). Each comparison has 14 degrees of freedom. Estimates of probability of null hypothesis (homogeneity) were carried out using a Monte Carlo simulation. Significance level is indicated.

| | 1996 | | 1997 | |
|---|---|---|---|---|
| | G | *P* | G | *P* |
| BP | 445.42 | < 0.01 | 200.21 | < 0.01 |
| BE | 270.93 | < 0.01 | 189.73 | < 0.01 |
| CP | 250.14 | < 0.01 | 166.89 | < 0.01 |
| CE | 225.25 | < 0.01 | 136.00 | < 0.01 |

**Table 8:** Results of R X C G tests of independence among genotype frequencies (***Mytilus edulis***, ***M. trossulus*** and hybrids) for sequential size-stages (life-history stages) during two consecutive years (1996, 1997). Spat I = 0.6-1.9 mm; spat II= 2.0-9.9 mm; spat III= 10.0-14.9 mm; adult I= 15.0-30.9 mm; adult II= 31.0-45.9 mm; adult III= 46.0-60.9 mm and adult IV= over 61 mm. Each comparison has 2 degrees of freedom. The critical values of the type I error $\alpha$ were adjusted using the Bonferroni method. Significance level is indicated (ns = non significant; * = significant). Estimates of probability of null hypothesis (homogeneity) were carried out using Monte Carlo simulations.

| 1996 | BP | | BE | | CP | | CE | |
|---|---|---|---|---|---|---|---|---|
| | G | P | G | P | G | P | G | P |
| Adults IV vs larvae | 209.88 | * | 108.27 | * | 81.29 | * | 56.65 | * |
| Larvae vs spat I | 3.56 | ns | 15.44 | * | 2.16 | ns | 0.44 | ns |
| Spat I vs spat II | 0.77 | ns | 0.33 | ns | 1.26 | ns | 6.80 | ns |
| Spat II vs spat III | 1.21 | ns | 0.77 | ns | 2.68 | ns | 0.09 | ns |
| Spat III vs adult I | 6.45 | ns | 4.72 | ns | 1.95 | ns | 1.57 | ns |
| Adult I vs adult II | 33.97 | * | 23.47 | * | 47.44 | * | 36.07 | * |
| Adult II vs adult III | 17.60 | * | 6.69 | ns | 6.84 | ns | 8.37 | ns |
| Adult III vs adult IV | 6.06 | ns | 18.40 | * | 7.40 | ns | 7.24 | ns |

| 1997 | BP | | BE | | CP | | CE | |
|---|---|---|---|---|---|---|---|---|
| | G | P | G | P | G | P | G | P |
| Adults IV vs larvae | 56.97 | * | 55.94 | * | 19.26 | * | 9.21 | ns |
| Larvae vs spat I | 2.62 | ns | 19.29 | * | 1.77 | ns | 6.75 | ns |
| Spat I vs spat II | 8.77 | ns | 9.75 | ns | 2.02 | ns | 4.57 | ns |
| Spat II vs spat III | 11.87 | * | 1.21 | ns | 1.48 | ns | 4.72 | ns |
| Spat III vs adult I | 5.13 | ns | 0.62 | ns | 2.99 | ns | 2.49 | ns |
| Adult I vs adult II | 24.09 | * | 18.60 | * | 17.56 | * | 26.94 | * |
| Adult II vs adult III | 2.82 | ns | 4.38 | ns | 14.86 | * | 9.76 | ns |
| Adult III vs adult IV | 2.59 | ns | 1.22 | ns | 1.15 | ns | 1.91 | ns |

The spat stage showed a decrease in the frequency of hybrids, especially at the exposed sites. The overall frequency of hybrids for the protected sites (BP,CP) was 18.28 % in 1995, 20.26 % in 1996 and 18.8 % in 1997, while for the exposed sites (BE,CE) it was 8.2 % in 1995, 6.34 % in 1996 and 7.8 % in 1997.

In the present study a total of 300 hybrids (including all adult mussel samples) (7.28 %) was identified using the two PCR-based nuclear markers. A higher frequency of hybrids seems to be associated with protected sites (Figures 10 to 16), since 8.91 % hybrids were found in the samples from the protected sites (BP and CP) while only 5.48 % hybrids were found at the samples from exposed sites (BE and CE).

There was an overall decrease in the relative frequency of *M. trossulus* between June and October 1996 with significant ($P<0.05$) G-tests at BP ($G=22.22$, d.f.=1); CP ($G=5.61$, d.f.=1) and CE ($G=5.82$, d.f.=1), while at BE the G-value was almost significant ($G=3.51$, $P=0.062$). This significant change in the frequency of *M. trossulus*, especially at the protected sites, indicates that there was a high mortality during summer which affected mainly *M. trossulus*.

## II.4 DISCUSSION

Planktonic mussel larvae were observed from late June to mid- October 1996, which is in accordance with previous observations for the time of spawning in Newfoundland, within the Strait of Belle Isle (Gilkinson, 1983) and at Bellevue (Thompson, 1984b) (see also Chapter 3). Most of the larvae from which DNA was successfully amplified and identified were *M. trossulus*, which dominated throughout the entire sampling period (Fig. 4), and was the only larva present during October and, at some sites, during September. *M. edulis* was the least common larva present during the sampling period, and at some sites was present for only a short time. Hybrid larvae (F1 and backcrosses) were present at intermediate frequencies and, like *M. edulis*, were present for a short period only, especially at the exposed sites. The most extended presence of *M. trossulus* planktonic larvae could be due

to a "dribble" spawning throughout the reproductive period (Seed and Suchanek, 1992) or to a delay of metamorphosis (Bayne, 1965, 1976b). Given the size of the larvae (Figures 4, E-H), the former explanation seems to be the more plausible, because there was variation in the mean size of the larvae present (see also Chapter 3).

Gene flow from the dispersal of long-lived planktotrophic larvae is generally predicted to homogenize marine populations over long distances (Scheltema, 1971, 1978; Edmonds et al., 1996). There is evidence supporting this prediction (Palumbi, 1992), but some exceptions have been reported (Burton, 1983; Palumbi, 1994). Larval dispersal could sometimes be restricted by behavioural mechanisms that may favour local recruitment (Burton and Feldman, 1981) or controlled by physical variables that affect water current patterns (Incze et al., 1990). Also, genetic differentiation may occur despite extensive dispersal due both to differential post-settlement mortality (Koehn et al., 1980) and to temporal variation in the genetic composition of larvae (Kordos and Burton, 1993). The pattern of relative frequencies of *M. trossulus*, *M. edulis* and hybrid larvae observed in this study indicated that there was little spatial (Table 1) or temporal (Table 2) variation in the composition of the planktonic larvae.

There was also a high frequency of *M. trossulus* in mussel spat at all sites sampled in the present study, a pattern that has also been reported in Nova Scotia (80 %; size range 4-44 mm, Hunt and Scheibling, 1996; 71% ; size range <5-9.9 mm, Hunt and Scheibling, 1998; 77-91% ; size <1.0 mm, Pedersen, 1991). Primary settlement on filamentous macroalgae has been reported by several authors, including Bayne (1965), Seed (1969, 1976), Eyster and Pechenik (1987), Seed and Suchanek (1992) and Cáceres-Martínez et al. (1994). This phenomenon has also been observed in Newfoundland by Gilkinson (1983) and in the present study, in which spat within a size range of 0.6-1.9 mm (some of the post-larvae still had the eye spot) were collected from filamentous macroalgae at each site. These small spat (also called "post-larvae" by authors such as Seed and Suchanek, 1992; Hunt and Scheibling, 1996, 1998; Cáceres-Martinez and Figueras, 1998) of the two species and the hybrids showed no spatial variation in their frequencies (Table 3), while at secondary

settlement (shell length > 2.0 mm) there was significant spatial variation in the frequency of *M. trossulus*, *M. edulis* and hybrids, with a higher frequency of *M. trossulus* at the most exposed sites (Table 3). This pattern was similar during the three consecutive years sampled (Table 4). Thus, according to this data, there is little evidence for temporal variation in recruitment in these areas.

The frequency of the three species showed no variation among the three different size ranges within the spat stage (Table 8). Post-settlement transport of juveniles can be attained by the production of long byssal threads which improve the passive hydrodynamic drag (Sigurdsson et al., 1976; Lane et al., 1985). These juveniles may use threads to drift in the water column, at least until they reach ~2.0 mm shell length (Sigurdsson et al., 1976; De Block & Tan-Maas, 1977; Lane et al., 1985). Bayne (1964) demonstrated that *M. edulis* enters a secondary pelagic phase at a size of about 2 mm by moving from subtrates such as filamentous algae to a more permanent attachment on adult mussel beds. This secondary settlement may produce a redistribution of the spat in the intertidal and subtidal zones. However, since no variation in their frequencies was detected while most of these spat (< 11 mm) were still able to move (Sigurdsson et al., 1976; Beukema and Vlas, 1989; Cáceres-Martinez et al., 1994; Cáceres-Martinez and Figueras, 1997), it can be assumed that there is little movement of post-larvae after secondary settlement, because spat >5mm shell length may disperse by crawling but are probably too heavy to drift on byssal threads (Hunt and Scheibling, 1996).

The frequency analysis showed little variation in the proportion of *M. trossulus*, *M. edulis* and hybrids between the larval stage and primary settlement (Table 8), although a significant decrease in frequency of hybrids was found at all sites sampled after pooling the data for number of both species, indicating that there is greater mortality in hybrids at this transitional stage from pelagic larva to spat. As Hilbish (1996) pointed out, "where larval dispersal is high there is virtually no capacity for populations to diverge by random processes, so significant genetic divergence among populations must be driven by selection". Hybrid zones are expected to create strong selection pressures because the production of

individuals of mixed ancestry increases variability in individual fitness (Barton and Hewitt, 1989; Mallet and Barton, 1989). Selection may therefore be only acting against some specific genotypes (Bert and Arnold, 1995), and selection against hybrids has been reported in the *Mercenaria mercenaria* and *M. campechiensis* hybrid zone (Bert et al., 1993; Bert and Arnold, 1995; Arnold et al., 1996) and also in the stone crab (*Menippe mercenaria* and *M. adina*) hybrid zone (Combs et al., 1997). Two types of selection against hybrids have been proposed for hybrid zones. First, selection operates intrinsically against hybrids (endogenous selection) in the tension-zone model (Barton, 1979, 1983; Barton and Hewitt, 1989), which predicts that the hybrid zone is maintained by a balance between production of and selection against hybrids resulting from the interspecific mixture. In this model selection will be homogeneous over the geographic space. Second, selection mediated by the environment (exogenous selection), proposed by the ecological selection-gradient model (Slatkin, 1973, 1975; Endler, 1977; Moore and Price, 1993), predicts that the environment determines fitness relationships between hybrids and parental genotypes.

In eastern Canada, there is an apparent scarcity of hybrids in adult mussels. Mallet and Carver (1995) reported <5% of hybrids identified electrophoretically using *Mpi* in Nova Scotia (but see Chapter 2). In the present study, the hybrids showed reduced viability at the spat stage at all sites sampled, in contrast to the larval stage, supporting the tension-zone model. However, the reduction in the frequency of hybrids also varies between sites among locations, showing lower viability at exposed sites (Figures 6-16), supporting the environmental gradient model. The overall low frequency of hybrids at the adult stage (< 8 %) and the results of artificial hybridization in the laboratory, which showed an increased proportion of abnormal larvae among interspecific crosses, indicate strong selection against hybrids, and show that these specific genotypes seem to be at a selective disadvantage, supporting the tension-zone model for the early stages of the mussel life-history.

The sympatric populations of *M. edulis* and *M. trossulus* studied exhibit a strong positive correlation between shell length of *M. edulis* and its abundance, and a negative correlation between shell length of *M. trossulus* and its abundance. The data indicate that the

competing hypothesis that there is year to year variation in the relative frequency of the species recruited can be ruled out. Strong intra-population length-dependent genetic variation has also been reported for most sympatric populations of *M. edulis* and *M. galloprovincialis* in England (Skibinski, 1983; Skibinski and Roderick, 1991). Alleles characteristically at high frequency in pure allopatric populations of *M. galloprovincialis* occur at highest frequency among larger mussels of sympatric populations. No clear evidence of growth differences between *M. edulis* and *M. galloprovincialis* has been reported (Skibinski, 1983; Skibinski and Roderick, 1991). Skibinski and Roderick (1991) concluded that differential mortality was largely responsible for this length-dependent variation observed in S.W. England. Factors that have been reported as probable causes of this differential viability in the *M. edulis-M. galloprovincialis* hybrid zone in western Europe include allozyme thermosensitivity differences between these species (Gardner and Skibinski, 1990b), predation mediated selection against *M. edulis* (Gardner and Skibinski, 1991), strength of attachment to the substrate (Gardner and Skibinski, 1991; Willis and Skibinski, 1992), genotype-dependent differences in parasitism (Coustau et al., 1991) and physiological differentiation (Hilbish et al., 1994). In the present study, a strong shell length-dependent species variation was also found. Differential growth between species may explain, in part, the increase in the frequency of *M. edulis* with increasing shell length observed in this study. However, in a Nova Scotia mussel population Mallet and Carver (1995) recorded only small differences in growth rate between *M. trossulus* and *M. edulis*, which may suggest that the observed changes in species frequency with shell length could be due to differential survival. Furthermore, the data available from the present study indicate that there is no year to year variation in the species settlement pattern, at least over a three year period. Thus selection seems to operate differentially on the two parental species within the zone. A similar trend was found at every site studied, but was more pronounced at the protected sites at both locations, suggesting that there may be differential mortality favouring *M. edulis* which could be enhanced by an environmental factor affecting *M. trossulus*, mostly at protected sites.

Several factors may contribute to mortality in mussels, including biological factors such as senescence, predation, parasitism, poor nutrition, reproduction, and/or physical factors such as wave action, ice scouring, overcrowding, silt, tidal currents, high temperature and low salinity. However, because viability is only reduced in *M. trossulus* found sympatrically with *M. edulis*, each of these factors could be acting differentially.

Shell thickness is an important component of fitness in bivalve molluscs, because the bivalve shell provides a defence against many potential predators (Blundon and Kennedy, 1982; Arnold, 1984). Other components of fitness, including age at first maturation and fecundity, are also directly or indirectly size-dependent. *M. trossulus* shells are lighter in weight and more fragile than *M. edulis* shells of the same length (Freeman et al., 1994; Mallet and Carver, 1995), which may be significant where predation by crabs and sea stars occurs.

In *Mytilus*, seasonal variation in mortality rate is common (Seed and Suchanek, 1992). High mortalities in summer have been recorded in mussels from Maine (Lutz, 1980), Nova Scotia (Freeman and Dickie, 1979; Mallet and Carver, 1995) and the Magdalen Islands (Myrand, 1990). High mortalities often occur after spawning in southwest England (Worrall and Widdows, 1984), and soon after sexual maturity and spawning in native mussels (*M. trossulus*) from British Columbia (Emmert et al., 1987; Bower, 1989). High mortality has also been observed in mussels from Newfoundland after spawning takes place in June-July (Thompson, 1991). In the present study a high summer mortality can be inferred from a drastic reduction in the relative frequency of *M. trossulus* from June to October 1996 (Figures 10 to 16), especially at the protected sites. This occurs predominantly in mussels over 15 mm in shell length, which are reproductively active (Suchanek, 1981; see also Chapter 3), and may indicate that post-spawned *M. trossulus* are under considerable stress, considering the increased water temperatures during summer. Mallet and Carver (1995) reported that during summer (after spawning) the dry tissue weight of the two species differed. In a 30 mm mussel, the dry weight of *M. edulis* was 0.28 g and the dry weight of *M. trossulus* was only 0.1 g, while in a 55 mm mussel dry weight was 0.75 g and 0.46 g in

*M. edulis* and *M. trossulus*, respectively. Spring and autumn tissue weight values were similar for the two mussel species (Mallet and Carver, 1995). A similar trend in dry tissue weight was found in the present study (see Chapter 3), and the mean gonadosomatic index (GSI) and gametic volume fraction (GVF) also differed between the two species at the pre-spawning stage, being higher in *M. trossulus* for mussels of similar shell length (Chapter 3). Furthermore, there is some evidence that *M. trossulus* is reproductively active (spawning) at a shorter shell length than *M. edulis* (Chapter 3). However, sexual maturity in *Mytilus* seems to be a function of age rather than size (Seed, 1969; Seed and Brown, 1977). If there is some differential growth in favour of *M. edulis* (Mallet and Carver, 1995), it may be that mussels of the same size are from different cohorts and that both species enter the reproduction stage at the same shell size but different age (Seed and Brown, 1977).

The physiology of the mussel is closely related to the reproductive cycle, which usually exhibits a discrete annual form, at least in eastern Canada (Thompson, 1984a). Like most bivalves, *Mytilus* stores energy largely in the form of carbohydrate, particularly glycogen, which is synthesised during the spring phytoplankton bloom in temperate areas (Seed and Suchanek, 1992). This stored energy is then used in gametogenesis and to support maintenance metabolism during winter, when less food is available (Hawkins and Bayne, 1992). The variation in the content of this stored energy may explain in part some of the mortality observed in natural populations. After spawning takes place, the mussel usually has little or no carbohydrate reserve, since the supply of glycogen has been depleted by the very high metabolic demands of gametogenesis (Bayne et al., 1982; Lowe et al., 1982). Bayne et al. (1983) were also able to demonstrate that high reproductive costs are associated with high mortality. More detailed studies on the physiology and biochemical composition of these two mussel species and their hybrids are necessary to determine if a "post-spawning stress" is the main cause of the very sharp fall in the frequency of *M. trossulus* after they reach sexual maturity in these sympatric populations.

The processes that maintain hybrid zones have been reviewed by Harrison (1990, 1993). In the *M. edulis* - *M. galloprovincialis* hybrid zone in Europe, Skibinski (1983) and

Gardner and Skibinski (1988) have suggested that the zone is maintained by a balance between immigration of *M. edulis* spat and selection against *M. edulis*. Some evidence of differential *M. edulis* mortality and a possible mechanism of selection has been obtained from populations in S.W. England ( Gardner and Skibinski, 1991; Skibinski and Roderick, 1991). According to Barton and Hewitt (1989) and Hewitt (1989) the mechanism that maintains most hybrid zones is a delicate equilibrium between dispersal and selection against hybrids. From the present study, there is evidence for the presence of "maladapted hybrid recombinants" within this hybrid zone between *M. edulis* and *M. trossulus* (see also Chapter 4). Hybrids produced by species which are genetically more divergent, such as *M. edulis-M. trossulus* (Koehn, 1991; McDonald et al., 1991; Toro, 1998), are more likely to have reduced fitness than hybrids produced by species which are genetically very similar, such as *M. edulis-M. galloprovincialis* (Gardner, 1992). This may explain in part the lower level of hybridization found between *M. edulis-M.trossulus* in the present study and between *M. trossulus-M. galloprovincialis* on the Pacific coast of North America (Sarver and Foltz, 1993; Suchanek et al., 1997) compared with the *M. edulis-M. galloprovincialis* hybrid zone in Europe, where levels of hybridization range from 25-80 % (Hilbish et al., 1994;Sanjuan et al., 1994; Comesaña and Sanjuan, 1997; Wilhelm and Hilbish, 1998).

In the present study, *M. edulis* is at a selective advantage, and one may expect that the structure of this hybrid zone is shifting towards this species, but the data indicate that this is not the case (although more evidence from long term data obtained from a dynamic cohort analysis are needed). The fact that *M. edulis* is favoured in large size classes, but does not predominate in the population at large, may be because the effect of selection favouring *M. edulis* in large individuals is counteracted by higher recruitment of *M. trossulus*. In the present study *M. trossulus* individuals as small as 10 mm were reproductively mature. Although these small mussels have a lower fecundity than larger individuals (Thompson, 1984a, b), they are found in larger numbers than *M. edulis* at the study locations (Figures 10 - 16). Therefore, it is probable that the reproductive output of the population is driven by the smaller size classes, where *M. trossulus* is more abundant (see also Chapter 3). This effect

could be magnified by the "dribble" spawning observed in *M. trossulus*, which may produce large amounts of "pure" *M. trossulus* larvae, in contrast to *M. edulis*, which has a more restricted spawning period (Chapter 3). The other possibility is that the recruits observed at these sites are derived from other populations formed by "pure" *M. trossulus* individuals, although this is very unlikely, because most mussel populations around Newfoundland contain individuals of both species (Bates and Innes, 1995; Innes pers. comm.). A similar situation has been reported in the south-west England mussel hybrid zone (*M. edulis - M. galloprovincialis)*, in which a strong viability selection against *M. edulis*-like genotypes in Whitsand Bay is balanced by immigration of larvae from "pure" populations of *M. edulis* (Gardner et al., 1993; Wilhelm and Hilbish, 1998). The *M. edulis-M. trossulus* hybrid zone in eastern Newfoundland is also very complicated. It appears that the tension zone model for the maintenance of this hybrid zone is operating at very early stages of the life-history of these mussels and that strong selection favouring *M. edulis* at later life-history stages is counteracted by massive recruitment of *M. trossulus*.

Finally, the present study applies molecular genetic techniques to early larval stages of marine bivalves, which may have implications for population genetic analysis of natural populations, because in exploiting PCR-techniques such studies can be extended to all stages and age-classes. RFLPs analysis of PCR-amplified DNA was used to identify individual ethanol-preserved mussel planktonic larvae, and the PCR primers used were highly specific to the target species. The DNA from around 87 % of the larvae was successfully amplified and identified. Some technical problems that may have played a role in those cases where DNA failed to amplify may include DNA degradation before preservation (larval death), technical errors, chemical impurities, and the possible inclusion of other types of bivalve larvae that may have been present in the area at the time of collection (e.g., *Modiolus modiolus*, *Placopecten magellanicus*), but which cannot be identified with the primers used.

# III. CHAPTER 2

## A combined molecular approach to ecological and genetic interactions within a mussel (*Mytilus edulis* - *M. trossulus*) hybrid zone

### III. 1. INTRODUCTION

The situation in which two species of mussels (*M. edulis* and *M. trossulus*) are found sympatrically in Atlantic Canada appears to be similar to that found in southwest England and the Atlantic coast of France, where two genetically distinct species of *Mytilus* (*M. edulis* and *M. galloprovincialis*) have overlapping ranges and at some locations occur sympatrically (Skibinski et al., 1978; Coustau et al., 1991; Gardner, 1994). A recent study on the genetic variation of these two species of mussels in Newfoundland showed that the distribution of a hybrid index based on three partially diagnostic loci (*Est-D*, *Pgm*, *Lap*) provided no evidence for hybridization (Bates and Innes, 1995). Two studies in Lunenburg Bay, Nova Scotia, estimated the degree of natural hybridization to be < 5% using the *Mpi* locus (Mallet and Carver, 1995) and 22.8 % using *Mpi* and *Est-D* loci (Saavedra et al., 1996). In the latter study *Mpi* and *Est-D* were used to classify the mussels as "pure" *M. edulis*, "pure" *M. trossulus*, or hybrids, and mtDNA variation was used to characterize the gender-associated mtDNA present in each individual. No "pure" *M. edulis* mtDNA type was found in "pure" *M. trossulus* individuals and vice-versa, suggesting that no mtDNA introgression between the two species occurs in nature. Furthermore, the study provides evidence that introgression is blocked early in the hybridization process, although the mechanism remains unknown. Saavedra et al. (1996) did not provide any information about the environmental characteristics of the sites sampled or the size of the mussels sampled. It is possible that these two factors play an important role in determining the proportions of the pure forms and their hybrids present in the sample.

The objective of the present study was to increase the chances of detecting F1, F2 and backcrosses between *M. edulis* and *M. trossulus* by using four molecular markers (two

PCR-based nuclear DNA markers and two allozyme markers) and to test for microgeographic differentiation. Mussels within a range of shell lengths were sampled to determine if both *Mytilus* species and their hybrids are distributed differentially according to size. A final objective was to examine further the possible intrinsic incompatibilities between *edulis* and *trossulus* nuclear and mitochondrial genomes in mussels of different sizes from different environments.

## III.2. MATERIALS AND METHODS

### III.2.1. STUDY SITES AND SAMPLING

Mussel populations (*M. edulis* Linnaeus, 1758; *M. trossulus* Gould, 1850) were sampled subtidally (approximately 1.5 m below mean low tide) by SCUBA at two locations (Bellevue and Chance Cove) on the east coast of Newfoundland, Canada (Fig. 1) during July, 1997. At each location, mussels were representatively sampled (hap hazzard) from a wave-exposed environment and from a protected, sheltered one. Mussels with shell lengths from 15 to 92 mm were used in the genetic analysis. The mussels were brought alive to the laboratory, dissected, and the sex of each individual determined by microscopic examination of the gonad. Gonad and digestive gland were stored separately at -80°C for PCR and allozyme analysis. A small piece of the mantle border tissue was stored in ethanol (95%) at -20 °C for PCR analysis.

### III.2.2. ALLOZYME ANALYSIS

Horizontal starch-gel electrophoresis was carried out on 11% gels (Sigma starch) at 4°C. The digestive gland was homogenized in an equal volume of 0.01 M dithiothreitol and centrifuged at 8000 x g for 7 min. The supernatant was used as the source for five enzyme loci that show different levels of diagnostic power for the two *Mytilus* taxa: esterase-D (*Est-D;* E.C. 3.1.1.), mannose-6-phosphate isomerase (*Mpi*; E.C. 5.3.1.8), leucine aminopeptidase

(*Lap*;E.C. 3.4.11.1), octopine dehydrogenase (*Odh*;E.C. 1.5.1.11) and phosphoglucomutase (*Pgm*; E.C. 5.4.2.2). Electrophoretic procedures were conducted following Bates and Innes (1995) for *Est-D, Lap* and *Pgm*, and Väinolä and Hvilsom (1991) for *Odh* and *Mpi*. Allele terminology was that used by McDonald and Koehn (1988) and McDonald et al. (1991). The *Mpi* locus appears to be completely diagnostic between *M. edulis* and *M. trossulus*, and *Est-D* is also a highly diagnostic locus for these two taxa (see McDonald and Koehn, 1988; Varvio et al., 1988; McDonald et al,. 1991; Väinolä and Hvilson, 1991). Nevertheless, *Odh* does not appear to be diagnostic as in Saavedra et al. (1996), perhaps because a different buffer system was used in the present study.

### III.2.3. DNA ANALYSIS

Mantle border tissue from each mussel was used for total DNA extraction as described in the Material and Methods section of Chapter 1. Two nuclear markers (*Glu-5* and *ITS*) and one mitochondrial DNA marker (*COIII*) were employed after PCR amplification. Similar DNA amplification conditions for both *Glu-5* (Rawson et al., 1996a) and *ITS* (Heath et al., 1995) markers were used (see Chapter 1), except that the initial denaturation period was 3 min (modified from Heath et al., 1995; Rawson et al., 1996a).

The mtDNA marker (*COIII*) is based on an 860-bp fragment of the cytochrome c oxidase subunit III gene, and the amplification protocol used was modified from Zouros et al. (1994a). Approximately 0.2 µg total DNA was incubated for 30 cycles at 94°C for 1 min, 54°C for 10 sec, and 72°C for 1 min. The initial denaturation period was 2 min and a final extension period was not used. The primer set used (FOR1 and REV1) amplifies the corresponding mtDNA fragment from the F and M types of both species. Aliquots of the amplification product were digested with *EcoRI* restriction enzyme to identify the various mtDNA genomes in males and females (Zouros et al., 1994a; Saavedra et al., 1996). For those males in which it was not possible to identify the M genome in mantle tissue, the previously frozen gonad was used for total DNA extraction.

### III.2.4. CLASSIFICATION OF MUSSELS

The two allozyme loci (*Mpi* and *Est-D*) and the two nuclear DNA markers (*Glu*-5 and *ITS*) were used to classify the mussels as "pure" *edulis*, "pure" *trossulus* or hybrids (F1-like, F2-like, backcross-like). The allozyme alleles 90 and 100 at the *Mpi* locus and allele 100 at the *Est-D* locus (typical alleles for *M. edulis*) and 84, 94 and 104 at the *Mpi* locus and allele 90 at the *Est-D* locus (typical alleles of *M. trossulus*) were used to classify individuals following Saavedra et al. (1996). The restriction analysis of the *ITS* PCR product with *Hhal* and the Glu-5 PCR assay produced species-specific banding patterns (see Chapter 1). Individuals with alleles at four loci belonging to the same taxon were classified as pure species. Hybrids were classified as follows: when an individual was found to be heterozygous for one *edulis* and one *trossulus* allele at all four diagnostic markers, it was considered to be an $F_1$. When alleles for two of the four loci were from one species, but alleles for the remaining two loci were from the other, the individual was considered an $F_2$. When alleles at one, two, or three loci belonged to one species, but the fourth locus contained an allele from each species, the individual was classified as a backcross biased towards one or the other species. This classification, based on the four markers, provides reliable information about the identity of any particular pure or hybrid mussel (see Saavedra et al., 1996; Boecklen and Howard, 1997).

### III.2.5. SIZE FREQUENCY ANALYSIS

Shell lengths of all sampled mussels were measured to the nearest 0.01 mm using electronic digital calipers. Size classes consisted of 5 mm shell length intervals. A species-specific hybrid index was used to study the relationship between genotype and size. The index was calculated according to Sanjuan et al. (1994) using the four markers for the *Mytilus* species. For all markers, each typical *trossulus* allele was given the value -1, each *edulis* allele the value +1 and all other alleles, i.e. those found in those species, the value 0. The hybrid index value for each individual consists of the sum of the eight values (two alleles

for each of four diagnostic markers). The index ranges between -8 (pure *trossulus*) and +8 (pure *edulis*). Individuals with value 0 (but only those tetra-heterozygotes) were considered as $F_1$. Those individuals with values between -8 and 0 were classified as *trossulus*-biased backcrosses, and those between 0 and +8 were classified as *edulis*-biased backcrosses. All genetic analyses were performed with BIOSYS-1 (Swofford and Selander, 1981) and Zaykin and Pudovkin (1993) computer programs.

## III. 3. RESULTS

The allele frequencies of the most diagnostic loci (*Est-D* and *Mpi*) varied among the four populations. The typical *M. trossulus* alleles (*Mpi 94* and *Est-D 90*) reached the highest frequencies in the Chance Cove exposed sample (CE) (Table 9). For example, the frequency of the *Mpi 94* allele was 0.390 for Bellevue protected and 0.765 for Chance Cove exposed. For *Est-D*, *Mpi* and *Pgm* loci, all samples showed a significant deficit of heterozygotes (significant positive F values) (Table 9), which suggests a Wahlund-like effect because it occurs in the enzyme loci where the differences in allele frequencies between pure *Mytilus edulis* and pure *M. trossulus* are larger.

The allele frequencies for all enzyme loci in each genotypic group for each sample are shown in Table 10. In all populations, the hybrid class had intermediate allele frequencies for *Est-D*, *Mpi*, and *Pgm* loci for those alleles which had larger differences in frequency between pure *edulis* and pure *trossulus*, even when two allozyme and two non-allozyme markers were used. For example, in Bellevue protected, the allele *Mpi* 94 had a frequency of 0.960 and 0.000 in pure *trossulus* and pure *edulis*, respectively, while its frequency in the hybrid class was 0.636. At Bellevue there was no significant difference ($G= 2.38$, $df= 2$, $p>0.05$) in the occurrence of the two species and hybrids between the protected and exposed sites.

**Table 9:** Allele frequencies at *Est-D*, *Lap*, *Mpi*, *Odh* and *Pgm* loci in four ***Mytilus*** populations in eastern Newfoundland: BP, Bellevue protected; CP, Chance Cove protected; BE, Bellevue exposed; CE, Chance Cove exposed. N: sample size, F: coefficient for heterozygote deficiency or excess.

| | Population | | | |
|---|---|---|---|---|
| Locus | BP | CP | BE | CE |
| *Est-D* | | | | |
| 80 | 0.005 | 0.010 | 0.005 | 0.010 |
| 85 | 0.000 | 0.000 | 0.010 | 0.000 |
| 90 | 0.369 | 0.591 | 0.505 | 0.737 |
| 95 | 0.035 | 0.015 | 0.015 | 0.030 |
| 100 | 0.581 | 0.374 | 0.460 | 0.212 |
| 105 | 0.005 | 0.005 | 0.000 | 0.010 |
| 110 | 0.005 | 0.005 | 0.005 | 0.000 |
| (N) | (99) | (99) | (100) | (99) |
| F | 0.65*** | 0.53*** | 0.64*** | 0.46*** |
| *Lap* | | | | |
| 90 | 0.000 | 0.000 | 0.000 | 0.015 |
| 92 | 0.035 | 0.066 | 0.010 | 0.070 |
| 94 | 0.220 | 0.265 | 0.250 | 0.305 |
| 96 | 0.330 | 0.408 | 0.395 | 0.435 |
| 98 | 0.405 | 0.250 | 0.340 | 0.170 |
| 100 | 0.010 | 0.010 | 0.005 | 0.005 |
| (N) | (100) | (98) | (100) | (100) |
| F | 0.41*** | 0.12 | 0.38* | 0.14 |
| *Mpi* | | | | |
| 84 | 0.010 | 0.020 | 0.010 | 0.025 |
| 90 | 0.090 | 0.045 | 0.080 | 0.030 |
| 94 | 0.390 | 0.635 | 0.495 | 0.765 |
| 100 | 0.505 | 0.300 | 0.405 | 0.165 |
| 104 | 0.005 | 0.000 | 0.010 | 0.015 |
| (N) | (100) | (100) | (100) | (100) |
| F | 0.64*** | 0.76*** | 0.69*** | 0.51*** |
| *Odh* | | | | |
| 90 | 0.015 | 0.050 | 0.035 | 0.040 |
| 95 | 0.000 | 0.005 | 0.005 | 0.010 |
| 100 | 0.935 | 0.870 | 0.885 | 0.895 |
| 105 | 0.005 | 0.005 | 0.025 | 0.010 |
| 110 | 0.045 | 0.070 | 0.050 | 0.040 |
| 120 | 0.000 | 0.000 | 0.000 | 0.005 |
| (N) | (100) | (100) | (100) | (100) |
| F | 0.27** | 0.24* | 0.06 | -0.02 |
| *Pgm* | | | | |
| 90 | 0.000 | 0.000 | 0.005 | 0.000 |
| 93 | 0.045 | 0.015 | 0.050 | 0.005 |
| 100 | 0.485 | 0.350 | 0.430 | 0.190 |
| 106 | 0.195 | 0.135 | 0.170 | 0.230 |
| 108 | 0.015 | 0.010 | 0.005 | 0.010 |
| 111 | 0.245 | 0.425 | 0.320 | 0.485 |
| 114 | 0.015 | 0.060 | 0.020 | 0.065 |
| 118 | 0.000 | 0.005 | 0.000 | 0.015 |
| (N) | (100) | (100) | (100) | (100) |
| F | 0.47*** | 0.44*** | 0.35*** | 0.26* |

* P<0.05; ** P<0.01; *** P<0.001 (significance levels for $\chi^2$ test)

At Chance Cove, however, there was a significant difference ($G=7.22$, $df=2$, $p<0.05$) between the frequencies of the two species and hybrids, with *M. trossulus* most frequent (61%) at the exposed site and *M. edulis* most frequent (44%) at the protected site. There was also a significant difference ($G=30.53$, $df=2$, $p<0.001$) in the frequencies of both species and hybrids between Chance Cove and Bellevue, pooling samples from exposed and protected sites. *M. trossulus* was most frequent (53%) at Chance Cove, *M. edulis* was most frequent (46%) at Bellevue and the frequency of hybrids was similar (26%, 24%) at both locations.

The distribution of mtDNA mitotypes among pure *M. edulis* and *M. trossulus* individuals from all sites combined is shown in Table 11. Mussels were first classified as pure forms or hybrids based on *Mpi* and *Est-D*, in order to permit comparisons with the results reported by Saavedra et al. (1996), and then classified using both allozyme loci plus the *ITS* and *Glu*-5 DNA markers (numbers in parentheses). The mussels were then characterized by one or two mitotype symbols (F-ed, F-tr, M-ed, M-tr) (Stewart et al., 1995) if they were found to be homoplasmic or heteroplasmic, respectively (Figure 18). The sex ratios (males:females) were 1:1.23 (*M edulis*) and 1:1.26 (*M. trossulus*) when classification was based on the four markers (*ITS*, *GLU*-5, *Mpi* and *Est-D*). All *M. edulis* and *M. trossulus* females were found to be homoplasmic (F-ed or F-tr, respectively) (Table 11). All *M. edulis* males were heteroplasmic for F and M *edulis* genome combinations (F-ed/M-ed), although two individuals (classified using the two allozyme loci) showed a heterospecific combination of F *edulis* genome and M *trossulus* genome (F-ed/M-tr) (Figure 18, line F). One of these heterospecific males was hybrid for both *ITS* and *Glu-5* nuclear-DNA markers, while the other was hybrid only for the *ITS* marker (Table 11). Both individuals were then reclassified as hybrids instead of pure forms when classification was based on four diagnostic markers.

All 84 pure *M. trossulus* females classified according to two allozyme loci were homoplasmic for the F-tr mitotype. However, based on the *ITS* and *Glu-5* nuclear-DNA markers, three individuals were hybrid for *ITS*, 11 were hybrid for *Glu-5*, one was hybrid for both *ITS* and *Glu-5*, and two were *M.edulis* for the *Glu-5* marker. Therefore, from the four markers, 15

**Table 10:** Allele frequencies at *Est-D*, *Lap*, *Mpi*, *Odh* and *Pgm* loci for mussels from 4 locations classified as "pure" ***Mytilus edulis*** (*edul*), "pure" ***M. trossulus*** (*tros*), or hybrids using four diagnostic markers (*Est-D*, *Mpi*, *ITS*, *Glu-5*). N: sample size.

| | Bellevue protected | | | Bellevue exposed | | | Chance Cove protected | | | Chance Cove exposed | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | edul | tros | hybr | edul | tros | hybr | edul | tros | hybr | edul | tros | hybr |
| Est-D | | | | | | | | | | | | |
| 80 | 0.010 | 0.000 | 0.000 | 0.000 | 0.016 | 0.000 | 0.000 | 0.023 | 0.000 | 0.000 | 0.008 | 0.019 |
| 85 | 0.000 | 0.000 | 0.000 | 0.012 | 0.016 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 90 | 0.000 | 0.960 | 0.586 | 0.000 | 0.938 | 0.759 | 0.000 | 0.965 | 0.607 | 0.000 | 0.958 | 0.635 |
| 95 | 0.019 | 0.040 | 0.068 | 0.000 | 0.031 | 0.019 | 0.018 | 0.012 | 0.018 | 0.000 | 0.025 | 0.058 |
| 100 | 0.951 | 0.000 | 0.364 | 0.976 | 0.000 | 0.222 | 0.946 | 0.000 | 0.375 | 0.964 | 0.000 | 0.288 |
| 105 | 0.010 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.018 | 0.000 | 0.000 | 0.036 | 0.008 | 0.000 |
| 110 | 0.010 | 0.000 | 0.000 | 0.012 | 0.000 | 0.000 | 0.018 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| (N) | 52 | 25 | 22 | 41 | 32 | 27 | 28 | 43 | 28 | 14 | 59 | 26 |
| Lap | | | | | | | | | | | | |
| 90 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.017 | 0.019 |
| 92 | 0.000 | 0.060 | 0.068 | 0.000 | 0.016 | 0.019 | 0.000 | 0.119 | 0.054 | 0.000 | 0.076 | 0.077 |
| 94 | 0.048 | 0.380 | 0.432 | 0.012 | 0.469 | 0.352 | 0.056 | 0.381 | 0.286 | 0.107 | 0.381 | 0.250 |
| 96 | 0.250 | 0.520 | 0.318 | 0.268 | 0.469 | 0.500 | 0.278 | 0.429 | 0.518 | 0.250 | 0.432 | 0.538 |
| 98 | 0.692 | 0.020 | 0.182 | 0.707 | 0.047 | 0.130 | 0.648 | 0.060 | 0.143 | 0.607 | 0.093 | 0.115 |
| 100 | 0.009 | 0.020 | 0.000 | 0.012 | 0.000 | 0.000 | 0.019 | 0.012 | 0.000 | 0.036 | 0.000 | 0.000 |
| (N) | 52 | 25 | 22 | 41 | 32 | 27 | 27 | 42 | 28 | 14 | 59 | 26 |
| Mpi | | | | | | | | | | | | |
| 84 | 0.000 | 0.020 | 0.023 | 0.000 | 0.016 | 0.019 | 0.000 | 0.047 | 0.000 | 0.000 | 0.034 | 0.019 |
| 90 | 0.144 | 0.000 | 0.068 | 0.183 | 0.000 | 0.019 | 0.125 | 0.000 | 0.036 | 0.179 | 0.000 | 0.019 |
| 94 | 0.000 | 0.960 | 0.636 | 0.000 | 0.953 | 0.704 | 0.000 | 0.953 | 0.768 | 0.000 | 0.958 | 0.750 |
| 100 | 0.856 | 0.000 | 0.273 | 0.817 | 0.000 | 0.259 | 0.875 | 0.000 | 0.196 | 0.821 | 0.000 | 0.192 |
| 104 | 0.000 | 0.020 | 0.000 | 0.000 | 0.031 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.008 | 0.019 |
| (N) | 52 | 25 | 22 | 41 | 32 | 27 | 28 | 43 | 28 | 14 | 59 | 26 |
| Odh | | | | | | | | | | | | |
| 90 | 0.000 | 0.060 | 0.000 | 0.037 | 0.063 | 0.000 | 0.089 | 0.047 | 0.018 | 0.000 | 0.068 | 0.000 |
| 95 | 0.000 | 0.000 | 0.000 | 0.012 | 0.000 | 0.000 | 0.018 | 0.000 | 0.000 | 0.036 | 0.000 | 0.019 |
| 100 | 0.962 | 0.920 | 0.886 | 0.878 | 0.891 | 0.889 | 0.750 | 0.930 | 0.893 | 0.857 | 0.881 | 0.942 |
| 105 | 0.010 | 0.000 | 0.000 | 0.000 | 0.031 | 0.056 | 0.000 | 0.000 | 0.018 | 0.000 | 0.008 | 0.019 |
| 110 | 0.029 | 0.020 | 0.114 | 0.073 | 0.016 | 0.056 | 0.143 | 0.023 | 0.071 | 0.071 | 0.042 | 0.019 |
| 120 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.036 | 0.000 | 0.000 |
| (N) | 52 | 25 | 22 | 41 | 32 | 27 | 28 | 42 | 28 | 14 | 59 | 26 |
| Pgm | | | | | | | | | | | | |
| 90 | 0.000 | 0.000 | 0.000 | 0.012 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 93 | 0.077 | 0.020 | 0.000 | 0.098 | 0.031 | 0.000 | 0.036 | 0.000 | 0.018 | 0.000 | 0.000 | 0.019 |
| 100 | 0.808 | 0.020 | 0.273 | 0.780 | 0.094 | 0.296 | 0.929 | 0.058 | 0.214 | 0.857 | 0.042 | 0.173 |
| 106 | 0.115 | 0.300 | 0.227 | 0.098 | 0.234 | 0.203 | 0.036 | 0.128 | 0.232 | 0.143 | 0.263 | 0.212 |
| 108 | 0.000 | 0.060 | 0.000 | 0.000 | 0.000 | 0.019 | 0.000 | 0.012 | 0.018 | 0.000 | 0.008 | 0.000 |
| 111 | 0.000 | 0.580 | 0.455 | 0.012 | 0.625 | 0.426 | 0.000 | 0.686 | 0.464 | 0.000 | 0.576 | 0.538 |
| 114 | 0.000 | 0.020 | 0.045 | 0.000 | 0.016 | 0.056 | 0.000 | 0.105 | 0.054 | 0.000 | 0.093 | 0.038 |
| 118 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.012 | 0.000 | 0.000 | 0.017 | 0.019 |
| (N) | 52 | 25 | 22 | 41 | 32 | 27 | 28 | 43 | 28 | 14 | 59 | 26 |

**Table 11:** Distribution by sex of the mtDNA genotypes among "pure" ***Mytilus edulis*** and ***M. trossulus*** as established by two allozyme loci (*Mpi*, *Est*) or as established by the two allozyme loci and the two nuclear DNA markers (in parentheses), pooling four populations.

| Mitotype | Pure *Mytilus edulis* | | Pure *Mytilus trossulus* | |
|---|---|---|---|---|
| | female | male | female | male |
| F-ed | 58 (56) | - | - | - |
| F-tr | - | - | 84 (69) | - |
| F-ed/M-ed | - | 71 (69) | - | 1[b] (0) |
| F-ed/M-tr | - | 2[a] (0) | - | 1[c] (0) |
| F-tr/M-tr | - | - | - | 64 (52) |
| M-trO/F-tr | - | - | - | 42 (35) |
| | 58 (56) | 73 (69) | 84 (69) | 108 (87) |

[a] From the two "pure" ***Mytilus edulis*** males that showed the heterospecific combination, one was hybrid for ITS and the other was hybrid for both nuclear-DNA markers

[b] This individual was ***edulis*** for both nuclear-DNA markers.

[c] This individual was hybrid for the ITS nuclear-DNA marker

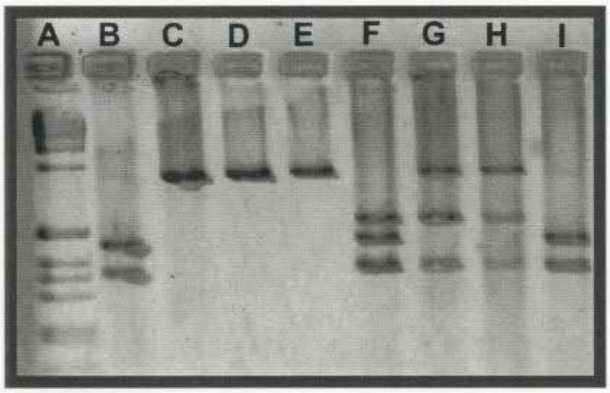



**Fig. 18.** Photo-negative of ethidium bromide stained 3% agarose gel transilluminated with ultraviolet light showing the mtDNA RFLPs profiles of the 860-bp *COIII* gene segment after digestion by the ***Eco*RI** restriction enzyme. B and I, ***Mytilus trossulus*** male scored as F-tr1/M-tr1; C, D, and E ***Mytilus trossulus*** male scored as M-trO; F, hybrid male (backcross to ***M. edulis***) scored as F-ed1/M-tr1; G and H, ***Mytilus edulis*** male scored as F-ed1/M-ed1. Molecular weight marker (Gibco BRL 1Kb ladder) (line A).

individuals were reclassified as hybrids. Of the 108 pure *trossulus* males (based on two allozyme loci), 64 were heteroplasmic for the F-tr/M-tr combination, and the remaining 42 showed a mitotype of only one 860-bp band for *EcoRI* and were classified as M-trO according to Saavedra et al. (1996). One individual showed the heteroplasmic combination of F and M *edulis* genomes (F-ed/M-ed), and was also found to be *edulis* for both *ITS* and *Glu-5* nuclear-DNA markers, and this individual was reclassified as an $F_2$ hybrid using the four diagnostic markers (*M. trossulus* for *Mpi* and *Est-D* and *M. edulis* for *ITS* and *Glu*-5). Another individual showed the heterospecific combination F-ed/M-tr and was found to be one of the three individuals classified as hybrids by the *ITS* marker. With the use of four diagnostic markers, no pure *M. edulis* or *M. trossulus* contained F or M mitochondrial DNA from the other species. Nevertheless, if the classification were based only on two allozyme loci, four male individuals would be identified as "pure" species having a heterospecific nuclear and mtDNA combination.

The distribution of mtDNA mitotypes among the hybrid individuals from all the samples is shown in Table 12. Of the three $F_1$-like female individuals (based on two diagnostic allozyme loci), one had a pattern that could be an M-ed/F-ed homospecific combination or a heterospecific combination (type F-ed/M-trO) (see Saavedra et al., 1996). This female had the *edulis* pattern for both *ITS* and *Glu-5* markers. Another two (one with F-ed and one with F-tr mitochondrial mitotypes) were *edulis* and *trossulus* for *ITS* and *Glu-5* nuclear markers, respectively. Thus according to the four markers these females were backcrosses. All five males classified as $F_1$ hybrids using *Mpi* and *Est-D* were also hybrids for *ITS* and *Glu-5* markers. Two of them had a clear heterospecific combination of F and M genomes (F-ed/M-tr, see Saavedra et al., 1996), whereas the other had only one 860-bp and for *EcoRI*. This pattern could be considered as heterospecific, combining the *trossulus* F genome and the *edulis* M genome, a pattern with only one 860 bp band (Saavedra et al., 1996).

**Table 12:** ***Mytilus*** spp. hybrids. Distribution of mtDNA genotypes by sex, based on *Mpi* and *Est-D* or using four diagnostic markers (in parentheses) for four populations pooled. Bc-tr, ***trossulus***-biased backcross; Bc-ed, ***edulis***-biased backcross.

| | F1-like | | F2-like | | Bc-tr | | Bc-ed | |
|---|---|---|---|---|---|---|---|---|
| mtDNA genotype | female | male | female | male | female | male | female | male |
| F-ed | 1 (0) | - | - | - | - | - | 12 (14) | - |
| F-tr | 1 (0) | - | 2 (0) | - | 16 (36) | - | - | - |
| F-ed/M-ed | - | - | - | 0 (1) | - | 2[b] (1) | - | 7 (8) |
| F-ed/M-tr | - | 2 (2) | - | - | - | 1 (2) | - | 0 (2) |
| F-ed/M-trO | 1[c] (0) | - | - | - | - | - | 0 (1) | 1[a] (1) |
| F-tr/M-ed | - | 3 (3) | - | - | - | - | - | - |
| F-tr/M-tr | - | - | - | - | - | 3 (15) | - | - |
| M-trO | - | - | - | 1 (0) | - | 6 (14) | - | - |
| | 3 (0) | 5 (5) | 2 (0) | 1 (1) | 16 (36) | 12 (32) | 12 (15) | 8 (11) |

[a] This individual could be M-trO and not M-ed because it is hybrid for the ITS and Glu-5 nuclear-DNA markers

[b] One individual is a male, hybrid for ITS, *trossulus* for GLU-5 and M-ed/F-ed, backcross *trossulus* biased; the other is a male, *edulis* for both nuclear-DNA markers, M-ed/F-ed and backcross *trossulus* biased.

[c] This individual is *edulis* for both nuclear-DNA markers

Thus of all mussels sampled (400 individuals) only five could be considered as true $F_1$ hybrids based on four diagnostic loci and one mtDNA marker. Among male individuals considered $F_2$-like and backcrosses to *edulis* and *trossulus*, only five had a heterospecific M and F mtDNA genome combination. There was one backcross to *trossulus* that had the homospecific F and M *edulis* mtDNA genome combination. The remaining $F_2$ and backcross individuals had the same species combination of mitochondrial and nuclear DNA. Nevertheless, it is worth noting the significant increase in the number of *trossulus*-biased backcrosses when classification of individuals was based on four diagnostic markers rather than two (Table 12). Most of these came from individuals classified as pure *trossulus* according to the two allozyme loci.

Among male individuals identified as backcrosses or $F_2$, an equal number of heterospecific and homospecific M and F mtDNA types is expected (Saavedra et al., 1996). Of the 44 backcross and $F_2$ males identified, only five were heterospecific for F and M mtDNA ($\chi^2$=26.3, df=1, $p<0.001$). Backcross females have a 0.25 chance of having a mitotype different from the majority of their nuclear genes (Saavedra et al., 1996). Of the 50 backcross females identified, 12.5 were expected to have a discordance between mtDNA and the majority of their nuclear genes, but none were observed ($\chi^2$=8.33, df=1, $p=<0.005$). A similar analysis for 24 backcross males carrying homospecific F and M mitotypes found one discordance where six were expected ($\chi^2$=2.78, df=1, $p>0.05$).

The relationship between frequency of the *M. edulis*, *M. trossulus*, and hybrid individuals (based on 4 markers) and shell length from the four populations is shown in Figure 19. *M.* trossulus was again the predominant species in the smallest size classes for all samples. At the Bellevue site *M. trossulus* was most frequent only in the smallest size categories (between 15 and 26 mm), whereas it was the predominant species in almost all size classes at Chance Cove. An increase in frequency of *M. edulis* individuals and a decrease in frequency of *M. trossulus* individuals with shell length was observed in both environments at both Bellevue and Chance Cove.

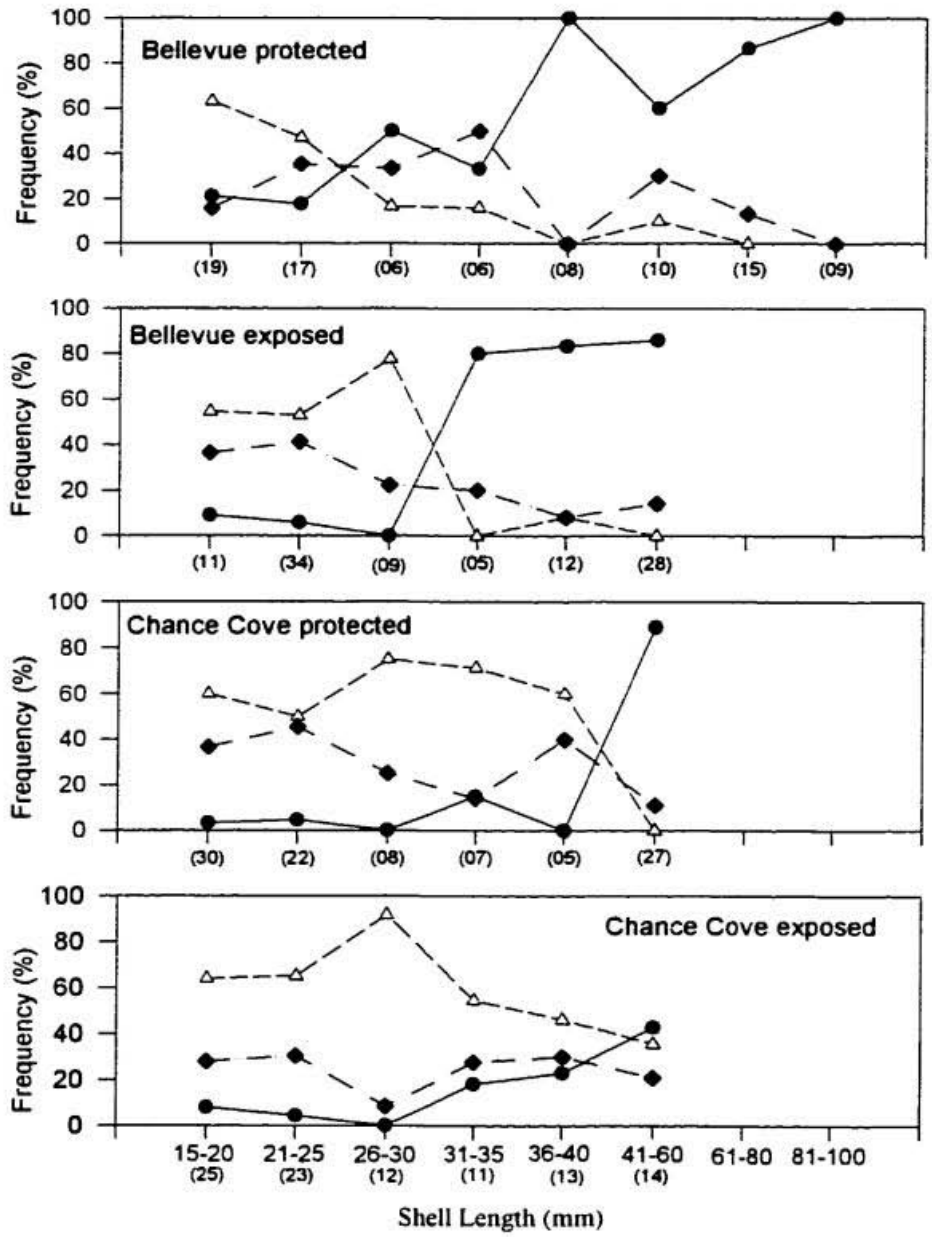



**Fig. 19.** *Mytilus* spp. Frequencies of putative *Mytilus edulis* (circles), *M. trossulus* (triangles) and hybrids (diamonds) plotted against shell length classes for four populations in eastern Newfoundland, from a sample taken in July 1997. Number of individuals in each length class in parentheses.

The change in frequency of both species was also gradual with increasing size (see Chapter 1), but at the Bellevue exposed site there was an abrupt change in the frequencies between the 26-30 mm and 31-35 mm size classes (Figure 19). The frequency of hybrids tended to decrease in the larger size classes (see also Chapter 1), although no clear pattern was observed.

The relationship between the hybrid index values and shell length for all individuals in four samples pooled is shown in Figure 20. Pure *M. trossulus* individuals (hybrid index value -8) were represented in small size classes, as were all the *trossulus*-biased backcrosses (values between -7 and 0). Pure *M. edulis* individuals (value of +8) and *edulis*-biased backcrosses (values between 0 and 7) were distributed throughout the entire size-range, and pure *M. edulis* dominated the largest size classes. When this size range was divided into two parts (from 15 to 35 mm and from 36 to 80 mm), there was a significantly different ($G=41.51$, $df=1$, $p<0.001$) distribution of the number of *edulis*-biased backcrosses (values between 0 and8) and *trossulus*-biased backcrosses (values between -8 and 0). Most of the backcross individuals (80% of the total backcross individuals) were clustered in the smallest size classes (< 35 mm), and of these, 88 % were *trossulus*-biased backcrosses. In the largest size classes (> 35 mm), 89% of the backcrosses were *edulis* biased. The six dots with a value of zero in the hybrid index correspond to 5 F1 and 1 F2 individuals.

## III. 4. DISCUSSION

Hybrid zones provide unique opportunities to study evolutionary processes that maintain reproductive isolation between species (Barton and Hewitt, 1985; Harrison, 1990). Hybrid zones between pairs of species within the *Mytilus edulis* species complex (*M. edulis*, *M. trossulus*, and *M. galloprovincialis*) have received an increasing amount of attention,

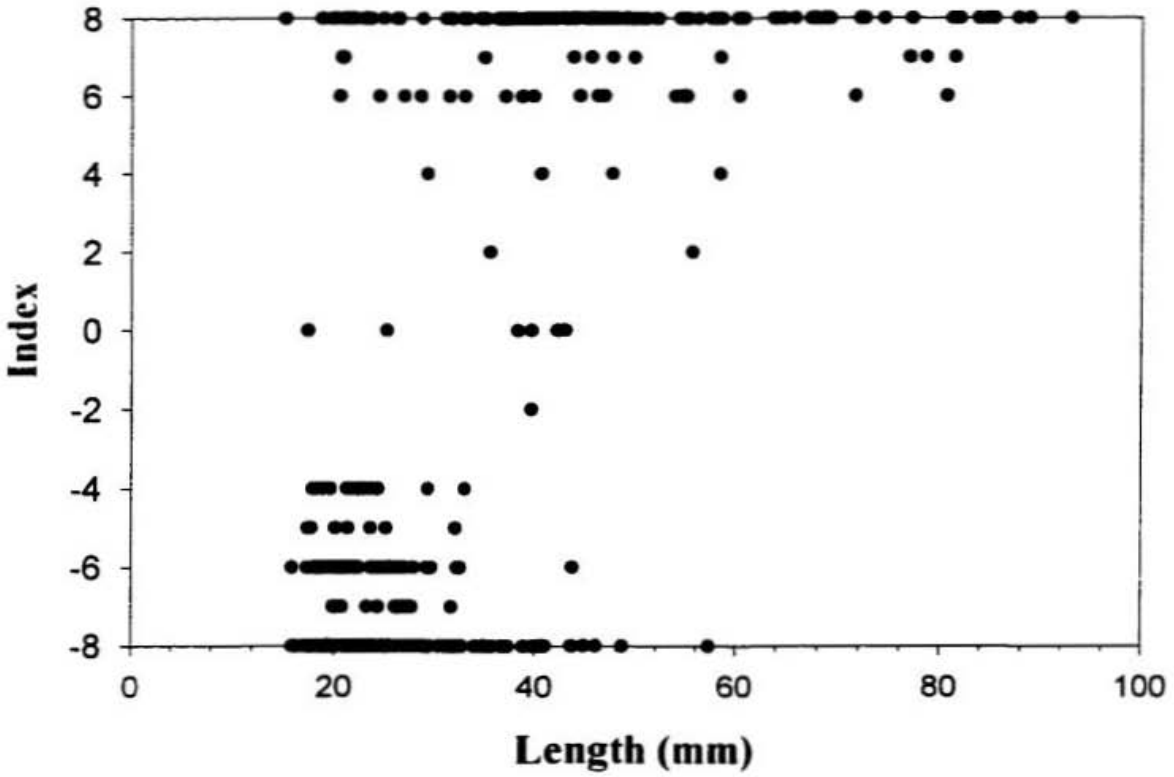



**Fig. 20.** *Mytilus* spp. Species-specific hybrid index values for all individuals (four populations pooled) plotted against shell length. Individuals with a value -8 were regarded as *M. trossulus*, those with a value +8 *M. edulis* and those with a value 0 (and tetraheterozygotes) as $F_1$. Individuals with values from -8 to 0 and 0 to 8 were regarded as *trossulus*-biased backcrosses and *edulis*-biased backcrosses, respectively.

particularly after the recent discovery of double uniparental inheritance (DUI) of the mitochondrial genome and the occurrence of separate maternal and paternal mtDNA lineages (Zouros et al., 1992;Skibinski et al., 1994; Zouros et al., 1994a, 1994b; Stewart et al., 1995; Saavedra et al., 1996). DUI adds an additional layer of complexity to assessing the fitness of hybrid individuals in which fitness may be reduced due to incompatible mixtures of genetic material from two species in the nuclear genome, between F and M mitotypes from different species in males, and incompatibility between mitochondrial and nuclear genomes derived from different species in both sexes. A critical requirement for such studies is the ability to identify individual species and their hybrids. Boecklen and Howard (1997) examined the relationship between the number of genetic markers and the error in discriminating among pure species, $F_1$, and backcross individuals within a hybrid zone. They concluded that as few as four markers were useful for most applications, with about 5% of backcross individuals being misclassified as either $F_1$ or pure species. In the present study, the addition of the two DNA markers to the two enzyme markers resulted in about 13 % of those individuals initially assigned to pure species being reclassified as hybrids. The largest change was a reclassification of pure *M. trossulus* individuals to *trossulus*-biased backcrossed individuals. Based on the four diagnostic markers, the Bellevue/Chance Cove area had a greater frequency of *M. trossulus* (41 %) than of *M. edulis* (33 %), with about 26 % of individuals of hybrid origin. This compares with a value of 23 % hybrids between *M. edulis* and *M. trossulus* detected by Saavedra et al. (1996) for a site in Nova Scotia, which may, however, be an underestimate since only two diagnostic markers were used. In these areas of Atlantic Canada, hybridization and introgression appear to be much lower than between *M. edulis* and *M. galloprovincialis* at various locations in Europe. For instance, Sanjuan et al. (1994) reported 25-50 % hybrids, Hilbish et al. (1994) about 80 % and Comesaña and Sanjuan (1997) between 27 and 49 % (see also Coustau et al.(1991) and Gardner (1994). Low levels of hybridization have also been reported for *M. trossulus* and *M. galloprovincialis* on the Pacific coast of North America (about 5.7% in Sarver and Foltz (1993); between 4-29% in Suchanek et al. (1997). However, in general it is difficult to make comparisons among studies

which use different numbers of markers which vary in their power to discriminate between pure species and hybrids.

Bates and Innes (1995) sampled mussels from the intertidal zone and found the highest frequency of *M. trossulus* at two wave-exposed sites and a higher frequency of *M. edulis* at several sheltered sites. In the present study, there was no consistent pattern in the distribution of *M. trossulus* or *M. edulis* according to wave exposure. Although *M. trossulus* was more common at the exposed Chance Cove site, no difference in the relative frequency of the two species was found between exposed and sheltered sites at Bellevue (but see Chapter 1). All samples were collected subtidally, which may decrease the likelihood of detecting any differences due to wave exposure. Furthermore, the observed differences between the two environments at Chance Cove may simply reflect the high degree of microgeographic variation in the occurrence of these species observed in a previous study (Bates and Innes, 1995).

None of the 281 individuals classified as pure species, based on four markers, contained mtDNA from the opposite species. This observation is consistent with the data of Saavedra et al. (1996) for a hybrid population of *M. edulis* and *M. trossulus* in Nova Scotia. The early block to the introgression of mtDNA between these species was also detected when only two markers were used, since only four males that were misclassified as members of a pure species contained mixtures of nuclear and mitochondrial genomes from each species. Furthermore, no $F_1$ hybrid females were detected and only five males were identified as $F_1$ hybrids, having heterozygous genotypes for the four nuclear markers and heterospecific mitotypes. Two of the $F_1$ hybrids had an *M. edulis* mother and an *M. trossulus* father, and three came from reciprocal matings. Only one $F_2$ hybrid was detected, probably due to the low frequency of matings expected among the very rare $F_1$ hybrids. Most of the hybrid individuals (94/100) were classified as backcrosses. This would be expected if only a few $F_1$ hybrids survive to reproduce and suggests that backcrossed individuals have a greater survival than $F_1$ individuals. *M. trossulus* biased backcrosses were more than twice as common as *M.*

*edulis* biased backcrosses, which probably reflects the greater frequency of pure *M. trossulus* in this area. Among the backcross individuals, males with heterospecific mitotypes and species-specific discordances between mitotype and the nuclear genome of both sexes occurred less frequently than would be expected if there were equal fertility and survival among all classes. These observations are consistent with those of Saavedra et al. (1996), who found approximately equal frequencies of both *M. edulis*-biased and *M. trossulus*-biased backcrosses in a sample, again probably a function of the approximately equal frequencies of the two species in their sample.

Samples from coexisting populations of *M. galloprovincialis* and *M. edulis* show a consistent pattern in which *M. galloprovincialis* is more frequent among larger individuals and *M. edulis* more frequent among smaller individuals (Gardner and Skibinski, 1988; Gardner et al., 1993). Higher growth and survival of *M. edulis* compared with *M. trossulus* may explain the increase in the frequency of *M. edulis* with increasing shell length observed in the Newfoundland populations. Mallet and Carver (1995) recorded only small differences in growth rate between *M. trossulus* and *M. edulis*, suggesting that the observed changes in species frequency with shell length may reflect differential survival. The various size classes include different cohorts of larvae settling in the area over several years. The observed increase in frequency of *M. edulis* with increasing shell length may simply reflect a much higher frequency of *M. edulis* in the oldest cohort at the time of settlement. Although the frequency of each species in the newly settled spat may vary from year to year and from site to site, the consistency of the pattern in each of the four samples, the distribution of *M. edulis* and *edulis*-biased backcrosses in the whole size range (Figure 20) and the regular occurrence of both species in this area (Koehn et al., 1984; Bates and Innes, 1995) do not support this explanation. Bates (1992) also found a higher frequency of *M. edulis* in the larger size classes and a higher frequency of *M. trossulus* in the smaller size classes at a different site in eastern Newfoundland. Interestingly, the presence of backcross individuals with a nuclear gene

composition biased towards one species or the other supports the trends observed in the pure species.

Factors responsible for blocking the introgression of mtDNA between *M. edulis* and *M. trossulus* in Atlantic Canada do not appear to be operating as strongly in the European *Mytilus* species hybrid zones. Populations of both *M. trossulus* and *M. galloprovincialis* contain individuals with *M. edulis* mtDNA (Rawson and Hilbish, 1998). The asymmetric introgression of mtDNA between *M. edulis* and *M. galloprovincialis* has been explained by previous observations of directional selection in the hybrid zone favouring alleles of *M. galloprovincialis* (Gardner and Skibinski, 1988; Skibinski and Roderick, 1991; Gardner et al., 1993). Thus hybrid individuals with *M. edulis* mtDNA and a predominance of *M. galloprovincialis* nuclear alleles would be favoured over hybrid individuals with *M. galloprovincialis* mtDNA and a predominance of *M. edulis* nuclear alleles. This mechanism might predict that any introgression of mtDNA in Atlantic Canada mussel populations would be in the direction of *M. trossulus* into *M. edulis*. Moreover, in small individuals we would expect a greater number of backcrosses with a *trossulus* nuclear genome and an *edulis* mtDNA genome (introgression in the direction of *M. edulis* into *M. trossulus*). Thus it seems that the incompatibility between mitochondrial and nuclear genomes is greater between *M. trossulus* and *M. edulis* than between *M. edulis* and *M. galloprovincialis*. The greater degree of mtDNA introgression observed in Europe than in North America may be due to a longer period of contact between these *Mytilus* species compared with the Atlantic Canada *M. edulis* and *M. trossulus*, or simply to a different degree to which natural selection is acting against each species on each continent. In addition, the different degree of divergence between the *Mytilus* species results in a different relationship between mitochondrial DNA and nuclear genomes for each *Mytilus* taxon (see also Discussion in Chapter 1). Further long-term studies are required of the different life history stages of both *M. trossulus* and *M. edulis* in relation to the environment, in order to identify the factors responsible for maintaining the integrity of each species.

# IV. CHAPTER 3

## Analysis of the reproductive cycles of *Mytilus edulis*, *M. trossulus* and their natural hybrids in eastern Newfoundland

### IV.1. INTRODUCTION

Most *Mytilus* populations, like those of many temperate water bivalves, have a seasonal pattern of reproduction. The seasonal reproductive period starts with a gametogenic phase, followed by the release of gametes (spawning) in which the reproductive follicles become partially or completely emptied. Apart from a few hermaphrodites the sexes in *Mytilus* are separate and most populations contain approximately equal numbers of males and females (Seed, 1976; Kautsky, 1982; Sprung, 1983). In *Mytilus* the gametogenic process occurs mainly in the mantle tissue, but reproductive tissue can also be found in the visceral mass and mesosoma (Bayne et al., 1978; Lowe et al., 1982; Newell et al., 1982;).

Several studies have been carried out on the reproductive cycle of *Mytilus edulis* (Thompson, 1979, 1984a, 1984b; Newell et al., 1982; Gilkinson, 1983; Hilbish and Zimmerman, 1988) and *M. trossulus* (Suchanek, 1981; Emmert et al., 1987; Blanchard and Feder, 1997) on the east and west coasts of North America. Previous studies of mussels on the northern Pacific coast were presumed to investigate the life history of *M. edulis*, but recent literature indicates that it is *M. trossulus* rather than *M. edulis* that ranges along the Pacific coast from California to Alaska (Heath et al., 1995; Suchanek et al., 1997).

Several authors have mentioned the importance of detecting the spawning events between two species of bivalves in a hybrid zone (Ahmad and Beardmore, 1976; Skibinski et al., 1980; Seed and Suchanek, 1992; Gardner, 1994; Eversole, 1997; Grant et al., 1998). It is known that any shift in the spawning events within a species or between two species will

significantly affect the fertilization success (Babcock et al., 1992; Levitan and Petersen, 1995). Gametogenic cycles in marine invertebrates are generally believed to be strongly influenced by seasonal variation in temperature (Seed, 1976). Therefore, mussel populations along a latitudinal (temperature) gradient are expected to show differences in their reproductive cycles (Sastry, 1979). Mussel populations inhabiting high latitudes are expected to have more synchronous spawnings, because of the less extended period of high water temperature. However, variation in nutrient supply (Newell et al., 1982; Arsenault and Himmelman, 1998) and the genotype of the individual (Rodhouse et al., 1986; Hilbish and Zimmerman, 1988) may also have a significant influence upon gametogenic cycles in many species of marine invertebrates. Moreover, external fertilization (such as in *Mytilus*) may create further problems in hybridization because higher gamete dispersal can reduce the chances of fertilization (Hodgson, 1988).

Several studies in which spawning events from several species are synchronous have been reported (McEuan, 1988; Gardner and Skibinski, 1990a; Babcock et al., 1992; Van Veghel, 1993). Among the main factors that can reduce the amount of hybridization in closely related species are habitat separation and the timing of spawning (Strathmann, 1981; Gardner and Skibinski, 1990a;Uehara et al., 1990; Gardner, 1992). Also, gametic barriers to hybridization are particularly important as reproductive isolating mechanisms (Lessios and Cunningham, 1990; Palumbi and Metz, 1991; Grant et al., 1998; see also Chapter 4). Despite all these possible isolating mechanisms, invertebrate hybrids are abundant at some locations, e.g., the quahogs *Mercenaria mercenaria* and *M. campechiensis* (Dillon and Manzi, 1989; Bert et al., 1993) and the sea urchins *Echinus esculentus* and *E. acutus* (Hagström and Lönning, 1961), composing 31-88% and 10-20% of the populations, respectively. Coustau et al. (1991) and Viard et al. (1994) also described the genetic structure of the *M. edulis-M. galloprovincialis* hybrid zone on the coast of France, and found that the degree of hybridization varied tremendously, depending on the site studied. More stable genotypic

structures have been determined for *Mytilus* hybrid zones in southwest England (Gardner and Skibinski, 1988; see also Chapter 1 and 2).

Studies carried out in S.W. England report the presence of asynchrony in the spawning events between *M. edulis* and *M. galloprovincialis* at certain sites within the hybrid zone (Skibinski et al., 1980; Skibinski, 1983; Gardner and Skibinski, 1990a). This asynchrony in the spawning activity of these species is more drastic in sympatric populations localized in the northern extremity of the hybrid zone (Gardner and Skibinski, 1990a). This observation may therefore explain why some studies within the S.W. England hybrid zone found a significant asynchrony in the spawning activity between the two mussel species, while others found very little difference in the timing of spawning (see review by Gardner, 1994). Lubet (1957) and Hrs-Brenko (1971) noted that the spawning activities of the two mussel types in France occurred simultaneously, which together with a high frequency of morphologically intermediate forms along the Atlantic coast of France (Seed, 1972) suggests that hybridization is common between these two species.

Several studies on the east coast of North America have described the presence of *M. trossulus* (Koehn et al., 1984; Bates and Innes, 1995; Mallet and Carver, 1995; Saavedra et al., 1996; Comesaña et al., 1998), rather than *M. edulis* alone, as previously thought (Seed, 1976). *M. edulis* and *M. trossulus* are found living sympatrically in Nova Scotia and Newfoundland, although information on the reproductive timing for *M. edulis*, *M. trossulus* and their hybrids on the east coast of North America is limited.

The objectives of the present study were to analyze and compare the reproductive cycles of *M. edulis*, *M. trossulus* and their hybrids at four sites in eastern Newfoundland. The gonadosomatic index was determined and, using stereological techniques, histological sections from the females and male gonads were studied by means of image analysis.

## IV.2. MATERIAL AND METHODS

### IV.2.1. STUDY SITES AND SAMPLING

At approximately 15 day intervals from May - October 1996, a representative sample of about 400-500 mussels was collected subtidally by SCUBA divers from each site at Bellevue (BP, BE) and Chance Cove (CP, CE) (Fig. 1). Mussels were immediately transported to the laboratory, where they were maintained in running filtered sea water within 0.5°C of the ambient temperature at Bellevue (Thompson, 1984b). During the three days following each field sampling, 40 mussels (38-42 mm shell length) from each site (BE, CP and CE) were then dissected carefully to separate the mantle (and in some cases the mesosome) (male and female) from other soft tissues. This size range was chosen to increase the chance of including both species and some of the hybrids (see Chapter 2). Mantles from 100 mussels from BP (shell length 4.22 to 101 mm) were carefully separated from the rest of the body tissues. This size range was chosen in order to relate gamete production to body size. In order to establish the genotype of the mussels (*M. edulis*, *M. trossulus* or hybrid), a very small piece of mantle border (approximately 20-30 mg) from each individual was stored in 95% ethanol at -20°C for later DNA analysis (see chapter 1).

### IV.2.2. LABORATORY ANALYSIS

#### IV.2.2.1. Histological analysis

One of the mantle lobes from each dissected mussel was subsampled by cutting a transverse section midway along the anteroposterior axis. This piece of the mantle was weighed and preserved in Bouin's fixative according to Lowe et al. (1982). The sample was

dehydrated in an ascending alcohol series, cleared in xylene and embedded in paraffin wax. Serial sections (7-μm) were cut, stained with hematoxylin and counterstained with eosin. Only one section of the mantle tissue from each individual was used, since previous studies have shown that the mantle in *Mytilus* is relatively homogeneous (Lowe et al., 1982; Newell et al., 1982; Bayne et al., 1985).

Terminology for stages of gametogenesis was adapted from King et al. (1989) and Kiyomoto et al. (1996) after partial modification. Gonadal cells were classified into the following four stages: Developing (follicles occupy a large part of the mantle; individuals restoring their gonads after a partial spawn are included in this category), ripe (follicles full of oocytes in female and packed lamellae of ripe spermatozoa in male), spent (follicles begin to collapse and degenerate), and resorbing-resting animals.

#### IV.2.2.2. Gonadosomatic index (GSI)

Following the removal of the tissue section for histological analysis, the remainder of the mantle was weighed, re-weighed after drying at 80° C to constant weight for 48 h and cooled in a desiccator. The ratio of wet to dry weight for this portion of the mantle was used to correct for the weight of the tissue section which had been removed for histological purposes. This adjustment allowed the total mantle dry weight to be estimated. The portion of the body excluding the mantle was also dried and weighed. The sum of body and mantle weights was used to calculate whole-mussel dry weight. The GSI of an individual was then calculated by dividing the gonad dry weight by the whole-mussel dry weight and multiplying by 100. A male GSI (MGSI) and a female GSI (FGSI) were also calculated.

#### IV.2.2.3. Stereological methods

In *Mytilus* the mantle tissue is composed of connective tissue (storage cells), blood spaces and germinal cells during the reproductive season (Lowe et al., 1982). All these cells

infiltrate the mantle tissue homogeneously (Bayne et al., 1985). In order to apply stereological techniques it is important that the tissue sections analyzed are representative of the whole tissue mass (Lowe et al., 1982). Therefore, the spatial homogeneity of the cell types within the mantle signifies that a section from any part of the mantle lobe is equally representative of the overall cellular composition.

The fractional area of the tissue that is composed of gametes (gamete volume fraction, GVF) was measured quantitatively using Optimas 6.2 image analysis software and a Nikon stereomicroscope, following standard stereological methods (Lowe et al., 1982). The colour image acquired was analyzed after adjusting the threshold by sampling area screen objects set by the operator (Heffernan and Walker, 1989). A threshold is a set of intensity values that separates pixels of interest from the rest of the image. The percentage of mantle volume occupied by the oocytes was calculated from the relation between the number of pixels occupied by the oocytes and the total pixels in the field. Five sections of 1,295,000 $\mu m^2$ taken randomly from each individual (histological slide) were measured. This procedure provided an estimate of the volume of the mantle that is composed of gametes. GVF can vary between zero, for a reproductively inactive individual, to values approaching 100 % for maximal reproductive condition, and provides a measure of the relative maturity of the gonad. However, it does not provide a quantitative estimate of reproductive output (i.e., an individual with very few gametes may have a high GVF if the gonad is small). To correct this problem, the proportion of the total weight devoted to gametes was also calculated. Total mantle dry weight was multiplied by the GVF to provide an estimate of the dry weight of gametes for each individual.

For each individual, 50 to 60 individual oocytes with nucleolus (10 from each histological section) were also analyzed using the image analyzer. For each oocyte, the area and longest axis were recorded. For direct measurements of eggs, mussels were induced to spawn. The mussels were washed in cold sea water (SW), placed in a shallow tray of filtered SW and subsequently exposed to rising temperatures (up to 22 °C). Once spawning was initiated, the individuals were placed in separate containers with sterile SW for completion

of spawning (see also Chapter 4). Any eggs released were collected, put into 10-ml tubes and fixed with 95% ethanol. The mussels from which eggs were collected were then genotyped and then the eggs from *M. edulis*, *M. trossulus* and hybrids measured using the image analysis system. These measurements produced an egg size-frequency distribution for each individual.

These results may be affected by two types of error, systematic and statistical. Systematic errors occur during fixation, tissue processing, or measurement of areas. The Bouin's fixative, xylene and ethanol do not cause significant change in oocyte profiles, but small sections of tissue (with or without oocytes) are generally lost in the dewaxing process. It is difficult to quantify this loss, but it should be constant because all samples were treated similarly. Statistical errors are normally minimized when sufficient measurements are taken. In this case 50 to 60 individual oocytes provided minimal errors, depending on the state of ripeness of the gonad and the degree of synchronization between individuals.

### IV.2.3. STATISTICAL ANALYSIS

Observed sex ratios were tested against a 1:1 ratio with the Chi-Square statistic. Normality of variables was determined using the Lillifors K-S (Wilkinson, 1991). Analysis of gonadosomatic index (GSI) values was performed by ANOVA ($\alpha$ =0.05) and Tukey's Studentized Range Test (SRT; $\alpha$ = 0.05). GVF and proportional gamete weight values were arcsine-transformed and analyzed by two-way analysis of variance (ANOVA). The two main effects were date of collection and genotype (*M. edulis, M. trossulus*). If there is a difference in the timing of reproductive events among the two species, the interaction between the two main effects in this analysis will be significant. Oocyte and egg size differences among species were tested using two way ANOVA (main effects: date of collection and genotype). All statistical analyses were carried out with Systat 5.1 (Wilkinson, 1991).

## IV.3. RESULTS

### IV.3.1. REPRODUCTIVE CYCLE

The histological sections of the male and female gonads for *M. edulis*, *M. trossulus* and their hybrids showed advanced gametogenesis at the first sampling (May 20). From late May to late June very few mussels were observed in the active spawning stage. Histological sections of female and male hybrids of *M. edulis* and *M. trossulus* showed normal gonadal development, ripening and spawning (Figures 21, 22). Ripe animals for both sexes were dominant in June for *M. edulis* and hybrids, while *M. trossulus* showed a more extended period of spawning (Figure 23). A large peak of spawning mussels was registered in late July at all sites sampled. Indeterminate *M. edulis* and hybrid mussels with no gametogenic activity predominated in late September and October. These gonad sections showed no follicles at all or only a few very contracted follicles between connective cells, and resorption of the undischarged eggs in follicles of the females. Some gonad sections from *M. trossulus* and some hybrids during August and September showed partially spawned female and male follicles. By the time of the final sampling in October, more than 50% of mussels were in the resorbing-resting stage.

### IV.3.1. SEX RATIO

#### IV.3.1.1. *Mytilus edulis*

Females outnumbered males. A total of 698 *M. edulis* mussels was sampled, of which 342 (49.0%) were females, 299 (42.8%) were males, six were hermaphroditic (0.86%) (Figure 24), and 51 (7.3%) were undifferentiated. The sex ratio (1.14F:1M, n = 641) did not differ significantly ($P>0.01$) from the expected ratio of 1:1.

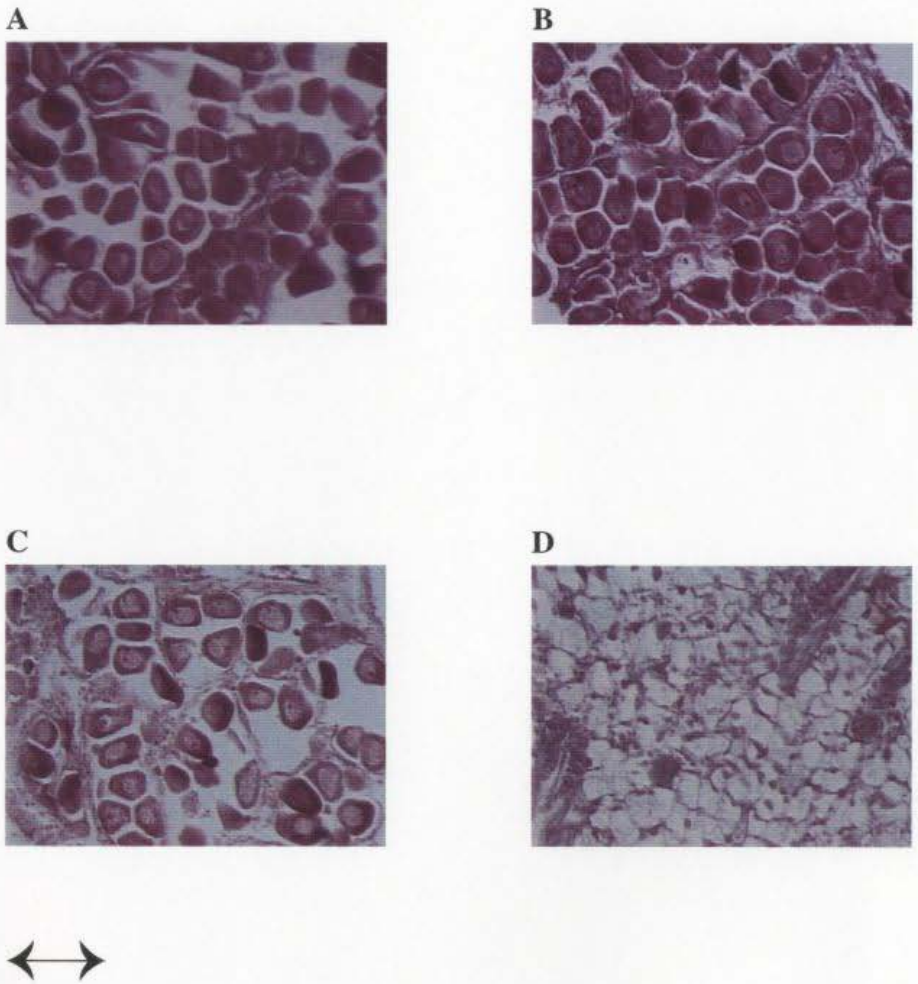



Fig. 21. Photomicrographs of gonadal stages of the hybrid female between ***M. edulis*** and ***M. trossulus***. (A) Stage 1; development of ova to maturity. (B) Stage 2; ripe stage. (C) Stage 3; active spawning where reproductive follicles are partially empty of ova. (D) Stage 4; spawned out condition where follicles are empty of ova and resorption of unspawned gametes is occurring. Scale bar (↔) = 100 µm.

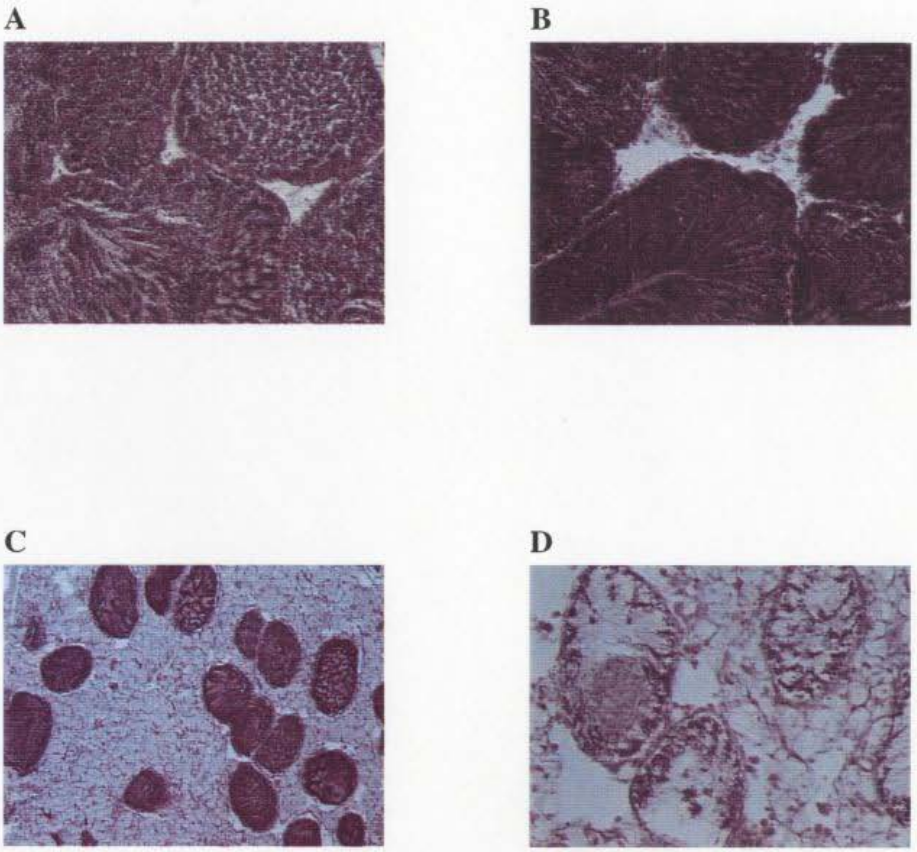



**Fig. 22.** Photomicrographs of gonadal stages of the hybrid male between ***Mytilus edulis*** and ***M. trossulus***. (A) Stage 1; spermatogenesis in the follicles. (B) Stage 2; mature spermatozoa in the follicles. (C) Stage 3; partially spent. (D) Stage 4; spawned out condition where follicles are empty and resorption of unspawned gametes is occurring.

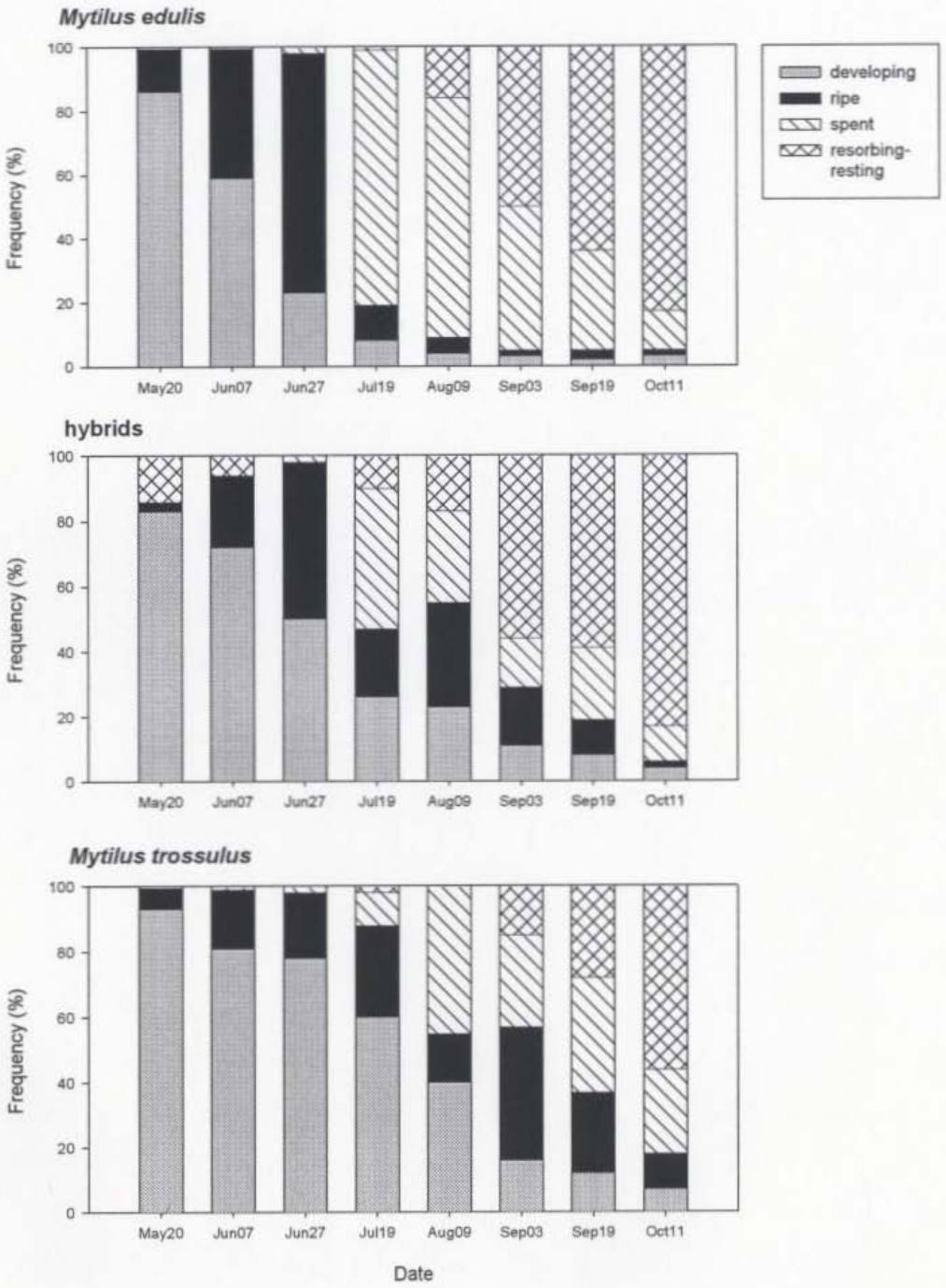



**Fig. 23.** Frequency distribution of gonadal maturation stages in *Mytilus edulis*, hybrid and *M. trossulus* mussels including both sexes during the 1996 reproductive season. For details of each stage see Material and Methods.

#### IV.3.1.2. *Mytilus trossulus*

Females again outnumbered males. A total of 782 *M. trossulus* mussels was sampled, of which 412 (52.7%) were females, 341 (43.6%) were males, five were hermaphroditic (0.6%), and 24 (3.1%) were undifferentiated. The sex ratio (1.2F:1M, n = 753) did not differ significantly (P>0.01) from the expected ratio of 1:1.

#### IV.3.1.3. Hybrids

In the hybrids males outnumbered females. A total of 280 *M. trossulus* mussels was sampled, of which 109 (38.93) were females, 143 (51.07%) were males, and 28 (10.0%) were undifferentiated. The sex ratio (1.31F:1M, n = 252) did not differ significantly (P>0.01) from the expected ratio of 1:1.

### IV.3.2. GONADOSOMATIC INDEX

This index is primarily affected by the accumulation and release of gonadal material as well as the utilization of stored energy products during the winter months. Both the male (MGSI) and female (FGSI) in *M. edulis*, *M. trossulus* and hybrids showed a steady decline in this index after the spawning in July (Figure 25), although *M. edulis* showed an increase during early autumn, after a more abrupt spawning occurred in late July, which may indicate storage of nutrients in the gonad (Blanchard and Feder, 1997).

### IV.3.3. GAMETE VOLUME FRACTION

The reproductive condition (GVF) of male and female mussels from each genotype was analyzed separately to determine the synchrony of their cycles. Comparison of the

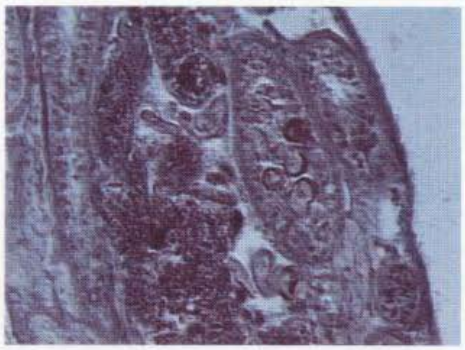


**Fig. 24.** Photomicrograph of gonad section from a hermaphrodite mussel. (***Mytilus edulis*** from Chance Cove protected).

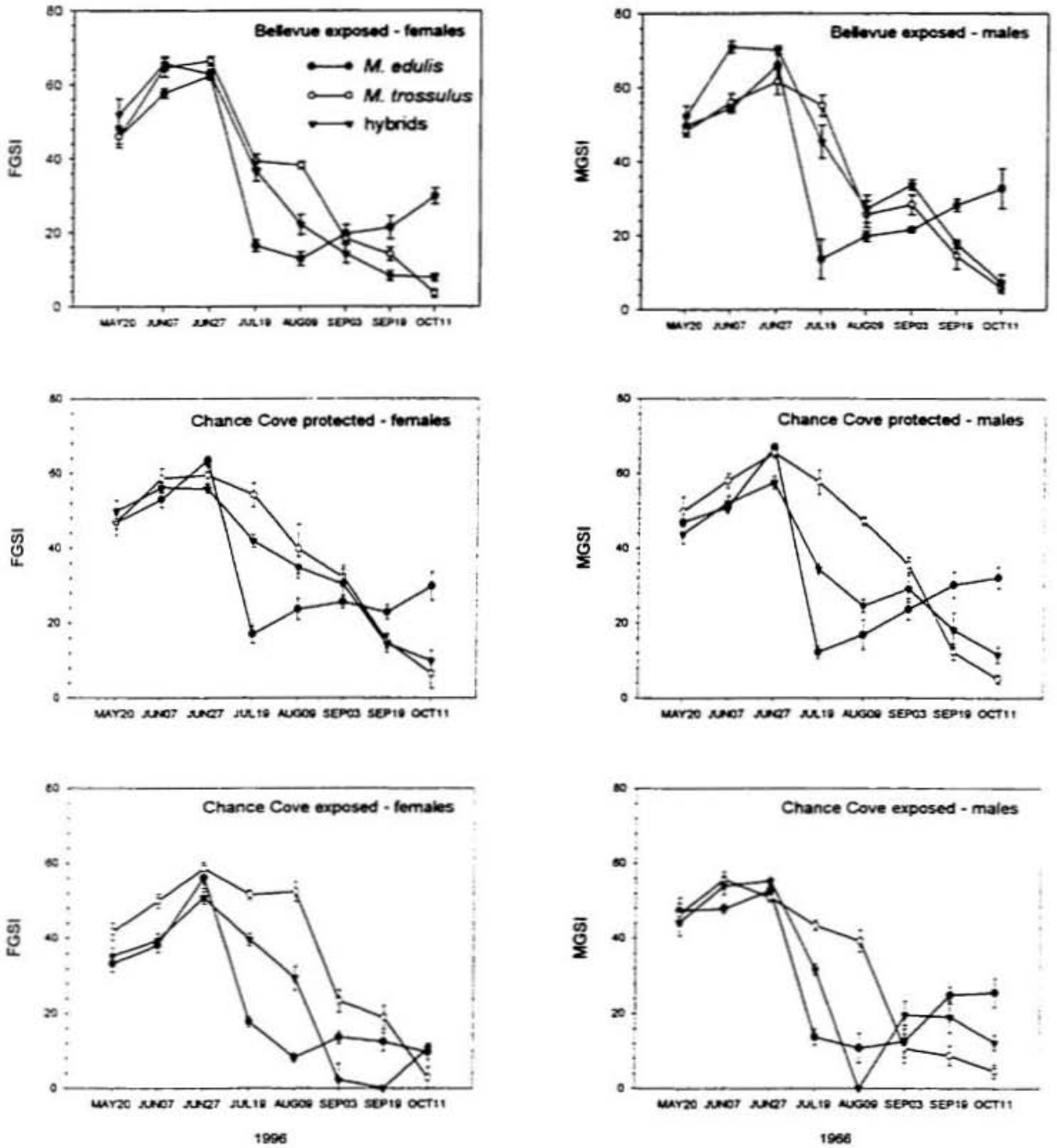



**Fig. 25.** Gametosomatic index (female = FGSI; male = MGSI) in *Mytilus* (*M. edulis*, hybrids, *M. trossulus*) during the 1996 reproductive season at three different sites in eastern Newfoundland.

relative volume fraction for each species indicated that gonadal development in *M. edulis* was different from *M. trossulus* and the hybrids (Figure 26). In mature female mussels (June 27), ripe sex cells made up comprised about 85% of the gonad in *M. trossulus*, which was significantly different from *M. edulis* and hybrids, in which GVF was generally less than 70% (Figure. 26). Subsequent reductions in mean GVF in August and September supported the observation of spawned individuals of these species. The GVF in October was nearly zero, indicating that the resorption of gametes was nearly complete.

Two way ANOVA (Table 13) indicate that there are significant differences ($P < 0.001$) among species (*M. edulis*, *M. trossulus* and hybrids) in the proportion of gonad occupied by gametes.GVF was higher in *M. trossulus* than in the hybrids or *M. edulis* (Fig. 26). There was also a significant date x species interaction ($P < 0.001$), indicating that the reproductive cycle, as indicated by GVF, differed among the species (Table 13, Figs. 23, 25, 26), owing to a more gradual spawning over several weeks by *M. trossulus* and some of the hybrids at all sites, while *M. edulis* showed an abrupt spawning during late July (Fig. 23). The difference in mean GVF between female *M. trossulus* and *M. edulis* (and their hybrids) was considerable (Fig. 26). On June 27, the mean GVF of 86.2% for *M. trossulus* was significantly greater (t-test, $P < 0.001$) than that of 67.9% for *M. edulis*.

A comparison among species between the dry weight of gametes just before spawning (June 27) (Fig. 27), revealed that *M. trossulus* had a significantly higher gamete weight per gonad than *M. edulis* (t-test, $P<0.05$) and hybrids ($P<0.05$), with the exception of the Chance Cove exposed site, in which no significant differences ($P>0.05$) were found among the species. The significant differences found during July 19 are due to the fact that *M. trossulus* and some hybrids spawned gradually over several weeks in comparison with *M. edulis*.

### IV.3.4. OOCYTE DIMENSIONS

The longest axis of the mature oocyte (June 27) in histological sections of *M. edulis*

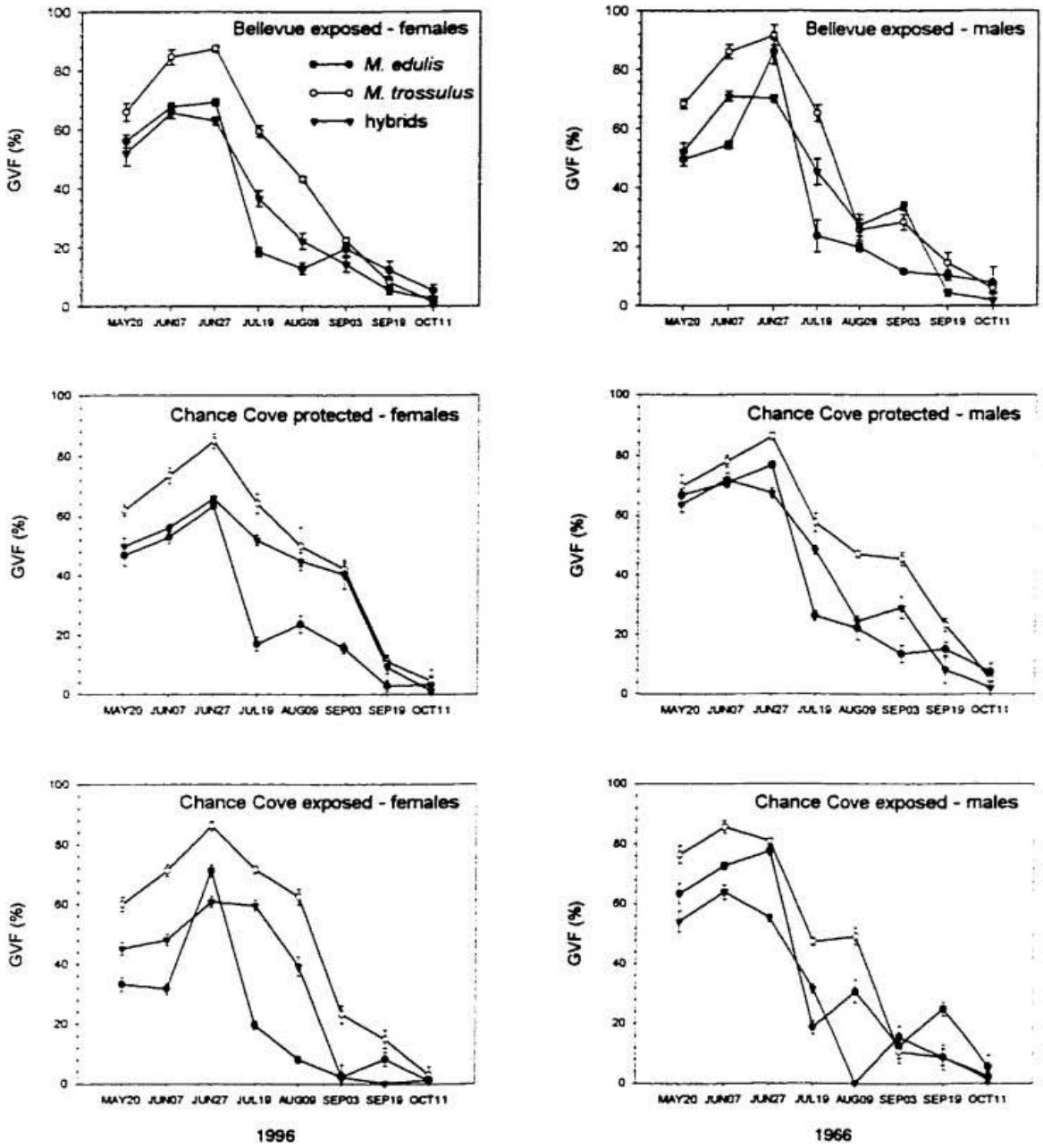



**Fig. 26.** Mean reproductive condition (GVF) ± S.E. for female and male *Mytilus (M. edulis*, hybrids, *M. trossulus*) at three different sites in eastern Newfoundland during the 1996 reproductive season..

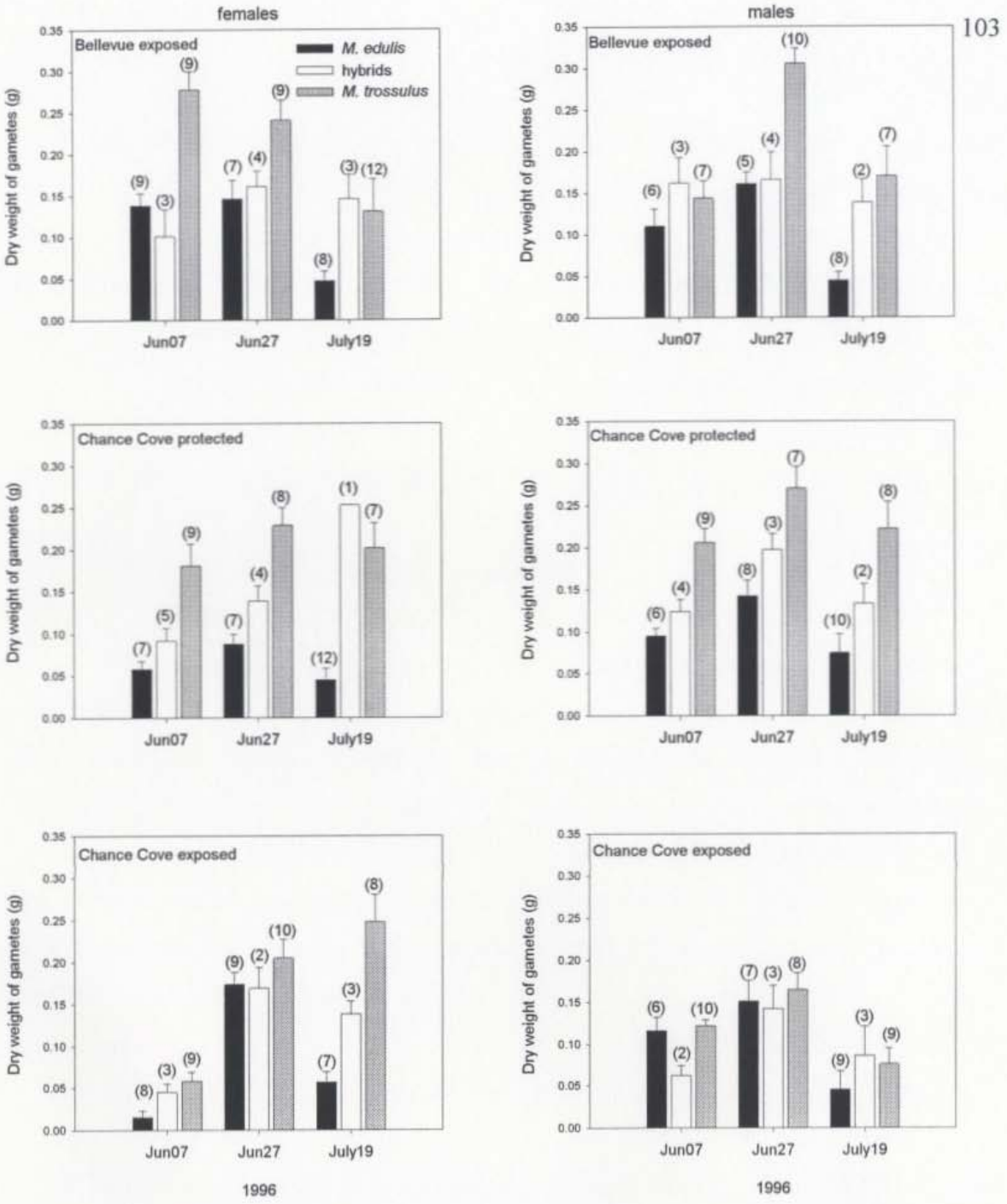



**Fig. 27.** Comparison of the dry weight of gametes among *M. edulis*, hybrids and *M. trossulus* at three sampling dates during the pre-spawning and spawning stage at three sites in Newfoundland. Number in parentheses indicates the number of mussels analyzed.

varied between 52.0 and 82.9 μm, with a mean of 62.1 μm. On the same sampling date *M. trossulus* mature oocytes ranged between 43.7 and 58 μm with a mean of 55.3 μm. The nested ANOVA carried out on values for egg areas measured from histological sections during the two sampling dates before spawning occurred showed in most cases a significant variation among mussels within species. The variation among species, however, is still significant, notwithstanding differences among mussels (Table 14, Figure 28).

There were significant differences ($P<0.001$) among species in the lengths and areas of eggs obtained by inducing spawning in the laboratory (Figures 28 C,D). *M. edulis* presented the largest eggs (Figure 28, Table 14).

### IV.3.5. SIZE AT FIRST MATURATION

The age at first potential reproduction was determined by direct observation and measurement of gonad weights for mussels of various sizes from the Bellevue protected site. The smallest mussels with differentiated sex were *M. trossulus*, a male of 6.9 mm and a female of 8.9 mm shell length, together with several other mussels less than 10 mm long which were observed during June at the Bellevue protected site. For hybrids and *M. edulis*, earliest gonad development and gamete storage in the mantle tissue occurred at about 12-15 mm shell length. The shell size at first maturation seems to be lower in *M. trossulus* than in hybrids and *M. edulis* at these locations.

### IV.3.6. FECUNDITY AND DRY WEIGHT OF THE SOFT TISSUES

Figure 29 shows that there was a significant relationship ($P < 0.01$) between the dry weight of the gonad and the total dry weight of the animal at four sampling dates from mussels (all species pooled) from the Bellevue protected site.

**Table 13.** Results of two way ANOVA for testing variation in the gamete volume fraction (GVF) in females and males among ***Mytilus*** species (***M. edulis***, hybrids and ***M. trossulus***) during the 1996 reproductive season at three sites (Bellevue exposed: BE; Chance Cove protected: CP; Chance Cove exposed: CE) in eastern Newfoundland..

| Site | Sex | Source | *df* | SS | F-ratio | *p* |
|---|---|---|---|---|---|---|
| BE | females | Date | 7 | 106849.94 | 135.237 | < 0.001 |
| | | Species | 2 | 4670.06 | 20.688 | < 0.001 |
| | | Date x species | 14 | 10225.42 | 6.471 | < 0.001 |
| | | Error | 326 | 36795.68 | | |
| BE | males | Date | 7 | 74941.77 | 178.566 | < 0.001 |
| | | Species | 2 | 6614.39 | 55.161 | < 0.001 |
| | | Date x species | 14 | 14577.07 | 17.637 | < 0.001 |
| | | Error | 211 | 12650.54 | | |
| CE | females | Date | 7 | 111086.03 | 135.211 | < 0.001 |
| | | Species | 2 | 3657.23 | 15.580 | < 0.001 |
| | | Date x species | 14 | 9220.34 | 5.611 | < 0.001 |
| | | Error | 267 | 31337.16 | | |
| CE | males | Date | 7 | 94502.32 | 93.389 | < 0.001 |
| | | Species | 2 | 3847.43 | 13.307 | < 0.001 |
| | | Date x species | 14 | 10051.80 | 4.967 | < 0.001 |
| | | Error | 251 | 36284.67 | | |
| CP | females | Date | 7 | 65220.06 | 59.719 | < 0.001 |
| | | Species | 2 | 5808.56 | 18.615 | < 0.001 |
| | | Date x species | 14 | 9075.22 | 4.155 | < 0.001 |
| | | Error | 281 | 43840.69 | | |
| CP | males | Date | 7 | 100775.64 | 99.833 | < 0.001 |
| | | Species | 2 | 5166.66 | 17.914 | < 0.001 |
| | | Date x species | 14 | 5211.71 | 2.581 | < 0.005 |
| | | Error | 266 | 38358.83 | | |

**Table 14.** Results of nested ANOVAs for testing variation in egg area and egg length among *Mytilus* species (***M. edulis***, hybrids and ***M. trossulus***).

| Date | Factors | Source | *df* | SS | F-ratio | *p* |
|---|---|---|---|---|---|---|
| June 07 | Egg area | Species | 2 | 11.110 | 99.818 | < 0.001 |
| | | Mussel {species} | 6 | 2.659 | 7.962 | < 0.001 |
| | | Error | 1341 | 74.632 | | |
| | Egg length | Species | 2 | 3.258 | 64.605 | < 0.001 |
| | | Mussel {species} | 6 | 0.767 | 5.070 | < 0.001 |
| | | Error | 1341 | 33.816 | | |
| June 27 | Egg area | Species | 2 | 22.859 | 311.254 | < 0.001 |
| | | Mussel {species} | 6 | 0.581 | 2.638 | 0.015 |
| | | Error | 1341 | 49.244 | | |
| | Egg length | Species | 2 | 4.774 | 134.827 | < 0.001 |
| | | Mussel {species} | 6 | 0.196 | 1.849 | 0.086 |
| | | Error | 1341 | 23.743 | | |
| Spawning | Egg area | Species | 2 | 22.430 | 297.113 | < 0.001 |
| | | Mussel {species} | 12 | 0.380 | 1.514 | 0.113 |
| | | Error | 735 | 15.375 | | |
| | Egg length | Species | 2 | 2.182 | 117.222 | < 0.001 |
| | | Mussel {species} | 12 | 0.240 | 2.152 | 0.012 |
| | | Error | 735 | 6.839 | | |

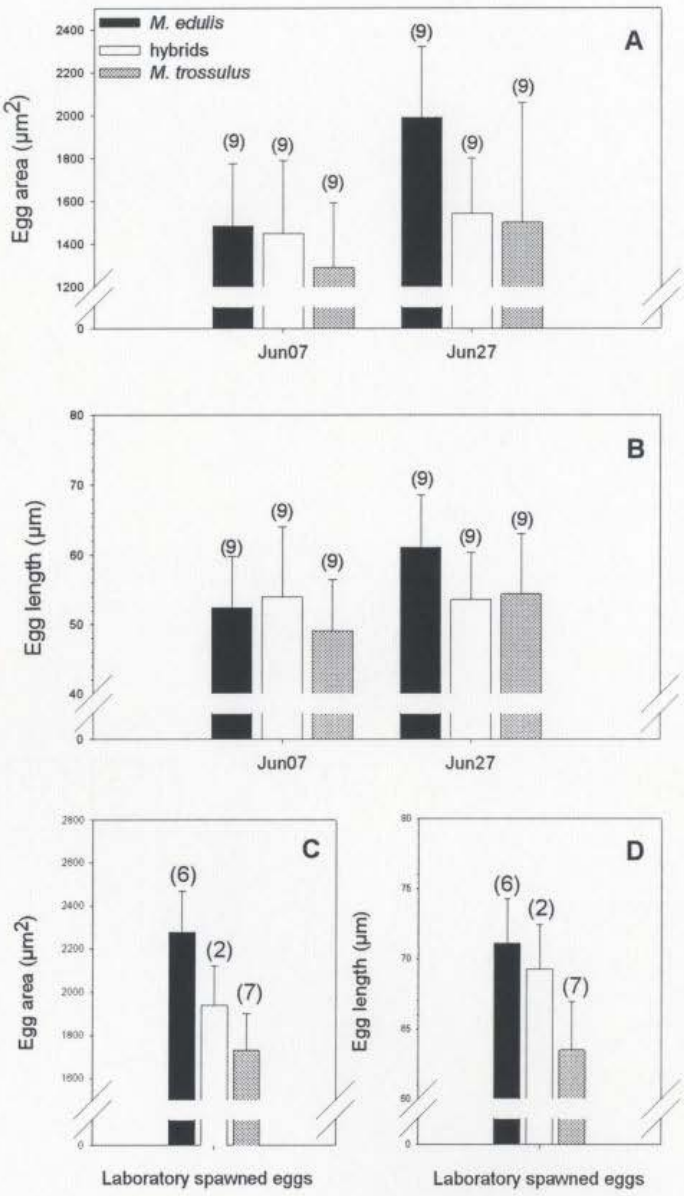



**Fig. 28.** Comparison of mean ± 1 S.D. of egg area µm$^2$ (A) and egg length µm (B) among *M. edulis*, *M. trossulus* and hybrids determined at two dates during the pre-spawning stage from histological slides and the determination of egg area (C) and egg length (D) from laboratory spawned eggs. Number in parentheses indicates the number of mussel used (50 eggs from each mussel were measured).

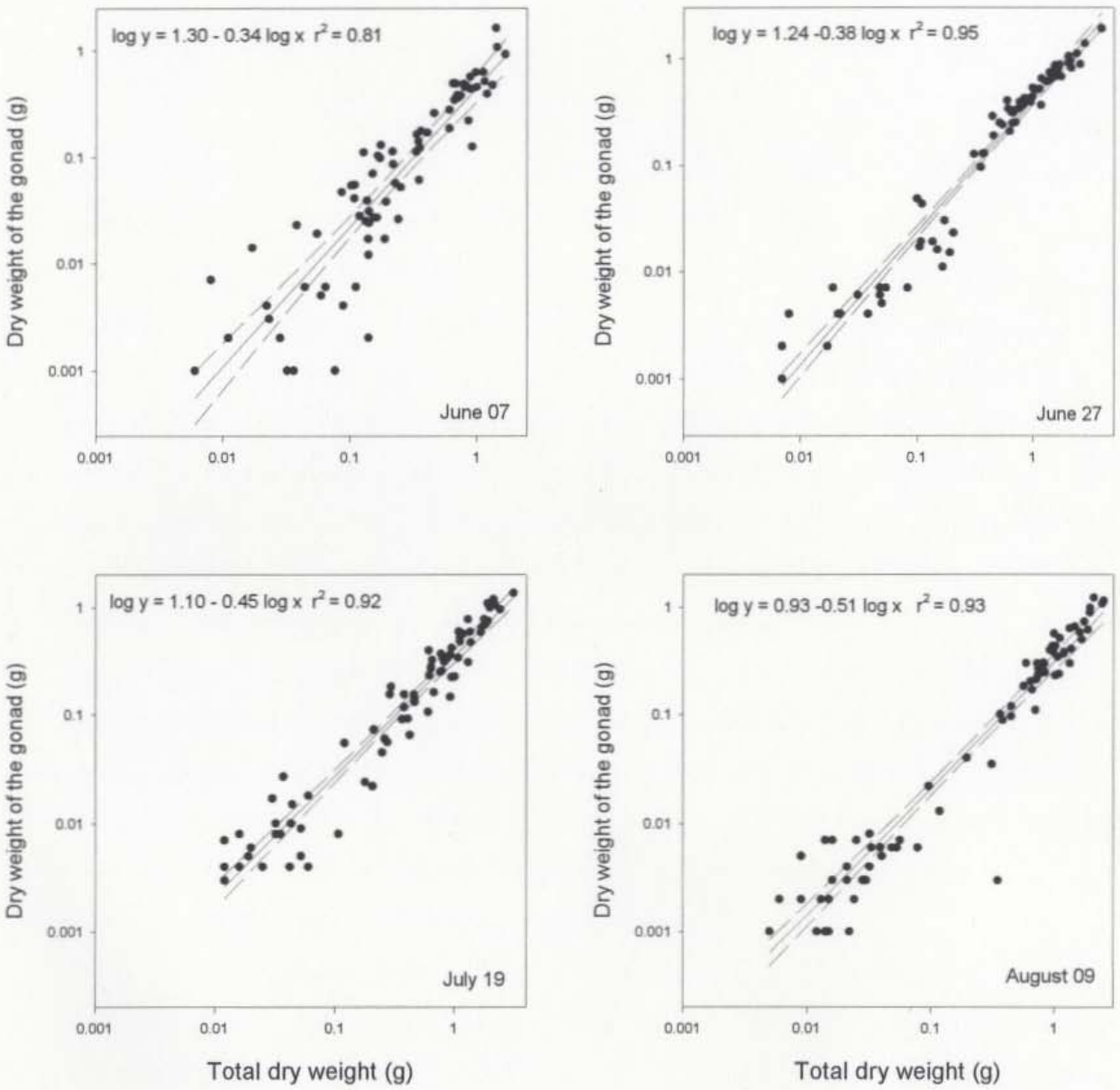



**Fig. 29.** Relationship between fecundity (estimated as the dry weight of the gonad) and the dry weight of the soft tissues in pooled *Mytilus* sp. From Bellevue protected, during the 1996 reproductive season. Lines show 95% C.I.

## IV.4. DISCUSSION

The sex ratio of *M. edulis*, *M. trossulus* and their hybrids in eastern Newfoundland does not significantly deviate from 1:1, as stated by Lubet (1959). The low frequency of hermaphroditism also agrees with Sunila (1981) and Lubet (1959).

Mussels from all three sites sampled showed a similar reproductive cycle, with gametogenesis progressing rapidly through spring and early summer and spawning taking place in late July. Similar observations were made on Bellevue mussels by Thompson (1984b), who also found that these mussels do not undergo gametogenesis throughout the winter and that reserves accumulated in the previous year do not appear to play a role in gamete development. Other populations of *M. trossulus* also present the same pattern in which gonad is synthesized in late winter and early spring in the Baltic (Kautsky, 1982) and in British Columbia (Emmett et al., 1987). Thus subtidal Baltic Sea mussels (Kautsky, 1982) and Newfoundland mussels (Thompson, 1984b) demonstrate an opportunistic reproductive strategy, as also observed for mussels from British Columbia (Emmett et al., 1987), where gametogenesis does not proceed during the winter months. However, Blanchard and Feder (1997) found that mussels (*M. trossulus*) from populations in Port Valdez, Alaska, follow a more conservative strategy, with gametogenic development throughout winter, while the spawning period is similar to that found in the present study.

No study of reproductive cycles of *M. edulis* and *M. trossulus* in terms of their importance in hybridization has been undertaken on the northeast coast of North America. The results of the present study indicate that *M. trossulus* was spawning over a prolonged period of time while *M. edulis* mussels presented a major spawning event in late July. Similar studies carried out few years earlier showed also that *M. edulis* is a synchronous spawner in Newfoundland (Thompson, 1984b). Hybrid mussels in this study exhibited spawning activity which was more similar to *M. trossulus*. Lubet et al. (1984) studied reproductive cycles of hybrids obtained from intra- and inter-specific crosses of *M. edulis* and *M. galloprovincialis* and found asynchrony in spawning. *M. edulis* spawning was more restricted

in time, while *M. galloprovincialis* showed a more prolonged spawning over several months and hybrids an intermediate pattern.

Results from Chapter 1 indicate that most hybrids are backcrosses, which is in accordance with the timing of the spawning events, where there is a small overlap in which some F1 hybrids could be produced. As already discussed in Chapter 1, only a few F1 hybrids are needed to spawn to form a large number of backcrosses with their parental species, especially when hybrids have an intermediate spawning with respect to the pure species.

The present study provides evidence that *M. trossulus* has a greater reproductive output than *M. edulis* in mussels between 38-42 mm shell length. (Figures 26, 27), which is consistent with the observations of Mallet and Carver (1995) for two mussel populations in Nova Scotia. However, the present study also suggests that the mean oocyte size is larger in *M. edulis* than in *M. trossulus*, with intermediate values in hybrids. Mussels produce a large number (up to 8 X $10^{10}$ eggs per individual *M. edulis*; (Bayne et al., 1978) of small eggs (around 70 µm diameter)). This may imply that *M. trossulus*, which produces a greater dry weight of gametes (Figure 27), releases larger numbers of eggs. The implications of this finding, besides a larger egg output by *M. trossulus* during spawning, are not clear. Mussel eggs have a relatively small amount of yolk (ca. 0.08 µg dry mass per egg; Bayne et al., 1978) representing a minimum investment per egg, in contrast to eggs which undergo direct or lecithotrophic development.

In the present study, *M. trossulus* was reproductively active at a relatively smaller shell length than *M. edulis*, the former devoting energy to reproduction earlier in the life cycle, although maturation size depends on rate of growth (Seed, 1969) and therefore may differ among species and locations. Nevertheless, this smaller size at first maturation in *M. trossulus* relative to *M. edulis* and hybrids may have some implications for growth and perhaps higher mortalities caused by predation (Theisen, 1968; Seed, 1969; Dare, 1976; Seed and Brown, 1978).

Previous studies have shown that the balance between reproductive output and growth in an individual animal changes with increasing age. The youngest mussels divert most

of their energy to growth, while most of the energy in older mussels is used for gametogenesis (Thompson, 1979). The results from Chapters 1 and 2, which show that mussel populations are composed mostly of *M. trossulus* in small size classes and *M. edulis* in large size classes, with a shift at approximately 30 to 40 mm, seems to be difficult to explain considering that large *M. edulis* are more fecund than small ones (Thompson, 1979). Furthermore, the significant relationship found in the present study between fecundity (estimated as a dry weight of the gonad) and the dry weight of the soft tissues represents more evidence of this general relationship between size and fecundity (Figure 29). However, there is a large number of small *M. trossulus* compared to a reduced number of large *M. edulis* (Figures10 to 16).

Gardner and Skibinski (1990a) found that mean genotypic fecundity of *M. galloprovincialis* was 2.8 times that of *M. edulis* at Croyde and 2.2 times greater at at Whitsand, because *M. galloprovincialis* has both greater mean length and greater mean fecundity per unit length than *M. edulis*. However, the study also estimated that the total population fecundity of *M. edulis* was 5 and 17 times that of *M. galloprovincialis* at Croyde and Whitsand, respectively; owing to the presence of larger numbers of small *M. edulis* compared with fewer large *M. galloprovincialis*. A similar mussel population structure in the present study, in which large numbers of *M. trossulus* are found in small size classes and lower numbers of *M. edulis* found in large size classes (Figures 10 to 16), is in accordance with the higher proportion of *M. trossulus* in the larval, spat and juvenile stages (Figures 4 to 9).

Summer mortalities in populations of sexually mature mussels on the west coast of North America have been observed by several authors (Heritage, 1983; Skidmore and Chew, 1985; Emett et al., 1987). A high mortality of *M. trossulus* was observed in the present study at all sites during late summer and early autumn (Chapter 1). Intense summer spawning activity in these populations coincides with the time of high mortality. Some of these mortalities may be caused by predation, although some studies have shown that metabolic stress, especially after spawning, could be partially responsible. Worrall and Widdows (1984)

studied the relationship between spawning and mortality in a population of mussels in the Lynher estuary, southwest England, which showed reduced "scope for growth" following spring spawning. The authors reported that mortality was detected after one month of spawning and that the mortality rate was greater in those mussels that showed higher reproductive effort. It is therefore possible that the late summer mortalities of *M. trossulus* observed in the present study, by Heritage (1983) and by Emmett et al. (1987) in British Columbia, and by Skidmore and Chew (1985) in Washington, are caused by reproductive stress. Further research is required on the effects of predation, disease and parasites on reproductive effort, reproductive value and reproductive cost (Bayne et al., 1983; Thompson, 1984b), combined with the determination of physiological variables such as "scope for growth" in these mixed populations of *M. edulis*, *M. trossulus* and hybrids, in order to understand the possible causes for these late summer mortalities in *M. trossulus*.

# V. CHAPTER 4.

## Fertilization success and early survival in pure and hybrid larvae of *Mytilus edulis* and *M. trossulus*

### V.1. INTRODUCTION

There is no evidence in the literature for the presence of unfit hybrid recombinants within *Mytilus* hybrid zones (Zouros et al., 1992; Beaumont et al., 1993). As discussed in Chapter 1, the degree of natural hybridization is generally higher in the *M. edulis* - *M. galloprovincialis* hybrid zone in Europe than in the *M. edulis* - *M. trossulus* hybrid zone on the east coast of North America. According to Harrison (1993) and Banks et al. (1994) the more genetically divergent the parental types, the lower will be the fitness of the hybrids produced. This difference in natural hybridization in these two mussel hybrid zones implies that *M. edulis* and *M. galloprovincialis* are more closely related (Gardner, 1994) than are *M. edulis* and *M. trossulus* (Rawson et al., 1996b). This has been corroborated by successful interbreeding between *M. edulis* and *M. galloprovincialis*, the production of viable offspring in back-crosses of F1 hybrids (Lubet et al., 1984; Beaumont et al., 1993) and the suggestion made by Gardner (1996) that *M. galloprovincialis* may be a subspecies of *M. edulis*, proposing the use of the trinomial form *M. edulis galloprovincialis*.

Zouros et al. (1992) were the first to hybridize *Mytilus edulis* and *M. trossulus* successfully, although no data were provided on the percentage of eggs which developed into larvae, the percentage of abnormal larvae and/or survival among the reciprocal crosses. Information on such variables in artificial hybridization between *M. edulis* and *M. trossulus* is therefore limited.

The objective of the present study was to compare in preliminary laboratory trials the success of pure lines and hybrids of *M. edulis* and *M. trossulus* during the crucial early stages of their development.

## V.2. MATERIAL AND METHODS

### V.2.1. MUSSELS AND EXPERIMENTAL DESIGN

Ripe mussels (*Mytilus* sp) (29-54 mm shell length) were collected from the Bellevue exposed site (Fig. 1) during the last week of May 1997 and held in ambient running sea water until required for spawning. Four spawning trials (June 9, June 16, June 30 and July 17) were carried out, using a factorial design to permit full reciprocal crosses.

Twenty mussels were exposed to air for about 1 h, then placed in filtered seawater at ambient temperature, one mussel per beaker, for induction of spawning by thermal shock. In some cases, eggs and sperm were held for about 45 minutes at 18°C before use to allow as many mussels as possible to spawn, thereby helping to synchronize the egg stage (Scarpa and Allen, 1992). Eggs from each female were rinsed with filtered (1 µm) UV irradiated sea water (FSW) and then resuspended in 1000 ml FSW at 18°C for fertilization. Sperm concentration was determined with a hemocytometer. The sperm to egg ratio used for fertilization was approximately 100:1 in all trials (Sprung and Bayne, 1984). Eggs from each cross and each pure line were rinsed after 10 min to remove excess sperm and resuspended in two 2-l glass flasks (pseudo-replicates) containing 1500 ml at a density 70-120 eggs/ml (Toro and Sastre, 1995). The larval cultures were then maintained at 16°C and after 72 hours a 15 ml sample was taken from each culture and fixed with 95% ethanol. The fixed sample was later examined in order to determine (a) the percentage of eggs which had developed into larvae and (b) the percentage of these larvae which exhibited any abnormality. Both assessments were derived from 3 samples from each replicate flask, counting the numbers of developed larvae and the number of morphologically normal and abnormal larvae within one field of view (100X magnification).

Veliger larvae were reared at 16°C (28-33 ppt salinity) in duplicate 2-l glass beakers at a concentration of 10-20 larvae/ml for a minimum of 8 days (in one trial on July 17 up to 18 days), using standard techniques, except that beakers were not aerated (Bayne, 1965;

Beaumont and Budd, 1983; Mallet et al., 1985; Toro and Sastre, 1995). Sea water was filtered, sterilized with UV light and changed every other day. At each seawater change, the contents of each beaker were passed separately through a 44 µm Nitex screen (empty beakers were treated with a dilute bleach solution and then rinsed with FSW). Larvae retained on the screen were rinsed with treated seawater and back-washed into the cleaned beakers, which were then re-filled with filtered seawater. At each water change, larvae were fed *Isochrysis galbana* at 25000-30000 cells/ml (Pechenik et al., 1990). The number of living larvae in the cultures was monitored before addition of food, and the volume of the water used in each culture was adjusted in order to maintain a density close to 10-15 larvae/ml. On the 6$^{th}$ day after fertilization (the 14$^{th}$ day in trial 4), the percentage of live veligers in each culture (the mean of 4 counts) was estimated within one field of view under the microscope at 100X magnification. The mean shell length of veliger larvae on the 16$^{th}$ day in trial 4 was estimated by measuring 30 randomly chosen larvae from each culture with image analyzer software (Optimas 6.2) (see Chapter 3).

Spawnings (20 mussels) and crosses were performed on June 9 (*M. edulis*: 1 female and 2 males; *M. trossulus*: 1female and 1 male), June 16 (*M. edulis*: 2 females and 2 males; *M. trossulus*: 1 female and 2 males), June 30 (*M. edulis*: 2 females and 1 male; *M. trossulus*: 2 females and 2 males) and July 17 (*M. edulis*: 1 female and 2 males; *M. trossulus*: 2 females and 4 males). The spawned mussels were typed using two PCR-based nuclear markers (Chapter 1) and two allozyme loci, *Mpi* and *Est* (Chapter 2), which are diagnostic for *M. edulis*, *M. trossulus* and the hybrids.

### V.2.2. STATISTICAL ANALYSIS

Before analysis, percentage abnormality and survival data were arcsine transformed and pooled between beakers and within pure and cross-fertilized groups, because these were pseudo-replicates (not true replicates). The non-parametric Kruskal-Wallis test was used to analyze the percentage abnormality at day 3 and the percentage survival at day 6 (day 14$^{th}$

in trail 4), because these data showed significant heterogeneity of variance (Cochran's test). Larval shell length data at day 16 were tested by nested analysis of variance, which confirmed that there were no significant differences in larval shell length between pseudo-replicates within groups ($P>0.05$).

## V.3. RESULTS

Fertilization occurred in all crosses and cleavage was observed within 30 to 40 minutes following mixing of eggs and sperm. No significant differences between pure lines and hybrids were detected in the percentage of eggs which developed into larvae (Table 15). However, the percentage of larvae which were abnormal by day 3 and the proportion of normal veligers which subsequently died during the early stages of growth were significantly greater in the hybrid crosses (Table 15).

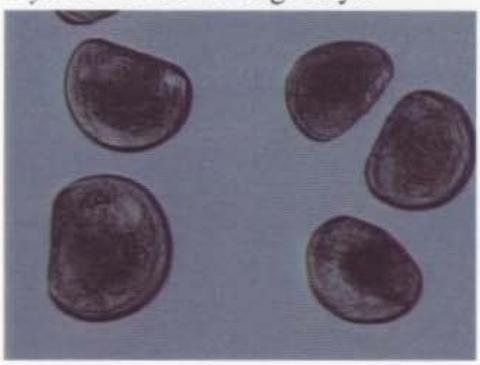
Figure 30 shows photomicrographs of normal larvae at D-stage and also the presence of abnormal larvae (which were more common in hybrid crosses) at the third day of culture. Most larvae of pure lines in all trials were healthy at day 6 (Figure 30), but most larval cultures from the first three trials only survived until day 9. In trial 4, all cultures were reared in good condition until day 18, but owing to contamination of the algal culture by ciliates, larvae from most crosses then died (Figure 30). The few larvae that survived in the different crosses were combined, cultured until day 25 (Fig. 30) and then discarded.

In trial 4, after an initially higher mortality, hybrid larvae from both reciprocal crosses grew significantly faster than those from pure lines (Table 16). There was, however, a significant difference in growth between hybrid larvae of the two reciprocal crosses, the *M. edulis* (female) crossed with *M. trossulus* (male) hybrids being the faster growing larvae (Table 16).

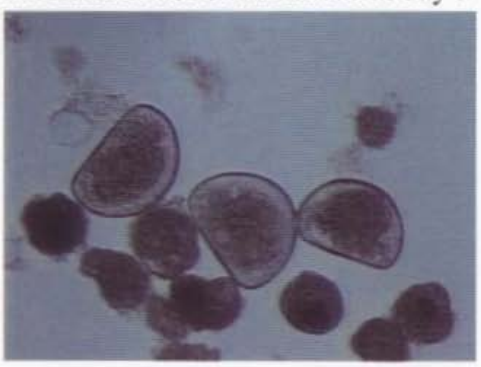
**Table 15.** Reciprocal crosses between ***M. edulis*** and ***M. trossulus***. Percentage of eggs which developed into larvae at day 3, the percentage of abnormal larvae at day 3 and the percentage of veliger larvae which survived to day 6 (day 14 in trial 4). E = pure ***M. edulis***, T = pure ***M. trossulus***, E x T = ***M. edulis*** female crossed with ***M. trossulus*** male, T x E = ***M. trossulus*** female crossed with ***M. edulis*** male. H = Kruskal Wallis test statistic between the mean performance of pure lines and that of hybrids. ns = non significant, ** = P<0.01.

| Trial | Percentage development (day 3) | | | | Percentage abnormality (day 3) | | | | Percentage survival (day 6) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E | T | E x T | T x E | E | T | E x T | T x E | E | T | E x T | T x E |
| 1 | 77.3 | 59.6 | 68.9 | 50.2 | 35.2 | 43.1 | 67.7 | 71.9 | 39.4 | 28.1 | 16.2 | 6.1 |
| 2 | 82.8 | 66.4 | 74.6 | 64.1 | 29.6 | 37.6 | 62.8 | 82.4 | 29.6 | 32.4 | 10.5 | 12.9 |
| 3 | 81.2 | 78.2 | 80.5 | 79.3 | 38.7 | 33.8 | 73.8 | 64.0 | 50.3 | 44.7 | 14.6 | 20.3 |
| 4 | 94.6 | 97.8 | 91.5 | 89.4 | 15.5 | 18.4 | 44.7 | 52.1 | 76.9 | 69.5 | 26.2 | 28.6 |
| mean | 79.73 ± 12.8 | | 74.81 ± 13.6 | | 31.48 ± 9.8 | | 64.92 ± 12.1 | | 46.36 ± 18.3 | | 16.92 ± 7.7 | |
| H | 0.540 ns | | | | 11.292 ** | | | | 10.599 ** | | | |

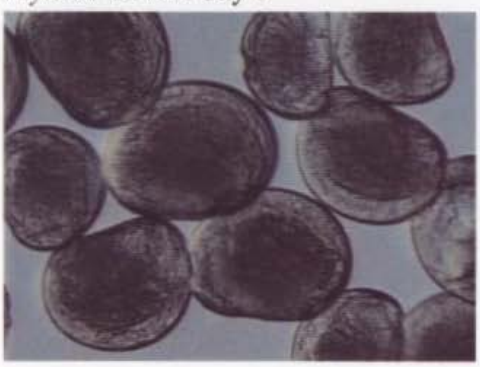
*Mytilus* larvae: D-stage day 3



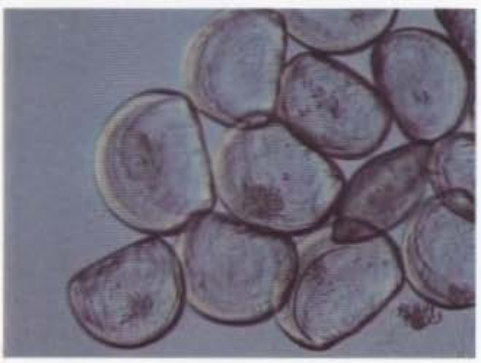
Presence of abnormal larvae at day 3



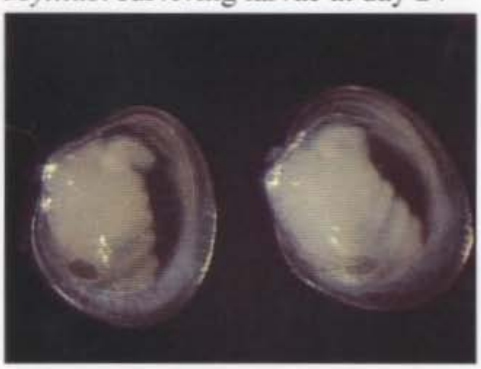
*Mytilus* larvae: day 6



Dead larvae: ciliate contamination



*Mytilus*: surviving larvae at day 24



*Mytilus*: surviving larvae at day 24

**Fig. 30.** *Mytilus* larvae: different stages showing normal, abnormal and dead larvae during the rearing of the larvae of pure and interspecific crosses in the laboratory trials.

**Table 16.** Mean shell length (µm ± SE) of pure and hybrid veligers of ***Mytilus*** after a period of growth in the laboratory (16 days), and results of nested ANOVA and Tukey's HSD test. For abbreviations, see Table 15. ns = non significant, *** P<0.001. Underline indicate homogeneity of means.

| E | T | E x T | T x E |
|---|---|---|---|
| 137.51 ± 2.38 | 133.79 ± 1.51 | 173.75 ± 1.88 | 152.61 ± 1.94 |

| Factor | Source | *df* | SS | F-ratio |
|---|---|---|---|---|
| Shell length | Cross | 3 | 12.502 | 99.856 *** |
| | Replicate {cross} | 4 | 0.019 | 0.116 ns |
| | Error | 1072 | | |

## V.4. DISCUSSION

Reports of naturally occurring hybrids between *M. edulis* and *M. trossulus* (Mallet and Carver, 1995; Saavedra et al., 1996; Comesaña et al., 1998), the results of previous experimental crosses (Zouros et al., 1992) and the data reported here all, confirm that the species are interfertile. In fact, there was no significant difference between pure lines and hybrids in the percentage of eggs which developed into larvae. However, hybrid crosses showed a higher mortality than pure lines crosses. This lower survivorship may reflect subtle differences in the time of sexual maturity or gamete quality in the two species, and one explanation, according to the results obtained in Chapter 3, may be that they have different optimum spawning times. However, the lower survivorship in F1 hybrid individuals could also be due to the incompatibility between mitochondrial and nuclear genomes, which seems to be greater between *M. trossulus* and *M. edulis* than between *M. edulis* and *M. galloprovincialis* (Comesaña et al., 1998). The results from Chapter 2 clearly show that naturally occurring hybrids consist mostly of backcrosses, which are *M. trossulus*-biased among small mussels and *M. edulis*-biased among large ones.

The progressively higher survival values in successive trials suggests that poor parental condition can adversely affect larval success, and emphasizing the need for choosing broodstock which is in good condition in order to carry out laboratory crosses. If most mussels of one species spawn even a few days before those of the other, species identity will be maintained into the next generation. A slight overlap of spawning times, or a few individuals of either species or just very few F1 hybrids spawning with the other, would explain the existence of naturally occurring hybrids and the presence of backcrosses. The results of Mallet and Carver (1995) and those from Chapter 1 and 2 suggest that hybridization between *M. edulis* and *M. trossulus* is not very common in the Atlantic Provinces compared with that between *M. edulis* and *M. galloprovincialis* in Europe.

The cumulative mortality of larvae after day 10 in trials 1, 2 and 3 may indicate inferior gametes (immature), particularly since none of the larval cultures were contaminated

with bacteria or ciliates. The production of large numbers of immature gametes by these mussels is also corroborated by the number of individuals which spawned mature gametes in each trial (5, 7, 7 and 9 out of 20, for trials 1, 2, 3 and 4 respectively). Some variation was evident among trials with respect to the percentage of developing eggs, larval abnormalities and survival in both pure and reciprocal crosses, although the patterns observed within trials were generally consistent.

Hybrid larvae from both reciprocal crosses grew significantly faster than larvae from pure lines, although the shell length values are within the range of those reported for pure *M. edulis* in trials carried out by Mallet et al. (1985) and trials under different feeding regimes undertaken by Bayne (1965). Because of the significantly higher size reached by hybrid larvae compared with pure lines of *M. edulis* and *M. trossulus*, hybrid vigour is suggested, although it is not clear what advantages this may impart. Similar results have been reported by Beaumont et al. (1993) for crosses between *M. edulis* and *M. galloprovincialis* and by Freeman et al. (1994) for crosses between *M. edulis* and *M. trossulus*. Growth of bivalves seems to be related to the genetic conformation of the individual (Newkirk, 1980, 1983). High levels of homozygosity due to inbreeding can significantly reduce growth rate (Beaumont and Abdul-Matin, 1994) and, conversely, high degrees of heterozygosity in artificial induced triploids (Beaumont and Budd, 1983; Beaumont and Kelly, 1989; Beaumont and Fairbrother, 1991) may enhance growth rate. Hybridization should increase the heterozygosity of individuals, which may in part explain the higher growth observed in hybrid larval cultures in the present study. Nevertheless, this faster growth of hybrid larvae has to be balanced against survivorship, which in most of the hybrid crosses was very low.

The results of this study provide evidence that there are no differences in the percentage of fertilized eggs between pure line crosses and hybrid crosses. However, the frequency of abnormal larvae significantly higher in the hybrid crosses. These observations suggest that there is little or no evidence of physiological barriers to fertilization, but that incompatibilities between mitochondrial and nuclear genomes may be causing a higher frequency of abnormalities and lower survival among F1 hybrid larvae (Rawson et al., 1996b;

Quesada et al., 1998). The data also suggest that F1 hybrid veligers may metamorphose earlier than pure line crosses.

Further research is required, including the study of pure line and reciprocal crosses, to assess the relative fitness of larval and juvenile hybrid mussels under different environmental conditions.

# VI. CHAPTER 5

## Morphological variation in the shell among *Mytilus edulis*, *M. trossulus* and their natural hybrids

### VI.1. INTRODUCTION

Early systematic taxonomical studies in mussels of the genus ***Mytilus*** have been largely based on shell morphological and morphometric characters (Gosling, 1992a; Seed, 1992). However, several studies on mussels have shown the enormous environmental plasticity of shell morphology (Seed, 1968, 1973) which may preclude the use of these characters in systematic studies. Recently, the availability of new molecular techniques has permitted the use of a combination of morphological attributes of the shell and allozyme genetic markers (; Beaumont et al., 1989; Coustau et al., 1991; Koehn, 1991; McDonald et al., 1991; Sarver and Foltz, 1993; Sanjuan et al., 1994, 1997;Bates and Innes, 1995; Gardner, 1996), nuclear and/or mtDNA markers (Inoue et al., 1995, 1997; Steward et al., 1995; Saavedra et al., 1996; Sanjuan et al., 1996; Suchanek et al., 1997; Toro, 1998) or a combination of allozyme and DNA markers (Beynon and Skibinski, 1996; Rawson et al., 1996a).

The main objective of the present study was to examine shells sampled from three different sites (Bellevue exposed, Chance Cove exposed and Chance Cove protected) in eastern Newfoundland (Fig. 1), in order to determine the degree of morphological differentiation between *M. edulis*, *M. trossulus* and their hybrids, coexisting (exposed to a common environment) at these habitats.

Canonical discriminant analysis of morphometric variation and two PCR-based nuclear DNA markers were used to test if *M. edulis*, *M. trossulus* and their hybrids have a similar shell morphology when exposed to a common environment.

## VI.2. MATERIAL AND METHODS

### VI.2.1. STUDY SITES AND SAMPLING

Mussel shells from adult mussels (*M. edulis*, *M. trossulus* and hybrids) that had been genotyped for two PCR-based nuclear markers from individuals collected and analysed in Chapter 3 were labeled for morphometric analysis. The mussels (38-42 mm shell length) were collected from June - October 1996 from the Bellevue exposed (n=280), Chance Cove protected (n=280) and Chance Cove exposed sites (n=280).

### VI.2.2. MORPHOMETRIC ANALYSIS

The 8 morphometric shell characters used to distinguish among three different forms of *Mytilus* (*M. edulis*, *M. trossulus* and hybrids) were as follows: (i) shell height; (ii) shell width; (iii) length of anterior adductor muscle scar; (iv) distance between the anterior edge of the posterior adductor muscle scar and the posterior margin of the shell; (v) distance between the ventral edge of the posterior adductor muscle scar and ventral shell margin; (vi) distance between the pallial line and the ventral shell margin midway along the shell; and (vii) distance between the umbo and posterior end of the ligament (Figure 31) (McDonald et al. 1991; Mallet and Carver, 1995). Each character was further standardized (shell length range of sampled individuals: 38 - 42 mm) by transforming the value to $\log_{10}$, and divided by $\log_{10}$ shell length (McDonald et al., 1991;Mallet and Carver, 1995). A canonical discriminant analysis was used to derive a canonical function that separated the three mussel types, using Systat V5.1 (Wilkinson, 1991).

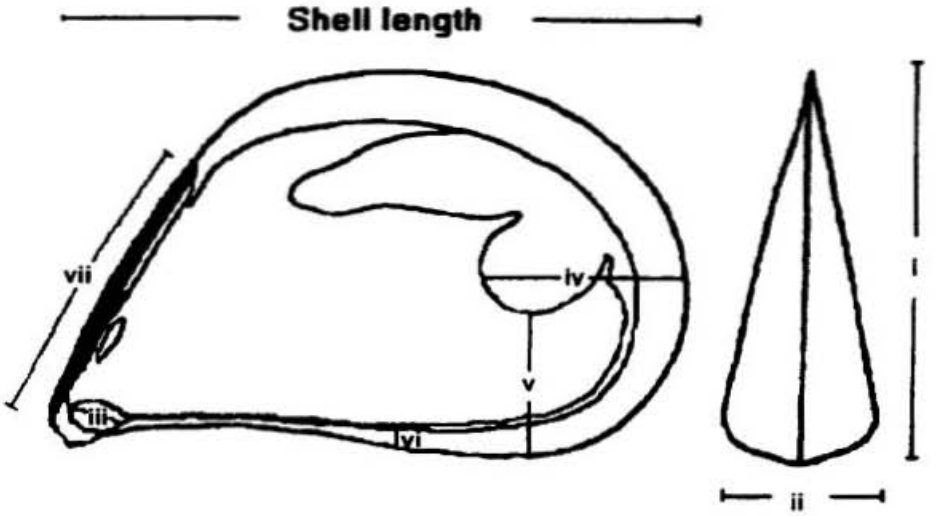



**Fig. 31.** The 7 morphometric mussel shell characters used in the canonical discriminant analysis: (**i**) shell height; (**ii**) shell width; (**iii**) length of anterior muscle scar; (**iv**) distance between the anterior edge of the posterior adductor muscle scar and the posterior margin of the shell; (**v**) distance between the ventral edge of the posterior adductor muscle scar and the ventral shell margin; (**vi**) distance between the pallial line and the ventral shell margin midway along the shell; and (**vii**) distance between umbo and posterior end of the ligament. (Modified from McDonald et al., 1991).

### VI.2.3. DNA ANALYSIS

DNA extraction and use of both DNA nuclear markers (*ITS* and *Glu*-5) to identify *M. edulis*, *M. trossulus* and their hybrids followed procedures described in Chapter 1.

## VI.3. RESULTS

The seven shell characters used in the discriminant function were able to distinguish between the two species of mussels (*M. edulis*, *M. trossulus*) previously identified using the PCR-based markers (Wilk's lambda: $P < 0.001$) (Figure 32, Table 17). The hybrids were scattered on the canonical plots, although a few of them were clustered between the two species (Figures 32, 33). The morphological differences were consistent between the two species over the three different sites (Figure 32), and maintained in most cases separate clusters despite a common environment within each site. In order to test if any micro-environmental differences were affecting shell morphology, canonical discriminant analyses which included *M. edulis*, *M. trossulus* and hybrid individuals from different sites were carried out (Figure 33, Table 18). The results showed similar morphological differences among the two mussel species and their hybrids, despite the fact that in these analyses each species was collected from a different micro-environment (site) (Figure 33, Table 18). Conversely, a canonical discriminant analyses was carried out for each species (*M. edulis*, *M. trossulus* and hybrids) at all sites. There was no significant difference in the morphology of each species from the different sites (Figure 34).

The standardized canonical coefficients showed that the characters shell height, distance between pallial line and the ventral shell margin, and shell width contributed most to discriminating among the three *Mytilus* forms (Tables 17, 18). Standardized canonical coefficients (Tables 17, 18) represent the amount by which canonical variates change for each change of one standard deviation in the individual character, and the character with

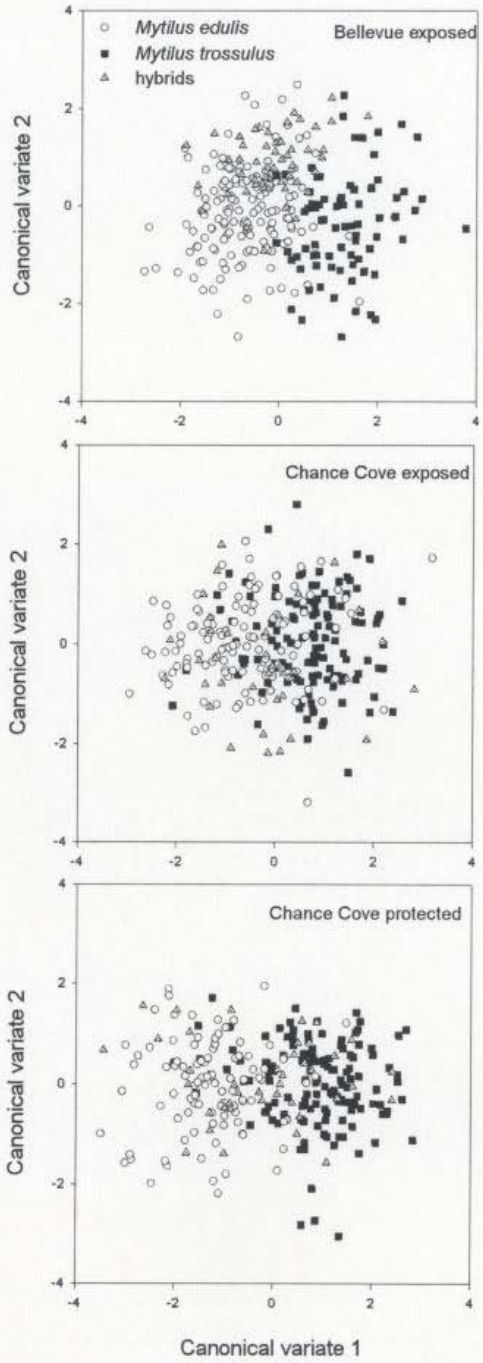



**Fig. 32.** First and second canonical variates of morphometric data from mussel populations sampled in Bellevue exposed, Chance Cove exposed and Chance Cove protected in eastern Newfoundland.

**Table 17.** Standardized canonical coefficients of the seven morphometric characters used in the discriminant analysis. Standardized coefficient for the first (St. can 1) and second (St. can 2) canonical variate and the probability from the F statistic (P) for the three groups analyzed (***Mytilus edulis***, ***M. trossulus*** and hybrids) at Bellevue exposed, Chance Cove exposed and Chance Cove protected. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Bellevue exposed

| Character | St. can 1 | St. can 2 | P |
|---|---|---|---|
| Shell height | -0.627 | 0.610 | *** |
| Shell width | 0.561 | 0.265 | *** |
| Length of anterior muscle scar | 0.013 | -0.059 | ns |
| Distance between anterior edge of the posterior adductor muscle scar and posterior margin of the shell | -0.077 | 0.498 | ** |
| Distance between ventral edge of the posterior adductor muscle scar and ventral shell margin | 0.200 | 0.139 | * |
| Distance between pallial line and the ventral shell margin | -0.555 | -0.357 | *** |
| Distance between the umbo and the posterior end of the ligament | 0.366 | 0.393 | *** |

Chance Cove exposed

| Character | St. can 1 | St. can 2 | P |
|---|---|---|---|
| Shell height | 0.805 | -0.109 | *** |
| Shell width | 0.074 | -0.421 | ns |
| Length of anterior muscle scar | 0.224 | 0.164 | *** |
| Distance between anterior edge of the posterior adductor muscle scar and posterior margin of the shell | 0.181 | -0.024 | *** |
| Distance between ventral edge of the posterior adductor muscle scar and ventral shell margin | 0.251 | 0.525 | ns |
| Distance between pallial line and the ventral shell margin | 0.276 | 0.466 | *** |
| Distance between the umbo and the posterior end of the ligament | -0.518 | 0.612 | *** |

Chance Cove protected

| Character | St. can 1 | St. can 2 | P |
|---|---|---|---|
| Shell height | 0.726 | 0.067 | *** |
| Shell width | -0.518 | 0.029 | *** |
| Length of anterior muscle scar | 0.171 | 1.035 | *** |
| Distance between anterior edge of the posterior adductor muscle scar and posterior margin of the shell | 0.075 | -0.701 | *** |
| Distance between ventral edge of the posterior adductor muscle scar and ventral shell margin | -0.481 | 0.033 | ns |
| Distance between pallial line and the ventral shell margin | 0.497 | -0.376 | *** |
| Distance between the umbo and the posterior end of the ligament | -0.270 | -0.071 | ** |

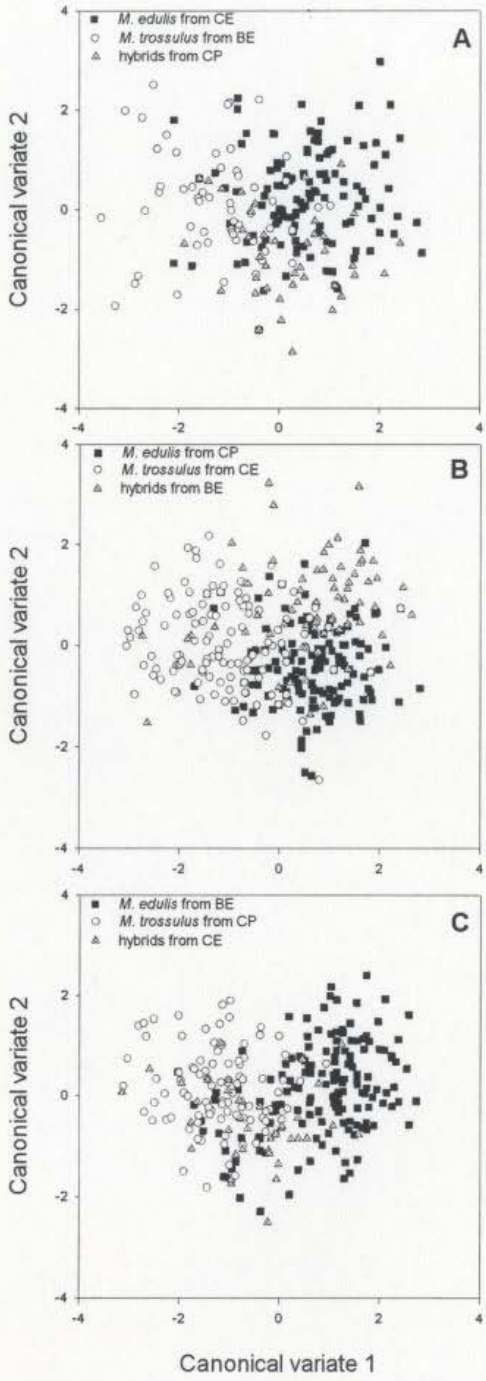



**Fig. 33.** First and second canonical variates of shell morphometric data from (A), *M. edulis* from Chance Cove exposed (CE), *M. trossulus* from Bellevue exposed (BE), hybrids from Chance Cove protected (CP); (B), *M. edulis* from CP, *M. trossulus* from CE and hybrids from BE, and (C), *M. edulis* from BE, *M. trossulus* from CP, and hybrids from CE.

**Table 18.** Standardized canonical coefficients of the seven morphometric characters used in the discriminant analysis. Standardized coefficient for the first (St. can 1) and second (St. can 2) canonical variate and the probability from the F statistic (P) from the analysis of mixed individuals (***Mytilus edulis***, ***M. trossulus*** and hybrids) from different populations (Bellevue exposed (BE), Chance Cove exposed (CE) and Chance Cove protected (CP)). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

***M. edulis*** from CP; ***M. trossulus*** from CE and hybrids from BE.

| Character | St. can 1 | St. can 2 | P |
|---|---|---|---|
| Shell height | 0.879 | -0.165 | *** |
| Shell width | 0.127 | 0.644 | *** |
| Length of anterior muscle scar | 0.209 | -0.182 | *** |
| Distance between anterior edge of the posterior adductor muscle scar and posterior margin of the shell | 0.197 | 0.482 | *** |
| Distance between ventral edge of the posterior adductor muscle scar and ventral shell margin | -0.162 | -0.027 | *** |
| Distance between pallial line and the ventral shell margin | 0.152 | 0.276 | *** |
| Distance between the umbo and the posterior end of the ligament | -0.628 | 0.367 | *** |

***M. edulis*** from CE; ***M. trossulus*** from BE and hybrids fromCP.

| Character | St. can 1 | St. can 2 | P |
|---|---|---|---|
| Shell height | 0.420 | 0.205 | *** |
| Shell width | -1.017 | 0.141 | *** |
| Length of anterior muscle scar | -0.085 | -0.705 | ** |
| Distance between anterior edge of the posterior adductor muscle scar and posterior margin of the shell | -0.067 | 0.377 | ns |
| Distance between ventral edge of the posterior adductor muscle scar and ventral shell margin | 0.079 | -0.824 | ** |
| Distance between pallial line and the ventral shell margin | 0.326 | 0.612 | ** |
| Distance between the umbo and the posterior end of the ligament | 0.097 | 0.068 | *** |

***M. edulis*** from BE; ***M. trossulus*** from CP and hybrids from CE.

| Character | St. can 1 | St. can 2 | P |
|---|---|---|---|
| Shell height | 0.246 | 0.387 | *** |
| Shell width | -0.086 | 0.387 | * |
| Length of anterior muscle scar | 0.007 | 0.422 | *** |
| Distance between anterior edge of the posterior adductor muscle scar and posterior margin of the shell | 0.216 | 0.163 | *** |
| Distance between ventral edge of the posterior adductor muscle scar and ventral shell margin | -0.418 | 0.552 | *** |
| Distance between pallial line and the ventral shell margin | 0.875 | -0.317 | *** |
| Distance between the umbo and the posterior end of the ligament | -0.099 | -0.014 | ns |

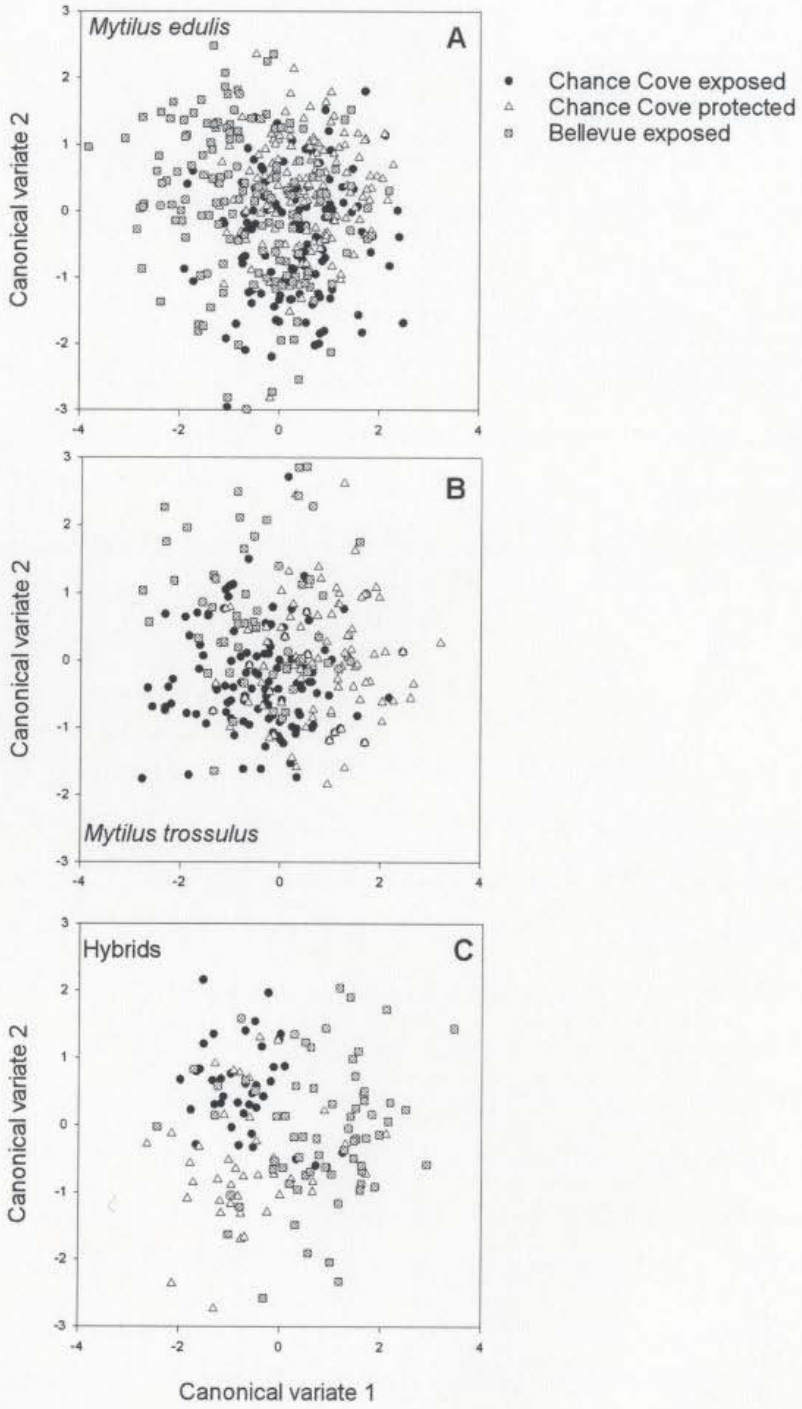



**Fig. 34.** First and second canonical variates of shell morphometric data from (A), *M. edulis* from Chance Cove exposed (CE), Chance Cove protected (CP), Bellevue exposed (BE), (B), *M. trossulus* from CE, CP, BE and (C) hybrids from CE, CP and BE.

the highest standardized coefficients contributes the most to the canonical function. For most individuals there is in general great overlap in canonical variates (Figure 32, 33, 34), but in sites such as Chance Cove protected and Bellevue exposed some individuals do provide non-overlapping clusters for each species.

Figure 35 shows the shell morphology and the colour of the inner surface of the shell for selected mussels with the most extreme canonical variate values for *M. edulis* and *M. trossulus* (sample collected at Bellevue protected in October 1995). The more elongated shell shape and the darkest colour of the inner surface of the shell in *M. trossulus* are the most obvious characteristics in this non representative (biased) sample of mussels.

## VI.4. DISCUSSION

Despite a common environment the three forms of *Mytilus* (*M. edulis*, *M. trossulus* and hybrids), differ in their shell morphology when described by a multivariate analysis of several shell characteristics, confirming previous claims that *M. edulis* and *M. trossulus* are morphologically different (McDonald et al., 1991; Mallet and Carver, 1995). However, these previous studies found a greater degree of morphological separation between *M. edulis* and *M. trossulus* than was apparent in the present study. Two main factors may account for the lower discriminatory power of the present study: the number of shell characters used, 7 rather than the 18 used by McDonald et al. (1991) and, perhaps more importantly, the sympatric origin of the populations in contrast to the allopatric single-species populations analyzed by McDonald et al. (1991). The enormous shell plasticity in mussels is well known (Seed, 1973, 1992), which in allopatric populations can be enhanced (Gardner, 1996) to produce larger differences in shell morphology due to specific local environmental conditions. All the sites sampled in the present study contained a mixture of both species, thus the exposure to common environmental conditions and hybridization (Chapter 1) may have resulted in an increase in morphological similarity. However, Mallet and Carver (1995)

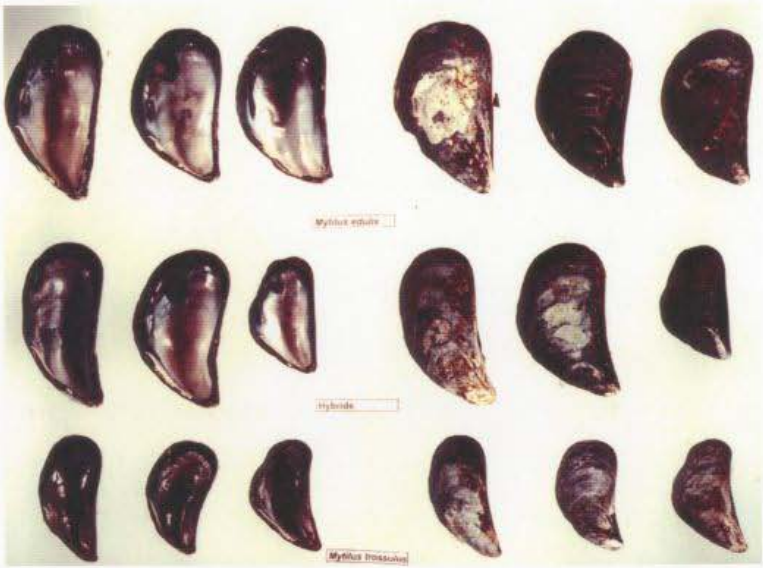



**Fig. 35.** Sample of the shell morphology from selected most extreme canonical variate values for ***Mytilus edulis*** and ***M. trossulus*** for a sample taken at Bellevue protected in October 1995, also showing the colour of the inner surface of the shell. First row: ***M. edulis***, second row: hybrids, third row: ***M. trossulus.***

reported a higher degree of discrimination between the two species (*M.edulis - M. trossulus*) using almost the same shell traits as those used in the present study, and their sample populations, which contained both species and hybrids, were reared in a common local environment (from a commercial mussel farm). According to Falconer (1989) the growth of individuals in a similar environment reduces the variability in quantitative traits.

In the present study, the incidence of *M. edulis-M. trossulus* hybrids is lower than that of *M. edulis-M. galloprovincialis* hybrids in southwest England (Sanjuan et al., 1994; Hilbish et al., 1994). Nevertheless, the identified hybrids in most samples in the present study show the importance of these individuals (most of them having intermediate values between the two mussel species) in morphological studies, based exclusively on variation in shell traits, because they may produce a canonical variates plot showing a single cluster for a *Mytilus* hybrid zone. Furthermore, morphological studies, based exclusively on shell trait variation, may occasionally be used to identify mussel species on a local basis, when the mussels are sharing the same environment, but such studies are less reliable over larger geographical scales (Gosling, 1992a, 1992b).

Recent studies of geographic variation in shell morphology in mussels, *M. edulis* and *M. trossulus* (Innes and Bates, 1999), and in the bay scallop, *Argopecten irradians* (Wilbur and Gaffney, 1997), recognized the effect which any difference in size among individuals may have where the aim is determining variation in morphology. Innes and Bates (1999) pointed out that if the shells analyzed differ in length between the species, this may accentuate differences among species, even after standardization of length by log transformation. The morphological separation among the three forms of *Mytilus* found in the present study is not confounded by differences in shell size of the mussels, because the size range of the individuals sampled for the study was restricted to 38-42 mm, and any further differences in size were corrected by the log-transformed length standardization (Mallet and Carver, 1995).

The multivariate analysis was able, to a certain degree, to distinguish between *M. edulis* and *M. trossulus*. Similar results were obtained when *M. edulis*, *M. trossulus* and hybrids were sampled from different sites, indicating that local environmental conditions

between the locations sampled were not large enough to influence shell morphology among the species.

# VII. GENERAL DISCUSSION

Previous studies on the *M. edulis - M. trossulus* hybrid zone on the Atlantic coast of North America (McDonald et al., 1991; Bates and Innes, 1995; Mallet and Carver, 1995; Saavedra et al., 1996) have confirmed the presence of the two species of mussels detected initially by Koehn et al. (1984). Some of these studies (Mallet and Carver, 1995; Saavedra et al., 1996) found that the degree of hybridization within Lunenburg Bay, Nova Scotia, was much less than the extent of hybridization occurring in the S.W. England hybrid zone between *M. edulis* and *M. galloprovincialis*. Bates and Innes (1995) showed that most populations of mussels on the east coast of Newfoundland consisted of a mixture of *M. edulis* and *M. trossulus*, although, the distribution of a hybrid index based on three partially diagnostic loci (*Est-D, Pgm, Lap*) provided no evidence for hybridization.

The present study, using four diagnostic markers (two allozyme loci and two PCR-based DNA markers), provided additional evidence for a mixture of the two species in populations from four sites in eastern Newfoundland. Furthermore, natural hybrids (F1, F2 and backcrosses) were detected at frequencies similar to those reported for Nova Scotia by Saavedra et al. (1996), and much lower than the values described for the *M. edulis* and *M. galloprovincialis* hybrid zone in Europe (Hilbish et al., 1994;Sanjuan et al., 1994; Comesaña and Sanjuan, 1997). This lower incidence of hybrids in Newfoundland could be partially related to differences found in the timing of spawning between the two species. *M. edulis* had a major spawning event in late July while *M. trossulus* showed a "dribble" type of spawning, resulting in a smaller chance for hybrid offspring to be produced than if spawning between the two species were synchronized.However, more importantly, interspecific crosses of *M. edulis* and *M. trossulus* carried out in the laboratory showed that although the percentage of activated eggs after fertilization did not show significant differences between any hybrid crosses and within species crosses, the percentage of abnormal larvae was higher in the former, suggesting also that hybrids are less fit than any of the pure species larvae. There have also been studies (Rawson et al., 1996b; Quesada et al., 1998) claiming that

some incompatibilities between mitochondrial and nuclear genomes in mussels could be causing a higher frequency of abnormalities and lower survival among F1 hybrid larvae. This suggestion is in accordance with results from the present study, in which most natural hybrids were backcrosses, *M. trossulus*-biased in small mussels and *M. edulis*-biased in large mussels. All these observations suggest that this *(M. edulis-M. trossulus)* hybrid zone is maintained by a balance between dispersal and selection against hybrids at early stages of the mussel life-cycle, which supports the tension zone model (Barton and Hewitt, 1985).

Another interesting finding of the present study, similar to the S.W England hybrid zone between *M. edulis* and *M. galloprovincialis*, is that the relative frequency of each species is strongly dependent upon size class. An increase in the frequency of *M. edulis* individuals and a decrease in the frequency of *M. trossulus* individuals with shell length was observed at all sites and locations sampled. This pattern could be attributable in part to a differential growth rate, but this aspect was not studied in the present investigation. However, Mallet and Carver (1995) reported only small differences in growth between *M. edulis* and *M. trossulus* in Nova Scotia, which may suggest that the observed changes in species frequency with shell length are due to differential selection. Differential survival was observed between June and October that affected mostly *M. trossulus*, which may be related to the post-spawning mortality often reported in mussels (Heritage, 1983; Worrall and Widdows, 1984; Skidmore and Chew, 1985; Emmett et al., 1987). The dominance of *M. trossulus* in the smallest size classes for all samples suggests that the larger number of recruits of this species may be indicative of a greater reproductive output. Indeed, a larger GVF was found in *M. trossulus* individuals than in *M. edulis* (hybrids being at intermediate values). Nevertheless, further studies should be carried out in order to establish any relationship between differential mortality between the species and reproductive and/or physiological aspects of their life-cycles.

The life-history variation study showed that at the larval and early juvenile stages of these mussels (*M. edulis*, *M. trossulus* and hybrids) there were no significant differences in micro-distribution (between locations or between sites within location). However, at the

adult stage significant differences in the frequency of these species were found, which may indicate differential survival of *M. edulis*, *M. trossulus* and hybrids related to environmental factors at these sites. These observations are in accordance with the environmental gradient model for the stability and maintenance of hybrid zones (Endler, 1977; Harrison and Rand, 1989; Cruzan and Arnold, 1993; Wilhelm and Hilbish, 1998). Therefore, it seems that both the tension zone model and the environmental gradient model are acting towards the stability and maintenance on this hybrid zone, but operating at different times within the life-history of these mussels. Finally, it is clear from multivariate analysis of shell morphometrics and two PCR-based markers that *M. edulis* and *M. trossulus* are two genetically and morphologically different forms. As discussed by other authors, this distinctness warrants taxonomic recognition at the species level based in the phylogenetic species concept (Koehn, 1991; McDonald et al., 1991).

In comparison with the European hybrid zone between *M. edulis* and *M. galloprovincialis* the information available on the Atlantic coast of North America hybrid zone between *M. edulis* and *M. trossulus* is very limited. Further studies on this hybrid zone should include an analysis of growth in the two species and their hybrids to support or disprove the hypothesis that the change in relative frequency of each species with size is due to differential mortality. Also, physiological evaluation, especially the "scope for growth" and biochemical examination of the soft tissues at different stages of the life cycle of these species should be analyzed to find evidence for any ecological differences, including nutrient storage cycles, reproductive effort, and differential tolerance to environmental variables. Carefully designed artificial crosses with larval, spat and juvenile rearing under controlled conditions will also be important to detect any differential survival and/or growth patterns at different stages of the life-cycle of *M. edulis*, *M. trossulus* and their hybrids.

# IX. CONCLUSIONS

1.- The presence of two pure mussel species *M. edulis* L. and *M. trossulus* Gould and their hybrids (F1, F2 and backcrosses) was detected using genetic markers at all sampling sites. Hybrids consisted mostly of backcrosses that were *M. trossulus*-biased among small mussels and *M. edulis*-biased among large ones.

2.- A significant decrease in the frequency of hybrids was found from larvae to juveniles at all sites sampled (after pooling the number of both species against hybrids) indicating that there was selective mortality against hybrids at this transitional stage from pelagic larva to spat, supporting the tension-zone model. However, hybrids also showed a lower viability at exposed sites, supporting the environmental gradient model. The overall low frequency of hybrids at the adult stage (< 8%) and the results of artificial hybridization in the laboratory, which showed an increased proportion of abnormal larvae among interspecific crosses, indicate strong selection against hybrids, and clearly show that these specific genotypes seem to be at a selective disadvantage, supporting the tension-zone model for the early stages of the mussel life-history.

3.- Significant spatial variation in the frequency of *M. edulis*, *M. trossulus* and hybrids was found, with a higher frequency of *M. trossulus* at the most exposed sites. This pattern was similar during the three consecutive years sampled, thus there was little evidence for interannual variation in recruitment in these areas.

4.- Five individuals were identified as F1 hybrids, having heterozygous genotypes for the four nuclear markers and heterospecific mitotypes, and only one F2 hybrid was detected, due to the low frequency of mating expected among the very rare F1 hybrids.

5.- None of the 281 individuals (scored with the PCR-based mtDNA marker (*COIII*) classified as pure species, based on four markers, contained mtDNA from the other species. Thus it seems that the incompatibility between mitochondrial and nuclear genomes is greater among *M. trossulus* and *M. edulis* than *M. edulis* and *M. galloprovincialis*. This greater degree of mtDNA introgression observed in Europe than in North America may be due to a more extended period of contact between these *Mytilus* species compared with Atlantic Canada *M. edulis* and *M. trossulus*, a greater divergence between these *Mytilus* species or perhaps that selection is operating differently against each species on each continent.

6.- The use of four diagnostic markers reduced the number of individuals misclassified. The addition of the two DNA markers to the two enzyme markers resulted in about 13 % of those individuals initially assigned to pure species being reclassified as hybrids. The largest change was a reclassification of pure *M. trossulus* individuals to *trossulus*-biased backcrossed individuals.

7.- In Atlantic Canada, hybridization between *M. edulis* and *M. trossulus* is much lower (8-26%) than between *M. edulis* and *M. galloprovincialis* at various locations in Europe (25-80%).

8.- The pre-spawning values of the gamete volume fraction and fecundity were significantly higher in *M. trossulus* than in *M. edulis* and hybrids. The spawning activity differed between species. *M. trossulus* spawned over a prolonged period (from early summer to early autumn) while *most M. edulis* individuals spawned simultaneously in late July. Hybrid mussels exhibited spawning activity which was more similar to *M. trossulus* than to *M. edulis*.

9.- Histological sections of female and male hybrids of *M. edulis* and *M. trossulus* showed normal gonadal development, ripening and spawning, and in interspecific crosses, the percentage of activated eggs after fertilization did not show significant differences with pure line crosses.

10.- The relative frequency of each species was strongly dependent upon size. *M. trossulus* was the predominant species in the smallest size classes for all samples. An increase in the frequency of *M. edulis* individuals and a decrease in the frequency of *M. trossulus* individuals with shell length was observed at all sites and locations sampled. The change in frequency of both species with increasing size was gradual, and the hybrid frequencies tended to decrease in large size classes. Other studies show only small differences in growth rate between *M. trossulus* and *M. edulis*, which may suggest that the observed changes in species frequency with shell length could be due to differential survival.

11.- High summer mortality can be inferred from a drastic reduction in the frequency of *M. trossulus* from June to October, especially at the protected sites. This occurs predominantly in mussels over 15 mm in shell length which are reproductively active, and may indicate that post-spawned *M. trossulus* are under considerable stress.

12.- This study demonstrates the feasibility of applying molecular genetic techniques to early larval stages of marine bivalves, which has implications for population genetic analysis of natural populations, because using PCR-techniques such studies can be extended to all stages and age classes.

13.- Both intrinsic genetic factors at early stages of the mussel life-history and extrinsic environmental factors at later stages influence the relative frequency of *M. edulis*, *M. trossulus* and their hybrids at these locations in eastern Newfoundland.

14.- The null hypothesis that there is no differences between *M. trossulus* and *M. edulis* life-history is refuted.

## IX. REFERENCES

Ahmad, M. and J.A. Beardmore. 1976. Genetic evidence that the "Padstow mussel" is *Mytilus galloprovincialis*. Mar. Biol., 35: 139-147.

Ahmed, M. and A.K. Sparks. 1970. Chromosome number, structure and autosomal polymorphism in the marine mussels *Mytilus edulis* and *Mytilus californianus*. Biol. Bull., 138: 1-13.

Aiken, D. 1993. Bivalve culture in Chile. World Aquaculture, 24: 7-19.

Arnold, M.L. 1992. Natural hybridization as an evolutionary process. Ann. Rev. Ecol. Syst., 23: 237-261.

Arnold, W.S. 1984. The effects of prey size, predator size, and sediment composition on the rate of predation of the blue crab, *Callinectes sapidus* Rathbun, on the hard clam, *Mercenaria mercenaria* (Linné). J. Exp. Mar. Biol. Ecol., 80: 207-219.

Arsenault, D.J. and J.H. Himmelman. 1998. Spawning of the Iceland scallop (*Chlamys islandica* Müller, 1776) in the northern Gulf of St. Lawrence and its relationship to temperature and phytoplankton abundance. Veliger: 41, 180-185.

Babcock, R.C., C. Mundy, J. Keesing and J. Oliver. 1992. Predictable and unpredictable spawning events: in situ behavioural data from free-spawning coral reef invertebrates. Inv. Reprod. Devel., 22: 213-228.

Banks, M.A., D.J. McGoldrick, W. Borgeson and D. Hedgecock. 1994. Gametic incompatibility and genetic divergence of Pacific and Kumamoto oysters, *Crassostrea gigas* and *C. sikamea*. Mar. Biol., 121: 127-135.

Barsotti, G. and C. Meluzzi. 1968. Osservazioni su *Mytilus edulis* L. e *Mytilus galloprovincialis* Lamarck. Conchiglie (Milan), 4: 50-58.

Barton, N.H. 1979. The dynamics of hybrid zones. Heredity, 43: 341-359.

Barton, N.H. 1983. Multilocus clines. Evolution, 37: 454-471.

Barton, N.H. and K.S. Gale. 1993. Genetic analysis of hybrid zones. In: Hybrid zones and the evolutionary process. R.G. Harrison (Ed), Oxford University Press, Oxford. 13-45.

Barton, N.H. and G.M. Hewitt. 1985. Analysis of hybrid zones. Ann. Rev. Ecol. Syst., 16: 113-148.

Barton, N.H. and G.M. Hewitt. 1989. Adaptation, speciation and hybrid zones. Nature, 341: 497-503.

Bates, J.A. 1992. Genetic and morphological variation in the mussel *Mytilus* in Newfoundland. M.Sc. thesis, Memorial University of Newfoundland. pp123.

Bates, J.A. and D.J. Innes. 1995. Genetic variation among populations of *Mytilus* spp. in eastern Newfoundland. Mar. Biol., 124: 417-424.

Bayne, B.L. 1964. Primary and secondary settlement in *Mytilus edulis* L. (Mollusca). J. Anim. Ecol., 33: 513-523.

Bayne, B.L. 1965. Growth and the delay of metamorphosis of the larvae of *Mytilus edulis* (L.). Ophelia, 2: 1-47.

Bayne, B.L. 1976a. Aspects of reproduction in bivalve molluscs. Estuarine Processes, 1: 432-448.

Bayne, B.L. 1976b. The biology of mussel larvae. In: Marine mussels their ecology and physiology. B.L. Bayne (Ed), Cambridge University Press. 81-120.

Bayne, B.L., A. Bubel, P.A. Gabbott, D.R. Livingstone, D.M. Lowe, and M.N. Moore. 1982. Glycogen utilization and gametogenesis in *Mytilus edulis* L. Mar. Biol. Lett., 3: 89-105.

Bayne, B.L., D.A. Brown, K. Burns, D.R. Dixon, A. Ivanovici, D.R. Livingstone, D.M. Lowe, M.N. Moore, A.R.D. Stebbing and J. Widdows. 1985. The effects of stress and pollution on marine animals. Praeger Press, New York, 384pp.

Bayne, B.L., D.L. Holland, M.N. Moore, D.M. Lowe and J. Widdows. 1978. Further studies on the effects of stress in the adult on the eggs of *Mytilus edulis*. J. Mar. Biol. Ass. U.K., 58: 825-841.

Bayne, B.L., P.N. Salkeld, and C.M. Worrall. 1983. Reproductive effort and value in different populations of the marine mussel, *Mytilus edulis* L. Oecologia, 59: 18-26.

Beaumont, A.R. 1991. Genetic studies of laboratory reared mussels, *Mytilus edulis*: heterozygosity deficiencies, heterozygosity and growth. Biol. J. Linn. Soc., 44: 273-285.

Beaumont, A.R., A.K.M. Abdul-Matin and R. Seed. 1993. Early development, survival and growth in pure and hybrid larvae of *Mytilus edulis* and *Mytilus galloprovincialis*. J. Moll. Stud., 59: 120-123.

Beaumont, A.R. and A.K.M. Abdul-Martin. 1994. Differences in morphology, survival and size between self- and cross-fertilized larvae of *Mytilus galloprovincialis*. J. Mar. Biol. Ass. U.K., 74: 445-448.

Beaumont, A.R. and M.D. Budd. 1983. Effects of self-fertilization and other factors on the early development of the scallop, *Pecten maximus*. Mar. Biol., 76: 285-289.

Beaumont A.R. and J.E. Fairbrother. 1991. Ploidy manipulation in molluscan shellfish: a review. J. Shellfish Res., 10: 1-18.

Beaumont, A.R. and K.S. Kelly. 1989. Production and growth of triploid *Mytilus edulis* larvae. J. Exp. Mar. Biol. Ecol., 132: 69-84.

Beaumont, A.R., R. Seed, and P. Garcia Martinez. 1989. Electrophoretic and morphometric criteria for the identification of the mussels *Mytilus edulis* and *M. galloprovincialis*. In: Reproduction, Genetics and Distribution of Marine organisms. J.S. Ryland and P.A. Tyler (Eds), Olsen & Olsen, Fredensborg. 251-258.

Bert, T.M. and W.S. Arnold. 1995. An empirical test of predictions of two competing models for the maintenance and fate of hybrid zones: both models are supported in a hard-clam hybrid zone. Evolution, 49: 276-289.

Bert, T.M., D.M. Hesselman, W.S. Arnold, W.S. Moore, H. Cruz-Lopez and D.C. Marelli. 1993. High frequency of gonadal neoplasia in a hard clam (*Mercenaria* spp.) hybrid zone. Mar. Biol., 117: 97-104.

Beukema, J.J. and J. Vlas. 1989. Tidal-current transport of thread-drifting postlarval juveniles of the bivalve *Macoma balthica* from the Wadden Sea. Mar. Ecol. Prog. Ser., 52: 193-200.

Beynon, C.M. and D.O.F. Skibinski. 1996. The evolutionary relationships between three species of mussels (*Mytilus*) based on anonymous DNA polymorphisms. J. Exp. Mar. Biol. Ecol., 203: 1-10.

Blanchard, A. and H.M. Feder. 1997. Reproductive timing and nutritional storage cycles of *Mytilus trossulus* Gould, 1850, in Port Valdez, Alaska, site of a marine oil terminal. Veliger, 40: 121-130.

Blot, M., B. Legendre and P. Albert. 1990. Restriction fragment length polymorphism of mitochondrial DNA in subantarctic mussels. J. Exp. Mar. Biol. Ecol., 141: 79-86.

Blundon, J.A. and V.S. Kennedy. 1982. Mechanical and behavioral aspects of blue crab, *Callinectes sapidus* (Rathbun), predation on Chesapeake Bay bivalves. J. Exp. Mar. Biol. Ecol., 65: 47-65.

Boecklen, W.J. and D.J. Howard. 1997. Genetic analysis of hybrid zones: numbers of markers and power of resolution. Ecology, 78: 2611-2616.

Bower, S.M. 1987. The summer mortality syndrome and haemocytic neoplasia in mussels (*Mytilus edulis*) from British Columbia. Can. Tech. Rep. Fish. Aquat. Sci. 1703. 65pp.

Brock, V. 1985. Immuno-electrophoretic studies of genetic relations between populations of *Mytilus edulis* and *M. galloprovincialis* from the Mediterranean, Baltic, East and West Atlantic, and East Pacific. Proc.19th EMBS, 19: 515-520.

Brown, T.J. 1998. A comparison of differential growth and species composition at three locations on the great northern peninsula, Newfoundland: a feasibility study for *Mytilus* spp. aquaculture. Bachelor of Science Thesis, Memorial University of Newfoundland. 1-34.

Buffery, C. 1993. The polymerase chain reaction. In: Molecular Biology and Biotechnology. J.M. Walker and E.B. Gingold (Eds), Royal Society of Chemistry, Cambridge. 51-62.

Burton, R.S. 1983. Protein polymorphisms and genetic differentiation of marine invertebrate populations. Mar. Biol. Lett., 1: 193-206.

Burton, R.S. and M.W. Feldman. 1981. Population genetics of ***Tigriopus californicus***. II. Differentiation among neighboring populations. Evolution, 35: 1192-1205.

Cáceres-Martínez, J. and A. Figueras. 1998. Distribution and abundance of mussel *(Mytilus galloprovincialis* Lmk) larvae and post-larvae in the Ria de Vigo (NW Spain). J. Exp. Mar. Biol. Ecol., 229: 277-287.

Cáceres-Martínez, J., J.A.F. Robledo and A. Figueras. 1994. Settlement and post-larval behaviour of *Mytilus galloprovincialis*: field and laboratory experiments. Mar. Ecol. Prog. Ser., 112: 107-117.

Combs, C.A., R.P. Henry, T.M. Bert, H.M. Perry and M. West. 1997. Increased metabolic cost of maintenance in hybrids of the stone crabs *Menippe mercenaria* and *M. adina*. Mar. Biol., 129: 53-61.

Comesaña, A.S. and A. Sanjuan. 1997. Microgeographic allozyme differentiation in the hybrid zone of *Mytilus galloprovincialis* Lmk. and *M. edulis* L. on the continental European coast. Helgoländer Meeresunters, 51: 107-124.

Comesaña, A.S., J.E. Toro, D.J. Innes and R.J. Thompson. 1998. A molecular approach to the ecology of a mussel (*Mytilus edulis* - *M. trossulus*) hybrid zone on the east coast of Newfoundland, Canada. Mar. Biol., 133: In Press.

Conrad, T.A. 1837. Description of new marine shells from upper California, collected by Thomas Nuttall, Esq. J. Acad. Nat. Sci. (Philad.), 7: 227-268.

Côrte-Real, H.B.S.M., D.R. Dixon and P.W.H. Holland. 1994a. Intron-targeted PCR: a new approach to survey neutral DNA polymorphism in bivalve populations. Mar. Biol., 120: 407-413.

Côrte-Real, H.B.S.M., P.W.H. Holland and D.R. Dixon. 1994b. Inheritance of a nuclear DNA polymorphism assayed in single bivalve larvae. Mar. Biol., 120: 415-420.

Coustau, C., F. Renaud and B. Delay. 1991. Genetic characterization of the hybridization between *Mytilus edulis* and *M. galloprovincialis* on the Atlantic coast of France. Mar. Biol., 111: 87-93.

Cracraft, J. 1989. Speciation and its ontology: The empirical consequences of alternative species concepts for undertanding patterns and processes of differentiation. In: Speciation and its consequences. D. Otte and J.A. Endler (Eds), Sinauer Associates, Sunderland, Massachusetts. 28-59.

Cragg, S.M. 1996. The phylogenetic significance of some anatomical features of bivalve veliger larvae. In: Origin and evolutionary radiation of the Mollusca. J. Taylor (Ed), Oxford University Press. 371-380.

Cruzan, M.B. and M.L. Arnold. 1993. Ecological and genetic associations in an *Iris* hybrid zone. Evolution, 47: 1432-1445.

Dare, P.J. 1976. Settlement, growth and production of the mussel *Mytilus edulis* L. in Morecambe Bay, England. Fish. Invest. Minist. Agric. Fish. Food G.B. Ser. II, 28: 1-25.

De Blok, J.W. and M. Tan-Maas. 1977. Function of byssus threads in young post-larval *Mytilus*. Nature, 267: 558.

Dillon, R.T. and J.J. Manzi. 1989. Genetics and shell morphology in a hybrid zone between the hard clams, *Mercenaria mercenaria* and *M. campechiensis*. Mar. Biol., 100: 217-222.

Dixon, D.R. and N. Flavell. 1986. A comparative study of the chromosomes of *Mytilus edulis* and *Mytilus galloprovincialis*. J. Mar. Biol. Ass. U.K., 66: 219-228.

Edmonds, S., P.E. Monberg and R.S. Burton. 1996. Allozyme and mitochondrial DNA evidence of population subdivision in the purple sea urchin *Strongylocentrotus purpuratus*. Mar. Biol., 126: 443-450.

Edwards, C.A. and D.O.F. Skibinski. 1987. Genetic variation of mitochondrial DNA in mussel (*Mytilus edulis* and *M. galloprovincialis*) populations from South West England and South Wales. Mar. Biol., 94: 547-556.

Emmett, B.K., K. Thompson and J.D. Popham. 1987. The reproductive and energy storage cycles of two populations of *Mytilus edulis* (Linné) from British Columbia. J. Shellfish Res., 6: 29-36.

Endler, J.A. 1977. Geographic variation, speciation, and clines. Princeton Univ. Press, Princeton, New Jersey, pp 336.

Eversole, A.G. 1997. Gametogenesis of *Mercenaria mercenaria*, *M. campechiensis* and their hybrids. Nautilus, 110: 107-110.

Eyster, L.S. and J.A. Pechenik. 1987. Attachment of *Mytilus edulis* L. larvae on algal and byssal filaments is enhanced by water agitation. J. Exp. Mar. Biol. Ecol., 114: 99-110.

Falconer, D.S. 1989. Introduction to Quantitative genetics. 3rd edn. Longman group Limited, Harlow, pp 438.

Fisher, C. and D.O.F. Skibinski. 1990. Sex biased mitochondrial DNA heteroplasmy in the marine mussel *Mytilus*. Proc. R. Soc. (Ser B), 242: 149-156.

Freeman, K.R. and L.M. Dickie. 1979. Growth and mortality of the blue mussel (*Mytilus edulis*) in relation to environmental indexing. J. Fish. Res. Bd Can., 36: 1238-1249.

Freeman, K.R., K.L. Perry, T.G. DiBacco and D.J. Scarratt. 1994. Observations on two mytilid species from a Nova Scotian mussel farm. Can. Tech. Rep. Fish. Aquat. Sci., 1969: 1-47.

Gardner, J.P.A. 1992. *Mytilus galloprovincialis* (Lmk) (Bivalvia, Mollusca): the taxonomic status of the Mediterranean mussel. Ophelia, 35: 219-243.

Gardner, J.P.A. 1994. The structure and dynamics of naturally occurring hybrid *Mytilus edulis* Linnaeus, 1758 and *Mytilus galloprovincialis* Lamarck, 1819 (Bivalvia: Mollusca) populations: review and interpretation. Arch. Hydrobiol. Suppl., 99: 37-71.

Gardner, J.P.A. 1996. The *Mytilus edulis* species complex in southwest England: effects of hybridization and introgression upon interlocus associations and morphometric variation. Mar. Biol., 125: 385-399.

Gardner, J.P.A. and D.O.F. Skibinski. 1988. Historical and size-dependent genetic variation in hybrid mussel populations. Heredity, 61: 93-105.

Gardner, J.P.A. and D.O.F. Skibinski. 1990a. Genotype-dependent fecundity and temporal variation of spawning in hybrid mussel (*Mytilus*) populations. Mar. Biol., 105: 153-162.

Gardner, J.P.A. and D.O.F. Skibinski. 1990b. Thermostability differences of allozyme loci in *Mytilus edulis*, *M. galloprovincialis* and hybrid mussels. Mar. Ecol. Prog. Ser., 64: 99-105.

Gardner, J.P.A. and D.O.F. Skibinski. 1991. Biological and physical factors influencing genotype-dependent mortality in hybrid mussel populations. Mar. Ecol. Prog. Ser., 71: 235-243.

Gardner, J.P.A., D.O.F. Skibinski and C.D. Bajdik. 1993. Shell growth and viability differences between the marine mussels *Mytilus edulis* (L.), *Mytilus galloprovincialis* (Lmk.) and their hybrids from two sympatric populations in S.W. England. Biol. Bull., 185: 405-416.

Gartner-Kepkay, K.E., L.M. Dickie and K.R. Freeman. 1980. Genetic differences and environments of mussel populations in the Maritime Provinces. Can. J. Fish. Aquat. Sci., 37: 775-782.

Gartner-Kepkay, K.E., E. Zouros, L.M. Dickie and K.R. Freeman. 1983. Genetic differentiation in the face of gene flow: a study of mussel populations from a single Nova Scotia embayment. Can. J. Fish. Aquat. Sci., 40: 443-451.

Geller, J.B. 1994. Sex-specific mitochondrial DNA haplotypes and hetroplasmy in *Mytilus trossulus* and *Mytilus galloprovincialis* populations. Mol. Mar. Biol. Biotechnol., 3: 334-337.

Geller, J.B., J.T. Carlton and D.A. Powers. 1994. PCR-based detection of mtDNA haplotypes of native and invading mussels on the northeastern Pacific coast: latitudinal pattern of invasion. Mar. Biol., 119: 243-249.

Gentili, M.R. and A.R. Beaumont. 1988. Environmental stress, heterozygosity, and growth rate in *Mytilus edulis* L. J. Exp. Mar. Biol. Ecol., 120: 145-153.

Gilkinson, K.D. 1983. Growth and reproduction of *Mytilus edulis* (Linnaeus) on two sides of the strait of Belle Isle. M.Sc. Thesis, Memorial University of Newfoundland. 158pp.

Gosling, E.M. 1984. The systematic status of *Mytilus galloprovincialis* in western Europe: a review. Malacologia, 25: 551-568.

Gosling, E.M. 1992a. Systematics and geographic distribution of *Mytilus*. In: The mussel *Mytilus:* ecology, physiology, genetics and culture. E.M. Gosling (Ed), Elsevier, Amsterdam. 1-20.

Gosling, E.M. 1992b. Genetics of *Mytilus*. In: The mussel *Mytilus*: ecology, physiology, genetics and culture. E.M. Gosling (Ed), Elsevier, Amsterdam. 309-382.

Gosling, E.M. 1994. Speciation and wide-scale genetic differentiation. In: Genetics and evolution of aquatic organisms. A.R. Beaumont (Ed), Chapman and Hall, London. 1-14.

Gosling, E.M. and D. McGrath. 1990. Genetic variability in exposed-shore mussels, *Mytilus* spp., along an environmental gradient. Mar. Biol., 104: 413-418.

Gosling, E.M. and N.P. Wilkins. 1981. Ecological genetics of the mussels *Mytilus edulis* and *M. galloprovincialis* on Irish coasts. Mar. Ecol. Prog. Ser., 4: 221-227.

Gould, A.A. 1850. Shells from the United States exploring expedition. Proc. Boston Soc. Nat. Hist., 3: 343-348.

Grant, C.M., S.H. Hooker, R.C. Babcock and R.G. Creese. 1998. Synchronous spawning and reproductive incompatibility of two bivalve species: *Paphies subtriangulata* and *Paphies australis*. Veliger, 41: 148-156.

Hagström, B.E. and S. Lönning. 1961. Morphological and experimental studies on the genus *Echinus*. Sarsia, 4: 21-31.

Harger, J.R.E. 1968. The role of behavioral traits in influencing the distribution of two species of sea mussel, *Mytilus edulis* and *Mytilus californianus*. Veliger, 11: 45-49.

Harger, J.R.E. 1970a. The effect of wave impact on some aspects of the biology of sea mussels. Veliger, 12: 401-414.

Harger, J.R.E. 1970b. The effects of species compositions on the survival of mixed populations of the sea mussels *Mytilus californianus* and *Mytilus edulis*. Veliger, 13: 147-152.

Harrison, R.G. 1990. Hybrid zones: windows on evolutionary process. Oxford Surveys in Evolutionary Biology, 7: 69-128.

Harrison, R.G. 1993. Hybrids and hybrid zones: historical perspective. In: Hybrid zones and evolutionary process. R.G. Harrison (Ed), Oxford University Press, Oxford. 3-13.

Harrison, R.G. and D.M. Rand. 1989. Mosaic hybrid zones and the nature of species boundaries. In: Speciation and its consequences. D. Otte and J.A. Endler (Eds), Sinauer, Sunderland, Massachusetts. 111-133.

Hawkins, A.J.S. and B.L. Bayne. 1992. Physiological interrelations, and the regulation of production. In: The mussel *Mytilus*: ecology, physiology, genetics and culture. E. Gosling (Ed), Elsevier, Amsterdam. 171-222.

Hawkins, A.J.S. and A.J. Day. 1996. The metabolic basis of genetic differences in growth efficiency among marine animals. J. Exp. Mar. Biol. Ecol., 203: 93-115.

Hawkins, A.J.S., A.J. Day, A. Gerard, Y. Naciri, C. Ledu, B.L. Bayne and M. Héral. 1994. A genetic and metabolic basis for faster growth among triploids induced by blocking meiosis I but not meiosis II in the larviparous European flat oyster, *Ostrea edulis* L. J. Exp. Mar. Biol. Ecol., 184: 21-40.

Healy, J.M. 1996. Molluscan sperm ultrastructure: correlation with taxonomic units within the Gastropoda, Cephalopoda and Bivalvia. In: Origin and evolutionary radiation of the Mollusca. J. Taylor (Ed), University Press, Oxford. 99-113.

Heath, D.D., P.D. Rawson and T.J. Hilbish. 1995. PCR-based nuclear markers identify alien blue mussel (*Mytilus* spp.) genotypes on the west coast of Canada. Can. J. Fish. Aquat. Sci., 52: 2621-2627.

Heath, D.D., D.R. Hatcher and T.J. Hilbish. 1996. Ecological interaction between sympatric *Mytilus* species on the West coast of Canada investigated using PCR markers. Molecular Ecology, 5: 443-447.

Hefferman, P.B. and R.L. Walker. 1989. Quantitative image analysis methods for use in histological studies of bivalve reproduction. J. Moll. Stud., 55: 135-137.

Heritage, G.D. 1983. A blue mussel (*Mytilus edulis* L.) culture pilot project in south coastal British Columbia. Can. Tech. Rep. Fish. Aquat. Sci., 1174: 1-27.

Hewitt, G.M. 1989. The subdivision of species by hybrid zones. In: Speciation and its consequences. D. Otte and D. Endler (Eds), Sinauer Associates, Sunderland. 85-110.

Hickman, R.W. 1992. Mussel cultivation. In: The mussel *Mytilus*: ecology, physiology, genetics and culture. E. Gosling (Ed), Elsevier Amsterdam. 465-510.

Hilbish, T.J. 1985. Demographic and temporal structure of an allele frequency cline in the mussel *Mytilus edulis*. Mar. Biol., 86: 163-171.

Hilbish, T.J. 1986. Growth trajectories of shell and soft tissue in bivalves: seasonal variation in *Mytilus edulis* L. J. Exp. Mar. Biol. Ecol., 96: 103-113.

Hilbish, T.J. 1996. Population genetics of marine species: the interaction of natural selection and historically differentiated populations. J. Exp. Mar. Biol. Ecol., 200: 67-83.

Hilbish, T.J. and R.K. Koehn. 1985. The physiological basis of natural selection at the Lap locus. Evolution, 39: 1302-1317.

Hilbish, T.J. and K.M. Zimmerman. 1988. Genetic and nutritional control of the gametogenic cycle in *Mytilus edulis*. Mar. Biol., 98: 223-228.

Hilbish, T.J., B.L. Bayne and A. Day. 1994. Genetics of physiological differentiation within the marine mussel genus ***Mytilus***. Evolution, 48: 267-286.

Hodgson, A.N. and R.T.F. Bernard. 1986. Observations on the ultrastructure of the spermatozoon of two mytilids from the south-west coast of England. J. Mar. Biol. Ass. U.K., 66: 385-390.

Hodgson, G. 1988. Potential gamete wastage in synchronously spawning coral due to hybrid inviability. Proceedings of the 6th International Coral Reef Symposium, 2: 707-714.

Hrs-Brenko, M. 1971. The reproductive cycle of the *Mytilus galloprovincialis* Lamk. in the Northern Adriatic Sea and *Mytilus edulis* L. at Long Island Sound. Thalassia. Jugoslavica., 7: 533-542.

Hu, Y.P., R.A. Lutz and R.C. Vrijenhoek. 1992. Electrophoretic identification and genetic analysis of bivalve larvae. Mar. Biol., 113: 227-230.

Hunt, H.L. and R.E. Scheibling. 1996. Physical and biological factors influencing mussel (*Mytilus trossulus*, *M. edulis*) settlement on a wave-exposed rocky shore. Mar. Ecol. Prog. Ser., 142: 135-145.

Hunt, H.L. and R.E. Scheibling. 1998. Spatial and temporal variability of patterns of colonization by mussels (*Mytilus trossulus, M. edulis*) on a wave-exposed rocky shore. Mar. Ecol. Prog. Ser., 167: 155-169.

Incze, L.S. and R.A. Lutz. 1980. Mussel culture: an east coast perspective. In: Mussel culture and harvest: a North American perspective. Elsevier Scientific Publishing, Amsterdam, Holland. 99-140.

Incze, L.S., P.B. Ortner and J.D. Schumacher. 1990. Microzooplankton, vertical mixing and advection in a larval fish patch. J. Plankton Res., 12: 365-379.

Innes D.J. and J. Bates. 1999. Morphological variation of *Mytilus edulis* and *Mytilus trossulus* in eastern Newfoundland. Mar. Biol., 133: In Press

Innes, D.J. and L.E. Haley. 1977. Genetic aspects of larval growth under reduced salinity in *Mytilus edulis*. Biol. Bull., 153: 312-321.

Inoue, K., S. Odo, T. Noda, S. Nakao, S. Takeyama, E. Yamaha, F. Yamazaki and S. Harayama. 1997. A possible hybrid zone in the *Mytilus edulis* complex in Japan revealed by PCR markers. Mar. Biol., 128: 91-95.

Inoue, K., J.H. Waite, M. Matsuoka, S. Odo and S. Harayama. 1995. Interspecific variations in adhesive protein sequences of *Mytilus edulis, Mytilus galloprovincialis*, and *Mytilus trossulus*. Biol. Bull., 189: 370-375.

Insua, A., J.P. Labat and C. Thiriot-Quievreux. 1994. Comparative analysis of Karyotypes and nuclear organizer regions in different populations of *Mytilus trossulus, Mytilus edulis* and *Mytilus galloprovincialis*. J. Moll. Stud., 60: 359-370.

Johannesson, K., N. Kautsky and M. Tedengren. 1990. Genotypic and phenotypic differences between Baltic and North Sea populations of *Mytilus edulis* evaluated through reciprocal transplantations. II. Genetic variation. Mar. Ecol. Prog. Ser., 59: 211-219.

Kafanov, A.I. and A.L. Drozdov. 1998. Comparative sperm morphology and phylogenetic classification of recent Mytiloidea (Bivalvia). Malacologia, 39: 129-139.

Kautsky, N. 1982. Quantitative studies on gonad cycle, fecundity, reproductive output and recruitment in a Baltic *Mytilus edulis* population. Mar. Biol., 68: 143-160.

Key, K.H.L. 1968. The concept of stasipatric speciation. Syst. Zool., 17: 14-22.

Kielley, M. 1997. Present situation on mussel culture in Canada. Proceedings of the First World-wide mussel conference, Vigo, Spain. 45-47.

King, P.A., D. McGrath and E.M. Gosling. 1989. Reproduction and settlement of *Mytilus edulis* on an exposed rocky shore in Galway Bay, west coast of Ireland. J. Mar. Biol. Ass. U.K., 69: 355-365.

Kiyomoto, M., A. Komaru, J. Scarpa, K.T. Wada, E. Danton and M. Awaji. 1996. Abnormal gametogenesis, male dominant sex ratio, and Sertoli cell morphology in induced triploid mussels, *Mytilus galloprovincialis*. Zool. Sci., 13: 393-402.

Koehn, R.K. 1991. The genetics and taxonomy of species in the genus *Mytilus*. Aquaculture, 94: 125-145.

Koehn, R.K., B.L. Bayne, M.N. Moore and J.F. Siebenaller. 1980. Salinity related physiological and genetic differences between populations of *Mytilus edulis*. Biol. J. Linn. Soc., 14: 319-334.

Koehn, R.K. and P.M. Gaffney. 1984. Genetic heterozygosity and growth rate in *Mytilus edulis*. Mar. Biol., 82: 1-7.

Koehn, R.K., J.G. Hall, D.J. Innes and A.J. Zera. 1984. Genetic differentiation of *Mytilus edulis* in eastern North America. Mar. Biol., 79: 117-126.

Koehn, R.K., R. Milkman and J. Mitton. 1976. Population genetics of marine pelecypods. IV. Selection, migration and genetic differentiation in the blue mussel *Mytilus edulis*. Evolution, 30: 2-32.

Koehn, R.K., R.I.E. Newell and F. Immerman. 1980. Maintenance of an aminopeptidase allele frequency cline by natural selection. Proc. Natl. Acad. Sci. USA, 77: 5385-5389.

Kordos, M.L. and R.S. Burton. 1993. Genetic differentiation of Texas Gulf coast populations of the blue crab *Callinectes sapidus*. Mar. Biol., 117: 227-233.

Lamarck, J.B.P.A. 1819. Histoire naturelle des animaux sans vertèbres. Vol. 6 A.S.B. Verdiere Libraire, Paris.

Lane, D.J.W., A.R. Beaumont and J.R. Hunter. 1985. Byssus drifting and the drifting threads of the young post-larval mussel *Mytilus edulis*. Mar. Biol., 84: 301-308.

Lessios, H.A. and C.W. Cunningham. 1990. Gametic incompatibility between species of the sea urchin *Echinometra* on the two sides of the isthmus of Panama. Evolution, 44: 933-941.

Levitan, D.R. and C. Petersen. 1995. Sperm limitation in the sea. Trends in Ecology and Evolution, 10: 228-231.

Linnaeus, C. 1758. Systema naturae per regna tria naturae. 10th Edition, Vol. 1. Regnum animale. Laurentii Salvi, Stockholm, 1384pp.

Liu, L.L., D.W. Foltz and W.B. Stickle. 1991. Genetic population structure of the southern oyster drill *Stramonita* (= *Thais*) *haemastoma*. Mar. Biol., 111: 71-79.

Loosanoff, V.L. and H.C. Davis. 1963. Rearing of bivalve molluscs. Adv. Mar. Biol., 1: 1-136.

Lowe, D.M., M.N. Moore and B.L. Bayne. 1982. Aspects of gametogenesis in the marine mussel *Mytilus edulis* L. J. Mar. Biol. Ass. U.K., 62: 133-145.

Lubet, P. 1957. Cycle sexuel de *Mytilus edulis* L. et de *Mytilus galloprovincialis* Lmk. dans le Bassin d' Arcachon (Gironde). Année biologique, 33: 19-29.

Lubet, P. 1959. Recherches sur le cycle sexual et l'émission des gamètes chez les Mytilidae et les Pectinidae. Revue des travaux. Institut des pêches maritimes, 23: 396-545.

Lubet, P., G. Prunus, M. Masson and D. Bucaille. 1984. Recherches experimentales sur l'hybridization de *Mytilus edulis* L. et *M. galloprovincialis* Lmk. (Mollusques, Lamellibranches). Bull. Soc. Zool. France, 109: 87-98.

Lutz, R.A. 1980. Mussel culture and harvest: A North American Pespective. Elsevier Science Publishers, B.V., Amsterdam, 305pp.

Lutz, R.A., L.S. Incze, B. Porter, and J.K. Stotz. 1980. Seasonal variation in the condition of raft-cultivated mussels (*Mytilus edulis* L.). Proc. World Maricult. Soc., 11: 162-168.

Mallet, A.L. and C.E.A. Carver. 1995. Comparative growth and survival patterns of *Mytilus trossulus* and *Mytilus edulis* in Atlantic Canada. Can. J. Fish. Aquat. Sci., 52: 1873-1880.

Mallet, A.L., E. Zouros, K.E. Gartner-Kepkay, K.R. Freeman and L.M. Dickie. 1985. Larval viability and heterozygote deficiency in populations of marine bivalves: evidence from pair mating of mussels. Mar. Biol., 87: 165-172.

Mallet, J. and N.H. Barton. 1989. Strong natural selection in a warning-color hybrid zone. Evolution, 43: 421-431.

Martinez-Lage, A., A. Gonzalez-Tizon and J. Mendez. 1995. Chromosomal markers in three species of the genus *Mytilus* (Mollusca: Bivalvia). Heredity, 74: 369-375.

Mason, J. 1976. Cultivation. In: marine mussels their ecology and physiology. B.L. Bayne (Ed), Cambridge University Press, London. 385-410.

Mayr, E. 1970. Populations, species and evolution. Belknap Press of Harvard University Press, Cambridge MA. 453pp.

Mayr, E. and P.D. Ashlock. 1991. Principles of systematic zoology. 2nd Ed. McGraw-Hill, Inc. New York. 475pp.

McDonald, J.H. and R.K. Koehn. 1988. The mussels *Mytilus galloprovincialis* and *M. trossulus* on the Pacific coast of North America. Mar. Biol., 99: 111-118.

McDonald, J.H., R. Seed and R.K. Koehn. 1991. Allozymes and morphometric characters of three species of *Mytilus* in the northern and southern hemispheres. Mar. Biol., 111: 323-333.

McEuan, F.S. 1988. Spawning behaviour of northeast Pacific sea cucumbers (Holothuroidea: Echinodermata). Mar. Biol., 98: 565-585.

Minchin, D. 1993. Multiple species mass spawning events in an Irish Sea Loch: the effect of temperature on spawning and recruitment of marine invertebrates. Inv. Reprod. Devel., 22: 229-238.

Moore, W.S. and J.T. Price. 1993. Nature of selection in the northern flicker hybrid zone and its implications for speciation theory. In: Hybrid zones and the evolutionary process. R.G. Harrison (Ed), Oxford University Press, Oxford. 196-225.

Moynihan, E.P. and G.A.T. Mahon. 1983. Quantitative karyotype analysis in the mussel *Mytilus edulis* L. Aquaculture, 33: 301-309.

Mullis, L.B. and F.A. Faloona. 1987. Specific synthesis of DNA *in vitro* via a polymerase catalyzed chain reaction. Methods in Enzymology, 155: 335-350.

Myrand, B. 1990. Can we increase mussel (*Mytilus edulis*) production in the Magdalen Islands (Quebec, Canada) by a better use of local stock-site combinations?. Bull. Aquacult. Assoc. Can., 90: 72-74.

New, M.B. 1997. Aquaculture and the capture fisheries, balancing the scales. World Aquaculture, June 1997: 11-30.

Newell, R.I.E., T.J. Hilbish, R.K. Koehn and C.J. Newell. 1982. Temporal variation in the reproductive cycle of *Mytilus edulis* L. (Bivalvia, Mytilidae) from localities on the east coast of the United States. Biol. Bull., 162: 299-310.

Newell, R.I.E. and R.J. Thompson. 1984. Reduced clearance rates associated with spawning in the mussel, *Mytilus edulis* (L.) (Bivalvia, Mytilidae). Mar. Biol. Lett., 5: 21-33.

Newkirk, G.F. 1980. Review of the genetics and the potential for selective breeding of commercially important bivalves. Aquaculture, 19: 209-228.

Newkirk, G.F. 1983. Applied breeding of commercially important molluscs: a summary of discussion. Aquaculture, 33: 415-422.

Olive, P.J.W. 1992. The adaptive significance of seasonal reproduction in marine invertebrates: the importance of distinguishing between models. Inv. Reprod. Devel., 22: 165-174.

Olson, R.R., J.A. Runstadler and T.D. Kocher. 1991. Whose larvae? Nature (Lond), 351: 357-358.

Palumbi, S.R. 1994. Genetic divergence, reproductive isolation, and marine speciation. Ann. Rev. Ecol. Syst., 25: 547-572.

Palumbi, S.R. and E.C. Metzz. 1991. Strong reproductive isolation between closely related tropical sea urchins (genus *Echinometra*). Molecular Biology and Evolution, 8: 227-239.

Pechenik, J.A., L.S. Eyster, J. Widdows and B.L. Bayne. 1990. The influence of food concentration and temperature on growth and differentiation of blue mussel larvae, *Mytilus edulis* L. J. Exp. Mar. Biol. Ecol., 136: 47-64.

Pulfrish, A. 1997. Seasonal variation in the occurrence of planktic bivalve larvae in the Schleswig-Holstein Wadden Sea. Helgoländer Meeressunters., 51: 23-39.

Quesada, H., C. Gallagher, D.O.F. Skibinski and D.A.G. Skibinski. 1998. Patterns of polymorphism and gene flow of gender-associated mitochondrial DNA lineages in European mussel populations. Molecular Ecology, 7: 1041-1051.

Ramorino, L. and B. Campos. 1983. Larvas y postlarvas de Mytilidae de Chile (Mollusca: Bivalvia). Rev. Biol. Mar. Valparaiso, 19: 143-192.

Rawson, P.D. and T.J. Hilbish. 1995. Distribution of the male and female mtDNA lineages in populations of blue mussels, *Mytilus trossulus* and *M. galloprovincialis*, along the Pacific coast of North America. Mar. Biol., 124: 245-250.

Rawson, P.D. and T.J. Hilbish. 1998. Asymmetric introgression of mitochondrial DNA among European populations of blue mussels (*Mytilus* spp). Evolution, 52: 100-108.

Rawson, P.D., K.L. Joyner, K. Meetze and T.J. Hilbish. 1996a. Evidence for intragenic recombination within a novel genetic marker that distinguishes mussels in the *Mytilus edulis* species complex. Heredity, 77: 599-607.

Rawson, P.D., C.L. Secor and T.J. Hilbish. 1996b. The effects of natural hybridization on the regulation of doubly uniparental mtDNA inheritance in blue mussels (*Mytilus* spp.) Genetics, 144: 241-248.

Rodhouse, P.G., J.H. McDonald, R.I.E. Newell and R.K. Koehn. 1986. Gamete production, somatic growth and multiple-locus enzyme heterozygosity in *Mytilus edulis*. Mar. Biol., 90: 209-214.

Roff, D.A. and P. Bentzen. 1989. The statistical analysis of mitochondrial DNA polymorphisms: $\chi^2$ and the problem of small samples. Mol. Biol. Evol., 6: 539-545.

Saavedra, C., D.T. Stewart, R.R. Stanwood and E. Zouros. 1996. Species-specific segregation of gender-associated mitochondrial DNA types in an area where two mussel species (*Mytilus edulis* and *M. trossulus*) hybridize. Genetics, 143: 1359-1367.

Sanjuan, A., A.S. Comesaña and A. De Carlos. 1996. Macrogeographic differentiation by mtDNA restriction site analysis in the S.W. European *Mytilus galloprovincialis* Lmk. J. Exp. Mar. Biol. Ecol., 198: 89-100.

Sanjuan, A., C. Zapata and G. Alvarez. 1994. *Mytilus galloprovincialis* and *M. edulis* on the coasts of the Iberian peninsula. Mar. Ecol. Prog. Ser., 113: 131-146.

Sanjuan, A., C. Zapata and G. Alvarez. 1997. Genetic differentiation in *Mytilus galloprovincialis* Lmk. throughout the world. Ophelia, 47: 13-31.

Sarver, S.K. and D.W. Foltz. 1993. Genetic population structure of a species' complex of blue mussels (*Mytilus* spp.). Mar. Biol., 117: 105-112.

Sarver, S.K., M.C. Landrum and D.W. Foltz. 1992. Genetic and taxonomy of ribbed mussels (*Geukensia* spp.). Mar. Biol., 113: 385-390.

Sastry, A.N. 1979. Pelecypoda (excluding Ostreidae). In: Reproduction of Marine invertebrates, Vol. 5. Molluscs: Pelecypods and lesser classes. A.C. Giese and J.S. Pearse (Eds), Academic Press New York. 113-292.

Scarpa, J. and S.K. Allen Jr. 1992. Comparative kinetics of meiosis in hybrid crosses of Pacific oyster *Crassostrea gigas* and Suminoe oyster *C. rivularis* with the American oyster *C. virginica*. J. Exp. Zool., 263: 316-322.

Scarpa, J., J.E. Toro and K.T. Wada. 1994. Direct comparison of six methods to induce triploidy in bivalves. Aquaculture, 119: 119-133.

Scheltema, R.S. 1971. Larval dispersal as a means of genetic exchange between geographically separated populations of shallow water benthic marine gastropods. Biol. Bull., 140: 284-322.

Scheltema, R.S. 1978. On the relationship between dispersal of pelagic veliger larvae and the evolution of marine prosobranch gastropods. In: Marine Organisms: Genetics, Ecology and Evolution. B. Battaglia and J.A. Beardmore (Eds), Plenum Press, New York. 303-322.

Seed, R. 1968. Factors influencing shell shape in the mussel *Mytilus edulis*. J. Mar. Biol. Assoc. U.K., 48: 561-584

Seed, R. 1969. The ecology of *Mytilus edulis* L. (Lamellibranchiata) on exposed rocky shores. 1. Breeding and settlement. Oecologia, 3: 277-316.

Seed, R. 1972. Morphological variation in *Mytilus* from the French coasts in relation to the occurrence and distribution of *Mytilus galloprovincialis* (Lmk.). Cahiers de Biologie Marine, 13: 357-384.

Seed, R. 1973. Absolute and allometric growth in the mussel *Mytilus edulis* L. (Mollusca: Bivalvia), Proc. Malac. Soc. Lond., 40: 343-357.

Seed, R. 1976. Ecology. In: Marine mussels their ecology and physiology. B.L. Bayne (Ed), Cambridge University Press. 13-66.

Seed, R. 1978. The systematics and evolution of *Mytilus galloprovincialis* Lmk. In: Marine Organisms: Genetics, Ecology and Evolution. B. Battaglia and J. Beardmore (Eds),. Plenum Press, New York. 447-468.

Seed, R. 1980. Shell growth and form in the Bivalvia. In: Skeletal growth of aquatic organisms. D.C. Rhoads and R.A. Lutz (Eds.), Plenum, 23-67.

Seed, R. 1992. Systematics evolution and distribution of mussels belonging to the genus *Mytilus*: an overview. Amer. Malac. Bull., 9: 123-137.

Seed, R. and R.A. Brown. 1977. A comparison of the reproductive cycle of *Modiolus modiolus* (L.), *Cerastoderma* (=*Cardium*) *edule* (L.) and *Mytilus edulis* in Strangford Lough, Northern Ireland. Oecologia, 30: 173-188.

Seed, R. and R.A. Brown. 1978. Growth as a strategy for survival in two marine bivalves, *Cerastoderma edule* and *Modiolus modiolus*. J. Anim. Ecol., 47: 283-292.

Seed, R. and T.H. Suchanek. 1992. Population and community ecology of *Mytilus*. In: The mussel *Mytilus*: ecology, physiology, genetics and culture. E.M. Gosling (Ed), Elsevier, Amsterdam. 87-169.

Shaw, D.D., A.D. Marchand, N. Contreras, M.L. Arnold, F. Groeters and B.C. Kohlmann. 1993. Genomic and environmental determinants of a narrow hybrid zone: Cause or coincidence?. In: Hybrid zones and the evolutionary process. R.G. Harrison. (Ed), Oxford University Press, Oxford. 165-195.

Sigurdsson, J.B., C.W. Titman and P.A. Davies. 1976. The dispersal of young post-larval bivalve molluscs by byssus threads. Nature, 262: 386-387.

Singh, S.M. and E. Zouros. 1978. Genetic variation associated with growth rate in the oyster (*Crassostrea virginica*). Evolution, 32: 342-353.

Skibinski, D.O.F. 1983. Natural selection in hybrid mussel populations. In: Protein polymorphism: adaptive and taxonomic significance. G.S. Oxford and D. Rollison, (Eds), Academic Press, London. 283-298.

Skibinski, D.O.F., M. Ahmad and J.A. Beardmore. 1978. Genetic evidence for naturally occurring hybrids between *Mytilus edulis* and *Mytilus galloprovincialis* Evolution, 32: 354-364.

Skibinski, D.O.F. and J.A. Beardmore. 1979. A genetic study of intergradation between *Mytilus edulis* and *M. galloprovincialis*. Experientia, 35: 1442-1444.

Skibinski, D.O.F., J.A. Beardmore and T.F. Cross. 1983. Aspects of the population genetics of *Mytilus* (Mytilidae: Mollusca) in the British Isles. Biol. J. Linn. Soc., 19: 137-183.

Skibinski, D.O.F., T.F. Cross and M. Ahmad. 1980. Electrophoretic investigations of systematic relationships in the marine mussels *Modiolus modiolus* L., *Mytilus edulis* L. and *Mytilus galloprovincialis* Lmk. Biol. J. Linn. Soc., 13: 65-73.

Skibinski, D.F.O., C. Gallagher and C.M. Beynon. 1994. Mitochondrial DNA inheritance. Nature, 368: 817-818.

Skibinski, D.O.F. and E.E. Roderick. 1991. Evidence of selective mortality in favor of the *Mytilus galloprovincialis* Lmk phenotype in British mussel populations. Biol. J. Linn. Soc., 42: 351-366.

Skidmore, D.A. and K.K. Chew. 1985. Mussel aquaculture in Puget Sound. Washington Sea Grant Rep. 85-4. University of Washington, Seattle, Wa. 56p.

Slatkin, M. 1973. Gene flow and selection in a cline. Genetics, 75: 733-756.

Slatkin, M. 1975. Gene flow and selection in a two locus system. Genetics, 81: 787-802.

Sokal, R.R. and F.J. Rohlf. 1981. Biometry. W.H. Freeman, San Francisco. 859pp.

Sokal, R.R. and F.J. Rohlf. 1987. Introduction to Biostatistics. 2th edition. W.H. Freeman and Company, New York, 363pp.

Soot-Ryen, T. 1955. A report on the family Mytilidae (Pelecypoda). Allan Hancock Pacific Expeditions, 20: 1-174.

Soot-Ryen, T. 1969. Family Mytilidae Rafinesque 1815. In: Treatise on invertebrate palaeontology, part N. Vol. 1, Mollusca 6. Bivalvia. R.C. Moore (Ed), Geological Society of America and University of Kansas Press, Lawrence. 271-280.

Sprung, M. 1983. Reproduction and fecundity of the mussel *Mytilus edulis* at Helgoland (North Sea). Helgoländer Meeresunters., 36: 243-255.

Sprung, M. and B.L. Bayne. 1984. Some practical aspects of fertilizing the eggs of the mussel *Mytilus edulis* L. Journal du Conseil International pour l'Exploration de la Mer, 41: 125-128.

Steward, D.T., C. Saavedra, R.R. Stanwood, A.O. Ball and E. Zouros. 1995. Male and female mitochondrial DNA lineages in the blue mussel (*Mytilus edulis*) species group. Mol. Biol. Evol., 12: 735-747.

Strathmann, R.R. 1985. Feeding and nonfeeding larval development and life-history evolution in marine invertebrates. Ann. Rev. Ecol. Syst., 16: 339-361.

Suchanek, T.H. 1978. The ecology of *Mytilus edulis* L. in exposed rocky intertidal communities. J. Exp. Mar. Biol. Ecol., 31: 105-120.

Suchanek, T.H. 1981. The role of disturbance in the evolution of life hystory strategies in the intertidal mussels *Mytilus edulis* and *Mytilus californianus*. Oecologia, 50: 143-152.

Suchanek, T.H., J.B. Geller, B.R. Kreiser and J.B. Mitton. 1997. Zoogeographic distributions of the sibling species *Mytilus galloprovincialis* and *M. trossulus* (Bivalvia:Mytilidae) and their hybrids in the North Pacific. Biol. Bull., 193: 187-194.

Sunila, I. 1981. Reproduction of *Mytilus edulis* L. (Bivalvia) in a brackish water area, the Gulf of Finland. Annals Zool. Fenn., 18: 121-128.

Sutherland, B., D. Stewart, E.E. Kenchington and E. Zouros. 1998. The fate of paternal mitochondrial DNA in developing female mussels, *Mytilus edulis*: implications for the mechanism of doubly uniparental inheritance of mitochondrial DNA. Genetics, 148: 341-347.

Swofford, D.L. and R.B. Selander. 1981. BIOSYS-1, FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. J. Hered., 72: 281-283.

Templeton, A.R. 1989. The meaning of species and speciation: A genetic perspective. In: Speciation and its consequences. D. Otte and J.A. Endler (Eds), Sinauer Associates, Sunderland, Massachusetts. 3-27.

Theisen, B.F. 1968. Growth and mortality of cultured mussels in the Danish Wadden Sea. Medd. Dan. Fisk. Havunders, 6: 47-78.

Thiriot-Quievreux, C. 1984. Chromosome analysis of three species of *Mytilus* Bivalvia: Mytilidae. Mar. Biol. Lett., 5: 255-273.

Thiriot-Quievreux, C. and N. Ayraud. 1982. Les caryotypes de quelques especes de bivalves et de gasteropodes marins. Mar. Biol., 70: 165-172.

Thompson, R.J. 1979. Fecundity and reproductive effort in the blue mussel (*Mytilus edulis*), the sea urchin (*Strongylocentrotus droebachiensis*), and the snow crab (*Chionoecetes opilio*) from populations in Nova Scotia and Newfoundland. Can. J. Fish Aquat. Sci., 36: 955-964.

Thompson, R.J. 1984a. Production, reproductive effort, reproductive value and reproductive cost in a population of the blue mussel *Mytilus edulis* from a subarctic environment. Mar. Ecol. Prog Ser., 16: 249-257.

Thompson, R.J. 1984b. The reproductive cycle and physiological ecology of the mussel *Mytilus edulis* in a subarctic, non-estuarine environment. Mar. Biol., 79: 277-288.

Thompson, R.J. 1991. Mortality in mussels: a physiological perspective. Proceedings; Atelier de travail sur la mortalité estivale des moules aux Iles-de-la-Madeleine. Quebec. 171-176.

Thorson, G. 1957. Bottom communities (sublittoral or shallow shelf). Memoirs of the Geological Society of America, 67: 461-534.

Toro, J.E. 1998. PCR-based nuclear and mtDNA markers and shell morphology as an approach to study the taxonomic status of the Chilean blue mussel (*Mytilus chilensis*) (Bivalvia). Aquatic Living Resources, 11: 347-353.

Toro, J.E. and L. Paredes. 1996a. Feeding trial with different families of Chilean blue mussel, *Mytilus chilensis* larvae: evidence for genotype-food interaction. Journal of Applied Aquaculture, 6: 81-87.

Toro, J.E. and L.I. Paredes. 1996b. Heritability estimates of larval shell length in the Chilean blue mussel *Mytilus chilensis*, under different food densities. Aquatic Living Resources, 9: 347-350.

Toro, J.E. and H.D. Sastre. 1995. Induced triploidy in the Chilean blue mussel, *Mytilus chilensis* (Hupe, 1854), and performance of triploid larvae. J. Shellfish Res., 14: 161-164.

Uehara, T., H. Asakura and Y. Arakaki. 1990. Fertilization blockage and hybridization among species of sea urchins. In: Advances in Invertebrate Reproduction. M. Hoshi and O. Yamashita (Eds), Vol. 5. Elsevier, Amsterdam, 305-310.

Väinölä, R. 1990. Allozyme differentiation between Baltic and North Sea *Mytilus* populations: a reassessment of evidence from transplantation. Mar. Ecol. Prog. Ser., 67: 305-308.

Väinölä, R. and M.M. Hvilsom. 1991. Genetic divergence and a hybrid zone between Baltic and North sea *Mytilus* populations (Mytilidae:Mollusca). Biol. J. Linn. Soc., 43: 127-148.

Varvio, S.L., R.K. Koehn and R. Vainola. 1988. Evolutionary genetics of the *Mytilus edulis* complex in North Atlantic region. Mar. Biol., 98: 51-60.

Viard, F., B. Delay, C. Coustau and F. Renaud. 1994. Evolution of the genetic structure of bivalve cohorts at hybridization sites of the *Mytilus edulis-M. galloprovincialis* complex. Mar. Biol., 119: 535-539.

Wendel, R.H. and D.W. Garton. 1995. Variation in genotype frequencies during the life history of the bivalve, *Dreissena polymorpha*. Evolution, 46: 1284-1288.

Wilbur, A.E. and P.M. Gaffney. 1997. A genetic basis for geographic variation in shell morphology in the bay scallop, *Argopecten irradians*. Mar. Biol., 128: 97-105.

Wiley, E.O. 1978. The evolutionary species concept reconsidered. Syst. Zool. 27: 17-26.

Wiley, E.O. 1981. Phylogenetics: the theory and practice of phylogenetic systematics. Wiley, New York, N.Y.

Wilhelm, R. and T.J. Hilbish. 1998. Assessment of natural selection in a hybrid population of mussels: evaluation of exogenous vs endogenous selection models. Mar. Biol., 131: 505-514.

Wilkins, N.P., K. Fujino and E.M. Gosling. 1983. The Mediterranean mussel *Mytilus galloprovincialis* Lmk. in Japan. Biol. J. Linn. Soc., 20: 365-374.

Wilkinson, L. 1991. SYSTAT. The system for statistics. Systat, Inc. Evaston, Illinois. 676pp.

Willis, G.L. and D.O.F. Skibinski. 1992. Variation in strength of attachment to the substrate explains differential mortality in hybrid mussels (*Mytilus galloprovincialis* and *Mytilus edulis*) populations. Mar. Biol., 112: 403-408.

Winter, J.E., J.E. Toro, J.M. Navarro, G.S. Valenzuela and O.R. Chaparro. 1984. Recent developments, status, and prospects of molluscan aquaculture on the Pacific coast of South America. Aquaculture, 39: 95-134.

Woodruff, D.S., K.C. Staub, E.S. Upatham, V. Viyanant and H.C. Yuan. 1988. Genetic variation in *Oncomelania hupensis*: *Schistostoma japonicum* transmitting snails in China and the Philippines are distinct species. Malacologia, 29: 347-361.

Worral, C.M. and J. Widdows. 1984. Investigation of factors influencing mortality in *Mytilus edulis* L. Mar. Biol. Lett., 5: 85-97.

Zouros, E., K.R. Freeman, A.O. Ball and G.H. Pogson. 1992. Direct evidence for extensive paternal mitochondrial DNA inheritance in the marine mussel *Mytilus*. Nature, 359: 412-414.

Zouros, E., A.O. Ball, C. Saavedra and K.R. Freeman. 1994a. An unusual type of mitochondrial DNA inheritance in the blue mussel *Mytilus*. Proc. Natl. Acad. Sci., 91: 7463-7467.

Zouros, E., A.O. Ball, C. Saavedra and K.R. Freeman. 1994b. Mitochondrial DNA inheritance. Nature, 368: 818.

Zaykin, D.V. and A.I. Pudovkin. 1993. Two programs to estimate chi-square values using pseudo-probability test. J. Hered., 84: 152.