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Gametogenic Cycles of Marine Mussels, Mytilus edulis and Mytilus trossulus, in Cobscook Bay, Maine

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GAMETOGENIC CYCLES OF MARINE MUSSELS, *MYTILUS EDULIS* AND *MYTILUS TROSSULUS*, IN COBSCOOK BAY, MAINE

By

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Thesis Advisor: Dr. Bruce J. Barber

An Abstract of the Thesis Presented
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The *Mytilus edulis* species complex includes three smooth-shelled blue mussels, *M. edulis* (Linnaeus 1758), *M. trossulus* (Gould 1850), and *M. galloprovincialis* (Lamarck 1819). When any two of theses species occur sympatrically, hybridization and backcrossing of hybrid and parental genotypes is evident. Despite introgression of genes between taxa their genetic integrity is maintained. To test the hypothesis that a temporal variation in species-specific spawning times is the mechanism limiting hybridization and maintaining genetic integrity in a *M. edulis* and *M. trossulus* hybrid zone in eastern Maine, mussels were sampled on monthly to semi-monthly intervals throughout 2000 from a low intertidal site in Cobscook Bay. Four polymerase-chain-reaction-based (PCR) genetic markers were used to differentiate between species. Gamete volume fraction (GVF) and oocyte area measurements indicated that both species are temporally synchronized with respect to gametogenesis and spawning. Thus low frequency of

hybridization and preservation of genetic identity is not the result of temporally separated spawning times. Limited hybridization may result from gametic incompatibility, differential environmental adaptation, or selective mortality of hybrids. Preferential collection of *M. edulis* seed for aquaculture in Cobscook Bay is thus not possible on the basis of spawning activity alone.

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Chapter 1

THE *MYTILUS EDULIS* SPECIES COMPLEX, GAMETOGENESIS, AND CULTURE

1.1. The *Mytilus edulis* Species Complex

Common blue mussels of temperate waters of the northern and southern hemispheres make up the *Mytilus edulis* species complex, which is composed of *M. edulis, M. galloprovincialis,* and M. *trossulus* (Koehn 1991; McDonald et al. 1991). Of the three, *M. edulis* is thought to be the ancestral form from which *M. galloprovincialis* and *M. trossulus* have diverged (Stanley 1972, Seed 1992). During glaciation events of the Pleistocene, *M. galloprovincialis* diverged in the Mediterranean (Barsotti and Meluzzi 1968) and *M. trossulus* diverged in more northerly latitudes (Varvio et al. 1988). *Mytilus trossulus* is of Pacific origin and via trans-Arctic movement has more recently expanded its range into the Northeastern Atlantic (Vermeij 1991). *Mytilus edulis* presumably retained a southern refuge on the east coast and expanded north as glaciation receded, resulting in a zone of secondary contact between species. Hybridization is observed in areas where any two of the three occur sympatrically and has created reluctance to observe them as three separate species (Seed 1974, 1978; Skibinski et al. 1983; McDonald and Koehn 1988; Skibinski 1983; Skibinski and Roderick 1991; McDonald et al. 1991; Gosling 1992). Due to the expansive distribution, morphological similarities, and lack of a completely diagnostic morphological character, confusion exists over the taxonomy of the *M. edulis* species complex.

1.1.1. Species Identification

Up to19 morphological characteristics have been used to distinguish among mussel species (McDonald et al.1991; Mallet and Carver 1995; Innes and Bates 1999). Some morphological characters produce a stronger phylogenetic signal than others, notably the adductor muscle scar, hinge plate length, and shell height (McDonald et al. 1991; Freeman et al. 1992; Mallet and Carver 1995). The consensus regarding morphology is that *Mytilus edulis* is more eccentric or elliptical while *M. trossulus* is more elongated (Innes and Bates 1999). Environmental factors (Seed 1968) or hybridization (McDonald et al. 1991) both contribute to the high level of plasticity in morphological characters, limiting the use of shell morphology for species identification. Using morphological measurements of seven shell characteristics, Mallet and Carver (1995) were unable to identify *M. edulis* and *M. trossulus* hybrids (Mallet and Carver 1995). McDonald et al. (1991) have shown that allopatric populations of *Mytilus* have a more distinct morphology than sympatric populations. Gardner (1996) suggests that for sympatric populations of *M. edulis* and *M. galloprovincialis* in southwest England, the morphological similarities were a result of common environmental conditions rather then a blending effect caused by hybridization.

Since morphological characters are not sufficient to distinguish among species and hybrids with a high degree of accuracy, the use of allozyme variation has been used (McDonald et al. 1991; Bates and Innes 1995; Comesaña et al 1999). Historically, mannose phosphate isomerase (Mpi) has been the most diagnostic allozyme marker for differentiation of *Mytilus edulis* and *M. trossulus* (Mallet and Carver 1995). Comesaña et

al. (1999) found that the Mpi enzyme was completely diagnostic for distinguishing between *M. edulis* and *M. trossulus*. This is contrary to Heath et al. (1995), who considered allozyme markers differentiated, but not completely diagnostic.

Recently developed techniques using polymerase chain reaction/restriction fragment length polymorphism-based (PCR/RFLP) genetic markers are now considered the most reliable method for distinguishing between *Mytilus edulis*, *M. trossulus* and their hybrids (Bates and Innes 1995; Innes and Bates 1999). Two such markers, polyphenolic adhesive protein (Glu-5′) and internal transcribed spacer (ITS) are nuclear DNA markers that have been used for species and hybrid identification (Heath et al. 1995; Rawson et. al. 1996; Comesaña 1999). Rawson and Hilbish (1995) used a mitochondrial subunit (mt16s) DNA haplotype to distinguish between *M. edulis* and *M. galloprovincialis*. *M. edulis* and *M. trossulus* populations in the Gulf of Maine have recently been differentiated using Glu-5′, ITS, mt16s, and the *Mytilus* anonymous marker (Mal-1) (Rawson et. al. 2001). To date, PCR-based genetic markers have provided the most diagnostic insight into the taxonomic structure of the *M. edulis* complex.

1.1.2. *Mytilus* Distributions in Eastern North America

An early survey of *Mytilus* populations on the east coast of North America using variation in allozyme frequency indicated the presence of a single species, *M. edulis* (Koehn et al. 1976). However, in a subsequent survey Koehn et al. (1984) identified two genetically distinct forms inhabiting Atlantic Canada with no evidence of hybridization. These two genetically distinct groups have since been verified and described as *M. edulis*

and *M. trossulus,* and form a zone of sympatry from northern Newfoundland south to the eastern coast of Maine (Varvio et. al. 1988; McDonald et. al. 1991; Bates and Innes 1995; Comesaña et. al. 1999; Rawson et. al. 2001). Overall, M. *edulis* is distributed from Cape Hatteras, North Carolina north to Newfoundland, and *M. trossulus*, from the Labrador coast south to eastern Maine.

Sympatric populations within the *Mytilus* complex hybridize in nature (Gosling 1992). In the Baltic Sea, *M. edulis* and *M. trossulus* hybridize so readily that they are considered semi-species (Väinölä and Hvilsom 1991). Within the European hybrid zone, the frequency of hybridization between *M. edulis* and *M. galloprovincialis* ranges from 25-80% (Sanjuan et. al. 1994; Comesaña and Sanjuan 1997; Hilbish et al. 1994). However, the frequency of hybridization between *M. edulis* and *M. trossulus* in the eastern Atlantic, even in sympatric populations, ranges from zero to only 26% (Koehn et. al. 1984; Varvio et. al. 1988; Bates and Innes 1995; Mallet and Carver 1995; Saavedra et. al. 1996; Comesaña et. al. 1999; Rawson et. al. 2001). In the eastern Pacific, average hybridization estimates between *M. trossulus* and *M. galloprovincialis* range from 5.7% (Sarver and Foltz 1993) to 22% (Rawson et al. 1999). Despite the different methodologies that have been used to discriminate between species, the frequency of hybridization in the western Atlantic and eastern Pacific have consistently been lower than in the Baltic and European hybrid zones.

1.1.3. *Mytilus trossulus* in Maine

Sowles et al. (1996), using allozyme electrophoresis at the *Mpi* locus found an appreciable frequency of *Mytilus trossulus* at Digby, Nova Scotia and Hospital Island,

New Brunswick, suggesting that blue mussel populations in the Gulf of Maine may be composed of two species, *M. edulis* and *M. trossulus*. It has since been confirmed that the Gulf of Maine contains high frequencies of *M. trossulus*, with the Cobscook Bay region in eastern Maine containing frequencies from 40 to 100% (Rawson et al. 2001).

1.1.4. The Southern Distributional Limit and Environmental Stress

Eastern Maine is the southern distributional limit of *M. trossulus*, and Rawson et al. (2001) have suggested a physical mechanism (the Eastern Maine Coastal Current) as the factor limiting larval dispersal of *M. trossulus* further southwest along the coast of Maine. The intensity of the Eastern Maine Coastal Current is highly variable (Townsend 1992) and may represent a semi-permeable barrier to larval dispersal. Environmental factors, notably temperature and food availability could also play a role in determining the distributional limits of *M. trossulus*. In adult mussels, sub-lethal levels of environmental stress are expressed as depressed growth rates, reduction in egg viability and subsequent larval survival, reduced fecundity, and a negative impact on the competitive ability of the organism (Bayne 1985). It is currently unknown if *M. trossulus* displays any signs of environmental stress near its southern distributional limit in eastern Maine.

1.2. Hybrid Zone Models and Maintenance of Genetic Identity

When hybridization is followed with backcrossing, introgression of nuclear and or mitochondrial genes occurs (Harrison 1991). Introgression of alleles from the genome of one taxa to another via hybridization results in one of four outcomes: the formation of a hybrid species, hybrids adapted to novel ecotones, reinforcement of reproductive barriers through natural selection against hybrid genotypes, or merging of the hybridizing taxon into a single evolutionary lineage (Arnold 1992).

Despite introgressive hybridization, members of the *Mytilus edulis* species complex maintain their genetic identity. The differential responses of taxa to environmental factors resulting in an assortative species composition that reflects the underling environmental heterogeneity, is known as a mosaic hybrid zone (Harrison and Rand 1989). These zones represent areas of secondary intergradation in which previously isolated taxa have become ecologically distinct, and the species-specific adaptation to particular habitat types promotes reinforcement of conspecific mating (Harrison and Rand 1989).

Sarver and Foltz (1993) suggest that *Mytilus galloprovincialis* and *M. trossulus* are ecologically distinct on the Pacific coast of North America and that environmental heterogeneity at least partly maintains genetic integrity. Gardner (1996) also infers some degree of ecological distinction between *M. galloprovincialis* and *M. edulis* in southwestern Europe by suggesting that hybridization is occurring at environmental discontinuities. Rawson et al. (2001), however, found little evidence to support

ecological distinction based on differential salinity and wave exposure (byssal strength) tolerance between *M. edulis* and *M. trossulus* in eastern Maine.

Reproductive isolation and maintenance of genetic identity has also been correlated with the degree of temporal variation in spawning events. In sympatric populations of *Mytilus galloprovincialis* and *M. edulis* in southwestern Europe, low hybridization is observed when spawning periods are out of phase with each other, while sites with a greater degree of spawning synchrony have a high degree of hybridization (Seed 1992; Gardner 1992). A comparative gametogenic study within the *M. edulis* and *M. trossulus* hybrid zone in eastern Maine has not yet been investigated.

Differential mortality and gamete compatibility could also be important in maintaining genetic integrity by facilitating conspecific mating. Gilg and Hilbish (2000) inferred that alleles specific to *M. galloprovincialis* increase in frequency with tidal as a result of genotype-dependant selection intensity on the settling spat. Differential genotypic selection structures the adult population leading to increased chances of conspecific mating due to the physical proximity of adults to one another. In the case of gamete compatibility, fertilization success is greatly reduced during interspecific crosses resulting from a mismatch in a protein recognition system at the egg-sperm interface. For sea urchins (*Echinometra* spp.), the protein bindin on the sperm and a glycoprotein receptor on the egg surface mediate sperm attachment; variation in the amino acid sequence of bindin results in reproductive failure between closely related species (Palumbi 1992; 1998). A similar phenomenon exists in abalone (*Haliotis* spp.) with the protein lysine, which is responsible for creating a hole in the vitelline envelope through which the sperm passes to fertilize the egg. A high degree of species specificity exists in

the ability of lysine to dissolve the vitelline envelope (Vacquier et al. 1990). In each case, gamete recognition proteins are responsible for maintaining assortative mating between closely related free-spawning marine invertebrates.

1.3. Reproduction

1.3.1. Gametogenesis

Mussels (*Mytilus* spp.) are dioecious and show no external signs of sexual dimorphism (Seed 1976). Spawning occurs through the release of gametes into the overlaying water column where external fertilization takes place. The initial larval phase is planktonic, lasting 2-7 weeks before settlement and metamorphosis occur (Bayne 1976; Seed 1976).

Regulation of gametogenesis and spawning in marine bivalves is considered a genetically controlled response to the environment (Barber and Blake 1991). The onset and duration of gametogenesis is proximally under neuroendrocrine control (Lubet 1955). Many exogenous factors contribute to the regulation of gametogenesis. Environmental stimuli are received and transmitted to nerve-ganglia, which release neurohormones controlling gametogenesis (Barber and Blake 1991). The most important environmental stimuli influencing bivalve reproduction are water temperature (Sastry 1979) and food availability (Newell et al. 1982). Barber and Blake (1991) discuss a threshold temperature, which must be met for the production of mature gametes to be achieved in scallops. Even under conditions of high nutrient availability, gamete maturity was halted

until a minimum threshold temperature was reached. In *Mytilus*, the production of mature gametes is independent of temperature and depends more on nutrient availability (Newell et. al. 1982; Thompson 1984).

The gametogenic cycle in marine bivalves may occur annually, semiannually or continuously depending on the species and environmental conditions (Sastry 1979; Newell et al. 1982). Development of the gonads and gamete production depend on the availability of nutrient supplies. Gametogenesis can be energetically supported from two sources: stored reserves in the mantle tissue (Bayne 1975; Gabbott 1976); recently ingested nutrients (Newell et al. 1982; Thompson 1984); or a combination of both (Barber and Blake 1991; Barber et. al. 1991). Hilbish and Zimmerman (1988) found that the onset of gametogenesis was delayed and gamete production was lower for *Mytilus edulis* having the Lap^{94} allele for leucine aminopeptidase (a protein-catabolizing enzyme). These results are indicative of the genetic influence on gametogenesis. However, the genotypic variation at the Lap^{94} locus is phenotypically expressed as a difference in assimilation efficiency. Therefore, gametogenesis, even though under genetic control, still depends on the assimilation of nutrients above those needed for metabolic maintenance.

Age also has an effect on gametogenesis. For *Mytilus edulis* and *M. galloprovincialis*, the allocation of energy to somatic or gametic growth is dependent on the age class and represents a possible source of variation in gonadal condition and spawning times (Gardner and Skibinski 1990).

1.3.2. Spawning

Marine bivalves have developed several reproductive strategies, including single mass spawning events, multiple spawning events, or continuous spawning of a few gametes over extended periods. Regardless of the particular strategy utilized, some degree of synchrony must be achieved to ensure a high fertilization rate. Both endogenous and exogenous factors play a role in spawning synchronization. The state of physiological maturity is an important endogenous factor since mature gametes must be available before spawning can be initiated. Exogenous factors potentially important in the initiation of spawning events are salinity, lunar phase, water temperature, food availability, and presence of gametes in the water column (Thompson 1984; Barber and Blake 1991; Cárceres-Martínez and Figueras 1997). The presence of gametes in the water column is thought to provide a chemical cue that stimulates spawning. Mass spawning has been observed in *Mytilus* populations coinciding with a peak in the chlorophyll-a concentration (Thompson 1984; Cárceres-Martínez and Figueras 1997). However, a peak in chlorophyll-a concentration is often associated with an increase in temperature. An abrupt change in water temperature is considered the most important exogenous factor controlling spawning synchrony and is often associated with changing tides (Thompson 1984, Barber and Blake 1991, Cárceres-Martínez and Figueras 1997).

1.3.3. Assessment of Reproductive Condition

Many methods have been used to qualitatively and quantitatively evaluate the gametogenic cycle in marine bivalves. Qualitative methods range from gross physical observations of the gonads to histologically-based staging. Staging is a subjective ranking based on the relative abundance of adipogranular tissue, vesicular connective tissue, and development of gametes within the gonad (Seed 1976; Lowe et al. 1982).

Quantitative measurements are based on seasonal changes in gonad weights or on histological analysis. The gonadal index, the seasonal variation in ash-free dry weight of the gonad, is an accurate measure of gametogenic cycles for bivalves that possess a discrete gonad that is easily dissected, such as scallops (Barber and Blake 1991). However, for other species, such as clams and oysters, the gonads are incorporated into the visceral mass and are not dissectible as a discrete unit. In mussels the gonad is primarily located within the mantle tissue which has been used for determining gonadal indices. A sharp decrease in the ash-free dry weight of the mantle tissue is assumed to represent a spawning event in mussels (Gardner and Skibinski 1990; Mallet and Carver 1995). Care must be taken with this approach, however, since a decrease in weight could also result from the resorption of gametes (Bayne et al. 1978).

Histologically-based measurements are a less subjective way to quantitatively evaluate the gametogenic cycle as well as provide important cytological information. A gonadal area index, a percentage of the total visceral mass occupied by gametes, has been used to compare the gametogenic events of marine bivalves and is considered an accurate indicator of gametogenic events (Barber et al. 1991; Barber 1996a). Newell et al. (1982)

used the gamete volume fraction (GVF) to evaluate spawning patterns for several populations of *Mytilus edulis*. To determine GVF, the area of developing gametes is measured and divided by the total gonadal area, producing a ratio of 0-1. A value of zero represents reproductive quiescence; a value of 1.0 the peak reproductive condition, and a sharp decrease in the mean GVF of the population indicates that spawning has occurred (Newell et al. 1982). An additional histological measure that has been used to determine gametogenic events in marine bivalves is the seasonal variation in oocyte diameter or area. This method has been successfully applied to a variety of species such as *Placopecten magellanicus* (Barber et al. 1988), *Mya arenaria* (Barber 1996b) and *Crassostrea gigas* (Lango-Reynoso 2000).

1.3.4. Gametogenic Variation in *Mytilus*

Observations have been made on the temporal variation in gametogenesis among mussels comprising the *Mytilus edulis* species complex. Newell et al. (1982) examined *M. edulis* populations from Delaware to southern Maine and noted site-specific variation in gonad development, which was attributed to variations in local environmental conditions. Measuring the changes in dry mantle weight of *M. edulis* and *M. trossulus* in Lunenburg Bay, Nova Scotia, spawning was determined to be nearly synchronous (Mallet and Carver 1995). However, studies at Croyde in southwest England and Whitesand Bay revealed an asynchronous spawning pattern between sympatric populations of *M. edulis* and *M. galloprovincialis* (Gardner and Skibinski 1990; Seed 1992; Secor et al. 2001).

Evaluation of *Mytilus edulis* populations from Stony Brook Harbor showed more somatic production was occurring in individuals less than three years old (<45mm), and more gametic production occurred in individuals greater than three years old (>45mm) (Rodhouse et al. 1986). This difference in the allocation of energy to gamete production could lead to differences in gonadal condition resulting in temporally varied spawning between size classes (Gardner and Skibinski 1990). Size dependant energy allocation could also contribute to variation in spawning between sites and populations with different size frequency distributions.

1.4. Mussel Culture in Maine

In 1998 the United States imported 31 million dollars of marine mussel products while exporting only 1.5 million dollars (NMFS 1999). Expansion of the mussel aquaculture industry in the U.S. could help reduce this trade deficit while creating new economic opportunities.

In Maine, mussel production is concentrated in the central and southern coast (DMR 1999). In 1998 production was valued at 1.06 million dollars (NMFS 1999). The industry relies predominately on wild harvest, and a small amount of bottom culture. Bottom culture utilizes seed harvested from natural beds, which are transplanted to bottom leases having desirable environmental conditions for growth. Industry expansion is limited by the availability of natural beds for wild harvest and suitable lease sites for bottom culture. A third, less utilized method of mussel culture is suspended raft culture, similar to that used in Spain (Mason 1976). This method also uses wild mussels in

Chapter 2

GAMETOGENIC CYCLES OF MARINE MUSSELS, *MYTILUS EDULIS* AND *MYTILUS TROSSULUS*, IN COBSCOOK BAY, MAINE

2.1. Introduction

Fertilization barriers initiate and maintain reproductive isolation between closely related organisms. Mate choice, habitat specialization and differential environmental tolerance, spawning synchrony, and gamete incompatibility are all thought to be important barrier generating processes which lead to speciation events in marine organisms (Palumbi 1994). However, reproductive barriers over a wide spectrum of marine invertebrates are often semi-permeable, with varying degrees of hybridization occurring between closely related taxa (Knowlton 1993). In free-spawning sympatric marine invertebrates, differential environmental adaptations, asynchronous spawning periods, and gamete interactions are the most likely mechanisms leading to varying degrees of conspecific mating.

In the northern hemisphere the *Mytilus edulis* species complex is composed of three free-spawning marine bivalves: *M. trossulus* in the Baltic Sea, northwestern Atlantic, and the Pacific; *M. edulis* in the eastern and western Atlantic; and *M. galloprovincialis* in the Mediterranean, Atlantic coast of southern Europe, northern Africa, and the Pacific coast of North America (Gosling 1984, 1992; Koehn 1991; McDonald et al. 1991; Suchanek et al. 1997). An early survey of *Mytilus* on the east coast of North America indicated the presence of only a single species, *M. edulis* (Koehn et al. 1976), but in a later study, Koehn et al. (1984) identified two genetically distinct

groups inhabiting Atlantic Canada. These two genetically distinct groups have since been verified as *M. edulis* and *M. trossulus* and form a zone of sympatry from northern Newfoundland south to the eastern coast of Maine (Varvio et al. 1988; McDonald et al. 1991; Bates and Innes 1995; Comesaña et al. 1999; Rawson et al. 2001).

Sympatric populations within the *Mytilus* complex hybridize in nature (Gosling 1992). In the Baltic Sea, *M. edulis* and *M. trossulus* hybridize so readily that they are considered semi-species (Väinölä and Hvilsom 1991). Within the European hybrid zone, the frequency of hybridization between *M. edulis* and *M. galloprovincialis* ranges from 25-80% (Sanjuan et. al. 1994; Comesaña and Sanjuan 1997; Hilbish et al. 1994). The frequency of hybridization between *M. edulis* and *M. trossulus* in the western Atlantic, however, ranges only from zero to 26% (Koehn et. al. 1984; Varvio et. al. 1988; Bates and Innes 1995; Mallet and Carver 1995; Saavedra et. al. 1996; Comesaña et. al. 1999; Rawson et. al. 2001). Despite the different methodologies used over time to discriminate between species (morphology, allozyme variation, mitochondrial and nuclear genetic markers), the frequency of hybridization on the Atlantic coast of North America is consistently lower than in the Baltic and European hybrid zones.

Assortative mating is a potential mechanism limiting hybridization and maintaining genetic identity in the face of gene flow within the *Mytilus edulis* complex. The differential responses of taxa to environmental factors resulting in an assortative species composition that reflects the underling environmental heterogeneity is known as a mosaic hybrid zone (Harrison and Rand 1989). Sarver and Foltz (1993) suggest that *Mytilus galloprovincialis* and *M. trossulus* are ecologically distinct on the Pacific coast of North America and that environmental heterogeneity is at least partly maintaining genetic

integrity. Gardner (1996) also infers some degree of ecological distinction between *M. galloprovincialis* and *M. edulis* in southwestern Europe by suggesting that hybridization is occurring at environmental discontinuities. Reproductive isolation and maintenance of genetic identity have also been correlated with the degree of temporal variation in spawning events. In sympatric populations of *M. galloprovincialis* and *M. edulis* in southwestern Europe, low hybridization is observed when spawning periods remain out of phase, while sites with a greater degree of synchrony have a higher degree of hybridization (Seed 1992; Gardner 1992). Interaction between gametes in the water column could potentially be important in limiting hybridization in a wide variety of free-spawning marine invertebrates.

The objective of this study was to determine whether the relatively low rate of hybridization occurring between *Mytilus edulis* and *M. trossulus* in eastern Maine could be attributed to a temporal variation in spawning. If spawning periods are distinct it might be possible for mussel farmers to preferentially collect the commercially preferred *M. edulis* seed for suspended raft culture.

2.2. Materials and Methods

2.2.1. Site Location and Collection

Approximately 120 (35 to 50mm in shell length) mussels were collected by hand from a sympatric, low intertidal population in East Bay (latitude 44° 56′ 30″ N; longitude 67° 07′ 50″ W Cobscook Bay, Washington County, Maine) throughout 2000. Samples were obtained monthly from January-April, October-December, and semi-monthly between May 4th and September 14th. Specimens were packed on ice and transported to The University of Maine for processing. Each mussel was opened and a piece of mantle tissue approximately 0.5cm^2 was removed and preserved in 95% ethanol for DNA extraction. The remainder of the animal was preserved in Dietrich's fixative (Gray 1954) for subsequent histological preparation. All preservation was completed within 24 hours of collection.

2.2.2. Genetic Characterization

DNA was extracted from gonadal tissue following the protocol of Rawson et al. (2001). Four PCR-based markers, polyphenolic adhesive protein (GLU-5′) (Rawson et al. 1996), internal transcribed spacer (ITS) (Heath et al. 1995), *Mytilus* anonymous locus-I (Mal-1) (Rawson et al. 2001), and one mitochondrial marker (mt16s-F) (Rawson and Hilbish 1995), were used to diagnostically identify *M. edulis*, *M. trossulus*, and hybrids. PCR products of the GLU-5′ nuclear marker were resolved directly in agarose to detect

polymorphism. Amplimers of ITS, Mal-1, and mt16s-F were subjected to restriction enzymes to produce restriction length fragment polymorphism (RFLP), thus creating species-specific banding patterns (Rawson et al. 2001).

Initially, the Glu-5′ marker was run on all samples. Results were used to identify 30 (40 on August 17th and 30th) individuals from each species for which the remaining markers were run. Individuals scored as a hybrid at any marker were eliminated from further analysis. The combined results of all four markers were used to pick 20 individuals (30 on August 17th and 30th) of each species for histological processing.

2.2.3. Histological Preparation

Each individual was dorso-ventrally cross-sectioned (2-3mm thick) with a razor blade just anterior of the byssal gland, dehydrated in an ascending alcohol series, cleared with Xylenes, and embedded in Paraplast (Barber 1996b). Five-micron thin cross-sections of each block were cut on a rotary microtome, placed on glass slides, stained with Shandon instant hematoxylin and eosin Y, and cover slipped for permanent preservation. Finished slides were used for subsequent quantitative measurements of gametogenesis.

2.2.4. Quantitative Assessment of Gametogenesis

Slides were examined using a compound microscope (Nikon LABPHOT-2) equipped with a video camera (Dage CCD 72). Images were digitized with a frame

grabber (Flash Point 128; Integral Technologies Inc.) and measurements made using image analysis software (Image Pro Plus; Media Cybernetics).

2.2.5. Gamete Volume Fraction (GVF)

The GVF of all individuals were calculated as the area of reproductive tissue present in one microscopic field divided by the entire area. This provided a ratio of reproductive tissue to all other mantle tissue ranging from 0-1. An overall mean of five random fields (300X) was calculated for each individual and used in subsequent statistical analysis.

2.2.6. Oocyte Area

In addition to the GVF, the mean oocyte area was calculated for each female. Cross-sectional areas of 50 oocytes with the nucleolus clearly visible were measured (1200X) for each individual. An overall mean was calculated from each individual and used in subsequent statistical analysis.

2.2.7. Statistics

GVF data were analyzed using a three-way ANOVA for sample date, species, and gender. Oocyte data were evaluated with a two-way ANOVA across sample date and species. Both data sets were evaluated at $\alpha=0.05$ using simultaneous Bonferroni pairwise

comparisons of sample level means. The number of males and females of each species analyzed in each sample date are provided in Table 1.

Statistical analyses were performed using Minitab 13.0. Minitab automatically adjusts the Bonferroni α level to compensate for the total number of possible pairwise comparisons. Since all possible combinations of pairwise comparisons were not of interest, the α level was readjusted to account for the appropriate number of comparisons used in the analysis.

2.3. Results

Gametogenic development in *Mytilus edulis* and *M. trossulus*, as determined using GVF, was very similar in Cobscook Bay throughout 2000 (Figure 1). All GVF values are reported as *M. edulis* and *M. trossulus*, respectively. Mean GVF increased for both species from February 20th (0.226, 0.141) through June 4th (0.894, 0.889). Maximum GVF measurements were reached by June 4th and maintained through July 17th, averaging 0.849 and 0.884. A sharp decrease in GVF occurred between July 17th (0.849, 0.880) and August 1st (0.475, 0.404), indicating that a major spawning event had occurred. After August 1st there was a more protracted and less pronounced decline in GVF through October 15th (0.153, 0.087). From October 15th through December 9th GVF measurements were constant.

The three-way ANOVA indicated that differences in GVF occurred between date ($F=142.53$, $P=0.000$) and gender ($F=59.24$, $P=0.000$), but not between species ($F=0.01$, $P=0.925$) (Table 2). Interactions occurred between date and species ($F=3.37$, $P=0.000$)

and between date and gender (F=2.63, P=0.001). Gametogenic cycles were the same for species (F=0.01, P=0.925); there were no significant interactions between date and gender (F=1.29, P=0.256) and date*species*gender (F=0.93 P=0.533). Though differences occurred between genders, spawning times were still synchronous. Bonferroni pairwise comparisons (α=0.05) indicated that significant decreases in GVF at both the species and gender levels corresponded with the initial spawning period between July 17th and August 1st.

GVF measurements indicated that females of both species lagged slightly in development, but developed to comparable levels as males (Figure 2). Spawning in females resulted in a greater loss in volume relative to males, and males had a yearly average GVF approximately 10% higher than females. For both *Mytilus edulis* and *M. trossulus*, Bonferroni pairwise comparisons (α=0.05) indicated significant differences in GVF between males and females on August 30th.

Similar results were found using mean oocyte areas to assess gametogenic events (Figure 3). All mean oocyte areas (μm^2) are reported as *Mytilus edulis* and *M. trossulus*, respectively. Mean oocyte area increased sharply for both species from March 21st (164.10, 111.08) through June 4th (589.42, 490.79). After June 4th, oocyte area gradually increased until a maximum was observed on July 17th (678.58, 530.11). A sharp decrease in mean oocyte area occurred between July 17th (678.58, 530.11) and August 1st (278.42, 184.67), suggesting that a major spawning event had taken place. After August 1st, there was an increase in oocyte area until August 30th for *M. edulis* (409.33) and September 14th for *M. trossulus* (457.26), followed by a less pronounced and protracted period of decline until December 9th (93.90, 77.95).

The two-way ANOVA indicated differences in mean oocyte area between date ($F=53.67$, $P=0.000$) and species ($F=4.04$, $P=0.045$) with an interaction between date and species ($F=2.18$, $P=0.006$) (Table 3). The difference between species was due to a variation in mean oocyte size rather than a variation in the timing of gametogenic events. Average yearly oocyte size of *Mytilus edulis* was 338.221 μm^2 and *M. trossulus* 308.221 μm^2. Bonferroni pairwise comparisons ($\alpha=0.05$) showed a significant decrease in oocyte area for both species between July 17th and August 1st, indicative of spawning. Additional significant decreases between dates were slightly out of phase, with the mean oocyte area of *M. edulis* decreasing from September 14th to October15th and that of *M. trossulus* from October 15th to November 17th. Bonferroni pairwise comparisons ($\alpha=0.05$) indicated that differences in mean oocyte size between species were significant only on October 15th.

2.4. Discussion

2.4.1. Biological Implications

These results clearly indicate that gametogenic cycles for the sympatric *Mytilus edulis* and *M. trossulus* population in Cobscook Bay were synchronous in 2000. This agrees with previous reports of Freeman (1992) and Mallet and Carver (1995) from Lunenburg Bay, Nova Scotia. There was one primary spawning event for both *Mytilus edulis* and *M. trossulus*, which occurred between July 17th and August 1st. [Though it is possible that each species could have spawned at different times within this two-week period, hybridization potential would seem to be high.] Following the initial spawning

event females displayed a slight increase in GVF. Histologically-based observations revealed follicles of loosely packed mature oocytes though no evidence of redevelopment was present. Some variation in timing was identified in females between August 17th and November 17th, after the initial spawning period. However, both *M. edulis* and *M. trossulus* males had declining GVF values suggesting that interspecific fertilization was still possible. This protracted period of decrease may have resulted from a slow release of small numbers of gametes by both species, but the observation of empty follicles, refractory gametes, and infiltration of phagocytes also indicated that resorption of gametes rather then redevelopment, was the predominant gametogenic feature at this time. The finding of synchronous spawning suggests that another biological mechanism is involved in limiting hybridization and maintaining genetic identity of *M. edulis* and *M. trossulus* in eastern Maine.

Gardner (1996) asserts that hybridization predominantly occurs along environmental discontinuities where neither pure genotype is superiorly adapted. The environmental factors considered most important for differential adaptation are temperature and salinity (Väinölä and Hvilsom 1991; Gosling 1992; Sarver and Foltz 1993; Gardner 1994; Mallet and Carver 1995). Three other factors have been implicated as potentially important to differential adaptation: habitat specific selection, strength of byssal attachment, and tidal height (Hilbish et al. 1994; Comesaña and Sanjuan 1997; Gilg and Hilbish 2000). Conclusive evidence is not available to explain the limited hybridization and maintenance of genetic identity seen in eastern Maine. Our results indicate that spawning times are synchronous and hybridization potential high, and a previous study concluded that physical factors such as salinity and wave exposure (byssal

strength) are not of importance in the region (Rawson at al. 2001). Postzygotic reproductive isolation that leads to assortative mating via nonviable or infertile hybrids would seem unlikely due to the presence of backcrossed genotypes.

Biological factors such as gamete recognition proteins have been shown to drastically reduce the hybridization potential between closely related taxa of marine invertebrates such as sea urchins and abalone. In urchins a mismatch between the sperm bearing protein bindin and a glycoprotein receptor on the egg surface results in reproductive failure (Palumbi 1992; 1998), while in abalone the protein lysine shows species-specific ability to penetrate the vitelline envelope (Vacquier et al. 1993). Prezygotic isolation mediated through gamete recognition proteins could produce an effective barrier to gene flow between these two species of mussels. Further studies may indicate whether a gamete recognition system in *Mytilus* is what promotes species-specific fertilization and maintains conspecific assortative mating. Though no direct evidence of gamete recognition exists within the *M. edulis* species complex, indirect evidence has been shown through low cross-fertilization success between *M. edulis* and *M. trossulus* (Rawson unpublished). Vacquier (1998) considers poor cross-fertilization success between two closely related species of free-spawning marine invertebrates to be indicative of species-specific differences in gamete recognition proteins.

Hybridization of *Mytilus trossulus* with *M. edulis* or with *M. galloprovincialis* on the coast of North America is substantially less than that seen in European populations between *M. edulis* and *M. galloprovincialis. Mytilus trossulus* is considered the most evolutionarily divergent, while *M. edulis* and *M. galloprovincialis* are more closely related to each other (McDonald et al. 1991; Rawson and Hilbish 1995a). This higher

degree of evolutionary divergence is expressed with more pronounced physiological and genetic differences between *M. trossulus* and the other two species. Variation in gamete recognition proteins of *M. trossulus* may also be contributing to its divergence from *M. edulis* and *M. galloprovincialis*, leading to reduced fertilization success in cross-fertilization events and maintenance of genetic identity.

Cobscook Bay in eastern Maine is near the southern most distributional limit of *M. trossulus* (Rawson et al. 2001) and may represent an area of environmental extremes for this more boreal species. In adult mussels, sub-lethal levels of environmental stress are expressed as depressed growth rates, reduction in egg size, a decrease in viability and subsequent larval survival, reduced fecundity, and a negative impact on the competitive ability of the organism (Bayne 1985). The results of this study showed that *M. edulis* had a larger mean oocyte size at maturity and spawned larger eggs than did *M. trossulus*. Given the fact that maximum GVF were similar for both species, it can be concluded that similarly sized *M. trossulus* individuals produced more, although smaller, eggs than *M. edulis*. Similarly, Gardner and Skibinski (1990) showed that *M. galloprovincialis* has a higher fecundity per unit length than *M. edulis* at Croyde in S.W. England. Despite this possible reproductive advantage of *M. galloprovincialis*, genotypic ratios between the two species have not changed over time, indicating intense immigration of *M. edulis* spat (Gardner and Skibinski 1990). Counts of eggs produced were not conducted in this study, yet it is important to consider that any difference in fecundity could lead to directional selection favoring the more fecund species. In laboratory studies, however, it was shown that larger eggs of *Mercenaria mercenaria* and *Argopecten irradians* produced larvae having significantly higher survival than those of smaller size (Kraeuter

et al. 1982). Studies of *M. edulis* held under sub-optimal conditions produced smaller eggs lower in lipid content resulting in lower growth rates in the larvae, longer development times, and lower survival than those held under optimal conditions (Bayne 1985). Without further analysis of oocyte size over its geographic range it is not possible to determine whether the smaller oocytes observed in *M. trossulus* represent a response to environmental stress or highlights a physiological (genetic) difference between these two closely related species.

2.4.2. Aquaculture Application

Based on the similar spawning times of *Mytilus edulis* and *M. trossulus* in Cobscook Bay, preferential collection of *M. edulis* seed in Cobscook Bay seems unlikely by simply varying the deployment time of the collection gear. However, it is unknown if the developmental time of larvae is similar between species. Spat settlement and species composition of the settling spat are likely to vary widely throughout the region, depending on local environmental conditions and available food. Detailed monitoring of the species composition of settling spat offers the best opportunity to identify collection sites consisting predominately of *M. edulis* within Cobscook Bay.

Table 1. Relative number of males, females and undifferentiated individuals of *Mytilus edulis* and *M. trossulus* sampled throughout 2000. Undifferentiated individuals were not used in statistical analysis.

	Mytilus edulis			*Mytilus trossulus*			
	Males	Females	Undifferentiated	Males	Females	Undifferentiated	Totals
19-Jan	7	9	4	5	5	1	31
20-Feb	9	6	5	8	11	1	40
21-Mar	11	7	2	11	8	1	40
17-Apr	7	11	2	9	10	1	40
4-May	8	10	2	8	12		40
18-May	12	8		11	9		40
4-Jun	8	12		8	11		39
18-Jun	11	9		13	7		40
30-Jun	8	11		9	11		39
17-Jul	12	8		12	8		40
1-Aug	10	10		9	11		40
17-Aug	19	10	1	17	11		58
30-Aug	15	14		13	16		58
14-Sep	7	10	3	13	4	3	40
15-Oct	9	11		7	5	8	40
17-Nov	10	9	1	7	7	4	38
9-Dec	6	12	1	6	8	6	39
Totals	169	167	21	166	154	25	702

Table 2. Gamete Volume Fraction. Results of a three-way ANOVA testing the effects of date, species and gender on the gametogenic cycles of *Mytilus edulis* and *M. trossulus* (**=P<0.01, ***=P<0.001)

Source	df	Mean Square	F Value
Date	16	4.4869	142.53***
Species	1	0.0003	0.01
Gender	1	1.8650	59.24***
Date*Species	16	0.1061	3.37***
Date*Gender	16	0.0827	2.63**
Species*Gender	1	0.0407	1.29
Date*Species*Gender	16	0.0293	0.93
Error	587		

Table 3. Mean Oocyte Area. Results of a two-way ANOVA testing the effects of date and species on the gametogenic cycles of *Mytilus edulis* and *M. trossulus* (*=P<0.05, **=P<0.01, ***=P<0.001)

Source	df	Mean Square	F Value
Date	16	1.6398	53.67***
Species	1	0.1236	4.04*
Date*Species	16	0.0665	2.18**
Error	285	0.0306	

Figure 1. Yearly cycle of mean (± 1 SD) gamete volume fraction for *Mytilus edulis* and *M. trossulus* from Cobscook Bay, Maine.

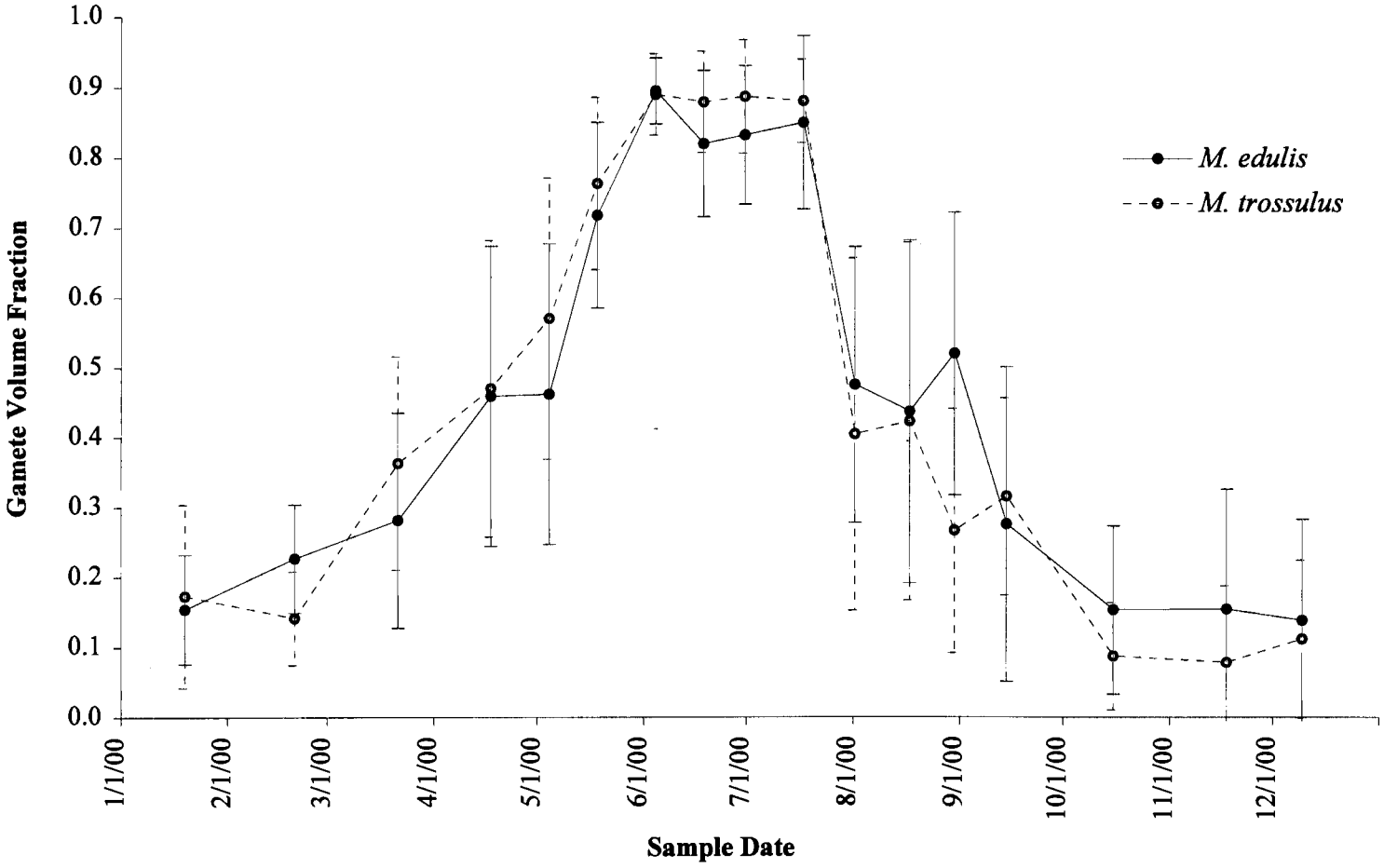


Figure 2a. Yearly cycle of mean (± 1 SD) gamete volume fraction for *Mytilus edulis* from Cobscook Bay, Maine.

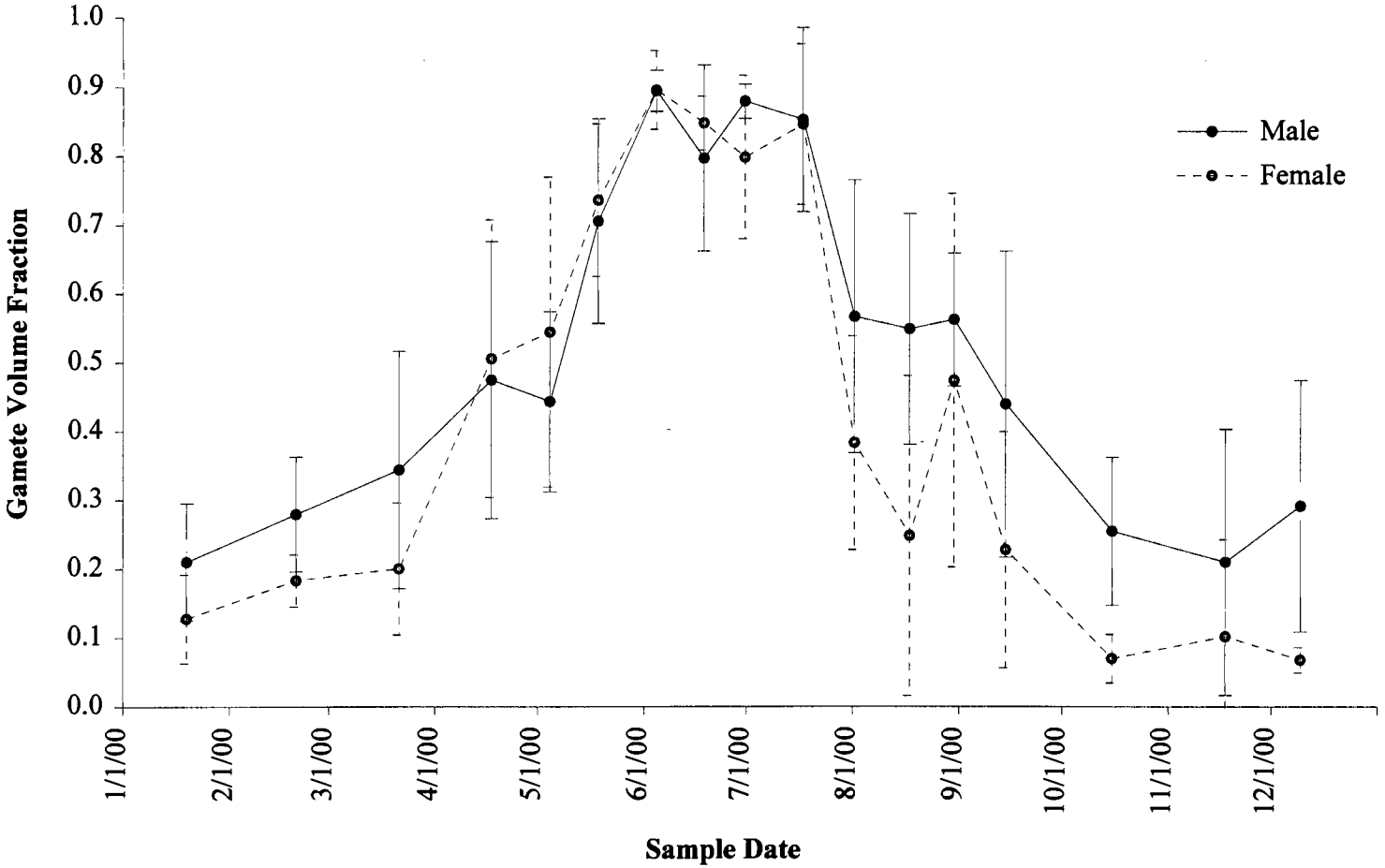


Figure 2b. Yearly cycle of mean (± 1 SD) gamete volume fraction for *Mytilus trossulus* from Cobscook Bay, Maine.

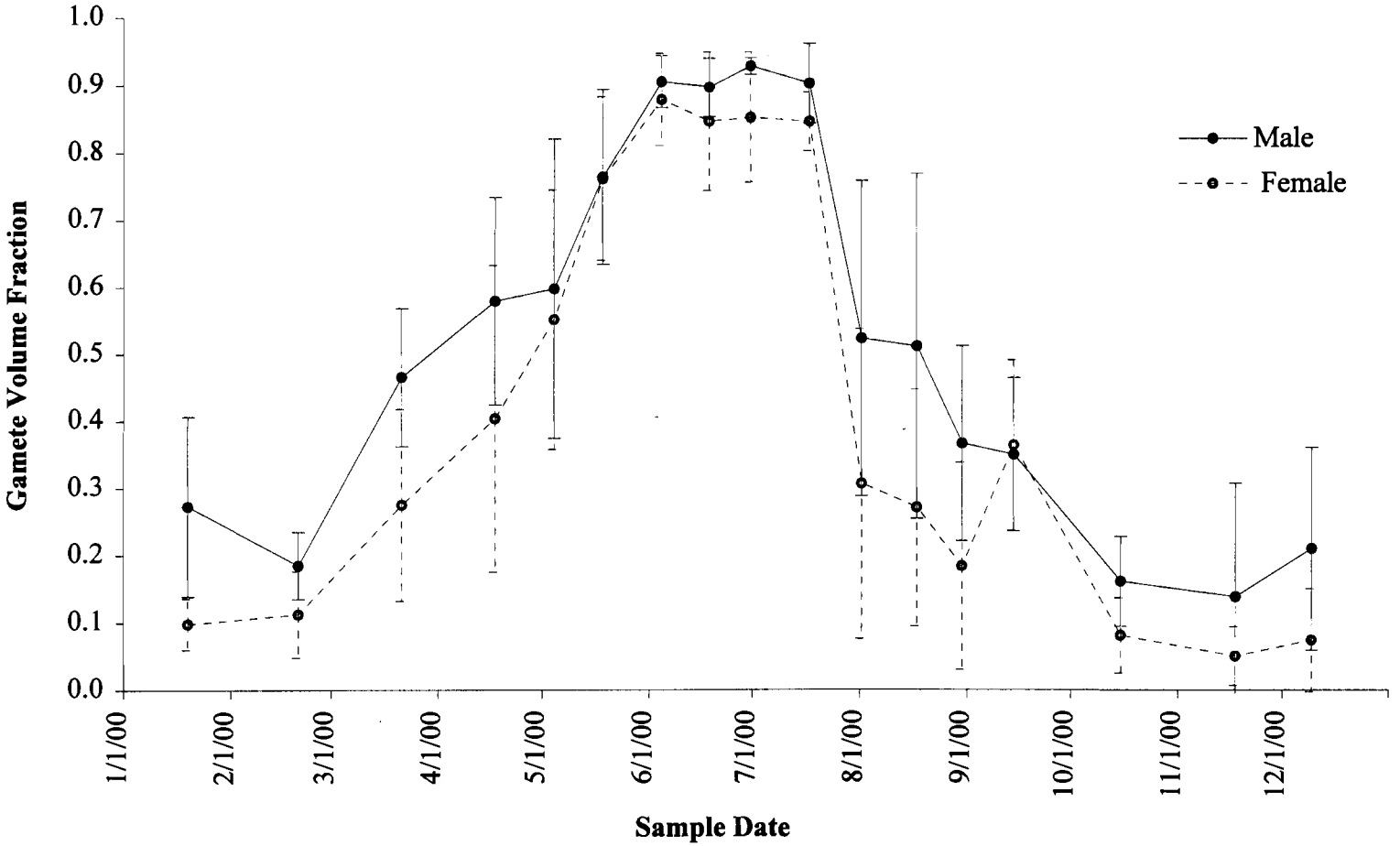
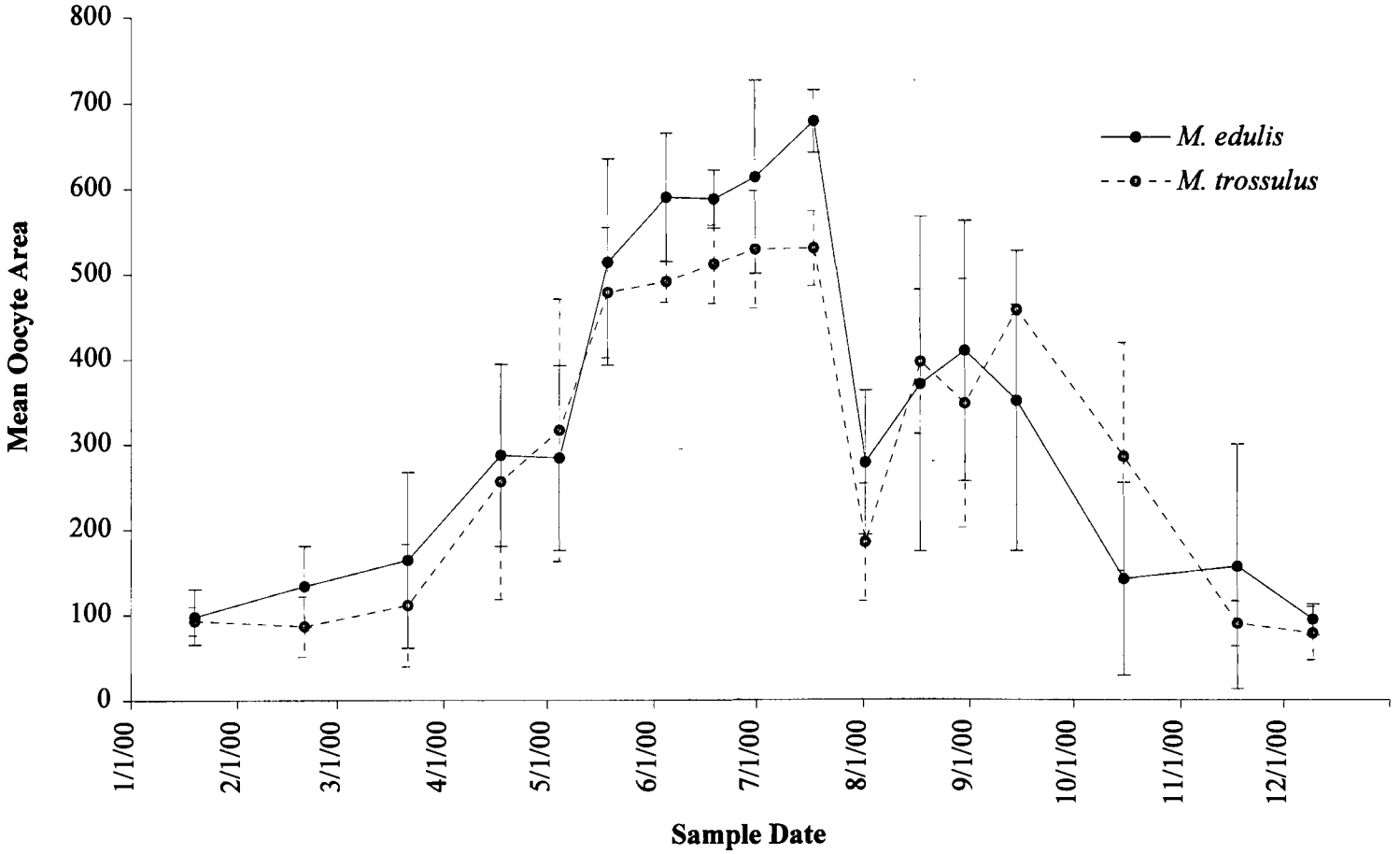


Figure 3. Yearly cycle of mean (± 1 SD) oocyte areas of *Mytilus edulis* and *Mytilus. trossulus* from Cobscook Bay, Maine.



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