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Reproductive isolation and reproductive output in two sympatric mussel species (*Mytilus edulis*, *M. trossulus*) and their hybrids from Newfoundland

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Abstract The mussels *Mytilus edulis* L. and *M. trossulus* Gould are found sympatrically in most areas of Newfoundland, with a low frequency of hybrids. To assess the potential for reproductive isolation, we sampled mussels from three sites in an eastern Newfoundland Bay from May–October 1996 to determine if there were differences in the reproductive cycles of the two species and their natural hybrids. In mussels with shell lengths of 38–42 mm, males and females with mature gametes were dominant in June for *M. edulis* and hybrids, while *M. trossulus* showed a lower frequency of individuals with mature gametes. *M. trossulus* and hybrids spawned over a prolonged period (from late spring to early autumn) compared with most *M. edulis* individuals that spawned over a period of 2–3 weeks in July. This asynchrony in spawning activity between the two species may partially explain the low frequency of hybrids found in previous studies of these mussel populations. Female and male hybrids between *M. edulis* and *M. trossulus* showed normal gonad development, ripening and spawning, providing an opportunity for the introgression of genes between the two species. *M. trossulus* had a higher reproductive output than *M. edulis* of similar shell length, while hybrids showed intermediate values of reproductive output. *M. trossulus* females produced smaller eggs than either *M. edulis* or hybrids. Differences in reproductive traits may partially explain the maintenance of the mussel hybrid zone in Newfoundland.

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Introduction

Hybrid zones occur where genetically distinct populations of individuals come into contact, mate, and produce offspring of mixed ancestry. The presence of naturally produced hybrids shows that reproductive isolating mechanisms are not sufficient to prevent hybridization. If hybrids are fertile, the potential exists for introgressive gene flow when hybrids backcross to one or both parental taxa. A major question in evolutionary biology is whether introgression can be an important source of new genetic variation leading to novel genotypes and adaptive evolution (Barton and Hewitt 1989; Harrison 1993; Arnold 1997). Thus it is important to understand factors influencing the generation and fate of hybrids. Depending on the survival and fertility of hybrids, selection may reinforce reproductive isolating mechanisms to maintain distinct taxa or break down barriers to hybridization, resulting in the fusion of taxa (Coyne and Orr 1997; Jiggins and Mallet 2000; Schluter 2001; Turelli et al. 2001). Alternatively, a stable hybrid zone may persist with varying degrees of introgression between the parental taxa (Barton and Hewitt 1989; Arnold and Hodges 1995; Rieseberg 1998).

The degree of reproductive isolation determines the rate of hybridization between closely related species. Reproductive isolation can act pre-zygotically through behavioral mechanisms (mating preference), spatial separation (habitat specialization), temporal separation (asynchronous reproduction), or barriers to fertilization (gamete incompatibility) (Palumbi 1994). Reproductive isolation can also act post-zygotically through reduced viability and fertility of hybrid offspring. Studies on the relative importance of various pre- and post-zygotic reproductive isolating mechanisms can contribute to a

better understanding of the factors responsible for the observed frequency of hybrid individuals in natural populations and the likely evolutionary fate of hybridizing species.

Sedentary invertebrate species that release gametes into the water column increase fertilization success through synchronous spawning of eggs and sperm. Therefore, closely related species may reduce hybridization and become reproductively isolated through asynchronous spawning between species (Palumbi 1994). However, species inhabiting higher latitudes generally have a similar spawning period owing to the strong seasonal occurrence of high water temperature and food availability (McEuan 1988; Gardner and Skibinski 1990; Babcock et al. 1992; Knowlton 1993; Van Veghel 1993). Furthermore, a short reproductive season may limit the role that asynchronous spawning can play in preventing hybridization between closely related species. Some studies have referred to the importance of establishing spawning times for species of hybridizing bivalves to assess the potential for producing hybrids (Ahmad and Beardmore 1976; Skibinski et al. 1980; Seed and Suchanek 1992; Gardner 1994; Eversole 1997; Grant et al. 1998). Although a difference in the spawning period between two species can also reduce the production of hybrids (Babcock et al. 1992; Levitan and Petersen 1995), other factors can reduce hybridization in closely related species, including habitat separation, gametic barriers to hybridization, and post-zygote non-viability (Strathmann 1981; Gardner and Skibinski 1990; Lessios and Cunningham 1990; Uehara et al. 1990; Palumbi and Metz 1991; Gardner 1992; Grant et al. 1998). In many cases the relationship between various reproductive isolating mechanisms and the observed frequency of hybrids is unknown. Despite this potential for reproductive isolation, hybrids are often abundant in populations of some marine invertebrate species. For example, sympatric populations of 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) are composed of approximately 31–88% and 10–20% hybrid individuals, respectively. Studies on the genetic structure of the *Mytilus edulis–M. galloprovincialis* hybrid zone on the coasts of France (Coustau et al. 1991; Viard et al. 1994) and England (Gardner 1996) have also revealed extensive hybridization.

In earlier studies, Lubet (1957) and Hrs-Brenko (1971) noted that spawning of *M. edulis* and *M. galloprovincialis* 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 and introgression are common between the two species in this area. Studies in SW England have reported asynchrony in spawning between *M. edulis* and *M. galloprovincialis* at certain sites within the hybrid zone (Skibinski et al. 1980; Skibinski 1983; Gardner and Skibinski 1990; Secor et al. 2001). Gardner and Skibinski (1990) found that the asynchrony was more pronounced at the Croyde site than at the Whitsand site, which may explain the greater degree of hybridization and introgression at Whitsand. However, Secor et al. (2001) used more sensitive measures of reproductive condition for Whitsand mussels and established small but significant differences in the spawning times of the two species.

Both *M. trossulus* and *M. edulis* occur on the east coast of North America (Koehn et al. 1984; Bates and Innes 1995; Mallet and Carver 1995; Saavedra et al. 1996; Comesaña et al. 1999; Penny and Hart 1999; Rawson et al. 2001), rather than *M. edulis* alone, as was previously thought (Seed 1976). *M. edulis* and *M. trossulus* are found sympatrically in Maine, Nova Scotia and Newfoundland. Recent studies in Nova Scotia (Saavedra et al. 1996) and eastern Newfoundland (Comesaña et al. 1999) revealed the presence of mussels of hybrid origin, but the frequency of hybrids appeared to be much lower (26% based on four loci) than that found between *M. edulis* and *M. galloprovincialis* in Europe (up to 80%; summarized in Gardner 1996 and Comesaña et al. 1999). Very few F_1 hybrids were observed between *M. edulis* and *M. trossulus*, with most of the hybrid mussels appearing to result from backcrossing to one species or the other (Saavedra et al. 1996; Comesaña et al. 1999). Although hybrids of *M. edulis* and *M. trossulus* have been observed in nature (Saavedra et al. 1996; Comesaña et al. 1999) and produced in the laboratory (Zouros et al. 1994), the role of post-zygotic mechanisms in limiting hybridization has not been examined empirically. Furthermore, asynchronous reproductive cycles may also decrease the frequency with which hybrids are produced in nature. Several studies have been carried out on the reproductive cycle of *M. edulis* (Thompson 1979, 1984a,b; Newell et al. 1982; 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, but there have been no studies comparing the reproductive cycles of *M. edulis* and *M. trossulus* in areas where they coexist.

Explaining the coexistence of the two species of *Mytilus* in a zone of hybridization requires information on the factors that limit hybridization and keep the two species distinct. Coexistence of the two species may involve a combination of asynchronous spawning and post-zygotic isolation to maintain genetically distinct species. Most *Mytilus* spp. populations, like those of many temperate bivalves, exhibit a seasonal pattern of reproduction, which starts with a gametogenic phase, is followed by the release of gametes (spawning) in which the reproductive follicles are partially or completely emptied. Gametogenesis 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). Apart from a few hermaphrodites, the sexes in *Mytilus* spp. are separate

and most populations contain approximately equal numbers of males and females (Seed 1976; Kautsky 1982; Sprung 1983). However, the occurrence of distinct male and female mtDNA genomes raises the possibility that sex ratios may be different in areas of hybridization (Saavedra et al. 1996, 1997).

The objective of the present study was to compare the reproductive cycles of *M. edulis*, *M. trossulus* and their hybrids in eastern Newfoundland, to determine if the observed low rate of hybridization can be explained in part by asynchronous reproductive cycles and to determine if differences in reproductive output play a role in maintaining their coexistence. In addition, sex ratios were tested for any deviation from unity resulting from hybridization and genetic incompatibility, which may disrupt the gender-associated inheritance of mtDNA.

Materials and methods

Study sites and sampling

The study was carried out at two locations in Trinity Bay, Newfoundland, where approximately 34% *Mytilus edulis*, 40% *M. trossulus* and 26% hybrids were observed by Comesaña et al. (1999). At approximately 15-day intervals from May to October 1996, a representative sample of 400–500 mussels was collected sub-tidally by SCUBA divers from an exposed site at Bellevue (BE) and an exposed and a protected site at Chance Cove (CE, CP, respectively) (see Fig. 1 in Comesaña et al. 1999). Mussels were immediately transported to the laboratory, where they were maintained in running, filtered seawater within 0.5°C of the ambient temperature at Bellevue. During the 3 days following each field sampling, an average of 34 mussels (38–42 mm shell length) from each site (BE, CE and CP) were then dissected carefully to separate the mantle (and in some individuals the mesosoma) from other soft tissues. This narrow size range was chosen to increase the

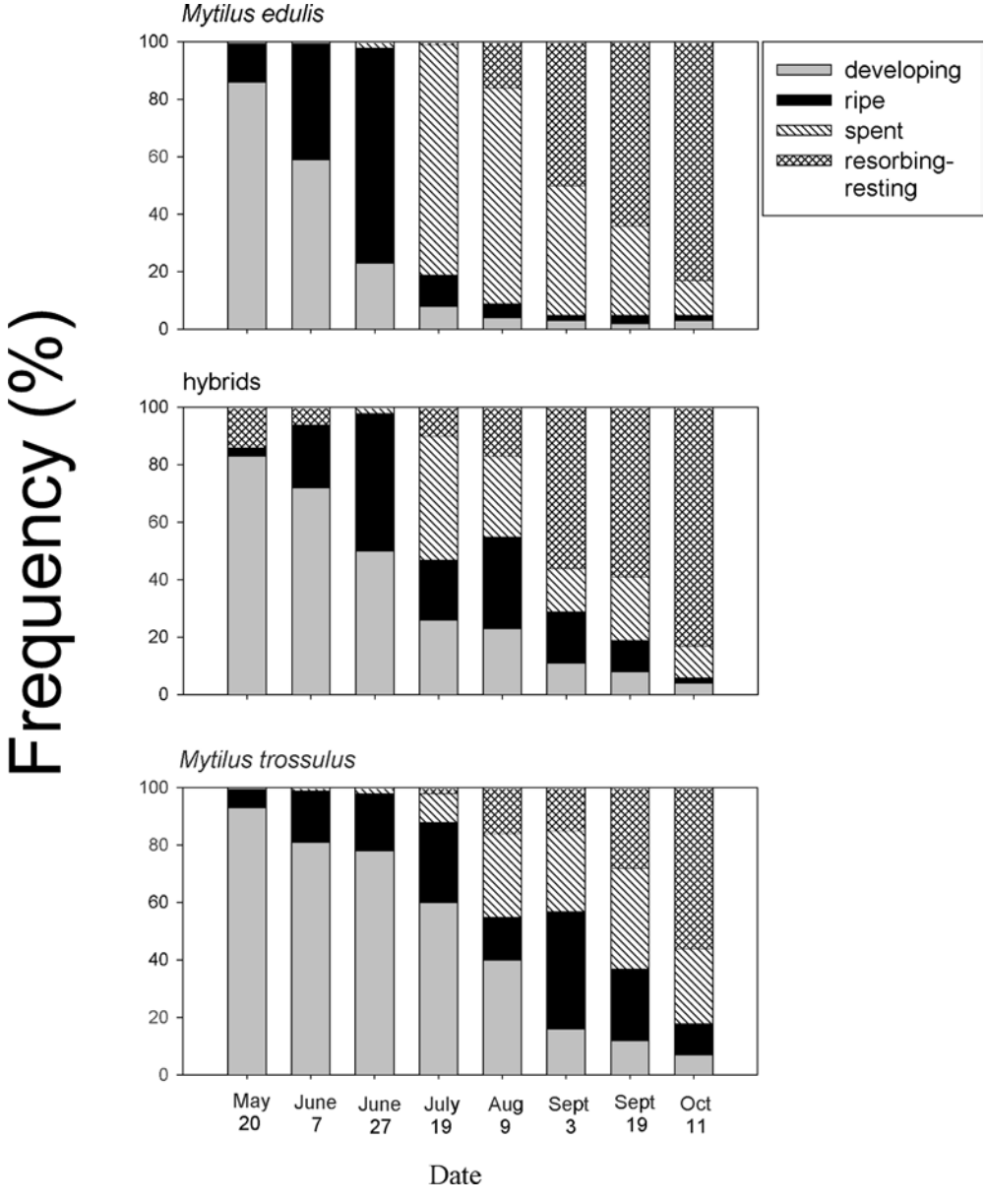

Fig. 1 *Mytilus* spp. Frequency distribution of gonad maturation stages in female and male *M. edulis*, *M. trossulus*, and hybrids during the 1996 reproductive season pooling both sexes and samples from three sites. Average $N = 100$ for each date. For details of each stage see "Materials and methods"

probability of including males and females of both species and some of the hybrids in the sample (Comesaña et al. 1999). A very small piece of mantle border (approximately 20–30 mg) from each individual was stored in 95% ethanol at –20°C to await identification of the two species and their hybrids using two diagnostic genetic markers (ITS and Glu) as described by Comesaña et al. (1999). Both ITS and Glu were used to classify 62% of the individuals sampled, the remaining 38% being classified with only the Glu marker to reduce costs. Using only the Glu marker misclassifies about 8% of the individuals and fails to discriminate between hybrids and the parent species (Comesaña et al. 1999). Additional mussels from a range of shell lengths were also sexed in order to determine the sex ratio and smallest size at maturity for the two species and their hybrids.

Histological analysis

One mantle lobe from each dissected mussel was sub-sampled by cutting a transverse section midway along the anteroposterior axis. This piece of the mantle was weighed, preserved in Bouin's fixative according to Lowe et al. (1982), 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 the mantle of *Mytilus* spp. is relatively homogeneous (Lowe et al. 1982; Newell et al. 1982; Bayne et al. 1985).

Terminology for the stages of gametogenesis was modified from King et al. (1989) and Kiyomoto et al. (1996). Each gonad was assigned to one of the following four stages: (1) developing (follicles occupy a large part of the mantle; individuals restoring their gonads after a partial spawning are included in this category), (2) ripe (follicles full of oocytes in female and packed lamellae of ripe spermatozoa in male), (3) spent (follicles begin to collapse and degenerate) and (4) resorbing-resting mussels. An average of 100 individuals was examined on each sampling date, pooling both sexes and samples from the three sites.

Gonadosomatic index (GSI)

Following the removal of the tissue section for histological analysis, the remainder of the mantle was weighed, dried at 80°C to constant weight, cooled in a desiccator and reweighed. The ratio of wet to dry weight for this portion of the mantle was used to correct for the weight of the tissue subsample 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 mantle dry weight by the whole-mussel dry weight and multiplying by 100. A GSI was calculated separately for males (MGSI) and females (FGSI).

Gamete volume fraction (GVF) and reproductive output

The fractional area of the tissue composed of gametes (GVF) was measured quantitatively with Optimas 6.2 image analysis software and a Nikon stereomicroscope, following standard stereological methods (Lowe et al. 1982). Since the gonad of *Mytilus* is homogenous with respect to the distribution of germinal cells and gametes (Bayne et al. 1985), the tissue sections examined were representative of the whole gonad, an important requirement for stereological analysis (Lowe et al. 1982). The color 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 the mantle volume occupied by gametes was calculated from the relationship between the number of pixels occupied by the gametes and the total pixels in the field. Two groups of five sections of 1.3 mm^2 were taken randomly from each individual (histological slide) to estimate the volume of the mantle that is composed of gametes. The average of each group of five sections produced two replicate estimates of the GVF for each mussel. GVF varies between 0% for a reproductively inactive mussel and 100% for a mussel in maximal reproductive condition, and also gives a measure of the relative maturity of the gonad. However, GVF does not estimate total reproductive output because an individual with a high GVF may have very few gametes if the gonad is small (Hilbish and Zimmerman 1988). To correct for this problem, total mantle dry weight was multiplied by the GVF to provide an estimate of the dry weight of gametes for each individual.

A total of 150 individual oocytes with a nucleolus from the histological sections were also examined by image analysis for three individuals from each species and hybrid. 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 seawater, placed in a shallow tray of filtered seawater, and subsequently exposed to rising temperatures (up to 22°C). Once spawning was initiated, the individuals were placed in separate containers with sterile seawater for completion of spawning. All 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 50 eggs from six *M. edulis*, seven *M. trossulus*, and two hybrids were measured by image analysis to estimate mean egg diameter.

Statistical analysis

Observed sex ratios were tested against a 1:1 ratio using a *G*-test (with Williams' correction) (Sokal and Rohlf 1981). The frequency of individuals in the four developmental stages was tested using a contingency *G*-test among the taxa (*M. edulis*, *M. trossulus* and hybrids) on each sampling date. Normality of reproductive variables (GSI, GVF) was determined by the Lillifors K–S test (Wilkinson 1991). Analysis of the GSI values was performed by two-way, fixed-factor ANOVA (date, taxon) after arcsine-square-root transformation, followed by Tukey's studentized range test (SRT; $\alpha = 0.05$) in cases where the *F*-value exceeded the critical value. Values of GVF were similarly transformed and analyzed by a two-way, fixed-factor ANOVA (date, taxon) for each sex and sampling date. Two estimates of GVF were made for each mussel, i.e. each mussel was nested within date and taxon. Only GVF data for the first five sampling dates (which span the greatest change in GVF) were analyzed. Probability values for both the GSI and GVF ANOVAs were adjusted ($\alpha = 0.05/6$) to reduce the chance of type I errors for each set of six analyses involving the three sites and the two sexes. If there were a difference in the timing of reproductive events among the taxa, the interaction between the two main effects (date, taxon) would be significant. Dry weight of gametes among the taxa was analyzed by a one-way ANOVA for each of the 18 date–sex combinations, with the significance test adjusted to $\alpha = 0.05/18$. Differences in oocyte and egg size among taxa were tested (ANOVA, mussel nested within taxon) following log transformation of the variates. All statistical analyses were carried out with SYSTAT 5.1 (Wilkinson 1991) and SAS v. 6.30.

Results

Sex ratio

For *Mytilus edulis*, 698 mussels were sampled, of which 342 (49.0%) were females, 299 (42.8%) were males, 6 were hermaphrodites (0.86%) and 51 (7.3%) were undifferentiated. The female:male sex ratio (1.14F:1M, $n = 641$) did not differ significantly ($P > 0.05$) from the expected ratio of 1:1. For *M. trossulus*, 782 mussels were sampled, of which 412 (52.7%) were females, 341

(43.6%) were males, 5 were hermaphrodites (0.6%) and 24 (3.1%) were undifferentiated. The sex ratio (1.20F:1M, $n = 753$) differed significantly ($P < 0.01$) from the expected 1:1 ratio, and females predominated. For hybrids, 280 mussels were sampled, of which 109 (38.93%) were females, 143 (51.1%) were males and 28 (10.0%) were undifferentiated. The sex ratio (1F:1.31M, $n = 252$) differed significantly ($P < 0.05$) from the expected 1:1 ratio, and males predominated.

Reproductive cycle

Histological sections of male and female gonads of *M. edulis*, *M. trossulus* and their hybrids showed advanced gametogenesis at the first sampling date (20 May). From late May to late June very few mussels had spawned (Fig. 1). Ripe mussels of both sexes were dominant in late June in *M. edulis*, while in *M. trossulus* there were few ripe mussels. Hybrids showed an intermediate proportion of ripe individuals (Fig. 1). A large proportion of spawned *M. edulis* was observed in late July, but there were few spawned *M. trossulus* and an intermediate proportion of spawned hybrids. The frequency of individuals in the four developmental stages was significantly different ($P < 0.05$, contingency *G*-tests) among *M. edulis*, *M. trossulus* and hybrids for all dates except the first (20 May) and last (11 October) (Fig. 1). A comparison of the reproductive cycles suggests an abrupt spawning for many *M. edulis* between 27 June and 19 July, compared with a more prolonged period of spawning in *M. trossulus* and an intermediate pattern in hybrids (Fig. 1). Mussels with no gametogenic activity predominated in late September and October in all taxa. These gonad sections contained no follicles at all or only a few very contracted follicles between connective cells, and showed resorption of the undischarged eggs in follicles of the females. By the final sampling in October, $> 50\%$ of all mussels were in the resorbing-resting stage. Both female and male hybrids showed normal gonad development, ripening and spawning. Insufficient hermaphrodites were collected to determine their reproductive cycle.

Gonadosomatic index

The GSI is primarily determined by the accumulation and release of gonad material, as well as the utilization of stored energy products during the winter. Two-way ANOVA (Table 1) showed a significant interaction ($P < 0.001$) between taxon (*M. edulis*, *M. trossulus*, hybrid) and sample date for males (MGSI) and females (FGSI) at each of the three sites, as well for the data pooled across the three sites ($P < 0.001$), indicating that GSI differed among the two species and their hybrids according to sampling date. Both the MGSI and FGSI in *M. edulis*, *M. trossulus* and hybrids showed a steady decline after spawning in July (Fig. 2). However, *M. edulis* showed a more abrupt decrease than *M. trossulus*, with an intermediate decline for the hybrids. *M. edulis* also showed a greater recovery of MGSI and FGSI during early autumn than either *M. trossulus* or hybrids (except for FGSI at the CE site), which may indicate an increased storage of nutrients in the gonad (Blanchard and Feder 1997).

Table 1 *Mytilus* spp. Two-way ANOVA testing variation in the gonadosomatic index (arcsine-transformed values) for females and males among *Mytilus* taxa (*M. edulis*, *M. trossulus*, hybrids) at three sites (*BE* Bellevue exposed; *CE* Chance Cove exposed; *CP* Chance Cove protected) in eastern Newfoundland (see Fig. 2)

Site	Sex	Source	*df*	MS	*F*	*P*
BE	Females	Date	7	0.5902	48.71	< 0.001
		Taxon	2	0.0239	1.98	> 0.05
		Date×Taxon	14	0.0927	7.65	< 0.001
		Error	126	0.0121		
BE	Males	Date	7	0.5574	59.47	< 0.001
		Taxon	2	0.0108	1.15	> 0.05
		Date×Taxon	14	0.0689	7.36	< 0.001
		Error	97	0.0094		
CE	Females	Date	7	0.4757	54.23	< 0.001
		Taxon	2	0.2688	30.64	< 0.001
		Date×Taxon	13	0.0774	8.83	< 0.001
		Error	125	0.0088		
CE	Males	Date	7	0.4415	31.98	< 0.001
		Taxon	2	0.0190	1.38	> 0.05
		Date×Taxon	13	0.0877	6.35	< 0.001
		Error	99	0.0138		
CP	Females	Date	7	0.4334	34.06	< 0.001
		Taxon	2	0.0140	1.10	> 0.05
		Date×Taxon	14	0.0907	7.13	< 0.001
		Error	128	0.0127		
CP	Males	Date	7	0.4171	54.59	< 0.001
		Taxon	2	0.0368	4.81	> 0.05
		Date×Taxon	14	0.0970	12.70	< 0.001
		Error	97	0.0076		

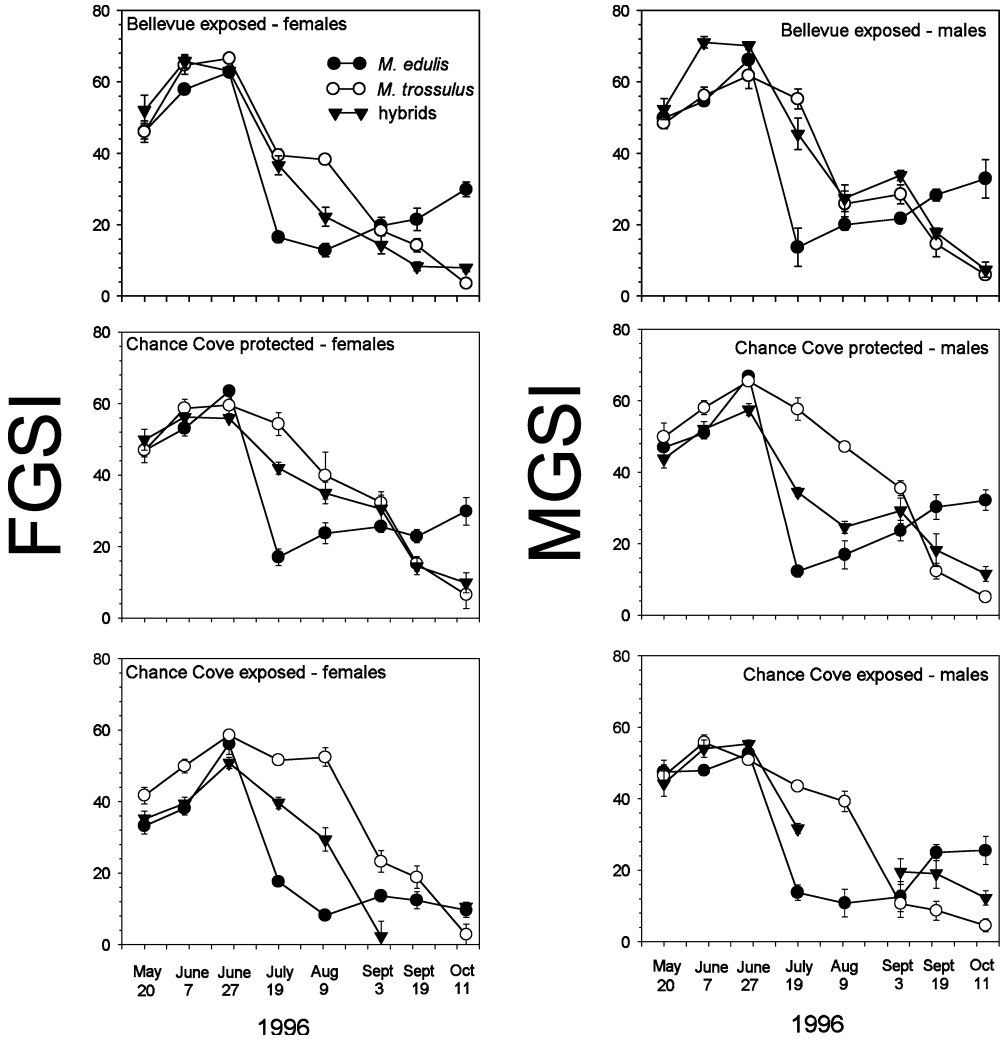

Fig. 2 *Mytilus* spp. Gonadosomatic index (±SE) for female (*FGSI*) and male (*MGSI*) *M. edulis*, *M. trossulus* and hybrids during the 1996 reproductive season at three sites in eastern Newfoundland. Average $N = 34$ for each site and date

Gamete volume fraction

The GVF of male and female mussels was analyzed separately at each site to determine reproductive synchrony among the taxa. Two-way ANOVA (Table 2) showed that all date by taxon interactions for females were significant, indicating that the gametogenic cycle, as measured by GVF, differed among the species and hybrids (Fig. 3) and was similar to the pattern observed for GSI (Fig. 2). Although GVF for males showed differences among taxa similar to those observed for females, the date-by-taxon interaction was non-significant (Table 2). *M. trossulus* and the hybrids at all sites released gametes over a 12–15 week period, while *M. edulis* spawned more rapidly for about 6 weeks (primarily between 27 June and 19 July samples; Fig. 3).

Reproductive output

M. trossulus showed the greatest mean GVF throughout most of the sampling period (Fig. 3). A comparison among the taxa for the dry weight of gametes revealed that *M. trossulus* had a significantly greater weight of gametes per gonad than *M. edulis* and hybrids for three of the six sex–site comparisons for each of 7 June, 27 June and 19 July (Fig. 4) as follows: 7 June BE females $F_{(2,18)} = 18.39$, $P < 0.0001$, CP females $F_{(2,18)} = 10.08$, $P < 0.01$, CP males $F_{(2,16)} = 15.26$, $P < 0.001$; 27 June BE males $F_{(2,16)} = 16.75$, $P < 0.001$, CP females $F_{(2,16)} = 17.57$, $P < 0.001$, CP males $F_{(2,15)} = 9.04$, $P < 0.01$; 19 July BE males $F_{(2,14)} = 6.87$, $P < 0.01$, CP females $F_{(1,17)} = 29.10$, $P < 0.001$, CE females $F_{(2,15)} = 15.86$, $P < 0.001$. The significant differences found on 19 July were due to the fact that some *M. trossulus* and hybrids had not yet spawned, whereas most *M. edulis* had done so.

Oocyte dimensions

There were no significant differences in mature oocyte diameter or area among the taxa for histological sections taken from the 7 June sample (Table 3; Fig. 5), but there was significant variation among taxa in the 27 June sample, with *M. edulis* exhibiting the greatest values (Table 3; Fig. 5). There were also significant differences among taxa in the diameter and area of eggs obtained by

Table 2 *Mytilus* spp. Two-way nested ANOVA (mussels nested within date and taxon) testing variation in the gamete volume fraction (arcsine-transformed values) for females and males among *Mytilus* taxa (*M. edulis*, *M. trossulus*, hybrids) at three sites (*BE* Bellevue exposed; *CE* Chance Cove exposed; *CP* Chance Cove protected) in eastern Newfoundland (see Fig. 3)

Site	Sex	Source	*df*	MS	*F*	*P*
BE	Females	Date	4	1.2871	82.43	< 0.001
		Taxon	2	0.9396	60.17	< 0.001
		Date×Taxon	8	0.0689	4.41	< 0.01
		Mussel (Date× Taxon)	72	0.0156	5.09	< 0.001
		Error	85	0.0031		
BE	Males	Date	4	1.5394	33.22	< 0.001
		Taxon	2	0.6391	13.79	< 0.001
		Date×Taxon	8	0.1043	2.25	> 0.05
		Mussel (Date× Taxon)	55	0.0463	50.28	< 0.001
		Error	70	0.0009		
CE	Females	Date	4	0.5765	32.20	< 0.001
		Taxon	2	2.9820	166.56	< 0.001
		Date×Taxon	8	0.1462	8.17	< 0.001
		Mussel (Date× Taxon)	74	0.0179	57.34	< 0.001
		Error	89	0.0003		
CE	Males	Date	4	1.0462	35.33	< 0.001
		Taxon	2	0.5359	18.10	< 0.001
		Date×Taxon	7	0.0570	1.93	> 0.05
		Mussel (Date× Taxon)	59	0.0296	312.25	< 0.001
		Error	73	0.0001		
CP	Females	Date	4	0.6293	19.96	< 0.001
		Taxon	2	1.4083	44.67	< 0.001
		Date×Taxon	8	0.1078	3.42	< 0.05
		Mussel (Date× Taxon)	75	0.0315	38.40	< 0.001
		Error	90	0.0008		
CP	Males	Date	4	1.1721	38.12	< 0.001
		Taxon	2	0.3477	11.31	< 0.001
		Date×Taxon	8	0.0688	2.24	> 0.05
		Mussel (Date× Taxon)	60	0.0307	394.96	< 0.001
		Error	75	0.0001		

spawning in the laboratory. *M. edulis* produced the largest eggs, followed by hybrids and *M. trossulus* (Fig.5; Table 3).

Size at first maturation

Size at maturity was determined by direct observation and measurement of gonad weights for mussels of various sizes. The smallest mussels that could be sexed were *M. trossulus*, a male of 6.9 mm and a female of 8.9 mm shell length, together with several other individuals < 10 mm long that were observed in the June samples. For hybrids and *M. edulis*, earliest gonad development and gamete storage in the mantle tissue occurred at about 12–15 mm shell length. Thus the shell length at first maturation appears to be smaller in *M. trossulus* than in hybrids and *M. edulis*.

Discussion

Sex determination and the unusual doubly uniparental inheritance (DUI) of the mtDNA in *Mytilus* spp. are interrelated, but the details of this relationship have yet to be determined. Recent studies have established that sex determination in *Mytilus* spp. is primarily under maternal control and that the sex ratio of progeny from different mothers can vary widely (Zouros et al. 1994; Saavedra et al. 1997). However, hybridization between *Mytilus* species results in a breakdown in DUI (Zouros et al. 1994) and may also affect sex determination and population sex ratio in a hybrid zone. In the present study, the greatest deviation from a 1:1 sex ratio was observed for individuals classified as hybrids in an area of hybridization between *M. edulis* and *M. trossulus* in eastern Newfoundland. With DUI, males are heteroplasmic, containing both maternally and paternally derived mtDNA, while females contain only maternal mtDNA. Hybridization can generate individual males that contain maternal mtDNA and nuclear DNA from one species, and paternal mtDNA and nuclear DNA from the other species. Any nuclear–cytoplasmic incompatibility may result in a greater mortality of hybrid males, shifting the sex ratio towards a female bias. However, hybrids showed a male-biased sex ratio, the opposite of the pattern predicted for

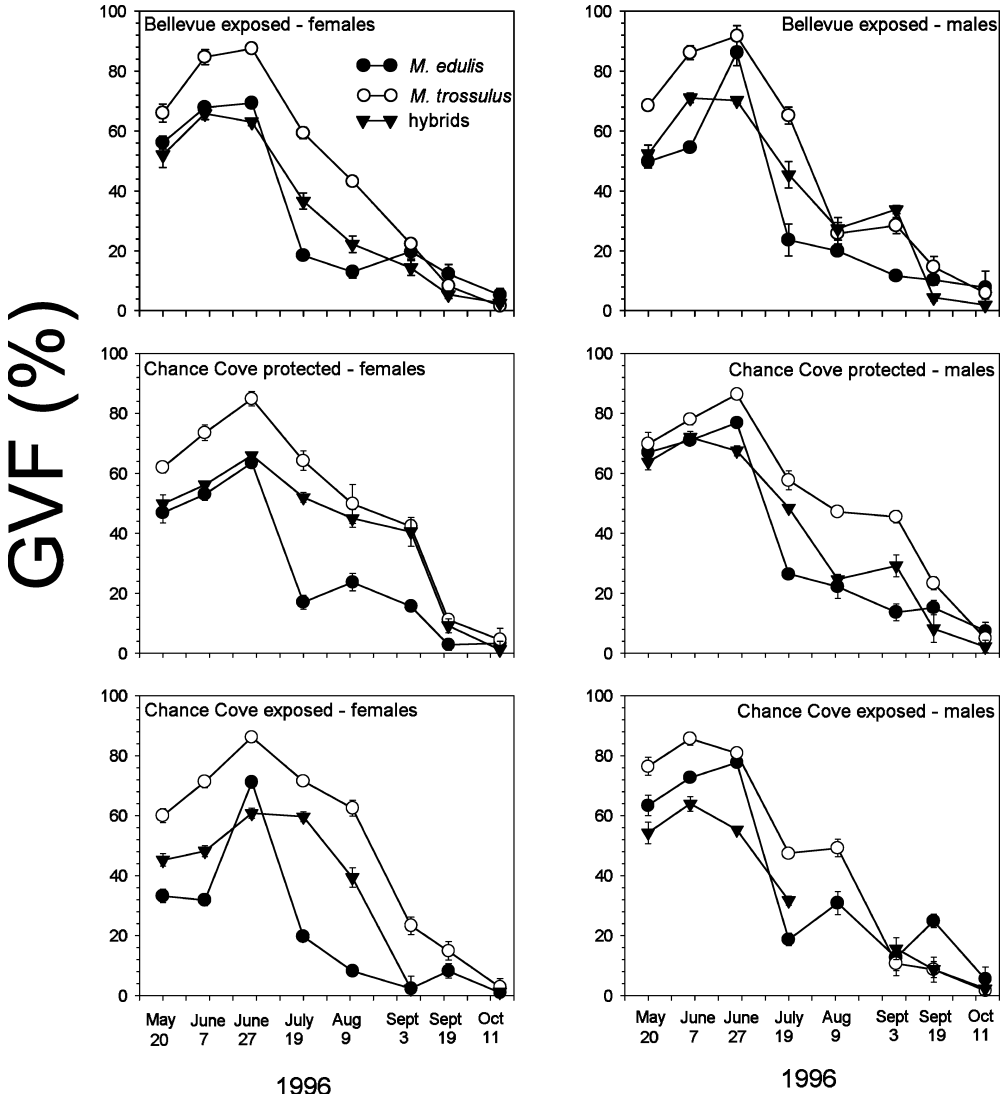

Fig. 3 *Mytilus* spp. Mean gamete volume fraction (*GVF*, ±SE) for female and male *M. edulis*, *M. trossulus* and hybrids at three sites in eastern Newfoundland during the 1996 reproductive season. Average $N = 34$ for each site and date

nuclear–cytoplasmic incompatibility in males. It is unclear if this biased sex ratio is a consequence of nuclear–cytoplasmic incompatibility through hybridization or a deviation simply due to sampling error (Palmer 2000). Furthermore, any bias in the sex ratio would be removed if a large fraction of the undifferentiated individuals were made up of the less common sex.

Mussels from all three sites 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 at a protected site 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. Some populations of *M. trossulus* also present the same pattern, in which gonad is synthesized in late winter and early spring, e.g. 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, which was also observed in mussels (*M. trossulus*) 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, followed a different pattern, with gametogenic development throughout winter (like *M. edulis* in the North Sea), while the spawning period is similar to that found in the present study.

There have been no previous studies of reproductive cycles of *M. edulis* and *M. trossulus* in the context of hybridization. In this study, *M. trossulus* from eastern Newfoundland spawned over a prolonged period of 12–15 weeks, while *M. edulis* mussels spawned almost completely over a 3-week period in July. Earlier studies also showed that *M. edulis* is a synchronous spawner in Newfoundland (Thompson 1984b). Hybrid mussels in the present study exhibited a spawning cycle that was more similar to *M. trossulus*. Lubet et al. (1984) found asynchronous spawning in hybrids obtained from

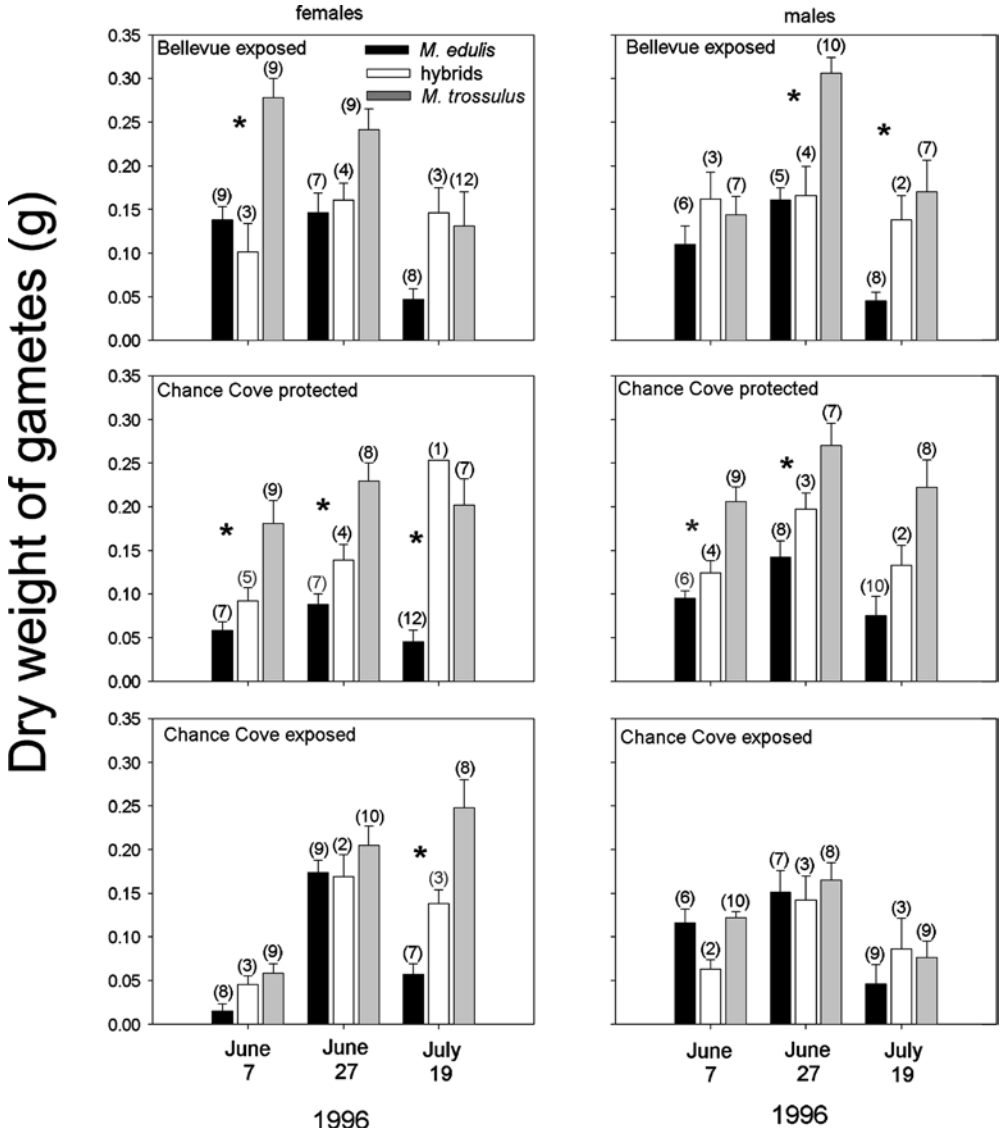

Fig. 4 *Mytilus* spp. Comparison of mean (±SE) dry weight of gametes among *M. edulis*, *M. trossulus* and hybrids on three sampling dates in the pre-spawning and spawning stages at three sites in eastern Newfoundland. Number of mussels analyzed in *parentheses*. *Asterisk* indicates a significant difference among taxa adjusted to 0.0028 (0.05/18) for the 18 tests to reduce the probability of a type I error

intra- and inter-specific crosses of *M. edulis* and *M. galloprovincialis*. *M. edulis* spawning was more restricted in time, while *M. galloprovincialis* showed a more prolonged spawning over several months, and hybrids an intermediate pattern.

There was a strong correlation between the measures of reproductive stages, GSI and GVF, which showed significant differences between the two species and hybrids. In general, *M. edulis* had a shorter spawning period than *M. trossulus*. GSI data also suggested that *M. edulis* began to recover from spawning earlier than *M. trossulus* or hybrids. Asynchronous spawning between *M. trossulus* and *M. edulis* would be expected to result in a low frequency of F_1 hybrids. For example, between 27 June and 19 July *M. edulis* males and females released large quantities of gametes into the water column, whereas *M. trossulus* released fewer gametes, suggesting that the probability of *M. edulis* sperm and eggs encountering each other would be greater than the probability of an encounter between sperm and eggs from the two different species. Comesaña et al. (1999) observed very few F_1 hybrids (based on four nuclear gene markers) at these sites, consistent with reproductive isolation due to a degree of asynchronous spawning between the species. However, the lack of F_1 hybrids could also be a result of other factors such as spatial isolation, assortative fertilization and genetic incompatibility.

Bates and Innes (1995) and Penny and Hart (1999) noted variation in the relative frequencies of *M. edulis* and *M. trossulus* at different sites in Newfoundland. Sites dominated by one species or the other will reduce the frequency of hybridization, owing to spatial separation. However, most sites in Newfoundland appear to have a mixture of both species, allowing opportunity for hybridization if spawning times overlap. Even with a large degree of overlap in spawning, hybridization may be reduced if there is assortative fertilization or a greater success of intra- compared with inter-specific fertilizations. Bierne et al. (2002) have recently demonstrated

Table 3 *Mytilus* spp. Results of nested ANOVA (mussel within taxon) for testing variation in egg area and egg diameter among *Mytilus* taxa (*M. edulis*, *M. trossulus* and hybrids) (see Fig. 5)

Date	Factors	Source	*df*	MS	*F*	*P*
7 June (histological section)	Egg area	Taxon	2	2.7987	1.88	> 0.05
		Mussel (Taxon)	6	1.4907	32.41	< 0.001
		Error	1341	0.0460		
	Egg diameter	Taxon	2	0.9937	2.45	> 0.05
		Mussel (Taxon)	6	0.4060	18.13	< 0.001
		Error	1341	0.0224		
27 June (histological section)	Egg area	Taxon	2	11.4297	117.98	< 0.001
		Mussel (Taxon)	6	0.0969	2.64	< 0.05
		Error	1341	0.3672		
	Egg diameter	Taxon	2	2.3872	72.92	< 0.001
		Mussel (Taxon)	6	0.0327	1.85	> 0.05
		Error	1341	0.0177		
Spawning (spawned eggs)	Egg area	Taxon	2	6.2151	196.20	< 0.001
		Mussel (Taxon)	12	0.0317	1.51	> 0.05
		Error	735	0.0209		
	Egg diameter	Taxon	2	1.0908	54.48	< 0.001
		Mussel (Taxon)	12	0.0200	2.15	< 0.05
		Error	735	0.0093		

that assortative fertilization can play a role in reducing the rate of hybridization between *M. edulis* and *M. galloprovincialis*. Post-fertilization genetic incompatibility can result in early death of hybrids and may also explain their reduced frequency. Both Saavedra et al. (1996) and Comesaña et al. (1999) found that some hybrid genotype combinations between *M. edulis* and *M. trossulus* were less frequent than expected, given equal fertilization and survival. Therefore, a combination of asynchrony in spawning time between the two species, assortative fertilization and genetic incompatibility probably prevents the formation of large numbers of F_1 hybrids between *M. edulis* and *M. trossulus*. Hybrids also appear to be rare at other sites in Newfoundland (authors' unpublished observations) and Maine (Rawson et al. 2001), suggesting that mechanisms reducing hybridization operate over a large area where these species are sympatric. In addition, temporal separation in spawning time between the species may vary among sites, so that the relative importance of various reproductive isolating mechanisms may also vary geographically.

A degree of overlap in the spawning period between hybrids and the two parental species provides the potential for introgression of genes between the two *Mytilus* species. Comesaña et al. (1999) showed that most hybrids of *M. edulis* and *M. trossulus* in the Newfoundland hybrid zone were backcrosses biased towards *M. trossulus*. Although only a few reproductively mature F_1 hybrids may be produced, they can spawn to form a large number of backcrosses with the parental species, especially when hybrids have a spawning period that overlaps with that of the pure species. The high frequency of *M. trossulus*-biased backcrosses appears to be due to the greater gamete output by the *M. trossulus* populations compared with the *M. edulis* populations at the study sites, although variation in viability among different backcross genotypes cannot be ruled out.

According to Bayne et al. (1978), an individual female mussel (*M. edulis*) can produce as many as $8{\times}10^{10}$ oocytes (70 μm diameter), depending on body size. In our study *M. trossulus* of shell length 38–42 mm exhibited a greater reproductive output than *M. edulis* of the same size, 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 oocyte is larger in *M. edulis* than in *M. trossulus*, with intermediate values for hybrids, which is in accordance with findings from the east coast of the USA (P. Rawson, personal communication). Furthermore, we found that *M. trossulus* had a greater total gamete output (based on dry weight of gametes). A greater total weight of gametes combined with a smaller egg size suggests that individual *M. trossulus* release more eggs than individual *M. edulis* of equal size. *M. trossulus* is capable of reproduction at a smaller shell length than *M. edulis*, 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 be a response to a higher rate of mortality for *M. trossulus* (Comesaña et al. 1999). The implications of this higher rate of mortality and greater reproductive output by *M. trossulus* are not clear, but the data suggest that the coexistence of the two species may be linked to life-history differences associated with age-specific reproduction and mortality. Further data are being collected to examine these aspects of the life history in more detail.

Gardner and Skibinski (1990) found that the mean fecundity of *M. galloprovincialis* was 2.8 times that of *M. edulis* at Croyde and 2.2 times greater at Whitsand, because *M. galloprovincialis* had 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

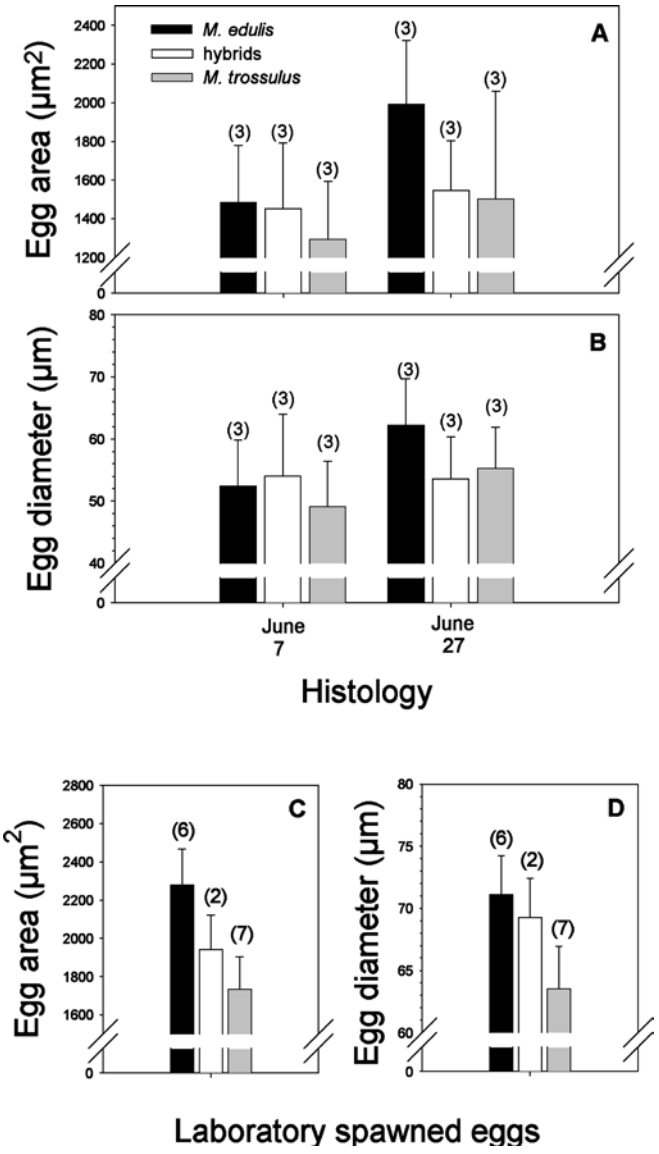

Fig. 5A–D *Mytilus* spp. Comparison of mean (±SD) of egg area (μm^2) (**A**) and egg diameter (μm) (**B**) among *M. edulis*, *M. trossulus* and hybrids determined from histological sections on two dates during the pre-spawning stage and also from laboratory spawned eggs: egg area (**C**) and egg diameter (**D**). Number of mussels used in *parentheses* (50 eggs from each mussel were measured)

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 but larger *M. galloprovincialis*. A similar mussel population structure was found at the present study sites. A higher frequency of *M. trossulus* was observed in the abundant small shell-length classes compared with a higher frequency of *M. edulis* in the much less abundant large shell-length classes (Comesaña et al. 1999; Toro 1999), suggesting a greater reproductive output by the *M. trossulus* population. A higher proportion of *M. trossulus* larval, spat and juvenile stages was observed at the study sites (Toro 1999), which is consistent with a higher proportion of *M. trossulus* contributing to the gamete pool, assuming that the larvae originated from a site with a similar population structure.

Reproductive isolation between *M. edulis* and *M. trossulus* is not complete, because hybrids occur in nature and can also be produced in the laboratory. The lower frequency of hybrids in Newfoundland compared with other mussel hybrid zones suggests that some pre- or post-zygotic reproductive isolating mechanisms may be operating. We observed significant differences in the reproductive cycle and the timing of spawning between the two species that could contribute towards reproductive isolation, but the overlap in spawning suggests that other factors may also play a role. Further studies are in progress to determine the roles played by pre-zygotic gamete and post-zygotic genetic incompatibility in explaining the observed rate of hybridization between *M. edulis* and *M. trossulus* in Newfoundland.

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