Synchronous reproduction may facilitate introgression in a hybrid mussel (*Mytilus*) population

By

Sandra D. Doherty

Supervisors of research

Dr. Elizabeth Gosling
Dr. Deirdre Brophy

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Contents

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Sandra D. Doherty

Abstract

Mussel populations on the Irish Atlantic coast comprise an interbreeding mixture of the blue mussel, *Mytilus edulis* (L.) and the Mediterranean mussel, *Mytilus galloprovincialis* (Lmk.). The occurrence of hybrid genotypes varies between sites but can be as high 80%. This study compares the reproductive cycle of *M. edulis, M. galloprovincialis* and their hybrids to determine if the extensive hybridisation observed at Irish Atlantic coast sites is linked to spawning synchrony between the two taxa. Mussels (40-45 mm size class) were collected monthly from a sheltered shore in Galway Bay from January to December 2005. Two major spawning events (March-June and September-October) were observed and gametogenesis took place throughout the year. The spawning cycles of the three taxa were largely overlapping. Small differences were observed in the timing of peak spawning which occurred in March and October in *M. galloprovincialis* and in May-June and September in *M. edulis*. Spawning of hybrid individuals was intermediate between the parental genotypes. Fecundity was slightly higher in *M. galloprovincialis* females compared to the other taxa (up to 30% difference, $p<0.05$). This apparent advantage is not shared by the sexes and is likely being offset by high numbers of hybrid genotypes releasing gametes during peak spawning of *M. galloprovincialis.* There was no evidence for increased mortality in hybrid males; sex ratios did not deviate from the 1:1 ratio. The results show that in this region of the hybrid zone the timing of reproduction does not present a barrier to gene flow between *M. edulis* and *M. galloprovincialis*. Nonetheless, small differences in the timing of peak spawning may increase the likelihood of conspecific fertilisation at certain times of the year. Hybrids outnumber the parental genotypes, undergo complete gametogenesis and show no evidence of depressed fitness (i.e. hybrids are reproductively competent suggesting a high degree of introgression.

1. Introduction

1.1 Taxonomy and distribution of *Mytilus edulis* and *M. galloprovincialis*

The Mytilidae family date back to the Devonian era and includes many important byssally attached genera such as *Choromytilus, Perna, Modiolus* and *Aulacomya*, as well as *Mytilus* itself (Seed and Suchanek 1992). The genus *Mytilus* is the most successful member of the family being the most diverse and widely distributed. It includes four mussels of distinct evolutionary lineages, *Mytilus edulis* (Linnaeus 1758), *Mytilus galloprovincialis* (Lamarck 1819) and *Mytilus trossulus* (Gould 1850) and *Mytilus californianus* (Conrad 1837). The *Mytilus* genus is considered to be of relatively recent origin with the first fossils appearing in the North Atlantic Pliocene strata ~ 5 mya (Vermeij 1992). *Mytilus edulis* is generally acknowledged to be the ancestral species from which all three mussel species evolved despite the fact that there is virtually no information on the evolution of the genus (Gosling 1992a).

The genus *Mytilus* exhibits an antitropical distribution pattern, typical of many marine species, with representative taxa in both the Atlantic and Pacific oceans (Hilbish et al. 2000). Southern hemisphere populations are more closely related to *M. edulis* and *M. galloprovincialis* than *M. trossulus* and are believed to have arisen from two migration events from the northern hemisphere via the Atlantic. The first main event took place during the Pleistocene and a second smaller event in the more recent geologic past (Hilbish et al. 2000). The divergence of the northern and southern hemisphere populations is believed to predate that of modern *M. edulis* and *M. galloprovincialis* (Gerard et al. 2008). The genus is also circumpolar in its distribution. In the Northern hemisphere *M. edulis* itself inhabits boreal to cool temperate waters on both sides of the Atlantic. It does not, as was previously thought, inhabit the Pacific coasts of America but has a distribution restricted to the east cost of

North America from the Canadian Maritimes to Cape Hatteras, North Carolina (Gosling 1992a). Its presence has also been verified in Iceland (Varvio et al. 1988; Smietanka et al. 2004). The species also inhabits temperate waters of the southern hemisphere (Mc Donald et al. 1991).

Mytilus galloprovincialis is cosmopolitan in its distribution, occurring in large disjunct populations in five geographic areas. It is native to the Mediterranean Sea and is believed to have originated there during one of the Pleistocene ice ages when the sea was cut off from the Atlantic Ocean (Gosling 1992a). It is also present on coasts of Portugal, France, and the British Isles as far north as Shetland and the Orkney Islands, the Black Sea, and populations on the Iberian Peninsula, previously recorded as *M. edulis*, have now been included in this taxon (Gosling 1992a). Gerard et al. (2008) have recently reported strong genetic structure in southern hemisphere *Mytilus*. Their study employed the *cytochrome oxidase subunit 1* locus and revealed three genetic entities 1) South America and Kerguelen Island, 2) Tasmania, 3) New Zealand. DNA markers have revealed the Kerguelen Island *Mytilus* are of mixed *M. edulis/ M. galloprovincialis* ancestry and *Mytilus* populations in Tasmania possess a predominantly *M. galloprovincialis* genomic background introgressed by *M. edulis* alleles (Borsa et al. 2007). As no evidence for a recent introduction was observed in these populations Borsa et al. (2007) have suggested the genomic reticulation, i.e. mixed ancestry, observed is due to genetic drift superimposed on ancient hybridisation and introgression. It is generally acknowledged that populations of *M. galloprovincialis* present in Japan, South Africa, and Hong Kong are the result of human introductions (McDonald 1991). These introductions have been accredited to the increase in trans-oceanic traffic during the 20th Century (Inoue et al. 1997). *M. galloprocincialis* is also well established on the Pacific coast of North America from

Mexico to British Columbia where it has been introduced for aquaculture (see Wonham 2004 for review). The debate as to whether *M. galloprovincialis* should be considered as a distinct species has been ongoing since the 1860s (Gosling 1992a). While its binomial nomenclature implies it is a sibling species of *M. edulis,* the systematic status of *M. galloprovincialis* remains controversial; it is considered by some to be a subspecies of the *M. edulis* complex (reviewed in Gosling 1984, 1992a). In areas where the taxa occur in sympatry they hybridise. Mc Donald et al. (1991) concluded, however, that the maintenance of distinct sets of alleles by the taxa, despite hybridisation, warrants taxonomic recognition at the species level. As *M. edulis* and *M. galloprovincialis* are the focus of this study *M. trossulus* will not be discussed from here on unless necessary.

1.2 Morphological variation in *M. edulis* and *M. galloprovincialis*

Mytilus edulis and *Mytilus galloprovincialis* have traditionally been separated by use of shell morphology characteristics (Fig. 1.1). In some areas however hybridisation has led to the presence of intermediate forms making identification on shell characters alone difficult (Gosling 1984). In a combined genetic and morphological study, Beaumont et al. (1989) found that of eight variables measured no single diagnostic character emerged. Other authors have also reported this difficulty (Gosling et al. 1984 and references therein). Mc Donald et al. (1991) found, that in "pure" samples there was considerable overlap between species in even the most informative of the individual characters.

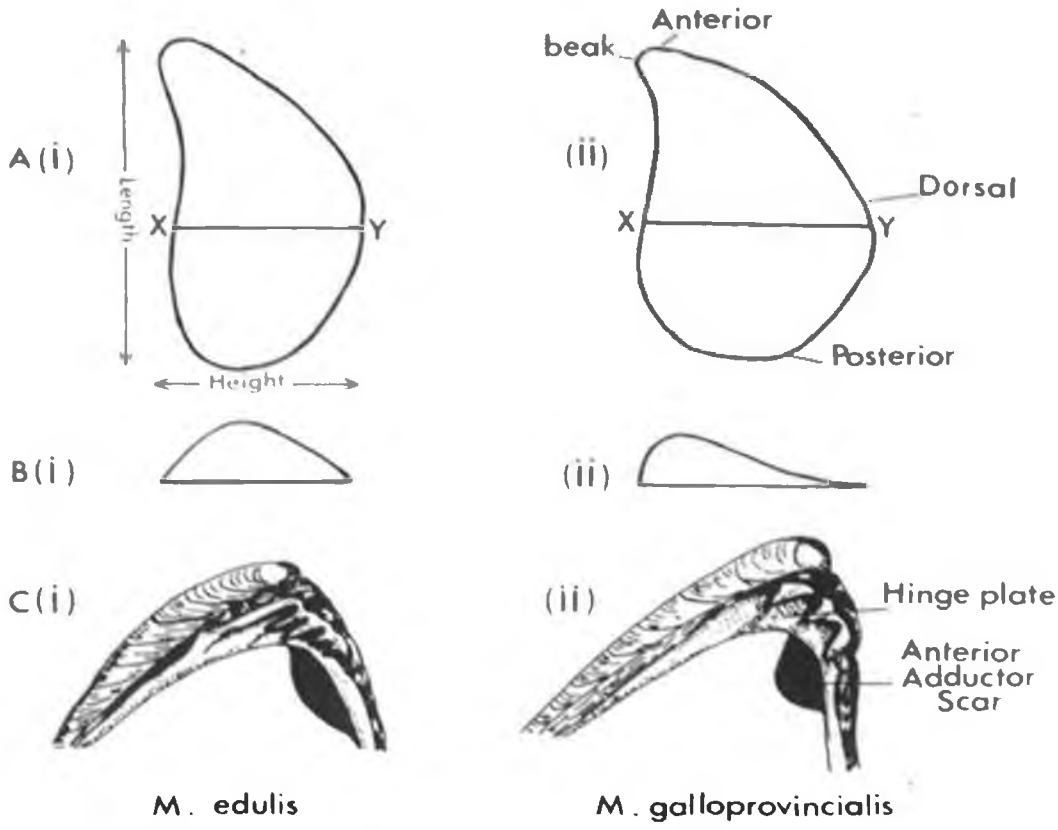

Fig. 1.1 – A (i) & (ii) & C (i) & (ii) some of the salient differences in shell morphology between *M. edulis* and *M. galloprovincialis*. B (i) & (II); transverse profiles through section XY of a single shell valve of *M. edulis* and *M. galloprovincialis* (Gosling 1984)

The shell of *M. galloprovincialis* tends to be higher and flatter than that of *M. edulis*, giving distinctly different transverse profiles in the two forms (Gosling 1984). The anterior end of the shell of *M. galloprovincialis* is distinctly beaked or incurved while that of *M. edulis* has a more snub-nosed appearance. In *M. galloprovincialis* the anterior edge of the shell merges smoothly into the dorsal edge, thus giving rise to a rounded convex profile, while that of *M. edulis* is angular where the anterior and dorsal edges meet (Gosling 1984). Both *M. edulis* and *M. galloprovincialis* exhibit considerable variation in external shell morphology. Interior shell characters such as the anterior adductor scar and hinge plate have therefore generally been regarded as

more reliable discriminators between the taxa (Gosling 1984; see Figs 1.1; 1.2). The high degree of phenotypic similarity exhibited in naturally hybridizing populations, suggests that the genes or gene complexes controlling morphological development in *M. edulis* and *M. galloprovincialis* are very similar, further emphasizing their close evolutionary relatedness (Gardner 1995).

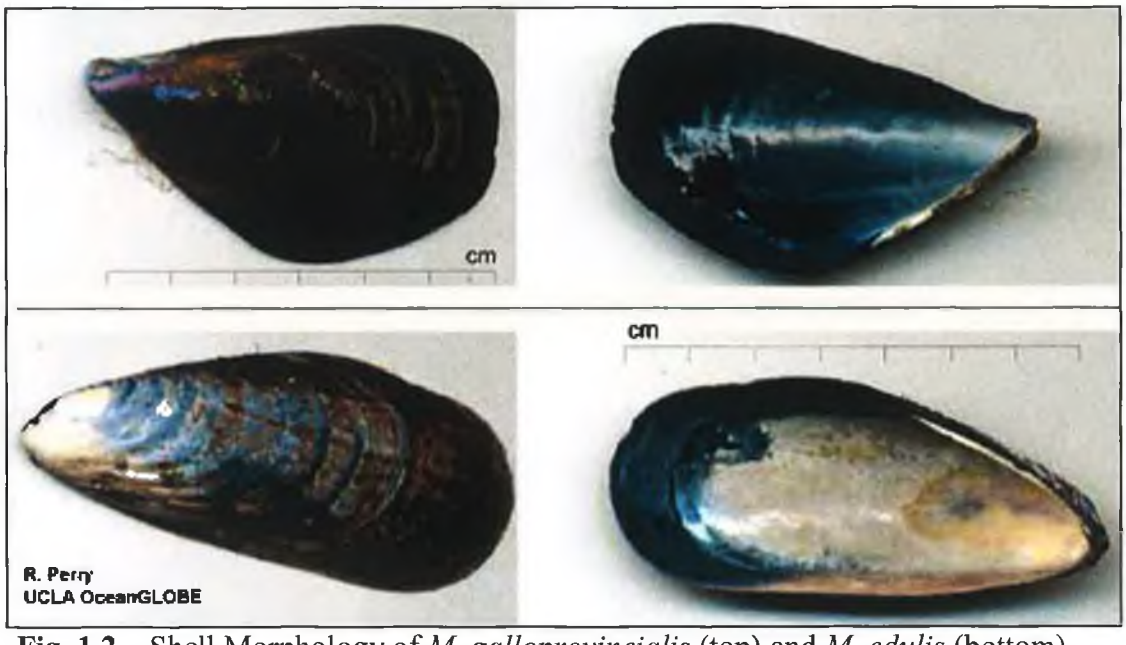

Fig. 1.2 – Shell Morphology of *M. galloprovincialis* (top) and *M. edulis* (bottom) (http://www.msc.ucla.edu/oceanglobe/specimenphotographs/animals/mollusca/Bivalviamollusksw2shells/Mytilusbothsppint&ext2speciesofmussels.JPG)

1.3 Molecular genetics of *M. edulis* and *M. galloprovincialis*

Recent advances in molecular systematics have greatly enhanced and altered our understanding of *Mytilus* ecology, evolution and biogeography (Suchanek et al. 1997). In addition to morphometric analysis, previous studies have employed allozyme electrophoresis to differentiate between the species (Gosling and Wilkins 1981; Beaumont et al. 1989; Gardner 1995). Nei's (1972) genetic identity (I) and distance (D) in allopatric populations have been calculated over 16 allozyme loci (Skibinski et al. 1980). The results obtained, (I = 0.850 and D = 0.172), are within the range expected for comparisons between subspecies of molluscs (Gardner 1995 and

references therein), indicating a close relatedness. However, even the most differentiated of these markers are only partially diagnostic between the species (Mc Donald et al. 1991; Gosling 1992b). Several types of DNA markers have been developed for *Mytilus* including the nuclear DNA markers *ITS* (Heath et al. 1995), *Glu5'* and *Me15/16* (Inoue et al 1995; Rawson et al. 1996), *Mac-1* (Ohresser et al. 1997; Daguin and Borsa 1999; Daguin et al. 2001) and *Efbis* (Bierne et al. 2000) and mitochondrial DNA markers e.g. COIII, 16S RNA, ND2. The development of these diagnostic DNA markers since the advent of PCR (polymerase chain reaction) technology has allowed for more precise estimations of hybrid frequencies within the *Mytilus* genus. For example, Daguin and Borsa (1999) reported that a sample of the Padstow mussel (SW England) assigned as *M. galloprovincialis* by allozyme and morphological data was in fact intermediate between *M. galloprovincialis* and *M. edulis* at the *Mac-1* locus. They also noted that while the *Mac-1* locus is diagnostic, or nearly so, between the two species it also exhibits sequence length polymorphisms within species that are only resolved on long-range polyacrylamide gels. The Me15 and Me16 primers developed by Inoue et al. (1995) however, produce species-specific bands, which can be visualised using the faster and cheaper technique of agarose gel electrophoresis. Microsatellite markers were first developed for *M. galloprovincialis* in 2002 (Presa et al. 2002). These workers reported that the seven loci cross amplified with *M. edulis* and *M. trossulus.* However, Gaardestrom et al. (2007) were unable to use these markers to identify Baltic *M. trossulus.* They subsequently characterised six microsatellite loci in Baltic *M. trossulus*, which cross-amplified with *M. edulis.* However, they have observed heterozygote deficiencies at all six loci, most likely due to null alleles. As this study was not concerned with genetic variation within or between mussel populations the *Me15/16* primers were used for the purpose of

species and hybrid identification. This allowed direct comparison of the reproductive cycles of the three taxa. Despite the diagnostic capability of this marker it does not allow differentiation of F_2 backcross hybrids.

1.4 Hybrid zones

Hybrid zones occur where the geographic range of closely related species overlap allowing them to cross-fertilise and produce hybrid offspring (Barton and Hewitt 1989). Such zones provide an ideal arena for studying the forces that drive divergence of populations (Buggs 2007). The occurrence of hybrids in nature indicates that reproductive isolating mechanisms are insufficient to prevent hybridisation (Barton and Hewitt 1989). Depending on the fertility and fitness of hybrids relative to the parental taxa, the potential exists for introgressive gene flow between species when hybrid individuals backcross with one or both parents. Information on the factors that limit hybridisation is essential to understanding the maintenance of hybrid zones and the continued separation of two species despite extensive gene flow. The coexistence of genetically distinct species may result through a combination of reproductive isolating mechanisms (RIMs). Pre-zygotic barriers to fertilisation can occur through mating preference, habitat specialisation, asynchronous reproduction and gamete incompatibility (Palumbi 1994). Alternatively, isolation may occur post-zygotically through gametic incompatibility, zygotic inviability or hybrid sterility. Despite the potential for reproductive isolation naturally occurring hybrids are often abundant.

Studies on the genetic structure of *Mytilus* populations have revealed extensive hybridisation across the geographical range of the genus (reviewed in Gosling 1992a). An *M. galloprovincialis-M. trossulus* hybrid zone extends from San Diego to Humboldt Bay on the Pacific coast of North America (McDonald and Koehn 1988;

Heath et al. 1995; Rawson et al. 1999; Wonham 2004). On the northwest Atlantic coast hybridisation takes place in a zone of contact between *M. edulis* and *M. trossulus* (Bates and Innes 1995; Saavedra et al. 1996; Rawson et al. 2001; Toro et al. 2004). On northeastern Atlantic shores an extensive hybrid zone between the blue mussel, *M. edulis,* and the Mediterranean mussel, *M. galloprovincialis,* spans more than 1400 km of coastline from western France to the north of Scotland (Gosling 1992b) and between the North Sea and Baltic Sea an area of hybridisation between *M. edulis* and *M. trossulus* has also been identified (Fig. 1.3) (Väinölä and Hvilsom 1991; Rignios et al. 2002). *M. trossulus* has recently been genetically identified in UK waters where it hybridises with both *M. edulis* and *M. galloprovincialis* (Beaumont et al. 2008). This is the first record of all three species and their hybrids occurring in one location in the North Atlantic region; *M. trossulus* is thought be a post glacial relict restricted to Loch Etive, Scotland, by its low salinity.

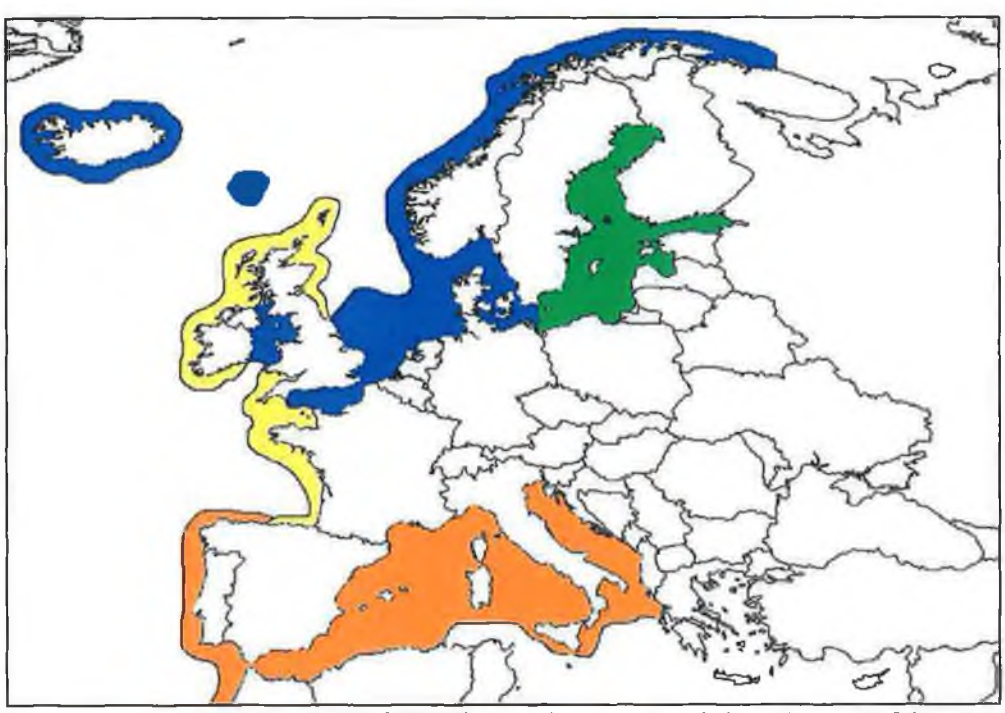

Fig. 1.3. European Distribution of *Mytilus*. (Blue = *M. edulis*, Green = *M trossulus*, Orange = *M. galloprovincialis*, Yellow = Zone of hybridisation between *M. edulis* and *M. galloprovincialis*).

Indeed, aquaculture practices in Europe and beyond may be, at least in part, responsible for range expansion of this invasive species.

A variety of factors may be responsible for the maintenance of *Mytilus* hybrid zones. These include asynchronous spawning (Secor et al. 2001), nuclear-cytoplasmic incompatibility in hybrids (Zouros et al. 1994), assortative fertilisation and habitat specialisation (Bierne et al. 2002a; Bierne et al. 2002b), preferential settlement (Gosling and McGrath 1990) and reduced reproductive output by hybrids (Toro et al. 2002).

1.5 Reproduction, Gametogenesis and Hybridisation in *Mytilus*

The reproductive cycle refers to a series of processes from the activation of the gonad, through gametogenesis to the release of ripe gametes, i.e. spawning, and the subsequent recession of the gonad (Seed and Suchanek 1992). Food reserves are accumulated in the gonad prior to the initiation of gametogenesis (Wilson and Seed 1974). Changes in the rate or duration of any of these closely linked processes will ultimately affect the entire reproductive cycle. The timing and duration of the reproductive cycle is controlled by interacting endogenous factors (hormonal cycle, nutrient reserves, genotype) triggered by environmental cues (temperature, food availability, salinity) (Seed 1992). The influence of one or other external factor as the initial trigger for activation of the gonad varies with locality. On the east coast of the United States Newell et al. (1982) found that the summer reproduction maxima of two populations at the same latitude experiencing the same temperature regime were separated by a 3-month interval. The differences observed were attributed to differences in the mussels food supply between habitats. In Baltic Sea mussels food abundance is considered as the primary factor controlling gonad growth (Kautsky

1982), while Bayne (1965) delayed the onset of gamete development by holding fed British mussels at temperatures down to 5°C. At the sampling site in this study gametogenesis was reported to occur throughout the entire year (King et al. 1989). *Mytilus* reproduction varies latitudinally; in southerly populations the onset of gametogenesis is generally later than in conspecifics in more northerly latitudes (Seed 1992). Consequently, it is plausible *M. edulis* and *M. galloprovincialis* on Irish coasts exhibit temporal differences in their reproductive cycles, thereby maintaining their genetic integrity despite some hybridisation. Previous reproductive studies have treated Irish mussel populations as panmictic populations of *M. edulis* alone. This is the first study to investigate the reproductive cycle of *Mytilus* on Irish coasts in the context of hybridisation.

M. edulis can become sexually mature in the year following settlement although the size at which this occurs is dependant on growth rate (Seed and Suchanek 1992). Gametogenesis occurs mainly in the mantle tissue, although reproductive tissue can also be found in the visceral mass and mesosoma (Bayne et al. 1978; Lowe et al. 1982). The many ducts of the reproductive system ramify throughout most of the body, each ending in a genital follicle (Seed 1992). Paired gonoducts open onto papillae, which are situated anterior to the posterior adductor muscle, between the mesosoma and inner gill lamellae. These gonoducts lead into five major canals with convoluted walls forming longitudinal ciliated ridges. These subsequently lead into a series of minor canals, which end in genital follicles. Oogonia and spermatagonia are budded off from the follicular germinal epithelium. Early oocytes are connected, for a time, to the epithelium by a slender stalk of cytoplasm, which eventually ruptures leaving the mature ovum free within the follicle. Spermatagonia give rise to spermatocytes, spermatids and finally spermatozoa. The

mature male gametes form dense lamellae, which converge towards the centre of the follicle (Seed and Suchanek 1992). The colour of the reproductive tissue can vary considerably, but generally speaking the female gonad is peach/orange while males are creamy-white (King et al. 1989). When spent the mantle tissue appears transparent with orange-brown spots often seen (Seed and Suchanek 1992).

The reproductive cycle can be assessed by several methods. The appearance of the gonad either macro- (colour, texture, thickness of the gonad) or microscopically (thin sections or squashes of gonads) can be monitored throughout the year. However, histological preparations of gonads at regular interval throughout the year provide the most reliable and detailed information (Seed 1992). From these sections the gonad somatic index (GSI), the ratio of gonad weight to body weight can then be calculated. The GSI increases as gametogenesis proceeds; a subsequent decrease can be inferred as spawning. Despite its usefulness the technique is a nominal rather than an interval measurement i.e. it does not give a quantative measure of gametes at various stages and so does not quantify the reproductive stages. Stereological methods, on the other hand, enable workers to quantifiably measure the volume of the gonad occupied by different cell types (oocytes, spermatocytes, storage cells etc). Point counts are carried out using test grids applied to sections of the mantle.

Mytilus are dioecious although sexual dimorphism does not occur; most populations exhibit a 1:1 male: female ratio (Seed 1972). Sex determination is under maternal control, and the sex ratio of progeny from different mothers can vary widely due the interrelationship of sex determination and the unusual mode of mitochondrial DNA inheritance, DUI (doubly uniparental inheritance). With this mode of inheritance females inherit only maternal mtDNA. Males, however, are heteroplasmic, containing both maternal and paternal mtDNA (Zouros et al. 1994; Saavedra et al.

1997). Pedigreed crosses have shown that females produce the same sex ratio of offspring regardless of the mate, and that this property is heritable (Kenchington et al. 2002). Hybridisation in *Mytilus* can lead to the breakdown in unity of sex ratios.

Asynchronous spawning between species may reinforce RIMs and thus reduce hybridisation. In higher latitudes however, the spawning cycle of species that reproduce by release of gametes into the water column are restricted by the strong seasonality of high water temperature and food availability (Seed 1976; Lowe et al. 1982; King et al. 1989; Gardner and Skibinski 1990; Toro et al. 2002). Studies on the reproductive cycles of mussel populations on the coast of southwest England have revealed that spawning times of the *M. edulis, M. galloprovincialis* and their hybrids are asynchronous (Skibinski et al. 1980; Skibinski et al.1983; Gardner and Skibinski 1990; Secor et al. 2001). On Atlantic coasts of France, Seed (1972) noted a high frequency of morphologically intermediate forms, which is not unexpected given that spawning of the two taxa in this area occurs simultaneously (Lubet 1957). In contrast, Bierne et al. (2002b) found evidence for temporal variation in settling cohorts of mussels in the Bay of Quiberon, France. They suggested this variation may be due to partial spawning asynchrony as *M. edulis* settled in the first settlement phase and a mixture of genotypes was observed in the second phase. An earlier study carried out in the *M. edulis-M. trossulus* hybrid zone in Newfoundland reported spawning asynchrony, with *M. trossulus* and hybrid individuals spawning over a prolonged period from late spring to early autumn, whereas *M. edulis* spawned over a 2-3 week period in July (Toro et al. 2002).

Objectives of study

The aim of the present study was to compare, for the first time, the reproductive cycles of *M. edulis, M. galloprovincialis* and their hybrids in Galway Bay to see whether the extensive hybridisation observed at Irish Atlantic coast sites is due to spawning synchrony between the two taxa. Reproductive output and fecundity were also examined to determine if there was a difference in reproductive effort between the two species and their hybrids. Also, sex ratios were compared to ascertain if any deviation from unity occurs as a result of hybridisation.

2. Methods

2.1 Study site and sampling

The study was carried out at a single location in Galway Bay. Ballynahown Quay, west of Galway city, (9°29′57.9′′W, 53°13′29.4′′N (Fig. 2.1) is a sheltered, rocky, sandy shore with a considerable amount of exposed rock interspersed throughout. The site is considered to have an exposure gradient of five on the Ballantine scale (Coghlan and Gosling 2007). This particular site was chosen based on the results of Coghlan and Gosling (2007), who observed higher numbers of *M. edulis* (17%) and larger size mussels at this site, compared to the nearby exposed shore site (5%), thus making it the most ideal for this study.

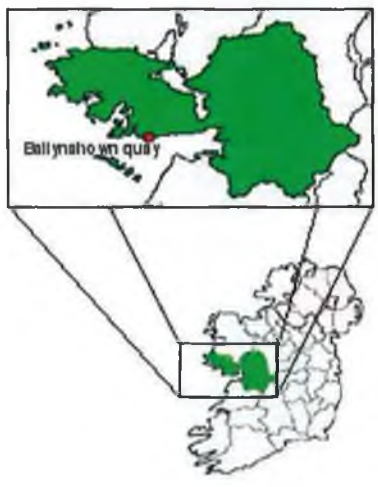

Fig. 2.1 - Ballynahown Quay, Co. Galway, Ireland.

Mussels (N_{total}=200) were collected monthly from January to December 2006. Sampling was carried out by stratified random sampling (Krebs 1999) on the low shore, due to the higher density of mussels in this area. Sampling was stratified by size to ensure that sufficient numbers of mature individuals of each genotype were

obtained for the reproductive analysis. Individuals of 40-45mm shell length were selected as it has been found (Coghlan, unpublished data) that the *M. edulis* genotype was most frequent in this size class (*M. edulis* = 7-17%, *M. galloprovincialis* = 28-36% and hybrid = 55-57%). Forty individuals were collected from five randomly selected points along a 30m transect. Samples were stored at 4° C until processing.

2.2 Sample preparation

All samples were dissected within five days of collection. During dissection the mantle was separated from the main body. A small section of mantle (2-3mm^3) was taken from the centre, i.e. the thickest part of the mantle, and fixed in 4% buffered formalin for histological analysis. In addition, a 2mm^3 section of posterior adductor muscle was removed for genetic analysis.

2.3 Gonad Somatic Index (Toro et al. 2002)

Gonad somatic index (GSI) is a quantative assessment of the reproductive stage of an individual. Seasonal changes in GSI reflect gonad development and a large decline in the mean GSI of a population is indicative of a spawning event. The whole of the mantle tissue was weighed, dried at 80°C to a constant weight, cooled in a desiccator and reweighed. The ratio of wet to dry weight for the remainder of the mantle was used to correct for the portion removed for histological analysis. The same procedure was carried out on the portion of the body excluding the mantle. The GSI of each individual was then calculated by dividing the mantle dry weight by the whole mussel dry weight and multiplying by 100. Male and female GSI was calculated separately, as MGSI and FGSI, respectively.

2.4 DNA extraction (Estoup et al. 1996)

Total genomic DNA was extracted from posterior adductor muscle and used as a template for PCR. Each section of muscle was placed in a 1.5 ml Eppendorf tube with 500 µl of 10% Chelex* solution (1 g Chelex to 10 ml sterile $_{dd}H_2O$) and 7 µl of proteinase K* (20 µl/ml), vortexed for 20 s and then placed in a rotary shaker and incubated at 56°C for 2 h. Samples were then vortexed again, 20 µl of ribonuclease A* (20 µl/ml) was added, and the samples were placed in a 36°C oven and shaken twice in one hour. Samples were then placed in a 100°C oven for 30 min and vortexed once after 15 min and then either used directly or stored at -20°C.

Prior to PCR processing each sample was spun at 10 000 rpm for 3 min to separate the mixture of supernatant and Chelex. Three µl of the supernatant was then added to the PCR mix taking care not to add any Chelex, as this would inhibit the reaction.

2.5 Molecular marker

2.5.1 *Me15/16*

The primers Me 15 and Me 16 (Table. 2.1) were designed by Inoue and colleagues (1995) to amplify a part of the non-repetitive region of foot protein 1. This genetic marker (*Me15/16*) is located within the nuclear gene and encodes a polyphenolic adhesive protein, a key component in bysuss thread formation. The size of the allele amplified is 180bp (E allele) in *M. edulis*, while in *M. galloprovincialis* the allele is 126bp (G allele) in length owing to a deletion of 54bp (Fig. 2.2). Hence, hybrid individuals appear as heterozygotes possessing one of each allele. Therefore, this marker exhibits a bi-allelic banding pattern when separated on an agarose gel. DNA,

* Sigma

from what are considered to be pure populations of *Mytilus edulis* Aarhus, Denmark (56°12'W 10° 20'N) and *Mytilus galloprovincialis* Rio Bensafrim, Lagos, Portugal (8°40`W 37° 6`N) were used as references.

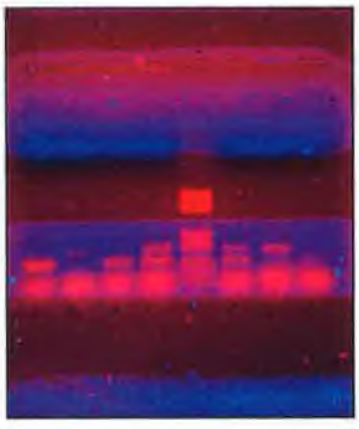

Fig. 2.2 - Agarose Gel. Bands represent; A) *M. galloprovincialis,* B) *M. edulis,* C) Hybrid, L) Ladder

Me 15 5′-CCA GTA TAC AAA CCT GTG AAG A-3′
Me 16 5′-TGT TGT CTT AAT AGG TTT GTA AGA-3′

Table 2.1 - Me 15 and Me 16 primer sequences.

2.6 Polymerase chain reaction (Coghlan and Gosling 2007)

PCR was carried out in a Biometra™ T1 Thermocycler. Amplification of *Me15/16* took place in a 13.2 µl reaction mixture containing 3µl of template DNA, 6µM of each primer♠, 2.5mM $MgCl_2$*, 0.15mM of each dNTP*, 0.6U Taq polymerase*, PCR buffer* and deionised water. Chemicals including the reaction mixture were kept on ice during the procedure to prevent activation of *Taq* polymerase. The reaction master mix was made up x40 to reduce sampling error when measuring small volumes (see Appendix Table 6.1). Amplifications were achieved by running 30 cycles of 94°C for 45 s, 56°C for 30 s and 70°C for 90 s, following a 4 min start at 95°C and a final

♠ MWG Biotech
* Bioline

extension of 6 min 40 s at 72°C. The soak file holds the samples, post amplification, at -1°C for an indefinite time (see Appendix Table 6.2). PCR products were either immediately run on 2% agarose gels or stored at -20°C.

2.7 Electrophoresis (Coghlan and Gosling 2007)

Agarose gels were run using a wide mini-sub cell GT electrophoresis cell (Bio-Rad). For each gel 100 ml of 1x TBE (Tris-Borate EDTA) and 2 g of agarose wide range/routine 3:1 powder (Sigma) was added (see Appendix Table 6.3). This solution was heated on a hotplate until the agarose powder had melted and the solution was clear, at which point 5 µl of ethidium bromide (EtBr 10 mg/ml) was added. This solution was then poured into a level gel mould, with two 20-sample combs after the gel had cooled slightly. The gel mould was then left to cool for 10 min before being placed at 4°C for 30 min.

The PCR samples were prepared for electrophoresis by adding 1 µl of 5x gel loading dye* to each tube. The agarose gel was placed in the electrophoresis chamber and submerged in 600 ml of 1x TBE running buffer. Of the 40 wells 37 were loaded with 13.5 µl of sample per well. One remaining well was loaded with 5µl of Hyperladder V*. A negative control i.e. PCR product loaded with sterile water in place of DNA, and a reference sample from a pure population of *M. galloprovincialis,* Madeira Islands, were also run on each gel.

One hundred volts was applied to the gel for ~1 h or until the loading solution had run ¾ of the way down the gel. The gel was then removed from the gel rig and visualised on an ultra violet (UV) light box (Hoefer, Mighty Bright) at high intensity (240 nm). Visualisation of the bands under UV light was possible due to the addition of EtBr to the gel. The resulting fluorescent banding was recorded using a digital

camera (Kodak, Easy Share CX4300); the image was then downloaded onto a desktop computer and enhanced using Adobe Photoshop™ 7.0. DNA, from what are considered to be pure populations of *Mytilus edulis* Aarhus, Denmark (56°12'W 10° 20'N) and *Mytilus galloprovincialis* Rio Bensafrim, Lagos, Portugal (8°40`W 37° 6`N) were used as references. At this stage, 30 individuals from each taxon were selected for further analysis.

2.8 Preparation of gonad tissue for cryo-sectioning

Following fixation in 4% buffered formalin each portion of gonad tissue was treated with a cryoprotectant solution to avoid cell damage due to ice crystals formation during freezing (Rush 2001). Slices of gonad were soaked in a series of sucrose solutions of increasing concentration (10%, 20% and 30%) in phosphate buffered saline (PBS); each solution also contained 2% dimethysulphoxide. The tissue slices were immersed in each solution for 15-30 min and were only transferred to the next sucrose gradient when the tissue had sunk to the bottom of the dish. Cryoprotectants are thought to increase viscosity at sub zero temperatures, thereby reducing the mobility of water molecules. The constrained water is prevented from forming ice crystal nuclei; hence ice crystal formation is inhibited (Rasmussen 1982). After cryo-protection the tissue was washed in PBS, mounted in cryo-m-bed mounting media™ and frozen in iso-pentane which had been cooled to -60°C with dry ice. Rapid freezing of the tissue in isopentane further reduces freezing artefacts (Barnard 1987). Six micron slices of gonad tissue were cut in horizontal sections on a Leica CM1900 cryostat. Lowe et al. (1982) have shown that no differential distribution of gametes exists in *Mytilus*. The mantle sections were cut horizontally for this study. For each individual one slide holding a minimum of five sections was prepared. Following

sectioning the slides were stained using haematoxylin and eosin in a Leica ST4040 linear stainer according to manufacturers guidelines (Appendix 6.4).

2.9 Reproductive staging

The prepared slides were observed under light microscopy for both sex determination, and assessment of gametogenesis. Ninety individuals each month were staged using the method of Wilson and Seed (1974). Individuals were assigned to one of four stages, 0 = spent, 1 = developing gonadal tissue, 2 = developing sex cells, 3 = ripe (see Figs 2.3a, b, c, d). Individuals included in the reproductive analysis were selected based on the results of the genetic analysis to ensure that each sample of 90 was comprised of 30 individuals of each genotype. However, every individual was assigned a two digit code using random number tables so that the genotype was not known at the time of staging. The occurrence of spent individuals in the sample or a marked reduction in numbers of ripe gametes between months was interpreted as an indication of spawning activity.

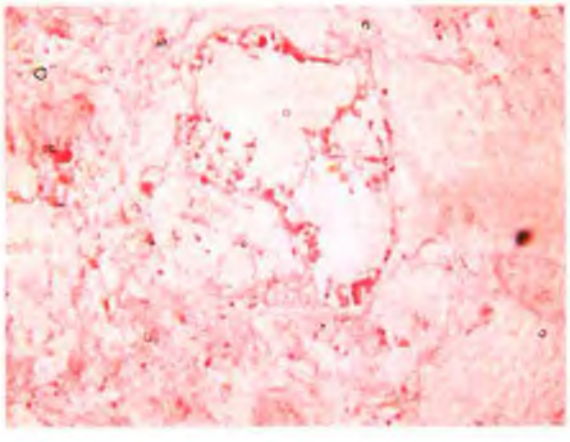

Fig. 2.3a ***Stage 0 – Spent:*** after the final spawning the follicles begin to collapse and degenerate. Any remaining gametes are re-absorbed and the animal enters resting stage.

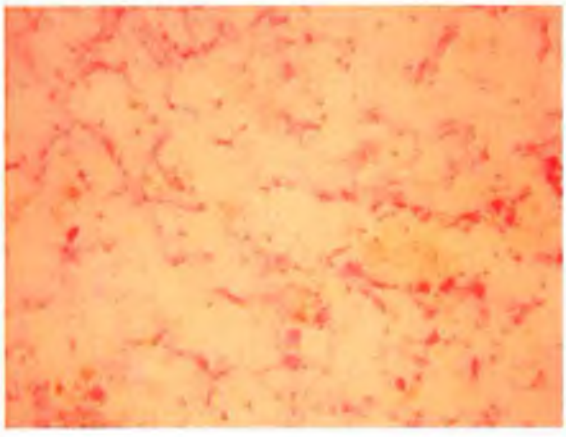

Fig. 2.3b ***Stage 1 – Resting/resorbing:*** sex determination is often difficult in this stage, notably at the early phase of gamete production. Indications of the onset of gametogenesis become apparent. Oogonia and spermatagonia are formed from the germinal epithelium line in the walls of the follicles.

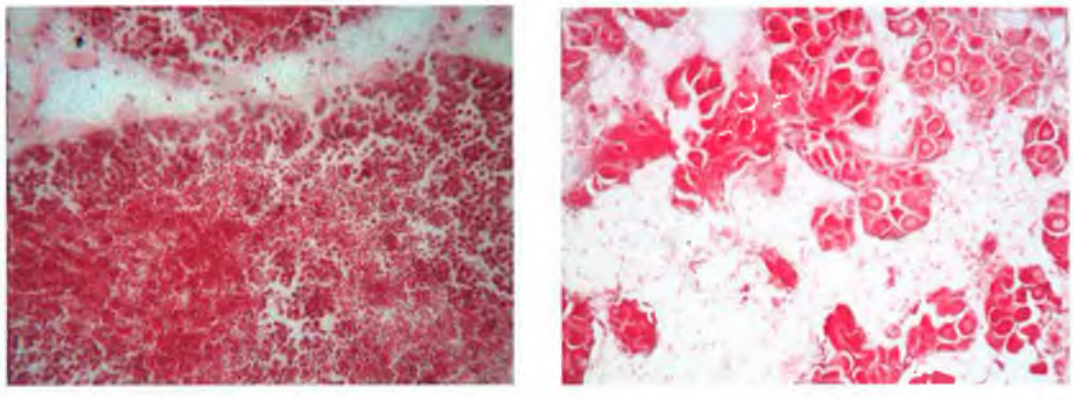

Fig. 2.3c ***Stage 2 – Developing sex cells***: follicles in both males and females now occupy a large part of the mantle. In males (left), masses of primary and secondary spermatocytes and spermatids fill the follicles, small darkly staining nuclei of spermatozoa are scattered among the larger cells. Female (right) oocytes have by now grown considerably and begun to accumulate yolk. Some larger oocytes remain attached to the epithelium via a slender stalk of cytoplasm.

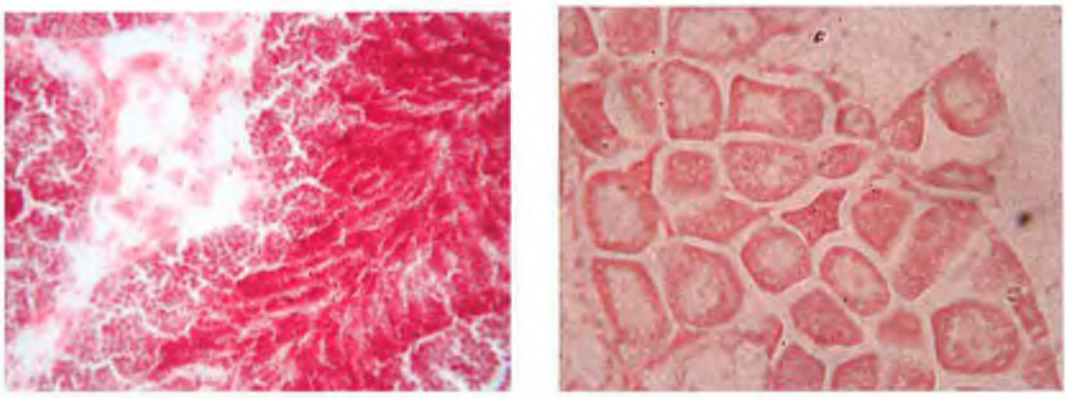

Fig. 2.3d ***Stage 3 – ripe:*** Male (left) follicles are now packed with spermatozoa arranged in lamellae which converge towards the lumen. Some residual spermatocytes and spermatids may still be present. In females (right), the oocytes have reached their maximum size and now lie tightly packed in the follicles. The pressure within the follicles compresses the oocytes which appear polyhedral in form. Connective tissue has now virtually disappeared due to the swollen follicles.

2.10 Gamete volume fraction (GVF) and reproductive output (RO)

The criterion for the use of stereology in counting tissue structures on random sections has been recommended by Weibel and Gomez (1962) and was followed for this study. Tissue sections were examined using an Olympus CX41 compound microscope interfaced with a PC and Olympus DP-soft image analysis software©. The fractional areas of morphologically ripe gametes were measured quantitatively (Lowe et al. 1982). A test grid of 30 points, measuring 180 x 165 μm was applied to 10 fields of view (400x) from each slide, giving 300 point counts for each animal, a quantity recommended by Weibel (1979) to obtain 95% confidence. Ten individuals of each genotype (five male and five female where possible) were randomly selected from each monthly sample for this part of the study. GVF varies from 0% for reproductively inactive gonads to 100% in ripe gonads. Only ripe gametes with a

visible nucleolus were counted. GVF was calculated by dividing the number of ripe gametes by 300 and multiplying by 100 to give the percentage of mantle occupied by ripe gametes. Reproductive output (RO) was calculated by multiplying total mantle dry weight by GVF to provide an estimate of the dry weight of gametes for each individual.

2.11 Oocyte area

A total of 150 oocytes with a nucleolus visible in the histological sections were measured using image analysis. Five ripe (stage 3) females were selected from each taxa and the area of ten oocytes from each individual was recorded. The average oocyte area was calculated for each individual prior to statistical analysis.

2.12 Statistical analysis

Observed sex ratios were examined using a Chi-square goodness of fit in Minitab to determine if any deviation from the expected 1:1 ratio has occurred (Preacher 2001). Chi-square analysis of contingency tables was used to test for variation in the frequency of individuals at each developmental stage between the three genotypes; Yates correction was applied where appropriate. Data from each sampling date was treated separately. Data for male and female GSI, GVF and RO were analysed separately. The data were screened to ensure that the assumptions of ANOVA (normality and homogeneity of variance) were met. Separate two-way ANOVAs were used to determine if GSI, GVF and RO differed between the taxa. Date and taxon were included as fixed factors in the analysis. One-way ANOVAs were used to compare the reproductive output among the taxa for each of the date-sex combinations. The analyses were repeated after the removal of spent (stage 0)

individuals from the data. Where significant differences were detected they were further interrogated using Tukey's pairwise comparisons. A one-way ANOVA was used to compare the oocyte area among the taxa. All statistical tests were carried out in Minitab™ v14.0.

Results

The genotype frequency at Ballynahown was in favour of hybrids (0.43±0.06) with *M. edulis* and *M. galloprovincialis* occurring at correspondingly lower frequencies: 0.26 ± 0.05 and 0.30 ±0.05, respectively. In pure populations of *M. edulis* and *M. galloprovincialis* screened with this marker the frequencies of the E and G alleles were each 1.0.

3.1 Sex ratio

Three hundred and sixty mussels of each taxon were collected over the sampling period (Table 3.1). The female: male sex ratio did not differ from the expected 1:1 for *M. edulis, M. galloprovincialis*, or hybrids (P>0.05, Pearson's chi square).

Table 3.1 *Mytilus sp.* Observed sex ratios of the three taxa.

Taxon	Male	Female	Undifferentiated individuals	N	M:F sex ratio
M. edulis	164	176	20	360	1:1.07
M. galloprovincialis	150	182	28	360	1:1.21
Hybrid	165	155	40	360	1:1.07

3.2 Reproductive cycle

Histological sections of male and female gonads of *M. edulis, M. galloprovincialis,* and hybrids revealed that gametogenesis takes place throughout the year (Fig. 3.1). A comparison of the reproductive cycles revealed broadly similar patterns in the three

taxa. The appearance of spent individuals (stage 0) in the sample indicated that spawning had recently occurred. This was observed in mussels collected in March, April, May, July, August, September and October. Spent individuals began to appear in the samples in March and were progressively more numerous between March and May. The proportion of ripe individuals (stage 3) increased until March and subsequently dropped. This indicates that the main spawning event occurred between March and May. Ripe individuals were absent from the samples in August. The proportion of spent and ripe individuals increased again between July and October indicating the occurrence of a second smaller spawning event in late summer/autumn. Complete evacuation of gonadal material was not observed in any month.

Although their reproductive cycles were broadly overlapping, some variation between taxa in the relative proportions of the developmental stages was observed in April, August and October. In April, samples of *M. galloprovincialis* contained more developing (stages 1 and 2) and fewer ripe individuals compared to samples of *M. edulis* and hybrids (Pearson Chi square = 54.57, $p < 0.05$; contribution to chi square = 12.36 and 31.60, respectively), indicating that on average, the release of gametes occurs slightly earlier in *M. galloprovincialis* than in the other taxa. In August samples of *M. galloprovincialis* contained more spent (stage 0) and less developing (stage 2) individuals compared to the other two taxa (contribution chi square = 20.83), suggesting that regeneration after the first spawning event may occur at a slower rate in *M. galloprovincialis* (contribution chi square = 20.83; Pearson Chi square = 40.39, $p < 0.05$). In that month, the hybrid sample was the most advanced in terms of reproductive development, with all gonads at stage 2 (contribution to chi square = 13.33). In October samples of *M. edulis* contained higher numbers of developing (stage 2) and lower numbers of ripe (stage 3) individuals than *M. galloprovincialis*

(Pearson Chi square = 54.57, $p < 0.05$; contribution to chi square = 11.67 and 8.00, respectively), indicating that the second spawning is more protracted in the latter taxon. Spent individuals were also present in the hybrid sample (contribution to chi square = 13.33), which indicates that this taxon is, on average, at a more advanced stage of development in October than *M. galloprovincialis*.

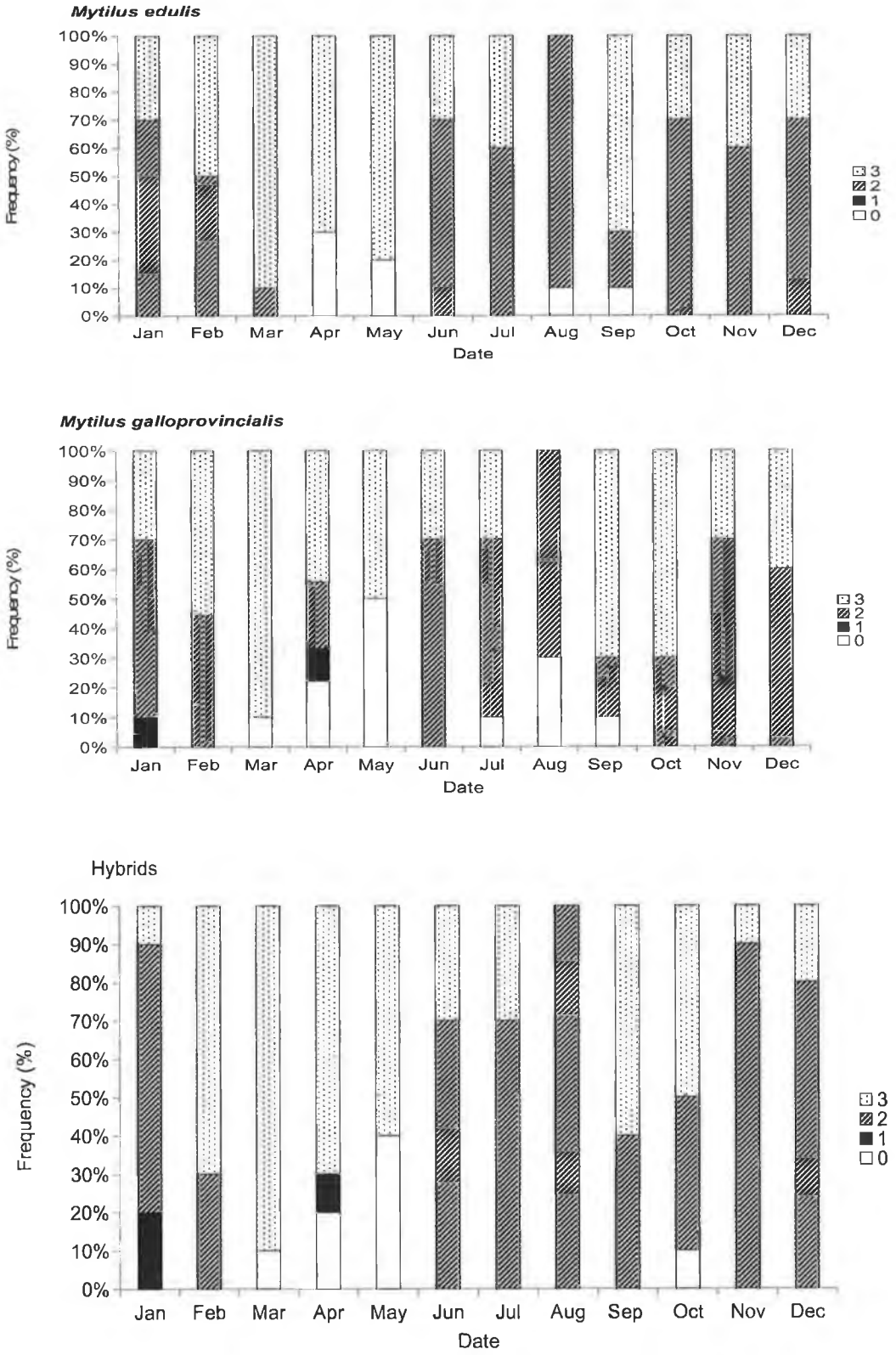

Fig. 3.1 *Mytilus* spp. Frequency distribution of individuals (sexes pooled) in the four developmental stages for 2007. N ≈ 90/month. 0 = spent, 1 = resting/resorbing, 2 = developing sex cells, 3 = ripe.

3.3 Gonad Somatic Index (GSI)

GSI of all taxa declined steadily from March until May (Fig. 3.2). Two-way fixed factor ANOVA revealed no significant interaction ($P>0.05$) between taxon and sampling date for either males (MGSI) or females (FGSI). This indicates that the rate of change in GSI during the year was consistent across the taxa and that gametogenesis was largely synchronous.

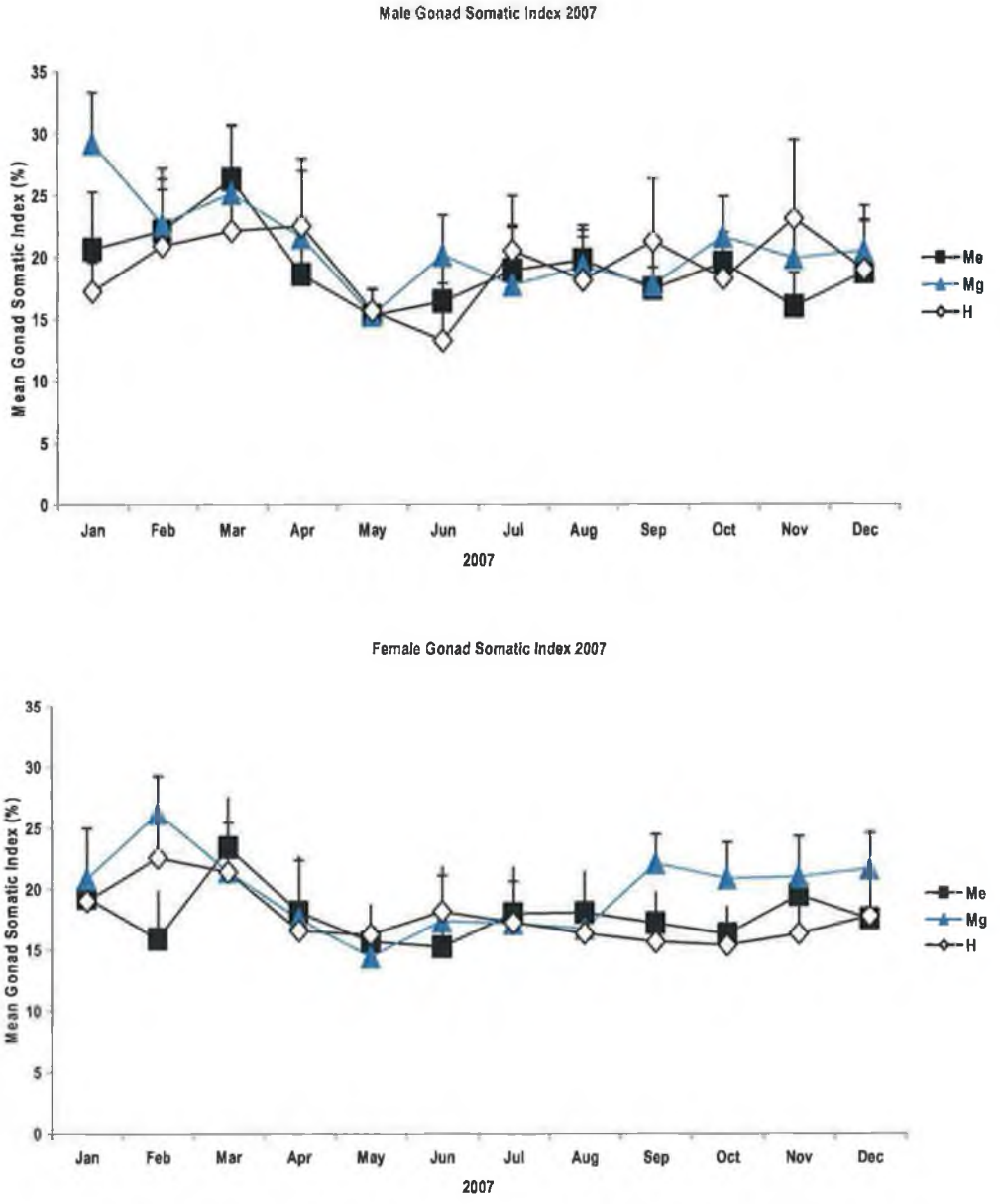

Fig. 3.2 *Mytilus* spp. Gonadosomatic index (± 95% CI) for male (MGSI) and female (FGSI) *M. edulis* (Me), *M. galloprovincialis* (Mg) and hybrids (H) during 2007. N ≈ 90/month.

Samples containing spent individuals were excluded and the analysis was repeated in order to compare GSI during development and before the release of gametes and to determine if reproductive investment varied between the taxa. There was no interaction between sample date and genotype in either sex. In females, there was a significant difference in GSI between the taxa (Table 3.2). *M. galloprovincialis* had a higher mean GSI than hybrids ($P<0.01$) but not *M. edulis* (Tukey's pairwise comparisons; see Table 3.3). In males, there were no significant differences in GSI between the taxa.

Table 3.2. ANOVA testing variation in male and female gonadosomatic index between stages 1, 2 and 3 in *M. edulis, M. galloprovincialis* and hybrids. No spent individuals were included in the analysis. (Adj MS = adjusted mean squares)

Sex	Source	*df*	Adj MS	*F*	*P*
Females	Date	6	96.57	2.20	0.04
	Taxon	2	294.04	6.71	0.002
	Date*Taxon	12	66.90	1.58	0.10
	Error	201	43.81		
Males	Date	6	6.75	0.16	0.99
	Taxon	2	15.43	0.35	0.70
	Date*Taxon	12	60.63	1.44	0.15
	Error	159	43.49		

Table 3.3. Pairwise comparisons of individual means FGSI of *M. edulis*, *M. galloprovincialis* and hybrids by Tukey's method.

		M. galloprovincialis	Hybrids
M. edulis	Diff of means	3.45	0.19
	SE of diff	1.12	1.12
	T - value	3.81	0.17
	P	0.0065	0.98
M. galloprovincialis	Diff of means		3.64
	SE of diff		1.12
	T-value		3.25
	P		0.04

3.4 Gamete volume fraction

Gamete volume fraction, the volume of the mantle tissue composed of gametes, was analysed separately for males (MGVF) and females (FGVF).

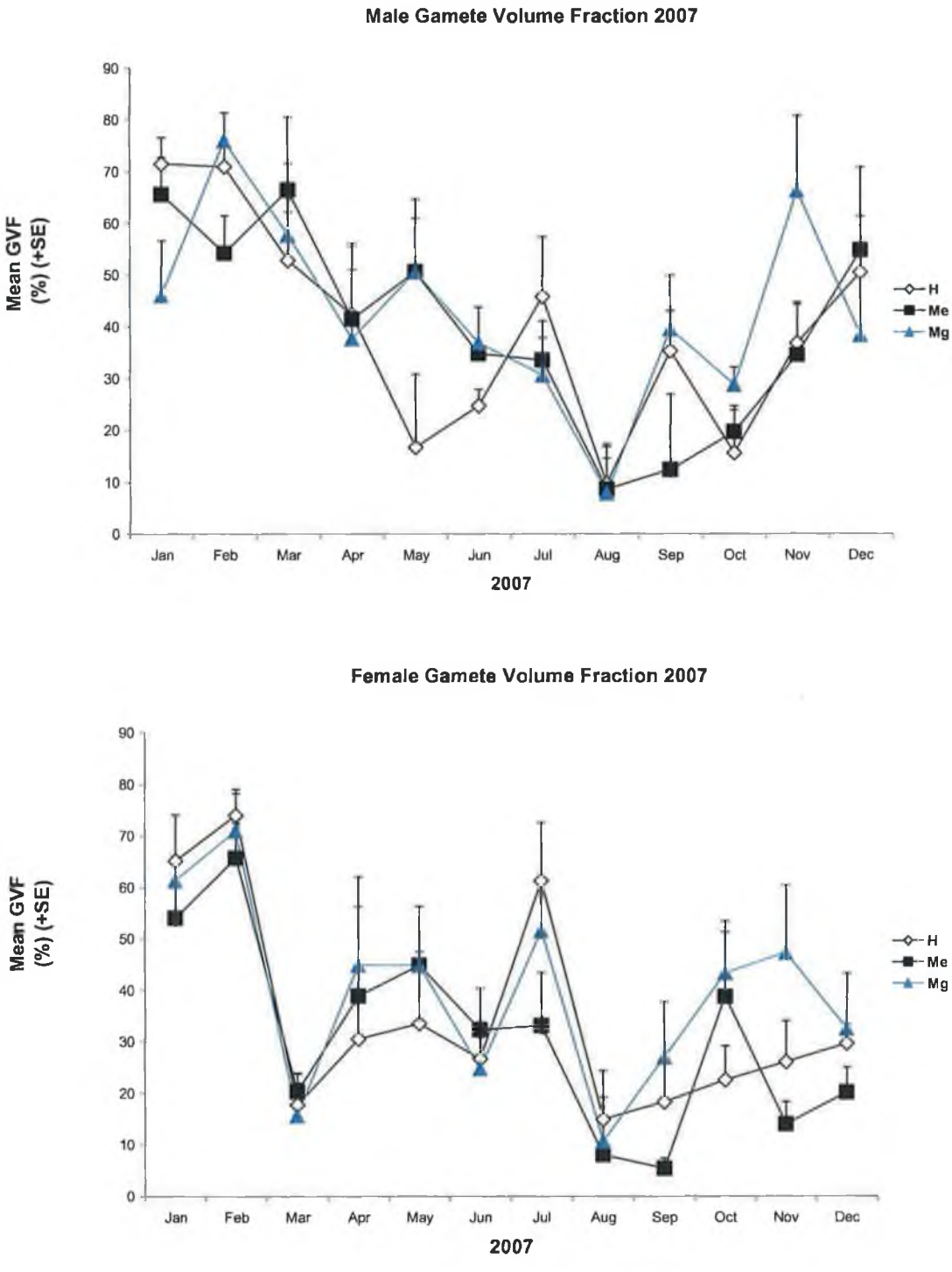

Fig. 3.3 *Mytilus* spp. Gamete volume fraction (± 95% CI) for male (MGVF) and female (FGVF) *M. edulis, M. galloprovincialis* and hybrids during 2007. N ≈ 90/month.

ANOVA (Table 3.4) showed that both FGVF and MGVF did not differ among the taxa, although in males the date* taxon interaction was significant. This result shows that the rate of change in MGVF is not consistent across the taxa. Posthoc tests with Bonferoni correction revealed this interaction was caused by the fact that peak MGVF values for hybrids (Jan > May, Aug and Sept, P > 0.05) and *M. edulis* (Jan > Aug and Sept, P < 0.05) were recorded in January whereas peak MGVF values for *M. galloprovincialis* (Feb > May, Aug and Oct, P > 0.05) were recorded in February. Also the lowest MGVF values were recorded for hybrids in May and in August for *M. edulis* and *M. galloprovincialis* (Fig 3.3).

Table 3.4. ANOVA testing variation in male and female gamete volume fraction (GVF) among *M. edulis, M. galloprovincialis* and hybrids

Sex	Source	*df*	Adj MS	*F*	*P*
Females	Date	11	4487.70	10.54	<0.001
	Taxon	2	1083.00	2.54	0.08
	Date*Taxon	22	386.00	0.91	0.59
	Error	143	425.60		
Males	Date	11	4260.10	9.08	<0.001
	Taxon	2	814.10	1.73	0.18
	Date*Taxon	22	1039.50	2.22	0.003
	Error	144	469.50		

Again, samples containing spent individuals were excluded and the analysis was repeated in order to compare reproductive investment across the taxa. ANOVA

revealed a significant difference in FGVF similar to that observed for FGSI, a significant date*taxon interaction was again observed for MGVF (Table 3.5). Post hoc tests showed that *M. galloprovincialis* had a higher mean FGVF than hybrids but not *M. edulis* (Table 3.6).

Table 3.5. ANOVA testing variation in male and female gamete volume fraction between stages 1, 2 and 3 in *M. edulis, M. galloprovincialis* and hybrids. No spent individuals were included in the analysis.

Sex	Source	*df*	Adj MS	*F*	*P*
Females	Date	6	6711.60	22.23	<0.001
	Taxon	2	1263.10	5.38	0.006
	Date*Taxon	12	250.80	0.83	0.62
	Error	84	301.90		
Males	Date	5	4460.70	9.62	<0.001
	Taxon	2	855.40	1.85	0.17
	Date*Taxon	10	914.40	1.97	0.049
	Error		463.50		

Table 3.6. Pairwise comparisons of individual means FGVF of *M. edulis, M. galloprovincialis* and hybrids by Tukey's method.

		M. galloprovincialis	Hybrids
M. edulis	Diff of means	7.57	-6.02
	SE of diff	4.11	4.11
	T-value	1.84	-1.46
	P	0.16	0.31
M. galloprovincialis	Diff of means		13.59
	SE of diff		4.11
	T-value		3.31
	P		0.04

3.5 Reproductive output

ANOVA of reproductive output (RO) revealed no significant interaction between date and taxon for either sex indicating that the rate of change in gamete weight was consistent across the taxa. No significant differences ($P > 0.05$) were observed between taxa when individuals from all sampling dates were analysed together.

However, when spent individuals were omitted a significant difference in female, but not male RO was observed (Table 3.7). Comparison of means revealed that *M. galloprovincialis* had the highest reproductive output of the three taxa (Table 3.8).

Table 3.7. ANOVA testing variation in male and female reproductive output (RO) among stage 1, 2 and 3 *M. edulis, M. galloprovincialis* and hybrids. No spent individuals were included in the analysis.

Sex	Source	*df*	Adj MS	*F*	*P*
Females	Date	6	14.92	5.57	<0.001
	Taxon	2	12.38	4.62	0.01
	Date*Taxon	12	3.20	1.23	0.28
	Error	96	2.68		
Males	Date	5	25.84	4.41	0.001
	Taxon	2	3.00	0.51	0.60
	Date*Taxon	10	6.06	1.03	0.43
	Error	72	5.86		

Table 3.8. Pairwise comparisons of individual means FRO of *M. edulis, M. galloprovincialis* and hybrids by Tukey's method.

		M. galloprovincialis	Hybrids
M. edulis	Diff of means	1.04	-0.06
	SE of diff	0.41	0.41
	T-value	2.57	-0.14
	P	0.031	0.99
M. galloprovincialis	Diff of means		0.99
	SE of diff		0.41
	T-value		2.43
	P		0.04

3.6 Oocyte area

As *M. galloprovincialis* had the highest FGSI, FGVF and FRO of the taxa a comparison of oocyte diameter by ANOVA (Table 3.9) was carried out to determine if this was due to the presence of bigger oocytes or if *M. galloprovincialis* produces more oocytes than the other taxa. Analysis revealed that oocyte size did not differ significantly between any of the taxa. This result indicates that *M. galloprovincialis* females produce more oocytes than either *M. edulis* or hybrids, giving *M. galloprovincialis* a reproductive advantage by means of higher fecundity.

Table 3.9. ANOVA testing variation in oocyte diameter among *M. edulis, M. galloprovincialis* and hybrids.

Source	*df*	Adj MS	*F*	*P*
Taxon	2	3.70×10^{19}	0.68	0.52
Error	12	5.43×10^{19}		

4. Discussion

The seasonality and duration of the reproductive cycle in *Mytilus* varies both spatially and temporally and has been shown to be directly linked to exogenous factors (Wilson and Seed 1974; Lowe et al. 1982). The results of the present study show that mussels at Ballynahown have broadly overlapping reproductive cycles, with gametogenesis taking place over the entire year. Two major spawning events took place, one from March to May and the second from July until October. It is not possible to generalise about the timing of spawning as these results are based on data from a single year. However, these findings are in agreement with previous studies. For example, two major spawning events have been observed in mussel populations on the west coast of Ireland: the first in spring and the second in summer, with gametogenesis occurring throughout most of the year (King et al. 1989: Coghlan and Gosling 2007). On an exposed shore situated ~ 900 m from the present study site, Coghlan and Gosling (2007) reported continuous settlement over a six-month period from May-October, with peak numbers settling in mid-July and late October, which coincides with the two main spawning events observed in this study. On the east coast of Ireland populations were shown to spawn simultaneously in late spring over three consecutive years, with spawning lasting 2-5 months (Wilson and Seed 1974). However, there was also inter-annual variation in the timing, with spawning taking place into winter months in some years (Seed and Brown 1975).

The spawning cycles of the three taxa were largely overlapping. Any evidence of spawning activity in one taxon coincided with evidence of spawning activity in at least one of the other taxa. The possibility that single-banded genotypic classes may in fact contain F_2 backcross individuals can not be ruled out. If some of the individuals

identified as pure *M. edulis* and *M. galloprovincialis* are actually backcrossed hybrids this could increase the apparent overlap in spawning times.

Small differences were observed in the timing of peak spawning in the three taxa. *M. galloprovincialis* released gametes from March-June, with peak spawning taking place in March. *M. edulis* also spawned from March-June but peak spawning occurred over the May-June period. The reduction in numbers of hybrid gametes was more gradual over the March-June period, suggesting a continuous gradual release of gametes during this time. A second spawning event took place from September to November in the three taxa. During this time, peak spawning was observed first in *M. edulis* in October. No evidence of a major release of gametes was observed in *M. galloprovincialis* until November. Hybrids showed some release of gametes in October but spawning peaked in November. Regardless of whether or not this overlap in spawning is consistent from year to year, it is clear that reproductive cycles of the taxa can overlap thus facilitating gene flow. A previous investigation of gene frequencies in settling spat at a nearby exposed shore found no evidence to suggest that *M. edulis* and *M. galloprovincialis* settle at different times in Galway Bay (Coghlan and Gosling, 2007). Therefore, the small differences in the timing of peak spawning observed in the current study are not reflected in different settlement patterns in the three taxa.

Although the observed overlap in spawning activity allows for cross fertilisation of gametes among the taxa, small differences in the timing of gametogenesis may increase the likelihood that oocytes are fertilised by conspecific sperm thus preventing the complete introgression of taxa. However, the influence of size on the timing of spawning must also be considered. Differences in spawning times among size classes of the same genotype have been reported in the SW England

hybrid zone (Gardner and Skibinski 1990). The current study has only included individuals of the 40-45mm size class. Taxon related differences in the timing of peak spawning in this size class may be counteracted by variation in spawning activity across size classes. Therefore, it is unlikely that the small differences in the spawning cycles of the taxa present significant barriers to gene flow.

In SW England where pure populations occur they show asynchronous spawning (Gilg et al. 2007). This may occur because allopatric populations of the two taxa experience different temperature and food regimes causing variation in the timing of gametogenesis and spawning. However, in sympatric populations spawning asynchrony has also been observed, which cannot be attributed to environmental differences (Gardner and Skibinski 1990; Secor et al. 2001). The overlapping spawning cycles observed in the current study may indicate that in this region of the hybrid zone the taxa are more highly introgressed than in SW England.

Timing of spawning also varies within the *M. edulis-M. trossulus* hybrid zone in the NW Atlantic. In a similar study to this carried out in Newfoundland the three taxa were found to have asynchronous spawning cycles and differential fecundity (Toro et al. 2002). The coexistence of these two species was later attributed to life-history differences associated with age-specific reproduction and mortality which are preventing complete introgression (Toro et al. 2004). Conversely, Maloy et al. (2003) have found no differences in the timing of gametogenesis and/or spawning between the species in Cobscook Bay, Maine.

In the months prior to spawning, *M. galloprovincialis* had a higher mean FGSI and FGVF than hybrids, but not *M. edulis*. Also, *M. galloprovincialis* females had a significantly greater dry weight of gametes, as measured by reproductive output, than the other two taxa. Oocyte size did not differ between the taxa, showing that the

observed difference in RO is due to the higher fecundity of *M. galloprovincialis*. The release of higher numbers of *M. galloprovincialis* oocytes should increase the fertilisation rate by sperm of all three taxa during periods of synchronous spawning with *M. edulis* and hybrids. The observed difference in fecundity is small (~35%) and so is probably offset by higher numbers (up to 54% at this site) of hybrid adults releasing gametes. The difference in fecundity between the taxa at this site is notably much less pronounced than that observed in SW England where fecundity in *M. galloprovincialis* exceeded the fecundity of the other taxa by a factor of 2.2-2.8 (Gardner and Skibinski 1990).

Barton and Hewitt (1989) have reported that in order for gene flow to be significantly reduced over much of the genome hybrids must be substantially less fit than parent species. Several features of the Ballynahown population refute the hypothesis of depressed hybrid fitness. Firstly, there is a high percentage (up to 54%) of hybrid genotypes in the adult population, which probably occurs as a result of the continuous spawning by hybrids during peak spawning periods of both parental species. Secondly, hybrids are fertile with the potential to backcross to parent species, also contributing to the high frequency of the hybrid genotype. Finally, sex ratios do not deviate from the expected 1:1 ratio. Deviations are observed when increased mortality in hybrid males, caused by nuclear-cytoplasmic incompatibility, shifts the sex ratio towards a female bias (Toro et al. 2002). These features highlight not just an absence of selective pressure against hybrid adults, but that ecological factors in this area of the zone actually favour hybrids. Therefore, any selection acting against the complete introgression of the parental species must be taking place either pre-zygotically through e.g., gamete incompatibility or assortative fertilisation, or post-zygotically via selection against hybrids in the early life history stages affecting larval

mortality or recruitment success. Springer and Crespi (2007) have reported divergence of the gamete recognition protein lysin-M7 in *M. galloprovincialis* in sympatric populations in the east Atlantic, which they suggest was driven by selection pressures following secondary Pleistocene contact between *M. edulis* and *M. galloprovincialis.* This divergence in gamete recognition proteins could reduce the rate of cross fertilisation due to gamete incompatibility. Bierne et al. (2002) in a study of laboratory crosses of *M. edulis* and *M. galloprovincialis,* found that assortative fertilisation occurred when gametes from both taxa were mixed that may be due to differences in gamete recognition systems. They also noted a significant reduction in hybrid viability during the larval stage, which was offset by hybrid heterosis in terms of growth rate. Beaumont et al. (2004) observed the opposite; they found that laboratory hybrid larvae grew slower than *M. edulis* or *M. galloprovincialis* with the latter having the fastest growth rate of the three. Although spawning and growth of laboratory larvae does not necessarily reflect what is happening in the wild, nevertheless, they have provided direct and valuable evidence of gamete compatibility between these species whether or not this is offset by some other form of selection.

In conclusion, the timing of spawning does not provide a mechanism for reproductive isolation of the three taxa, at this site at least, as periods of non-overlap are short. Large overlap in the spawning cycles of the three taxa provides ample opportunity for introgressive gene flow. Nonetheless, small differences in the timing of peak spawning may increase the likelihood of conspecific fertilisation at certain times of the year. The apparent advantage of higher fecundity in *M. galloprovincialis* is clearly being offset by some condition that favours hybrids. There seems to be no advantage for *M. edulis* at this site as it is the least frequent genotype. This supports the findings of Gosling et al. (2008) who reported low frequencies of *M. edulis* on

Irish Atlantic coasts. It is likely that mussels at this site are highly introgressed since heterozygotes outnumber each parental species and there does not appear to be any barriers to fertilisation among the taxa. Further work employing additional molecular markers is needed to investigate the extent of introgression in these mussels.

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6. Appendices

Table 6.1. PCR master mix for *Me15/16*

Chemicals	x 1 (µl)	x 40 (µl)
Distilled, sterile water	3.1	124
10x Buffer*	1.0	40
$MgCl_2$* (2.5 mM)	1.5	60
dNTPs* (0.6 mM)	2.0	80
Forward Oligo Primer$ (6 mM)	1.0	40
Reverse Oligo Primer$ (6 mM)	1.0	40
RedTaq™Polymerase*(1ul = unit)	0.6	24
Total	10.2	408

*Chemical produced by Sigma

$ MWG Biotech AG

Table 6.2. Thermocycler incubator files.

Incubator files	Temperature (°C)	Time
Time delay file (Denaturing)	95	4 min
Step cycle file (Denaturing)	94	45 s
(Annealing)	56	30 s
(Primer extension)	70	90 s
Final extension time delay file	72	6 min 40s
Soak file	-1	~

Table 6.3. Agarose gel electrophoresis
10x Tris-borate EDTA (TBE) electrophoresis and gel buffer, pH 8.3

Trizma base	108 g
Boric acid	55 g
EDTA, disodium salt	9.5 g
Distilled H2O	750 ml

Table 6.4.

Haematoxylin and eosin histological staining protocol for the Leica ST4040 Linear

Station no.	Stain
1	Neo-clear
2	Neo-clear
3	Neo-clear
4	Neo-clear
5	100% Alcohol
6	100% Alcohol
7	90% Alcohol
8	75% Alcohol
9	Running water
10	Distilled water
11	Haemalum
12	Haemalum
13	Running water
14	Running water
15	25% HCL in water
16	Running water
17	75% Alcohol
18	Eosin
19	Eosin
20	90% Alcohol
21	90% Alcohol
22	100% Alcohol
23	100% Alcohol
24	100% Alcohol
25	Neo-clear
26	Neo-clear
27	Neo-clear

Immersion time: 1 minute 10 seconds
Draining time: 5 seconds

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