Aspects of the biology of *Mya arenaria* and *Ensis* spp. (Mollusca; Bivalvia) in the Irish Sea and adjacent areas

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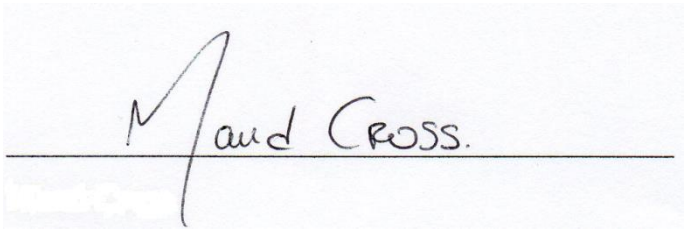
Declaration

This thesis is the work of Maud Cross only, and has not been submitted for another degree, either at University College, Cork, or elsewhere.



Maud Cross.

Abstract

This study was undertaken to investigate the general biology, including the reproductive cycle and health status, of two clam taxa in Irish waters, with particular reference to the Irish Sea area. Monthly samples of the soft shell clam, *Mya arenaria*, were collected from Bannow Bay, Co. Wexford, Ireland, for sixteen months, and of the razor clam, *Ensis* spp. from the Skerries region (Irish Sea) between June 2010 and September 2011. In 2010, *M. arenaria* in Bannow Bay matured over the summer months, with both sexes either ripe or spawning by August. The gonads of both sexes of *E. siliqua* developed over autumn and winter 2010, with the first spawning individuals being recorded in January 2011. Two unusually cold winters, followed by a warmer than average spring, appear to have affected *M. arenaria* and *E. siliqua* gametogenesis at these sites. It was noted that wet weight of *E. siliqua* dropped significantly in the summer of both 2010 and 2011, after spawning, which may impact on the economic viability of fishing during this period. Additional samples of *M. arenaria* were collected at Flaxfort (Ireland), and *Ensis* spp. at Oxwich (Wales), and the pathology of all clams was examined using both histological and molecular methods. No pathogenic conditions were observed in *M. arenaria* while Prokaryote inclusions, trematode parasites, *Nematopsis* spp. and inflammatory pathologies were observed at low incidences in razor clams from Ireland but not from Wales; the first time these conditions have been reported in *Ensis* spp. in northern European waters. *Mya arenaria* from sites in Europe and eastern and western North America were investigated for genetic variation using both mitochondrial (cytochrome oxidase I (*CO*I) and 16S ribosomal RNA genes) and nuclear markers (10 microsatellite loci). Both mitochondrial *CO*1 and all nuclear markers showed reduced levels of variation in certain European samples, with significant differences in haplotype and allelic composition between most samples, particularly those from the two different continents, but with the same common haplotypes or alleles throughout the range. The appearance of certain unique rare haplotypes and microsatellite alleles in the European samples suggest a complicated origin involving North American colonization but also possible southern European Pleistocene refugia. Specimens of *Ensis* spp. were obtained from five coastal areas around Ireland and Wales and species-specific PCR primers were used to amplify the internal transcribed spacer region 1 (ITS1) and the mitochondrial DNA *CO*1 gene

and all but 15 razor clams were identified as *Ensis siliqua*. Future investigations should focus on continued monitoring of reproductive biology and pathology of the two clam taxa (in particular, to assess the influence of environmental change), and on genetics of southern European *M. arenaria* and sequencing the CO1 gene in *Ensis* individuals to clarify species identity

Chapter 1. Introduction

The economic and ecological values of fishing

The waters around Ireland are some of the most productive fishing grounds in the world (Marine Institute, 2006). Ireland currently produces almost half of the UK and Ireland's shellfish with total shellfish exports from Ireland increasing each year (BIM, 2006). Between 2000 and 2005 the overall value of the Irish seafood market increased by 14%, the home market by 23% and exports by 7%, while seafood exports increased from €331 million to €354 million (BIM, 2006). More recently total shellfish exports from Ireland increased in value by 18% to €135.5m, from 2009 to 2010, and by another 14% to €378 million at the end of 2010 (BIM, 2011a). The OECD / FAO 2010-2011 outlook forecasts positive long term growth in the global seafood market, as demands for healthy proteins rise (BIM, 2011b). In order to meet these demands, worldwide aquaculture production is increasing rapidly, with aquaculture volumes in Ireland predicted to grow at the rate of 2.8% per annum, while natural beds of shellfish are under pressure (Darriba *et al*., 2005; Hauton *et al*., 2007; BIM, 2011a).

The future commercial exploitation of shellfish species will need careful management, to preserve natural populations, avoid depletion of wild stocks, and to develop long-term sustainable fishing and aquaculture practices (Arias *et al*., 2011). To manage an exploited species, knowledge of the reproductive cycle of the species is essential, as it provides valuable data for recruitment, age and growth studies, leading to a greater understanding of the species life history in a specific area, and the effects of environmental irregularities on these characteristics (Morsan and Kroeck, 2005). Data on the extent of molecular genetic structure and genetic variability in different populations of shellfish species throughout their geographical range is also crucial to identify and preserve natural populations, and to develop sustainable aquaculture programmes (Arias *et al*., 2011).

Ireland's marine resource consists of 900,000 km^2 of seabed (O'Connor *et al*., 2005). While important for economical reasons, the ecological impacts of fishing also need to be considered in this zone, some parts of which have been established as "Biologically Sensitive Areas (BSAs)" (Marine Institute, 2006). Many northeast Atlantic continental-shelf species reach their southern or northern limits around the coast of Ireland, making it well placed for the study of the reproductive limits of

marine species, taking into consideration reduced biodiversity due to isolation from Britain and continental Europe, past influence of glacial periods, and the potential effect of future climate variability (Hiscock *et al*., 2004; Reise *et* al., 2006). Both the English Channel and St. George's Channel are significant barriers to extension of distribution from Britain to Ireland, and the Irish Sea itself may prove too great a distance for the survival of larvae of some benthic animals (Crisp & Southward, 1958; Hiscock *et al.*, 2004).

Over-fishing natural resources of the marine environment is increasingly becoming a problem around the world. Past incidences include the exhaustion of cod populations in the Eastern North Atlantic (Baird *et al*., 1991), the depletion of razor clam numbers in a few short years in the Irish Sea (South Wales Sea Fisheries Committee, 1999; Marine Institute, 2010; Fahy, 2012) and the Iberian Peninsula (Darriba *et al*., 2005), and scallop numbers around the Isle of Man (Brand *et al*., 1991; Hill *et* al., 1999; Beukers-Stewart *et al*., 2003), where the latter fishery is now dependent on the strength of the recruiting year-class each season. This situation leaves the scallop fisheries in a precarious balance each year, reliant upon maximum recruitment, growth and survival of each cohort. Across Europe, concern is growing with regards to weakening of certain bivalve stocks, due to the declining numbers of breeding adult specimens, the deteriorating genetic variation involved in over-fishing of viable populations, and of collateral environmental damage caused by hydraulic dredge fisheries for bivalves, such as impacts on the by-catch and seabed structure (Linnane *et al.*, 2000; BIM, 2011b; Hauton *et al*., 2007).

The effects of trawls on the seabed, amplified by the advance in technological developments of trawling gears and the expanding number of fishing vessels, are increasingly gaining international public and political importance (Linnane *et al*., 2000). Clear changes as a result of extreme physical disturbance, including scraping, penetration, and pressure have become apparent in heavily dredged areas (Hill *et al.,* 1999). These include sediment suspension, habitat destruction, burying, mortality in benthos, exposure to predators, removal of non-target invertebrate benthic species, organic matter translocation, increased polychaete to mollusc ratios, loss of some fragile species, and an increase in the predominance of scavenger/predatory species (Mayer *et al*., 1991; Linnane *et al*., 2000).

An emerging problem is that of climate variability and its effect on marine organisms. Past work has indicated that annual seawater temperatures may rise in European, including Irish, waters in the future (Hiscock *et al.*, 2004; Christensen *et al*., 2007). In the Celtic/Biscay shelf, sea water temperatures are predicted to increase between 1.5 and 5°C over the next 100 years (Philipart *et al*., 2011), with global temperature hypothesised to increase between 1.8 and 4°C by the end of the 21st century (Matozzo and Marin, 2011). Inshore sea temperatures are thought to have begun showing significant short-term variations already, with a trend towards higher temperatures, and more variable conditions than in the past (Hiscock *et al*., 2004).

The possibility of changing sea and air temperatures, increased precipitation, more extreme weather events, ocean acidification and rising sea levels are additional pressures to the ones already experienced by marine fish and shellfish stocks, such as loss of habitat, pollution, over fishing, disturbance and introduced species (Brander, 2010; Ni Longphuirt, *et al.,* 2010). Within the next century, climate change has the potential to impact marine organisms in Ireland and the UK (Callaway *et al.,* 2012). Changes in rainfall patterns, increasing ocean acidification, and a greater frequency of extreme weather, could influence the stress and health status of bivalve species in the Irish Sea, and influence the success of predatory and competitor species, either native or introduced (Callaway *et al.,* 2012). As the biology of marine bivalves is influenced by both endogenous and exogenous factors, among which temperature is one of the most important, the warming of sea- and air-surface temperatures being predicted to cause changes in the distribution and abundance of marine bivalve species (Gaspar & Monteiro, 1999; Darriba *et al.,* 2005; Joaquim *et al.,* 2008; Moura *et al.,* 2008; Kröncke *et al.,* 2011). Higher air temperatures would be expected to speed growth and increase fecundity in southern species but would stress cold-water species (Genner *et al.,* 2004; Hiscock *et al*., 2004). The effects of ocean acidification are of particular concern for calcifying organisms, such as marine molluscs, as the lowering of calcium carbonate saturations states impacts shell-forming marine organisms (Doney *et al.,* 2009; Cheung *et al.,* 2012).

The increase in the occurrence of Harmful Algal Blooms (HABs) is believed to be related to changing climate temperatures and the accelerated eutrophication of coastal waters from human activities (Anderson *et al.,* 2002; Heil *et al.,* 2005). Past blooms have resulted in prolonged closures in many Irish sites, with some sites being

closed for several months, resulting in economic losses for producers and processors, and major mortalities of benthic and pelagic marine organisms (Marine Institute, Bord Iascaigh Mhara & Taighde Mara Teo 2006; Ottoway *et al.,* 1979; Silke *et al.,* 2005). Tanker oil spills such as those from the Sea Empress in Wales (1996), the Aegean Sea in west Spain (1992), and the Prestige in Galicia (2002) and polluting chemicals and particles from industrial, agricultural and municipal waste, into riverine, transitional and marine waters have also been demonstrated to place enormous pressure on the marine environment, causing serious negative impacts on the structure and functioning of ecosystems, and affecting both wild and cultured stocks (Grainger *et al.,* 1984; Mc Govern *et al.,* 2011; Roose *et al.,* 2011).

Bivalves and their fishery around Ireland

The Irish coastline is close to 7,500km long and is an extremely productive fishing region (O'Connor *et al.,* 2005; Marine Institute, 2006). Exports of all molluscan species increased in volume by 10% from 2009 to 2010, by another 15% from May 2010 to May 2011, and continue to increase (BIM, 2011a). Six species of wild bivalve shellfish are currently commercially fished in Ireland (Shellfish Stocks and Fisheries Review, 2011), while numerous molluscs are hand caught for personal consumption and use as bait. Of those commercially fished the native scallop, *Pecten maximus* (Linnaeus, 1758), is presently of highest value at €5.90 per individual (Shellfish Stocks and Fisheries Review, 2011). Scallop fishing extends back to at least the 16th century in Ireland. The commercial fishery of wild scallops began in inshore waters off the southeast coast, in the 1970s and gradually expanded offshore and into the south Irish Sea. Irish vessels currently fish for scallops in both inshore and offshore waters off the southeast coast of Ireland, in the Irish Sea and English Channel (Tully *et al.,* 2006; Marine Institute, Bord Iascaigh Mhara & Údarás na Gaeltachta, 2008).

Aquaculture of mussel and oyster species commenced in Ireland when native stocks produced insufficient numbers for demand, for both national and overseas markets. Due to volume, the most valuable species produced are the mussels *Mytilus edulis* (Linnaeus, 1758) and *Mytilus galloprovincialis* (Lamarck, 1819), which are commercially fished all around Ireland (though *M. galloprovincialis* is not present on the east coast). Seed is relaid on bottom plots in shallow regions, and raft and long-line systems are used in the deeper waters of the west coast (Maguire *et al.,*

2007; Marine Institute, Bord Iascaigh Mhara & Údarás na Gaeltachta, 2008). Populations of the native oyster *Ostrea edulis* (Linnaeus, 1758) have declined significantly throughout Europe since the 1970s (Tully & Clarke, 2012). Events such as over-fishing, colder than average winters, habitat loss and infection by *Bonamia ostreae* have had significant deleterious effects on Irish stocks of this species (McArdle *et al.*, 1991; Kennedy & Roberts, 1999; Laing *et al.*, 2006; Smyth *et al.*, 2009), while aquaculture of the introduced Pacific oyster, *Crassostrea gigas* (Thunberg, 1793), has been affected by summer mortality, thought to be due mainly to the herpes virus OSHV-I and variants (Malham *et al.*, 2009). For the cockle species *Cerastoderma edule*, landing fluctuates due to recruitment problems related to surfacing events (West *et al.*, 1979; Marine Institute, 2010).

Two clam species are commercially fished in Ireland; the surf clam, *Spisula solida* (Linnaeus, 1758), and the razor clam, *Ensis siliqua* (Linnaeus, 1758). The Manila clam is indigenous to the Indo-Pacific region and was introduced to Ireland in 1982 for aquaculture purposes (Drummond *et al.*, 2006). Razor clams are currently fished on the east coast of Ireland (Marine Institute, Bord Iascaigh Mhara & Údarás na Gaeltachta, 2008). While not presently commercially fished in Irish or European waters, the soft shell clam, *Mya arenaria*, also has potential to be of great economic value in Ireland, as has occurred on the east coasts of the United States and Canada (Beal, 2002; Connell *et al.*, 2007; Weston & Buttner, 2010a).

Mya arenaria

The soft sediment bivalve *Mya arenaria* (Linnaeus, 1758) occurs over a wide geographical range, and often plays a dominating role in benthic communities (Strasser, 1999). The soft shell clam is a euryhaline osmoconforming bivalve which feeds by filtering seawater (Newell & Hidu, 1986). The diet consists of a variety of flagellates, blue-green algae, pelagic and benthic diatoms, and chlorococcales (Kamermans, 1994; Strasser, 1999). *Mya arenaria* shows high niche overlap with other common bivalves such as *Cerastoderma edule*, *Macoma balthica* and *Scrobicularia plana*, all relying on the same food sources, and preyed upon by the same set of predators in their early life stages (Reise, 1985; Kamermans, 1994; Strasser, 1999). *Mya arenaria* is rarely collected for food or bait in European waters,

but it is an ecologically important food source for some fish, crustacean and bird species (Zwarts and Wanink 1989; Strasser, 1999).

Mya arenaria is a commercially important bivalve for fisheries and aquaculture in North America, where it is predominantly fished by hand (Beal, 2002; Weston & Buttner, 2010a). This species has been harvested commercially year–round since the mid-1800s (Wallace, 1997). Annual U.S. commercial landings of softshell clams from 1977 to 1981 averaged 4.2 million kg, worth $15 million, while landing values of $198 million were generated in 1984 (Newell & Hidu, 1986; Hidu & Newell, 1989). In the following decade however, soft shell clam landings steadily declined (Beal, 1994), most likely due to over-fishing, decreasing water quality and an increase in disease (Brousseau & Baglivo, 1991; Farley *et al.*, 1991). The value of soft shell clams has increased again in the last decade. In 2010 the average total weight of landed *M. arenaria* in Canada was over 300 thousand kg, at a value of $564,000, while total landings of this species in 2011 in the U.S. were estimated at 2 million kg, worth over $21m (NOAA, 2012).

Commercial harvesting of *M. arenaria* in Atlantic North America occurs from the Quebec North Shore and Prince Edward Island, Canada, to the Chesapeake Bay, Maryland, USA (Weston & Buttner, 2010a) while aquaculture of soft shell clams in this area is mainly undertaken north of New York. Private commercial and public aquaculture initiatives currently exist in New England, particularly Maine and Massachusetts, and in Quebec and New Brunswick, Canada (Beal, 1988; Buttner *et al.*, 2008; Weston & Buttner, 2010b). Selected coastal flats, harvested by commercial shellfishers, are targeted for restoration and enhancement of wild populations of soft shell clams in public aquaculture ventures, and private aquaculture involves the acquisition and maintenance of coastal leases, usually two acres in size (Weston & Buttner, 2010a). *Mya arenaria* juveniles can be produced in large numbers using standard hatchery techniques (Buttner *et al.*, 2008) and purchased to stock these flats and leases (Weston & Buttner, 2010a).

The culture of *M. arenaria* involves the collection of broodstock clams from local flats, which are kept in cages, socks, bands or sediment, to simulate their natural environment, and then conditioned to spawn (Buttner & Weston, 2010). The resulting larval clams are reared in a series of containers in continuous flow or static systems until they are large enough to be moved to local areas (Flimlin & Beal,

1993; Buttner & Weston, 2010; Weston & Buttner, 2010b). The most effective way to reduce shellfish losses in the field is to exclude predators from the bivalves, using a variety of materials including plastic or nylon screen flexible netting, the Predator Exclusion Device (PED), clam tents and physical removal of predator species (Flimlin & Beal, 1993; Leavitt, 1998; Leavitt & Burt, 2007; Buttner *et al.*, 2008). In eastern North American waters, predators of soft shell clams can vary with the clams size, and include the green crab, *Carcinus maenas,* the mud crabs *Dsypanopeus sayi* and *Panopeus herbstii*, carnivorous gastropod- whelks in the genus *Busycotypus*, moon snails *Neverita duplicatus* and *Euspira heros,* echinoderms such as the starfish species *Asterias forbesi* and *Asterias vulgaris*, the milky ribbon worm, *Cerebratulus lacteus*, and vertebrate predators such as skates and rays, e.g. the cownose ray, *Rhinoptera bonascus* (Flimlin & Beal, 1993; Leavitt & Burt, 2007).

Mya arenaria is not currently fished commercially in European waters, though it may have the potential to be an economically important species in the Irish Sea in the future, as it is thought to be widespread throughout the region. If this does come to fruition, knowledge of the soft shell clam's life history, population genetics and health status in the Irish Sea would be essential to future management of the species and the establishment of sustainable fisheries. This knowledge is also invaluable when investigating the ecological status of this species, and its interaction with other soft sediment organisms.

Ensis siliqua

Thirteen species of the *Ensis* genus are currently recognised world-wide (Bouchet & Gofas, 2013). The razor clam, *Ensis siliqua*, is a suspension feeder, consuming dinoflagellates, flagellates and phytoplankton such as *Tetraselmis suecica* and *Monochrysis lutheri* species (Bamio, 2011). When cultured, this species will flourish on a diet of *Tetraselmis suecica*, *Isochrysis albana*, *Pavlova lutheri*, *Chaetoceros calcitrans*, *Phaeodactylum tricornutum* and *Skeletonema costatum* (Da Costa *et al.,* 2010). *Ensis siliqua* have been reported as living in areas also supporting the clam species *Mactra stultorum*, *Pharus legumen* and *Lutraria angustior* (Palmer, 2010). These latter two species were recorded to have replaced *E. siliqua* as the dominant species in the Gormanstown area, a razor bed in the Irish Sea, razor calm stocks had declined due to fishing (Fahy & Carroll, 2007).

Ensis siliqua is widely exploited in Europe, Spain, Portugal, The Netherlands, the United Kingdom and Ireland (Arias *et al.*, 2012; DaCosta *et al.*, 2010: Fernandez-Tajes *et al.*, 2012; Ruiz *et al.*, 2012; Varela *et al.*, 2012). A small, but lucrative, export fishery in razor clams also emerged in Scottish waters in the late 2000s (Hauton *et al.*, 2007). This species is sold in both canned and fresh forms in European countries (Fernandez-Tajes *et al.*, 2012), and imported mainly to Spain, where it is a highly appreciated seafood, and also to Italy, France, Portugal and The Netherlands (Fernandez-Tajes & Mendez, 2007; Arias-Perez *et al.*, 2012). In 1982 Spain was the principal source of *Ensis* species, reporting landings of 1500 tonnes. This had decreased to 7 tonnes by 1984. Portugal harvested the largest number of razor clams in Europe in 1988, and continued for ten years, recording landings of 2600 tonnes in 1995. In the late 1990s Ireland attained the highest yield of wild razor clams in Europe, landing more than half of the north-east Atlantic tonnage (~ 400 tonnes/year) from 1999 to 2001 (Fahy & Carroll, 2007). The demand for and economic value of razor clams has increased significantly since 2000, making them a highly valuable seafood species with the potential for commercial aquaculture in many European countries (Wootton *et al.*, 2003; DaCosta *et al.*, 2010). In 2004 alone, the import value of the razor clam market attained a quote of €550 million, and captures of around 1500 tonnes per year were reported in Europe in 2012 (Fernandez-Tajes & Mendez, 2007; Fernandez-Tajes *et al.*, 2012).

Ensis siliqua are not currently cultured in Europe, but research projects have been carried out in Spain since 2000, to investigate the biology of this species and, the procedures involved in their aquaculture (Darriba, 2006). Thus far, research has identified two major problems in the development of aquaculture systems; the low survival of *E. siliqua* during post-larval and seed cultures, and the requirement of a substrate to allow burrowing of older individuals, to prevent shell gaping and consequent shellfish stress and morbidity (DaCosta *et al.*, 2010). While experiments to determine the most suitable substrate for spat survival are on-going (DaCosta *et al.*, 2010), past research on the aquaculture of the American jacknife razor clam, *Ensis directus*, may provide insights into the conditions required for successful culture of *Ensis siliqua* in Europe (Freudendahl & Nielsen, 2005; www.seagrant.umaine.edu).

Four species of the genus *Ensis* occur in Ireland, *E. siliqua, E. arcuatus, E. minor* and *E. ensis*, with the vast bulk of landings comprising of *E. siliqua* (Fahy & Carroll, 2007). *Ensis siliqua* is widely distributed along the east coast of Ireland (Fahy, 1999). Currently, there are no commercial landings on the west coast, and the number of sustainable populations in this area is unknown. The fishery for razor clams on the east coast of Ireland commenced in 1997 from the Gormanstown razor clam bed, off the coast of Dublin (South Wales Sea Fisheries Committee, 1999; www.marine.ie, 2010). This class A area yielded more than half the razor clams landed in Europe between 1999 and 2001, and led the world in wild-caught landings of razor clams, with close to 400 tonnes landed per year during this time (Fahy & Carroll, 2007). However, landings to Ireland decreased by 43% in weight and 41% in value between 2000 and 2003, due to over-fishing of the beds, and over-supply in the market (Fahy, 2012). Landing statistics for part of 2003 recovered (from 200 to 350 tonnes per year, to exceed those of the year before, and the value of razor clam landings in 2004 in the Republic of Ireland was €1.4 million (www.marine.ie, 2010). The dominant species, and most commercially viable razor clam, *Ensis siliqua,* declined over a period of seven years, from comprising 90% to 50% of the Gormanstown landings in 2005 (Fahy & Carroll, 2007). Since the decline in landings from the Gormanstown bed, other areas have been opened to harvesting (www.marine.ie, 2010). The Skerries is an east coast area of razor clam harvesting which is currently yielding most landings of *Ensis siliqua* in the Republic of Ireland (Fahy & Carroll, 2007; Dore & Nolan, 2008).

Ensis siliqua was traditionally fished by hand or using rakes or small dredges (Varela *et al.,* 2012). These methods have been replaced by the more efficient hydraulic dredging, which has the potential to remove the majority of a clam population, leading to over-exploitation and the loss of sustainable fisheries (Gaspar & Monteiro, 1998; Tuck *et al.,* 2000; Fahy & Gaffney, 2001; Fernandez-Tajes *et al.,* 2007; Varela *et al.,* 2012). In Northern Europe, many razor clam populations are already threatened, while natural beds of *E. siliqua* in northwest Spain are in a critical condition due to over-fishing (Darriba *et al.,* 2005; Hauton *et al.,* 2007). Adding to the complexity of the issue, it is difficult to estimate the real landing values of *E. siliqua* in Europe, as the official data do not always relate to the actual amounts of razor clams fished, due to illegal catches and the misidentification of different razor clam species (Arias-Perez *et al.,* 2012). Because of the increasing

global demand for shellfish, including *Ensis siliqua*, natural beds of this species are under pressure (Darriba *et al.,* 2005; Hauton *et al.,* 2007). The future commercial exploitation of these species will need careful management to ensure sustainability and avoid fishery over-depletion.

Figure 1. The Skerries fishing region of the Irish Sea. Map of the approximate location of the Skerries fishing region in the Irish Sea, situated on the east coast of Ireland. (Adapted from google images: http://www.google.com.)

Clam biology in the Irish Sea - The gaps in our knowledge.

Because of the large global demand for shellfish, natural marine beds are under pressure (Darriba *et al.,* 2005; Hauton *et al.,* 2007) and aquaculture, the fastest growing food production industry, is viewed as a potential mechanism to meet the growing demand (Dumbauld *et al.,* 2009; Byron *et al.,* 2011; Gjedrem *et al.,* 2012). Bivalves are prime candidates for selective breeding programmes due to their high economic value, control over the complete life cycle of many species, and the potential to breed improved strains of molluscs (Gosling, 2003). Past exploitation of the oyster species *Crassostrea gigas* and *Ostrea edulis,* and the cockle species *Cerastoderma edule* has revealed the benefits and difficulties inherent in translocation, selective breeding and intensive monoculture of a shellfish species, and the economic risks of relying on one species for fishing. The soft shell clam has

been successfully exploited on the east coast of the United States, and has the potential to be commercially fished in the Irish Sea, while *Ensis siliqua* is currently fished in the Republic of Ireland. Little information is currently available on the biology, health status and population genetics of these two species in Ireland, all of which would be essential to the future exploitation and management of these bivalves.

According to Morsan and Kroesck (2005), knowledge of the biology, including the reproductive cycle of a species is a prerequisite for long-term management of a fishery as it provides valuable data for age and growth studies, and the distribution and abundance patterns of juvenile and adult populations (Hooker & Creese, 1995; Darriba *et al.,* 2004). These data are also vital in aquaculture ventures. When shellfish are translocated from other regions it is important to know their provenance and pre-translocation histories, including what pathogens occur in a particular area, as translocated species are more likely to cause damaging effects with regards to disease, since the diseases that they carry are likely to be genetically different or exotic to local taxa (Culloty *et al.,* 2004; Holmer *et al.,* 2008). Problems can also arise when translocated species flourish in a new area, at the expense of native fauna (Gosling, 2003; Rajagopal *et al.,* 2005; Mantovani *et al.,* 2006).

Knowledge of the extent to which any population is isolated from or interconnected with others is also a basic requirement for the management of populations (Arias-Perez *et al.,* 2012). To preserve natural populations of a species, while still being fished, and to allow for stock enhancement through transplanting individuals from other regions, information on the levels of genetic variation and population differentiation throughout the range of a species is a prerequisite (Beaumont & Bruford, 1999; Gosling, 2003; Fernandez-Tajes *et al.,* 2007; Arias *et al.,* 2011). Recent improvements in genetic analysis have resulted in a rapid expansion of the power of molecular markers to address these questions (Selkoe & Toonen, 2006). Two factors have contributed to the technical advancements of molecular ecology in the last two decades. One is that laboratory techniques have become streamlined and less expensive, enabling the use of large numbers of samples and many loci, and the other is improvements in computing technology which have inspired the use of intensive statistical approaches (Selkoe & Toonen, 2006).

Molecular studies, using genetic markers, can identify discreet stocks and patterns of historical and contemporary gene flow, in species displaying high dispersal capabilities, such as the planktonic larval stage of marine bivalves, which can potentially disperse over large distances (Waples, 1998; Kenchington *et al.*, 2006; Baker *et al.*, 2008). Genetic approaches also help establish the approximate timing of species divergence, provide insight into population demographic processes, the role of selection, phylogenetic relationships among species, and the series of evolutionary events leading to biogeographic patterns (Palumbi, 1996; Baker *et al.*, 2008). Molecular markers are heritable characters, in the form of polymorphic proteins (allozymes) or DNA sequences, with multiple states at each character which reflect differences in DNA sequences, and can be used as indicators of genome-wide variation, and subsequently, population and evolutionary processes (Sunnucks, 2000; Beebee & Rowe, 2008). In contrast to allozymes, the use of DNA as molecular markers is more economical and efficient, as it can be extracted from old material, is more convenient for collection and storage of samples, is generally more variable, and easy to amplify through the polymerase chain reaction (PCR) (Sunnucks, 2000; Selkoe & Toonen, 2006).

The differences in transmission between nuclear and mitochondrial DNA can reveal different aspects of population biology and history. Cells of most eukaryotes contain nuclear DNA inherited from both parents, and mitochondrial DNA (mtDNA) that is usually inherited maternally only (Sunnucks, 2000). While overall mutation rates tend to be higher for the mitochondrial rather than the nuclear genome, the mitochondrial genome does not usually recombine and may not evolve sufficiently rapidly to infer levels of contemporary gene flow (Hellberg *et al.*, 2002; Lukoschek *et al.*, 2008). However, mitochondrial DNA analysis has proved invaluable in addressing historical issues of species movement, due to its haploid nature, lack of recombination, and propensity to accumulate mutations (Avise, 2000; Gysels *et al.*, 2004; Holmer *et al.*, 2008).

Polymorphic microsatellites are widely considered more powerful for resolving population structure than mtDNA markers, particularly for recently diverged lineages or geographically proximate populations, as they have greater information content than less polymorphic markers, allowing fine-scale studies (Schlötterer, 2000; Chistiakov *et al.*, 2006; Lukoschek *et al.*, 2008; Kenchington *et al.*, 2009; Arias-Perez *et al.*, 2012). These markers have revealed the existence of

genetic structuring in species previously thought to be homogeneous with markers such as allozymes or mtDNA (Jorgensen *et al.*, 2005, Cabranes *et al.*, 2008; Diz & Presa, 2008; Was *et al.*, 2008; Arias-Perez *et al.*, 2012).

Microsatellites (also known as variable number tandem repeats - VNTR) are tandem repeats of 1–6 nucleotides found at high frequency in the nuclear genomes of most taxa (Hellberg *et al.*, 2002; Selkoe & Toonen, 2006). Microsatellite loci are often highly variable with respect to the number of these small repeats (Hellberg *et al.*, 2002), as they mutate frequently by slippage and proofreading errors during DNA replication (Selkoe & Toonen, 2006). Because alleles differ in length, they can be distinguished by high resolution gel electrophoresis, which allows rapid genotyping of many individuals at many loci for a fraction of the price of sequencing DNA (Hellberg *et al.*, 2002; Selkoe & Toonen, 2006). Microsatellite markers are widely used in the field of population genetics of marine organisms because of their high variability, relatively small size, high frequency throughout the genome and rapid detection methods (Chistiakov *et al.*, 2006; Holmer *et al.*, 2008).
However, the use of microsatellite markers can be hindered by complications such as species-specific marker isolation, unclear mutational mechanisms, hidden allelic diversity, and problems with amplification (Selkoe & Toonen, 2006). In this respect, it is more beneficial to apply a combination of markers when assessing the genetic variability of a species.

SUSFISH

The current thesis is part of an EU Interreg funded study (SUSFISH) which aims to produce guidelines for future fisheries management and policy of the shellfish industry in Ireland and the Irish Sea for the next 50-100 years, with particular reference to the potential effects of future climate change. The current thesis contributes to the SUSFISH project by investigating aspects of the reproductive biology, health status, and population biology, of two clam species found in the Irish Sea, and the possible effects of a changing climate on these species.

Aims, Strategy and Outline of the Thesis

The objective of the current thesis is to obtain a better understanding of the biology, health status and population genetics of the two clam species *Mya arenaria* and *Ensis siliqua* in the Irish Sea and surrounding areas. The following outlines how the research was undertaken to gain such an understanding.

Aim 1. To investigate the biology, including the reproductive cycle, of native populations of *Mya arenaria* and *Ensis siliqua* in the Irish Sea.

Background:

Little is known about the biology of the softshell clam in Europe, despite it being a commercial species in the United States, and so possibly having some potential future commercial value in Europe. Previous work in the United States has revealed details of the reproductive biology of this species in the West Atlantic and some areas in Europe. To date, details of the reproductive cycle of *M. arenaria* in the Irish Sea have not been recorded (Chapter 2). *Ensis siliqua* is a commercially important species in Europe, and is exploited in many countries, including Ireland, where it is sold by wet weight. Past work has outlined the gametogenic cycle of *E. siliqua* in the Iberian Peninsula. Knowledge of the reproductive cycle of this clam species in the Irish Sea will aid management and future exploitation (Chapter 3).

Aim 2. To examine the health status of *M. arenaria* and *E.* siliqua, and to identify any pathogens present, using both histological and molecular methods.

Background:

Several diseases and parasites have been documented in *M. arenaria* on the Atlantic west coast, which have been associated with the increasing mortalities of soft shell clams in that area. To date, no pathological conditions have been recorded in this species in their European range. A range of protozoans, ciliates, trematodes and neoplastic disorders have recently been identified in *Ensis siliqua* on the Spanish coast, but there has been no investigation of these conditions in razor clams further north in Europe. For both clam species, no studies have previously been carried out in the Irish Sea (Chapter 4).

Aim 3. To study the population genetics of Irish Sea soft shell clams and to determine how this Irish stock fits into their entire distributional range.

Background:

The softshell clam is currently widespread on the east and west coasts of North America and western European shores where the origin of the species, whether introduced or relict, has been debated. In support of re-introduction, either from Eastern North America, or a European refugium, previous genetic work based upon mitochondrial DNA data, has shown a reduced level of haplotype diversity in European populations compared to those from eastern North America. Therefore, the current study aims to explore the genetic variation in *Mya arenaria* from a number of European sites, using ten polymorphic microsatellite markers and sequence analysis of mitochondrial DNA (Chapter 5).

Aim 4. To identify the *Ensis* spp. sampled from different areas in the Irish Sea, using a molecular approach to determine the distribution of different species around the Irish coast.

Background:

Past work on *Ensis* species in the Irish Sea and around Ireland has morphologically identified *Ensis siliqua*, *Ensis arcuatus* and *Ensis directus* to be present. As morphological identification of these bivalves can be very difficult and there has been a number of misidentifications in the past, the more accurate method of genetic based approaches has recently been employed to resolve this matter in the Iberian Peninsula. To date, there has been no identification of razor clam species in the Irish Sea using these novel methods (Chapter 6).

Finally, Chapter 7 summarises the results of the four different aims of this thesis. The implication of these results and their contribution to the general knowledge of clam biology in the Irish Sea is addressed, along with suggested future research.

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Chapter 2.

The reproductive biology of the softshell clam, *Mya arenaria*, in Ireland, and the possible impacts of climate variability.

Abstract

Little is known about the biology of the softshell clam in Europe, despite it being identified as a potential species to culture for food in the future. Monthly samples of the soft-shell clam, *Mya arenaria*, were collected intertidally from Co. Wexford, Ireland, over a period of sixteen months. The mean weight of sampled individuals was 74±4.9g and mean length was 8.2±0.2cm. Histological examination revealed a female to male ratio of 1:1.15. In 2010, *M. arenaria* at this site matured over the summer months, with both sexes either ripe or spawning by August. A single spawning event was recorded in 2010, completed by November. Two unusually cold winters, followed by a warmer than average spring appear to have affected *M. arenaria's* gametogenesis in this area, potentially affecting the time of spawning, fertilisation success, and recruitment of this species. No hermaphrodites were observed in the samples collected, nor were any pathogens observed. Timing of development and spawning is compared with that on the coasts of eastern North America and with other European coasts.

Key words: Bivalve, climate change, gametogenesis, Ireland, *Mya arenaria*, spawning, temperature.

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2.1. Introduction

The soft-shell clam, *Mya arenaria*, is widely distributed in coastal and intertidal soft substrates in boreal waters and is often a dominant species in benthic communities (Lasota *et al*., 2004). *Mya arenaria* currently occupies a wide geographical range in the northern hemisphere, on the east and west coasts of North America, where it is commercially important for fisheries and aquaculture (Beal 2002; Connell *et al*., 2007). In 2008 alone the National Marine Fisheries Service of U.S.A. reported approximately 1.73 million kg of *M. arenaria* harvested, worth in excess of €16 million (Weston and Buttner, 2010).

The present European distribution of *M. arenaria* ranges from Northern Norway to Portugal, including the Black Sea (Zaitsev & Mamaev, 1997; Strasser, 1999; Conde *et al*., 2010), with recent reports of its introduction to the Mediterranean Sea (Weston & Buttner, 2010). *Mya arenaria* is rarely collected for food or bait in European waters, but it is an ecologically important food source for fish such as plaice (*Pleuronectes platessa)* and flounder (*Platichtys flesus*) (Strasser, 1999), shrimp, sandworms, crabs and wading birds, such as oystercatchers (*Haematopus ostralegus*) and curlew (*Numenius arquata*) (Zwarts & Wanink, 1989). Due to the softshell clams' ability to survive and reproduce in a variety of differing areas such as mud and gravel, it could be an ideal species to culture in European waters in the future. *Mya arenaria* are widespread around all Irish and British coasts (Eno *et al.*, 1997; O'Riordan, 2002), but little information is currently available on reproductive biology in these areas.

Previous work in the United States has revealed that the sexes of *M. arenaria* are usually separate, with a sex ratio of 50:50 (Newell & Hidu, 1986; Brousseau, 1987). On eastern North American coasts *M. arenaria* usually begins spawning in May or June and can continue for two to three months. Though most sampled populations of *M. arenaria* spawn once a year (Ropes & Stickney, 1965), there have been examples of two spawning periods; in spring and autumn (Pfitzenmeyer, 1965; Brousseau, 1987). In European waters single spawning events have been recorded in the Black Sea (Began, 1979), the Wadden Sea (Gunther, 1992; Cardoso *et al*., 2009), the west coast of Sweden (Möller & Rosenberg, 1983), and on the east coast of Denmark (Munch-Petersen, 1973), while biannual spawning events were observed in

Oslofjord, Norway (Winther & Gray, 1985), and the south coast of England (Warwick & Price, 1975). To date, details of the reproductive cycle of *M. arenaria* in the Irish Sea have not been recorded.

Environmental conditions such as water temperature, food availability, and the presence of pollution have been shown to affect gametogenesis and spawning of *M. arenaria* (Brousseau, 1987; Gauthier-Clerc *et al*., 2002), and an increase or decrease in Sea Surface Temperature (SST) can affect the distribution and abundance of some bivalve species (Kröncke *et al*., 2011). There have been records of spawning at different temperatures across the native range of *M. arenaria*, from 4°C to 25°C (Pfitzenmeyer, 1965; Brousseau, 1978). In the Celtic/Biscay shelf, sea water temperatures are predicted to increase between 1.5 and 5°C over the next 100 years (Philipart *et al*., 2011), with global sea water temperature hypothesized to increase 1.8 to 4°C by the end of the 21st century (Matozzo & Marin, 2011). However, the rate of change of temperature is highly variable spatially (Wethey *et al*., 2011), with extreme episodes of unusual temperatures occurring in some areas. Some studies are indicating that annual seawater temperatures may rise in European, including Irish, waters in the future (Hiscock *et al*., 2004; Christensen *et al.*, 2007), while the winter of 2009-10 in Ireland was the coldest in 50 years, with December 2010 being the coldest on record (MetEireann, 2010).

This study was undertaken due to the lack of knowledge of the reproductive biology of *Mya arenaria* in Irish waters, and their potential as a future commercial food species. The potential effects of climate change on this species can be investigated in the short term (the effect of cold winters) and provide a baseline for longer term climate change studies in European waters. The aims of the current research were to investigate, using histological analysis, the annual reproductive cycle of *M. arenaria* in Ireland under natural conditions, in an area close to the centre of the introduced European range and to determine whether there are differences in the reproductive cycle between that recorded in the Irish Sea and other European sites, and whether patterns are similar to those observed in the native range in eastern North America.

2.2. Materials and Methods

Study Site

Bannow Bay, Co. Wexford is an estuary on the southeast coast of Ireland, located at the interface between the Celtic and Irish Seas (52° 27'N 006° 47'W). It covers an area of *ca* 1050 ha, is 8km long and between 1 and 3km wide (Malham *et al*., 2009). The intertidal habitat consists of mud and sand flats, and approximately 75% of the bay is uncovered at average low tide. The tidal range at Bannow Bay is 0.6 to 3.8 metres. There is oyster cultivation on the eastern side of the estuary, and the surrounding land mainly supports dairy and arable farming.

Sampling

Soft-shell clam, *M. arenaria*, specimens (n≈30) were collected by digging in the lower intertidal (52° 13' 33 N 006° 47' 51 W) each month from March 2010 to June 2011, generally during the first week of each month at low Spring tides. Specimens were located in the muddy sediment by identifying the characteristic "key-hole" shaped opening left by the siphons. Digging beside these siphon holes allowed for the collection of individuals without breaking the brittle shells. *Mya arenaria* were usually found at a depth of 30cm, though could be as deep as 40cm during the colder months. The first 30 siphon holes identified were dug out and the individuals collected below these apertures were used. Most key-hole shaped openings were produced by larger individuals, and very few small *M. arenaria* were encountered while digging. Density estimates were not obtained as the substrate containing *M. arenaria*, consisting of mud and gravel, was not suitable for collecting density data. *Mya arenaria* were transported to a cold room (4°C) within four hours of collection and left over night for dissection the next morning. Each clam was numbered and the total wet weight (g), shell length from anterior to posterior end (cm), and width from dorsal side to ventral side (cm) of each individual were recorded. No sample could be taken in December 2010 due to severe weather conditions preventing travel to the sampling area.

A continuous temperature monitor, the Stowaway® Tidbit™ Weatherproof & Waterproof Temperature Logger, was deployed underwater 10 m from the *M. arenaria* collection site in Bannow Bay from June 2010. The monitor recorded

ambient temperature at 16 minute intervals, and the monthly mean was calculated from this date to the end of the study. Data were obtained from the monitor using an Optic base Station/Coupler Kit, and BoxCar® Pro 4.3 Starter Kit Software. As temperature for the site was only available from June 2010, air temperature data from January 2008 to July 2011 was also obtained from Met Eireann for Johnston Castle, which is approximately 20 km from Bannow Bay. Degree days per month were calculated by multiplying the mean daily temperature by the number of days in each month.

Histological Techniques

The soft tissue of each clam was dissected within 24 hours of collection from the fresh individuals. Two cuts through the body of the animal provided a transverse section of the visceral mass, which contained the gonad, renal gland and digestive tract, and sections of the gill and mantle. The tissue was fixed in Davidson's solution for 48 hours and stored at 4°C. Slides were prepared using standard histological techniques, where tissues were dehydrated in alcohol, cleared in xylene, embedded in paraffin wax, sectioned at 7µm, and stained with Harris' hematoxylin and eosin before being mounted on clean standard microscope slides (Porter, 1974). The prepared microscope slides were examined using 10x, 20x, and 40x magnification, to determine sex and stage of reproductive development. Individuals were also screened for the presence of any parasites and abnormal conditions or pathologies.

Staging of gonadal development

Clam reproductive maturity was categorised into five stages using the maturity scale described by Brousseau (1987), whereby maturity stages were designated as 'Indifferent, Developing, Ripe, Spawning and Spent'. When more than one developmental stage was present in a single individual, the maturity was scored based on the most prevalent stage.

The indifferent stage of female *Mya arenaria* is identified when the distinctive female inclusions are visible in the follicle cells and extremely small primary oocytes are present in the alveolar membrane. The developing stage is recognised by an increase in the number and the size of oocytes. A central lumen is present in each follicle, into which protrude the stalked oocytes. In the ripe female

there are many mature, spherical oocytes which appear to be free within the follicular lumen. In the spawned stage, the mature oocytes are gradually discharged. Emptying follicles and the cessation of oogenesis in all follicles is characteristic of this stage. In the spent stage, unspent oocytes in the early phases of cytolysis are present. These appear in the lumen as large, darkly staining bodies with obscure nuclei. Follicle cells begin to reinvade the follicles from the basal membrane.

During the indifferent stage of male *Mya arenaria* the follicles contain the aberrant forms, multinucleated non-pycnotic cysts and pycnotic cells. The basal membrane and follicle cells are the dominant structural elements, with a few primary spermatocytes or spermatogonia visible at the periphery of the lumen. In the developing stage, the maturation and proliferation of the spermatocytes takes place. In the ripe male clam the follicle is filled with dense radiating bands of spermatozoa, the tails of which project into the central lumen. In the spawned stage the follicle is characterised by fewer spermatozoa than the ripe clam. A few spermatozoa remain in the radiating bands but the rows of follicle cells gradually increase to replace the spawned spermatozoa. In the spent male, the follicles are almost completely filled with follicle cells and the reduced lumen contains a few sex cells (Brousseau, 1987).

2.3. Results

Measurements

Of the 432 individuals collected over 16 months from March 2010 to June 2011, the average weight of all individuals was 74g ±4.9g (fig. 1) with the lightest individual collected weighing 3.1g, and the heaviest 200g. Over the months sampled, mean monthly values ranged from 60.5g ±4g to 103g ±5.4g. The average length of all *M. arenaria* collected was 8.2cm ±0.2cm (fig. 2). Individuals collected ranged from 2.6 to 11.6cm in length, with the mean monthly lengths ranging from 7.4cm ±0.1cm to 9.4cm ±0.1cm. Due to the nature of collection, only soft shell clams with visible siphon holes were collected. Very few spat were observed at this area while sampling. All lengths and weights of *Mya arenaria* were observed in various stages of gametogenesis apart from males under 5.9cm.

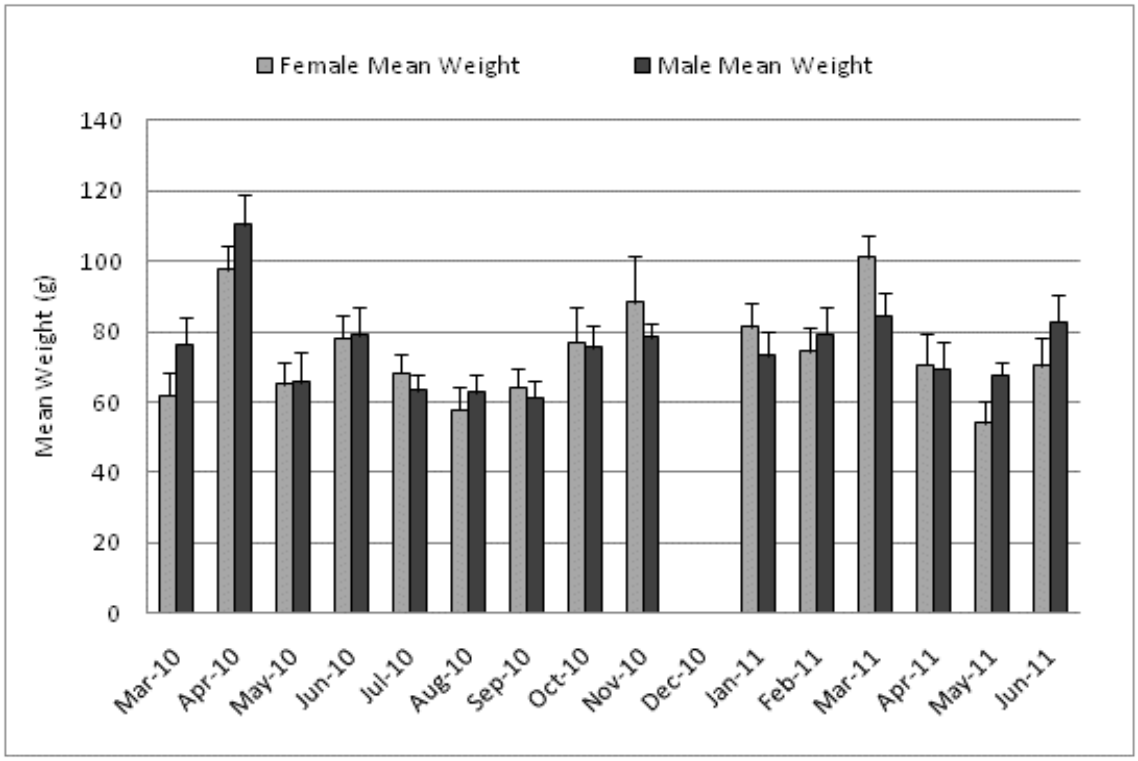

Figure 1: Mean weight (± S.D.) of female and male *Mya arenaria* sampled at Bannow Bay from March 2010 to June 2011.

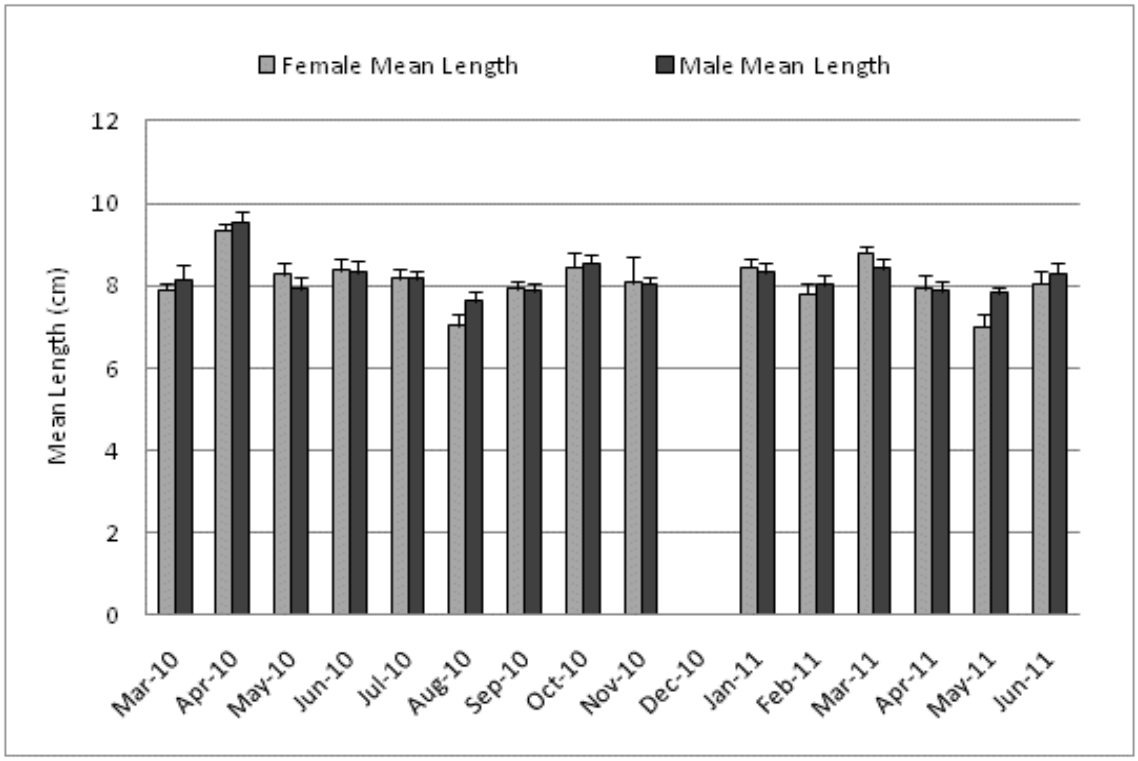

Figure 2: Mean length (± S.D.) of female and male *Mya arenaria* sampled at Bannow Bay from March 2010 to June 2011.

The average weight and length of female individuals were 74g ±3.5g and 8.1 cm ±0.2cm respectively, while the average weights and lengths of male individuals were 75g ±3.2g and 8.2 cm ±0.1cm. On average, female individuals were both slightly lighter and shorter than male *M. arenaria*, though this was not statistically significant when using a T-test (t=0.53, *df* = 28, p>0.1). Only measurements of undamaged shells were included in the results.

Histology

Sex Ratio

Of the softshell clams examined, 199 (46%) were female, 229 (53%) were male, and four (1%) were immature or the sex could not be determined (fig. 3). A Chi-squared (χ^2), with a Yates Correction, was used to analyse sex ratios. The overall female:male sex ratio of 1:1.15 did not show a significant divergence from a 50:50 ratio (χ^2 =2.57, *df* = 1, *P* > 0.05). Within months, it was revealed that there was no statistically significant divergence from the 50:50 ratio of sexes (χ^2 =0.133, *df* = 1, *P* > 0.05 to χ^2 =2.882, *df* = 1, *P* > 0.05), though the closest to showing a significant divergence was the October 2010 sample, with 10 female and 20 male *M. arenaria* (χ^2 =3.333, *df* = 1, *P* > 0.05).

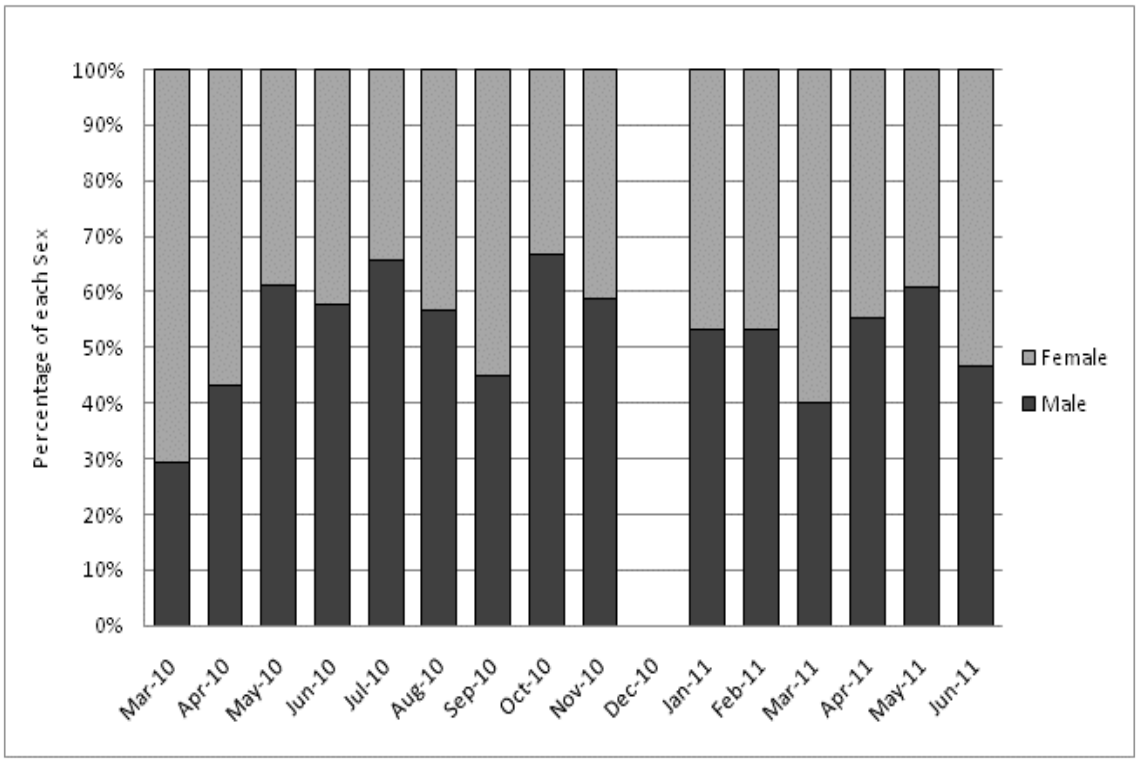

Figure 3: Sex ratio of *Mya arenaria* sampled at Bannow Bay over study period.

Sexual Cycle

Female

During the course of the study all five of the stages of gametogenesis described by Brousseau (1987) were observed (fig. 4). In 2010, the sampled female *M. arenaria* showed a less defined cycle than the male individuals, with development, ripening and spawning taking place from March to October (fig. 5). Fifty percent of individuals were spent in May 2010, following a large number (76%) ripe in March, and 41% spawning in April. The percentage of females developing rose from March to July 2010, with all individuals either ripe or spawning by August 2010 (fig. 5). The majority of spawning individuals were present in September (62%), with all spawning completed by November 2010. October and November samples contained the highest percentage of spent females, at 70% and 67%, respectively, with some indifferent individuals also present (10%).

In 2011 most females were developing over the winter months of January and February (~90%), with some spent individuals present (~10%). Female *M. arenaria* began ripening in March 2011, similar to 2010, but at a much lower percentage (39%). The majority of individuals (~60%) were developing from March to June 2011, with some at the ripe stage (~30%) but none were spawning, in contrast to the same period in 2010. There were spent females present in April, May and June of 2011, and individuals of indifferent stage present in April (9%).

Female Male

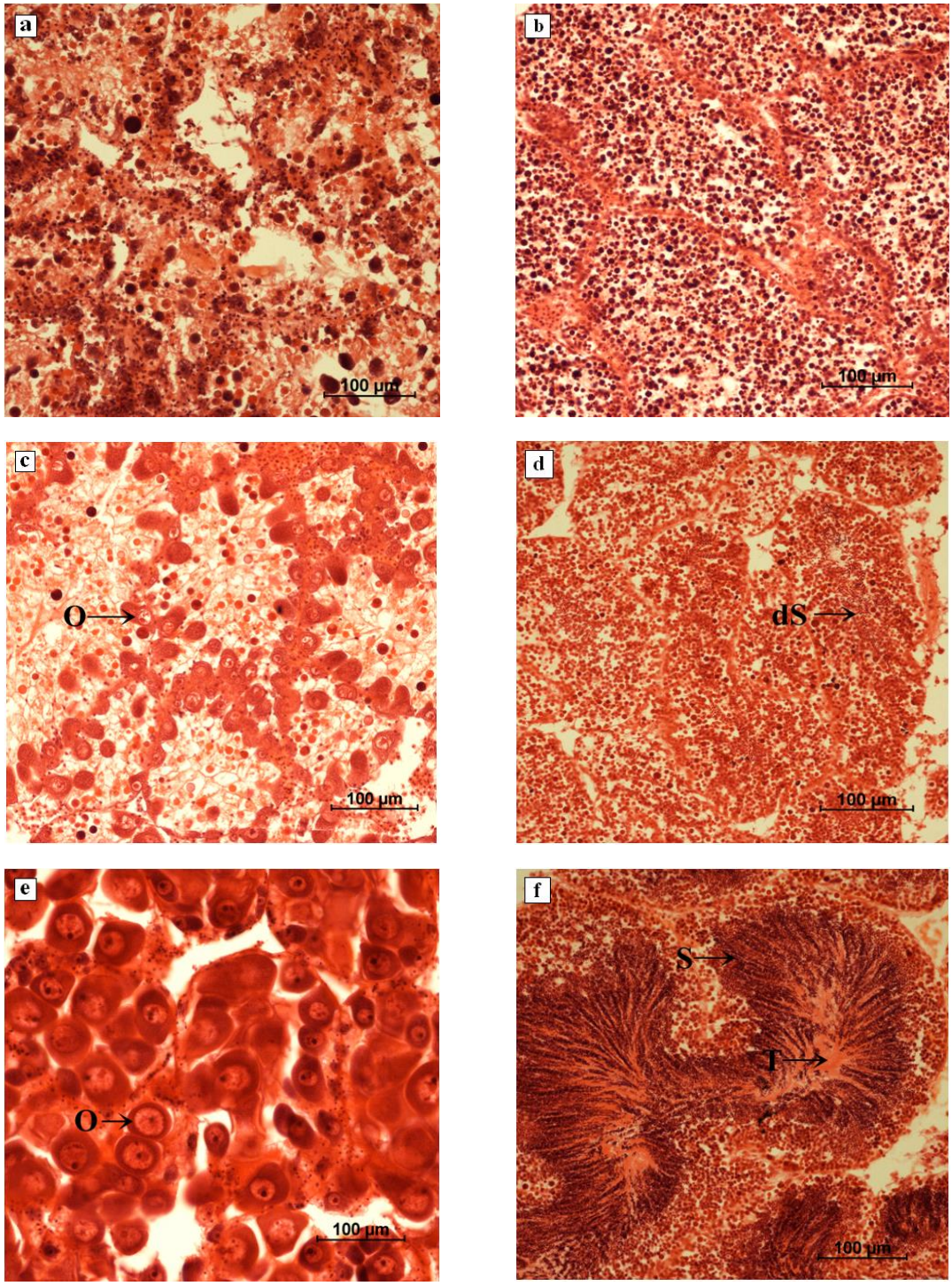

Figure 4. The different stages of the reproductive cycle of *M. arenaria*. (a–b) Indifferent female and male gonads, (c–d) developing gonads of female and male clams, (e-f) ripe gonads of female and male clam. Showing oocytes (O), developing spermatocytes (dS), spermatozoan (S), tails of spermatozoa projecting into central lumen (T), reduced lumen of gonad (L). All slides 20X.

Female Male

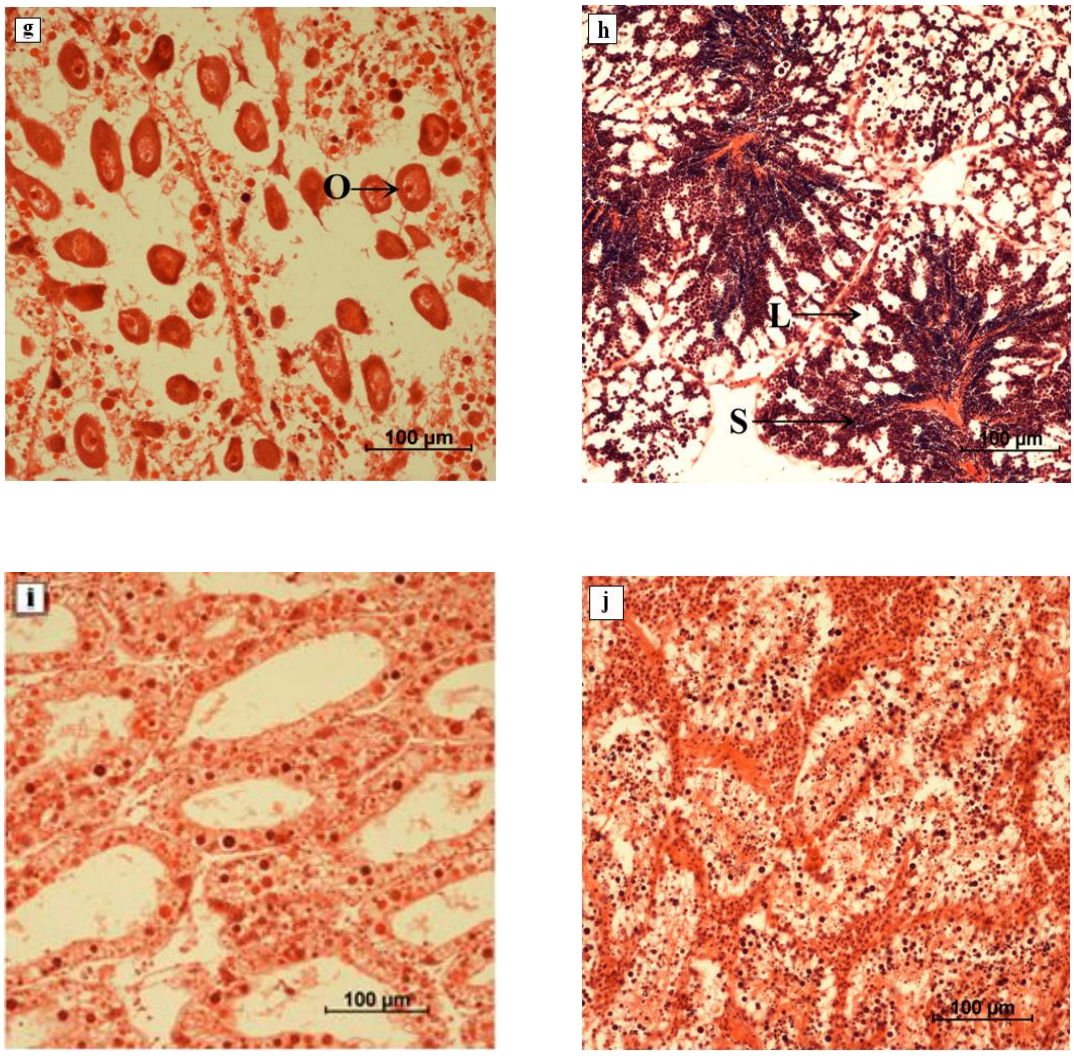

Figure 4 continued. The different stages of the reproductive cycle of *M. arenaria* continued. (g–h) spawning gonads of female and male clams, (i–j) spent gonads of female and male clams. Showing oocytes (O), developing spermatocytes (dS), spermatozoan (S), tails of spermatozoa projecting into central lumen (T), reduced lumen of gonad (L). All slides 20X.

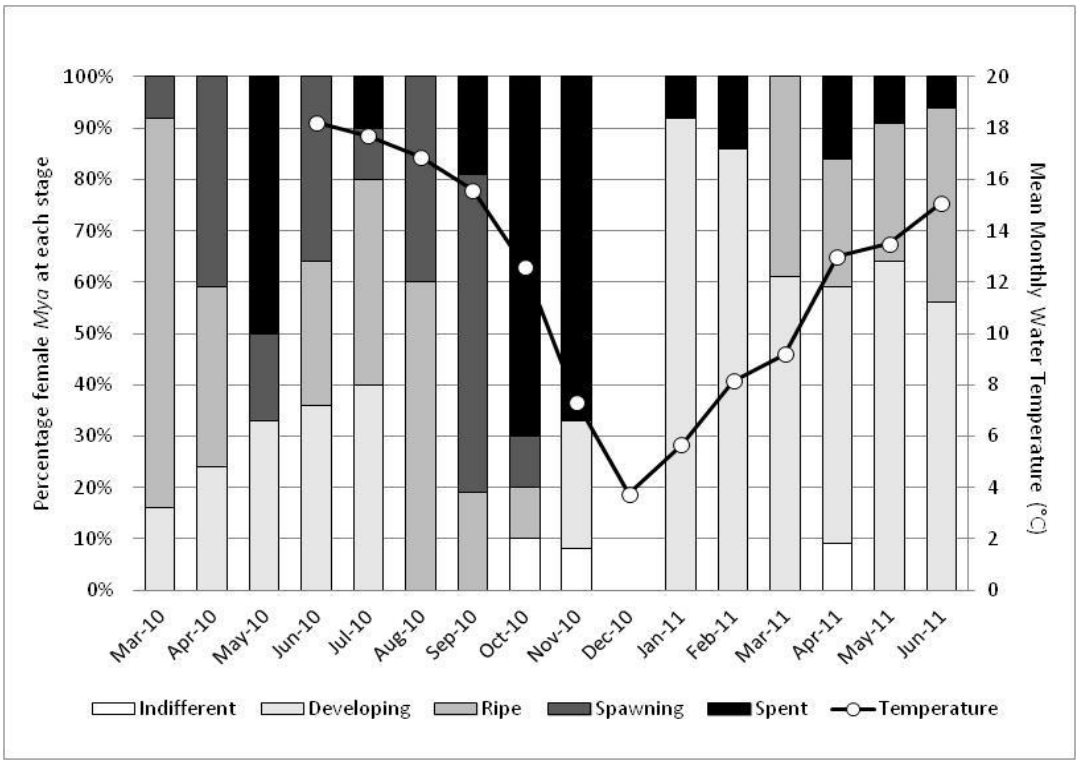

Figure 5: Stages of gametogenesis observed in female clams over the study period.

Male

In the collected samples, all male *M. arenaria* were indifferent in March 2010 (100%), which steadily decreased until June (13%) (fig. 6). Development of the male gonads began to take place in April (8%) and spawning was observed for the final time in October 2010. The number of developing individuals rose to 66% in June and 47% in July 2010. Ripe individuals were first present in May (22%), and by August all males were either ripe (47%) or spawning (53%). Spawning was completed by November 2010, with the majority of individuals spent in September (38%) and October (50%) samples. All males collected from November 2010 to February 2011 were in the resting indifferent stage, with development beginning earlier, in March 2011, as compared to the previous year, when developing individuals were not detected until April. Ripe individuals were present in May 2011, similar to the previous year, though some spawning individuals were present in this month, compared to a lack of spawning individuals until August, in 2010. There was a higher number of indifferent individuals present in June 2011 (29%) as opposed to June 2010 (13%).

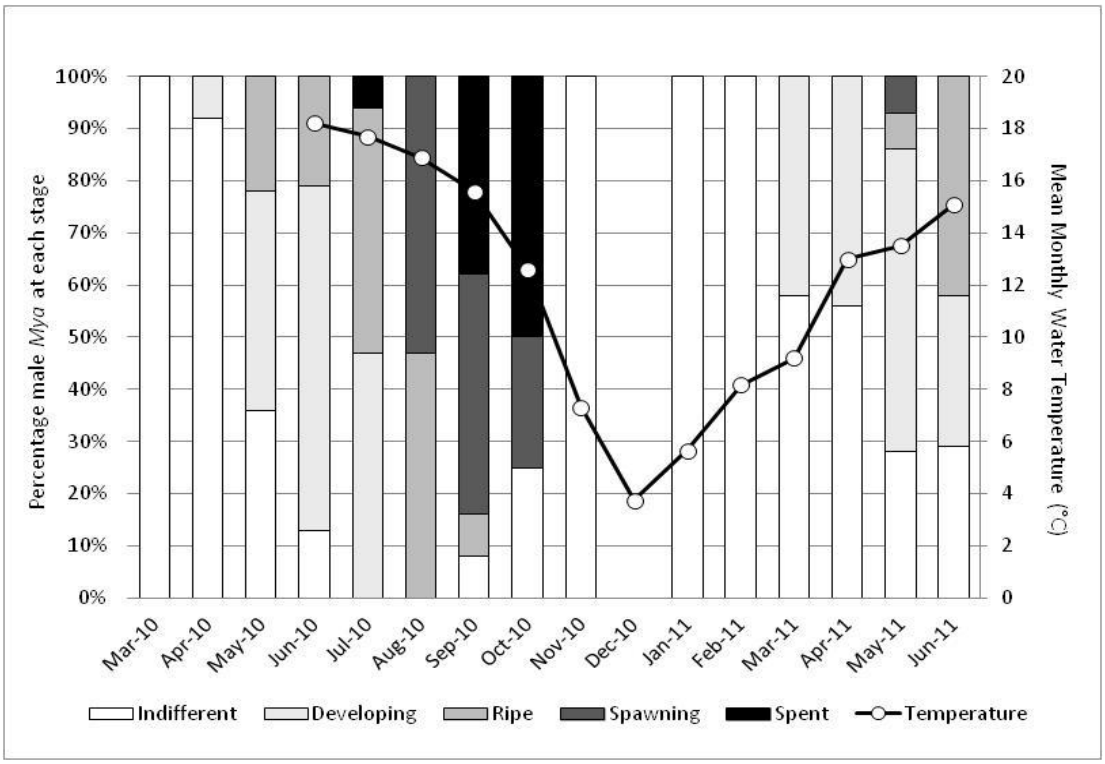

Figure 6: Stages of gametogenesis observed in male clams over the study period.

The smallest reproducing female and males sampled were 5.1cm (17.8g) and 6.1cm (29.0g) respectively. The only immature clam collected was 2.6cm in length, and 3.1g in weight.

Disease

Screening of the histological sections of the visceral mass, gill and mantle of sampled *Mya arenaria* revealed no parasites or pathological indications of disease, such as hematopoietic neoplasia, in any of the individuals sampled in Bannow Bay over the study period.

Temperature

The mean monthly temperature recorded by the Stowaway® Tidbit™ Temperature Logger, under water, at Bannow Bay was highest in June 2010, at 18°C and decreased each subsequent month to 3°C in December 2010 (Table 1). The highest recorded temperature points in 2010 were 27.97°C on June 26th and 27.31°C on July 12th, and the lowest was -1.66°C on December 24th and 25th. The lowest water temperature recorded in 2011 was recorded at -0.44°C on 29th January. Mean

Table 1: The mean, minimum, maximum monthly sea water temperatures, and degree days per month, recorded by a continuous temperature monitor in Bannow Bay from June 2010 to June 2011.

Month	**Mean Temperature**	**Minimum Temperature**	**Maximum Temperature**	**Degree Days/Month**
Jun-10	18.21	10.34	27.97	546.30
Jul-10	17.70	13.51	27.31	548.70
Aug-10	16.89	9.48	26.32	523.59
Sep-10	15.59	8.62	24.06	467.70
Oct-10	12.59	5.73	18.21	390.29
Nov-10	7.31	-1.35	16.43	219.3
Dec-10	3.75	-1.66	8.33	116.25
Jan-11	5.66	-0.44	9.48	175.46
Feb-11	8.16	4.60	11.80	228.48
Mar-11	9.20	4.90	16.00	285.20
Apr-11	13.00	8.00	22.20	403.00
May-11	13.50	8.60	20.60	418.50
Jun-11	15.10	10.30	26.40	453.00

monthly water temperatures rose from 5.66°C in January 2011 to 15.1°C in June, peaking at 26.4°C on 3rd June 2011 (Table 1).

Air temperature readings at Johnstown Castle from January 2008 to July 2011 showed a decrease in the monthly minimum temperatures recorded during the winter months, from 4.4°C in January 2008 to 2.3°C in January 2009, and -0.15°C in December 2010. The mean monthly winter temperatures also showed a decrease during this time, from 6.9°C in January 2008 to 4.9°C in January 2009, and 2.2°C in December 2010 (fig. 7).

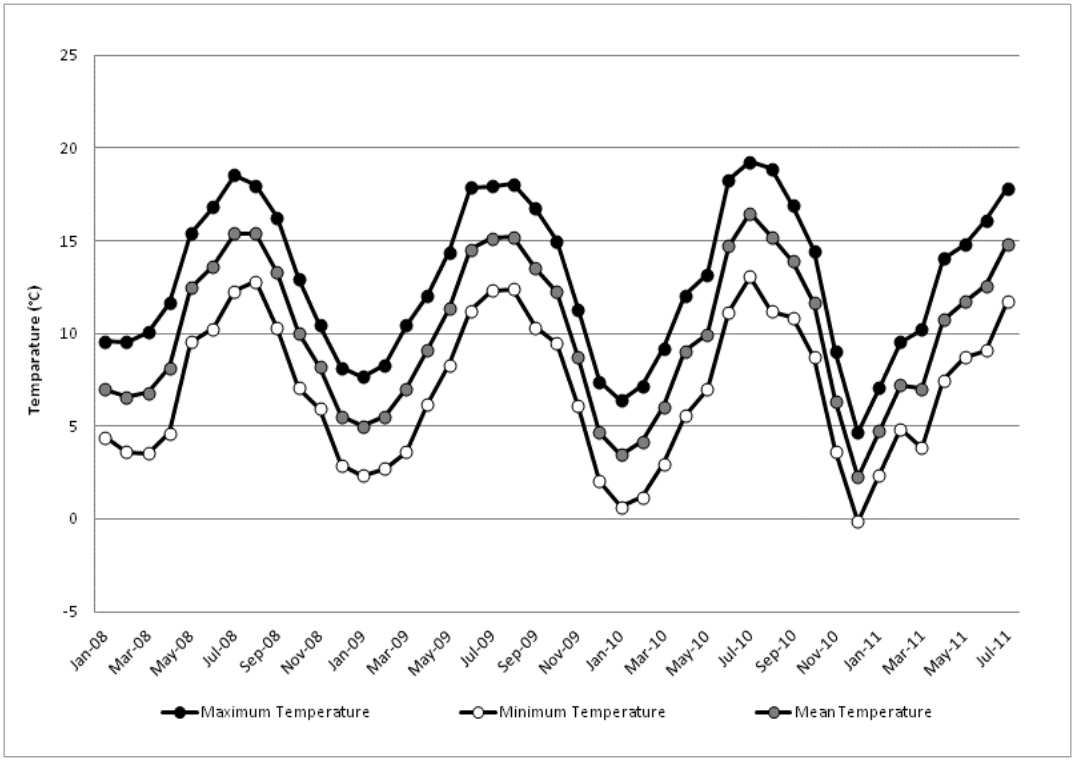

Figure 7: Air temperature reading for Johnstown Castle, January 2008 to July 2011, showing mean, maximum and minimum temperature for each month.

In 2008 the mean monthly air temperatures at Johnstown Castle rose from 6.9°C and 6.5°C in January and February, respectively, to 15.4°C in July and August, peaking at 18.5°C in July 2008. Mean monthly air temperatures fell to 4.9°C in January 2009, and increased to 15.2°C in August, with the maximum temperature of 2009 recorded at 18°C in August. In 2010 the mean monthly air temperature of 3.5°C was recorded in January, rising to 16.5°C in July, and peaking at 19.2°C. In 2011 the mean monthly air temperatures at Johnstown Castle were 1.7°C warmer, on average, from January to June, than the same months in 2010. The maximum temperature recorded in 2011 up to the end of the field trial was 17.8°C in July (fig. 7).

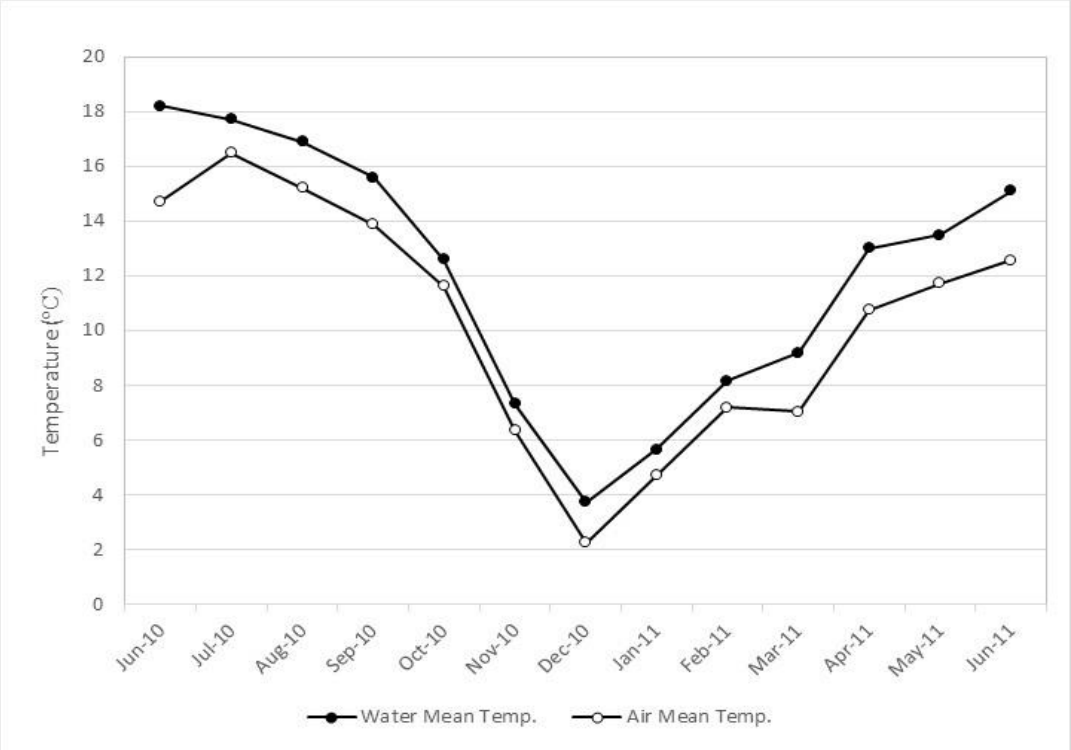

Figure 8: Air temperature reading for Johnstown Castle and water temperature reading for Bannow Bay, June 2010 to June 2011.

From June 2010 to June 2011, the mean monthly air temperature, taken at Johnstown Castle, was, on average, 1.7°C cooler than the mean monthly water temperature, taken at Bannow Bay, Co. Wexford, Ireland. Figure 8 shows a similar trend between the air and water temperatures taken at these areas during this period.

Table 2: The timing of spawning seasons, by latitude, of European *Mya arenaria* as reported in literature.

Area	Latitude	Tidal Level	Spawning	Month	Authors
Oslofjord, Norway	59°91'N 10°37'E	Intertidal	Biannual	June and September	Winther & Gray, 1985
West coast of Sweden	57°37'N 35°51'E	Intertidal	Annual	June/July	Moller & Rosenberg, 1983
Wadden Sea	55°00'N 6°7'W	Intertidal	Annual	May/June	Gunther, 1992
Dutch Wadden Sea	55°00'N 6°7'W	Subtidal and Intertidal	Annual	July-November	Cardoso *et al.*, 2009
Denmark	55°55'N 12°0'E	Subtidal	Annual	May/June	Munch-Peterson, 1973
S.E. Ireland	52° 13'N 06° 47'W	Intertidal	Annual	August/ September	Current paper
S.E. England	50°37'N 4°25'W	Intertidal	Biannual	March and late Summer	Warwick & Price, 1975
Black Sea	42°30'N 35°00'E	N/A	Annual	Late May	Began, 1979

2.4. Discussion

Of the sampled *Mya arenaria*, the average length of all individuals was 8.17±0.18cm, with the shortest measuring 2.6cm and the longest 11.5cm. Previous studies in North American (Brousseau, 1979) and European (Winther & Gray, 1985) waters have revealed maximum lengths of individuals between 10 and 17cm (Cowles, 2007; Cohen, 2011). Past work has revealed that softshell clams smaller than 2cm in length are usually immature, with indistinguishable gonads (Brousseau, 1978, 1987). First reproduction usually occurs at a size of 2-5cm in length (Strasser, 1999; DFO, 2011) which, depending on environmental conditions, can mean the individual is anything from 1 to 4 years of age (Strasser, 1999). Newell & Hidu (1986) suggested that the size of the individual is more important than age when it comes to the initiation of a spawning event. Data collected in the present study correlate with this, in that the smallest reproducing female and male were 5.1 and

6.1cm respectively. The sex of the smallest clam sampled (2.6cm) was not discernible as the clam was immature.

The overall female:male sex ratio of 1:1.15 in the present study did not show a significant divergence from the 50:50 ratio previously described in Denmark (Munch-Petersen, 1973) and Oslofjord, Norway (Winther & Gray, 1985), and on north American coasts in Massachusetts and Connecticut (Brousseau, 1978, 1987), in Maryland (Shaw, 1962), and in Washington (Porter, 1974). There was no evidence of hermaphroditism in the present study, in keeping with recordings of low incidences of hermaphroditism in past studies (Coe & Turner Jr., 1938; Munch-Petersen, 1973; Brousseau, 1978). All stages of gametogenic development of *M. arenaria* previously described by Brousseau (1987) were observed over the period of sampling. *Mya arenaria* matured over the summer months of 2010, with both sexes either ripe or spawning by August, similar in timing to the spawning period recorded in New England, USA (Ropes & Stickney, 1965), in Massachusetts, U.S.A. (Stevenson, 1906; Belding, 1930), in Denmark (Thorson, 1946), and in the Dutch Wadden Sea (Cardoso *et al*., 2009).

Previous studies of *M. arenaria* in both North America and continental Europe have shown that softshell clams can spawn either once or twice during one year, with the majority of spawning taking place during June, July and August (Brousseau, 1987; Strasser, 1999; Cohen, 2011). The data from the present study demonstrated that male *Mya arenaria* from Bannow Bay had one spawning period at the end of summer 2010. These data are in keeping with previous observations in the north east Atlantice.g. on the west coast of Sweden (Möller & Rosenberg, 1983), on the east coast of Denmark (Munch-Petersen, 1973), and in the Wadden Sea (Gunther, 1992; Cardoso *et al*., 2009) and also in the Black Sea (Began, 1979). The female *M. arenaria* of Bannow Bay show a less tightly defined spawning period than the males, with spawning individuals recorded from March to October, and peaks in spawning taking place in April, June and August/September 2010. However, all female individuals were either ripe or spawning in August 2010, with a large percentage in these stages in September, and most individuals were spent in October and November. Based on the data collected at Bannow Bay, Ireland, and previous research across the North Atlantic, it would seem that the reproductive cycle of *Mya*

arenaria is affected by more than just latitudinal distribution of individuals (Newell & Hidu, 1982; Brousseau, 1987).

In eastern Atlantic waters the majority of spawning periods have been recorded in the summer months of May and June (Table 2). Where *M. arenaria* were recorded as spawning biannually, in Oslofjord, Norway and S.E. England, the second spawning took place in or around September. In 2010, the present study revealed spawning at a similar time of March to July for female softshell clams, and August to October for both female and male clams. The less tightly defined spawning period of female *M. arenaria* compared to male individuals has not been recorded previously in European waters, and was not repeated in 2011, when no spawning had taken place in female *M. arenaria* by June, when the field study concluded.

Environmental conditions including food availability and temperature have been shown to affect gametogenesis and spawning of *M. arenaria* (Brousseau, 1978). It is currently unknown whether a 'critical' water temperature is more important at a certain point in gonadal development, to progress maturation, or to begin the spawning event itself (Loosanoff & Davis, 1950; Brousseau, 1978). It is generally considered that spawning of *M. arenaria* begins when water temperature rises to 10°C (Nelson, 1928; Weston & Buttner, 2010), though this can depend on the area, as a spawning peak was recorded at 4-6°C in Massachusetts (Brousseau, 1978) while Belding (1930) reported a spawning peak at 22°C in the same area. When *M. arenaria* are forced to spawn in culture, water temperature needs to reach 22 to 24°C before spawning begins (Buttner & Weston, 2010). The male *M. arenaria* of this study seemd to begin spawning in August 2010, which had an average daily water temperature of 16.9°C. However, the two preceding months of June and July had average daily water temperatures of 18.2 and 17.7°C respectively, with 22°C and 24°C first recorded in late May 2010. The majority of spawning of female *M. arenaria* also took place in August and September 2010, with a small group spawning in May. In 2011, water temperatures first rose to 10°C in the last week of February, and male development began in early March. Female development was in progress during the winter months, but ripe individuals were first detected in March 2011. The critical spawning temperature of 22°C was first recorded in 2011 on 2nd June. The critical upper temperature of 28°C (Strasser, 1999;

Weston & Buttner, 2010) was never reached in the waters of Bannow Bay in 2010, though a peak of 27°C was recorded in July 2010 for a short period of time.

The winters of 2009/10 and 2010/11 were exceptionally cold in Ireland, with air temperatures decreasing to -17°C in northern areas in January 2011 (MetEireann, 2010). Air temperature data, collected at Johnstown Castle (20km from Bannow Bay) for the period January 2008 to July 2011 reveal that the lowest recorded temperatures dropped each winter from 3°C in March 2008, to 2.34°C in January 2009, 0.65°C in January 2011, and to -0.2°C in December 2010. Unusually cold intervals similar to this have led to mass mortalities of bivalve species, and contraction of species range in the past (Crisp, 1964). A disparity in gametogenic development timing between the sexes may be a critical factor reducing recruitment in the species, as it results in less overlap of months where both sexes are spawning simultaneously. In 2010, female and male *M. arenaria* of Bannow Bay were spawning synchronously in the months of August, September and October, while the gametes released by female individuals from March to July 2010 would have remained unfertilised.

Following the period of cold winters, air temperature readings from Johnstown Castle reveal monthly mean temperatures were greater in 2011 compared to 2010, from January to May. In the present study, the effects of two unusually cold winters, followed by a warmer than average spring, would seem to have affected the timing of development in *M. arenaria* individuals in 2011, compared to 2010. In the males of the species, the variable temperatures appear to have advanced the progression of gonad development, with 42 and 44% of males developing in March and April 2011, though none or few individuals were developing in the same months in the previous year. Ripe males were first recorded in May of each year, with spawning taking place in 2010 in August, though spawning was observed as early as May in 2011. In contrast, the development of female *M. arenaria* would seem to have been inhibited in 2011, with the majority of individuals still developing in March, compared to the majority of females already being ripe in March 2010. Also, though spawning individuals were present from March in 2010, by June 2011 only ripe individuals were still present in the sample, with many (56%) still developing. Overall, the cold winters followed by a warmer than average spring appeared to have accelerated gametogenesis in males in 2011, compared to 2010, and had the opposite

effect in female *Mya arenaria.* This asynchronous spawning may be a critical factor reducing recruitment in the species if climatic variability continues and male and female *Mya arenaria* continue to spawn at different times.

No disease or pathogens have been observed in the Irish Sea *M. arenaria*, despite the fact that other bivalves such as *Cerastoderma edule* collected monthly in the same area, and screened using identical methods, have revealed a heavy prevalence of trematodes, neoplasia and *Nematopsis* spp. (Morgan *et al.*, 2012). To date, only low levels of disease or pathogens have been reported in *Mya arenaria* on European shores (Strasser *et al*., 1999), despite a number of diseases and parasites being present in the *M. arenaria* populations of North America, particularly hematopoietic neoplasia, which was not observed in this study (Earle & Crisley, 1975; Gibbons & Blogoslawski, 1989; Hidu & Newell, 1989; Leavitt *et al*., 1997; McLaughlin & Faisal, 1999; Dungan *et al*., 2002). This could be due to a lower density of these animals, than in the intensively cultured areas of North America (Renault & Novoa, 2004).

In summary, the *Mya arenaria* of Bannow Bay of south east Ireland matured over the summer months of 2010, with both sexes either ripe or spawning by August in a single spawning episode, and all spawning being completed by November. Gametogenic development of female *M. arenaria* would seem to be inhibited in 2011, compared to 2010, while development of male clams has been advanced. Progression of developmental stages suggests that a discrepancy between the sexes spawning events might have occurred in 2011, potentially affecting subsequent recruitment in Bannow Bay *M. arenaria*.

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Chapter 3.

The reproductive biology of the exploited razor clam, *Ensis siliqua,* in the Irish Sea.

Abstract

Knowledge of the reproductive cycle of a species is a prerequisite for sustainable management of a fishery. The infaunal marine bivalve, *Ensis siliqua*, is a commercially important species in Europe, and is fished in many countries, including Ireland, where it is sold by wet weight. Seasonal variations in the reproductive cycle of subtidal razor clams from the Skerries region of the Irish Sea, an important fisheries area, were examined between June 2010 and September 2011 while monitoring weight. Histological examination revealed that the *E. siliqua* sex-ratio was not different from parity, and no hermaphrodites were observed in the samples collected. In the summer months of 2010 all female clams were either spent or in early development, with just a small percentage of males still spawning. The gonads of both sexes developed over the autumn and winter months of 2010, with the first spawning individuals recorded in January 2011. Spawning peaked in March 2011, but unlike in 2010, spawning continued through June and July with all animals spent in August 2011. The earlier and longer spawning period found in this species in 2011 compared to 2010 may have been due to the colder than normal temperatures observed during the winter of 2010, plus the relatively warmer temperatures of Spring 2011, which could have affected the gametogenic development of *Ensis siliqua* in the Irish Sea. It was noted that wet weight dropped significantly in the summer months of both years, immediately after the spawning period which may impact on the economic viability of fishing for this species during this period. Timing of development and spawning is compared with other sites in the Irish Sea and elsewhere in Europe, including the Iberian Peninsula.

Key words: Bivalve, *Ensis siliqua*, gametogenesis, Ireland, spawning.

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3.1. Introduction

Ensis siliqua is a native species in Europe, and is distributed along the European Atlantic coast from the Norwegian Sea and the Baltic, south to the Mediterranean and along the Atlantic coast of Morocco (Gaspar & Monteiro, 1998; Darriba *et al*., 2005; Fernández-Tajes &Méndez, 2007; Varela *et al*., 2007; Costa *et al*., 2010). It is abundant in the British Isles, and widely distributed along the east coast of Ireland (Fahy, 1999). Commonly known as the razor clam or pod razor, *E. siliqua* inhabits fine sand, silt or muddy sediments along the coast of Europe, and can be found at depths ranging from 0 to 58m, though they are thought to be most abundant at 3-7m below Chart Datum (Gaspar and Monteiro, 1998; Fahy, 1999; Costa *et al*., 2010; Encyclopaedia of Life, 2010). The valves of *E. siliqua* shells usually reach a maximum of 21cm in length (Holme, 1951; Conchological, 2008; Encyclopaedia of Life, 2010), though larger individuals, of up to 24cm, have been recorded in Northern Europe (Muir & Moore, 2003). This clam species has a very large and powerful foot and is capable of rapid vertical burrowing in the fine sediments that it favours (Fahy & Carroll, 2007; Fernández-Tajes *et al*., 2007; Encyclopaedia of Life, 2010). Razor clams are usually found to have a sex ratio of 1:1, with a very low incidence of hermaphroditism (Gaspar & Monteiro, 1998; South Wales Sea Fisheries Committee, 1999).

Ensis siliqua is currently harvested by commercial fisheries in Spain, Portugal, and Ireland (Costa *et al*., 2010) and is regarded as an increasingly valuable fishery resource, with potential for commercial aquaculture in many European countries (Wootton *et al*., 2003; Fernández-Tajes *et al*., 2007; Varela *et al*., 2007; Arias-Pérez *et al*., 2011). By 2004, the importation rates of razor clams were quite significant in Europe, representing a total value of €550 million, with Spain, Italy, France, Portugal and the Netherlands being the most significant importers (Fernández-Tajes & Méndez, 2007; Fernández-Tajes *et al*., 2007).

In 1997, a razor clam fishery began in the Republic of Ireland when a large bed of *Ensis* spp. which measured 21km^2, was discovered outside Gormanstown, off the Meath coast (Fahy, 1999; Fahy & Carroll, 2007). In 1998, landings of *Ensis* were in the order of c.500tonnes and valued at €1,000,000 EU, making Ireland the largest supplier of *Ensis* in Europe in the space of two years (South Wales Sea Fisheries Committee, 1999). This continued until 2000, as the Republic of Ireland led the

world in wild-caught landings (Hauton *et al*., 2007). Though official landing details of razor clams are unavailable for subsequent years, it is thought that the Irish fishery experienced problems in *Ensis* landings caused by over-fishing, poor recruitment and winter mortality in some beds (Hauton *et al*., 2007).

Of the four species of the genus *Ensis* that occur in Ireland – *E. siliqua, E. arcuatus, E. minor* and *E. ensis* - *E. siliqua* makes up the vast bulk of landings (Fahy & Carroll, 2007). Because of the large global demand for shellfish, including *Ensis siliqua*, natural beds of this species are under pressure (Darriba *et al*., 2005; Hauton *et al*., 2007). The future commercial exploitation of these species will need careful management to ensure sustainability and avoid fishery over-depletion. To manage an exploited species, knowledge of the reproductive cycle of the species is essential, as it provides valuable data for recruitment, age and growth studies (Morsan & Kroeck, 2005). In particular, the effect of the gametogenic cycle on the weight of individuals would have an economic impact, as *E. siliqua* is currently sold by wet weight.

Previous work on this species has outlined the reproductive cycle of *E. siliqua* in other areas. In Southern Portugal *E. siliqua* gametogenesis has been recorded as beginning in December with spawning individuals first observed in May and all individuals spent by July (Gaspar & Monteiro, 1998). Similar gonadal development and spawning times of *E. siliqua* were reported in razor clams sampled in the Gormanstown Bed of the Irish Sea by Fahy in 1999. However, site specific variation in this cycle appears to exist, since *E. siliqua* individuals from North western Spain, which were examined histologically in 2000, supplied evidence of gametogenesis beginning in November and spawning taking place in April in razor clams from this area (Darriba *et al*., 2005). In all of these studies the gametogenic cycle of this species was found to be annual, with a long sexual rest period during the summer and autumn months. Less detailed reports of *E. siliqua* spawning periods in March and April in Plymouth, United Kingdom (Lebour, 1938), and July and August in North Wales (Henderson & Richardson, 1994), and the Clyde Sea of Scotland (Muir & Moore, 2003) have also been recorded.

Environmental conditions, including temperature, have been shown to influence the gametogenesis and spawning of a range of bivalve species such as *M. arenaria* (Brousseau, 1978; Gauthier-Clerc *et al*., 2002; Cross *et al*., 2012), *Crassostrea gigas* (Ruiz *et al*., 1992a), *Ostrea edulis* (Ruiz *et al*., 1992b ; Cano *et al*., 1997), *Pecten maximus* (Pazos *et al*., 1997), *Pinna rugosa* (Ceballos-Vazquez *et*

al., 2000), *Argopecten ventricosus* (Luna-Gonzalez *et al*., 2000), and *Ensis arcuatus* (Darriba *et al*., 2004) by affecting the timing and length of the spawning period, or causing a disparity between the spawning of either sex. In the Celtic/Biscay shelf, sea water temperatures are predicted to increase between 1.5 and 5°C over the next 100 years (Philipart *et al*., 2011), with global temperature hypothesised to increase 1.8 to 4°C by the end of the 21st century (Matozzo & Marin, 2011). The potential effect of changing waters temperatures, either seasonal or long-term, on the gametogenesis and spawning of the razor clam, *Ensis siliqua,* in Europe has not been addressed previously.

To allow for the on-going management and future exploitation of *E. siliqua* in Irish waters, the main objective of the present study was to determine the current reproductive cycle of this clam in the Irish Sea and examine any relationship between gametogenesis, individual weight, length, and temperature, on a monthly and seasonal scale.

3.2. Materials and Methods

Study Site

The Skerries region of the Irish Sea is located off the coast of Dublin city, in the region of N 52°13’06.9”, W 006°47’38.1”. Thirty live *Ensis siliqua,* which had been fished from a reliable, subtidal sitein the Skerries region were obtained monthly from June 2010 to September 2011, from a commercial shellfish wholesaler on the east coast of Ireland. The clams were identified as *E. siliqua* using the method described by Fernandez-Tajes *et al.* (2010), in which amplification of the internal transcribed spacer 1 (ITS-1) of razor clams is used to differentiate diverse species (Cross *et al., in prep*). No sample could be obtained in November 2010 due to severe weather conditions preventing trawling in the sampling area.

Histological Techniques

The total wet weight (g) and shell length (cm) of each individual clam were recorded. The soft tissue of each clam was dissected within 24 hours of collection. A section of the body of the animal was cut out, which contained the gonad, renal gland and digestive tract, and sections of the gill and mantle. The tissue was fixed in Davidson’s solution for 48 hours and stored at 4°C. Slides were prepared using

standard histological techniques, where tissues were dehydrated in alcohol, cleared in xylene, embedded in paraffin wax, sectioned at 7µm, and stained with Harris' Hematoxylin and Eosin before being mounted (Porter, 1974). The prepared microscope slides were examined using 10X, 20X, and 40X magnifications, to determine sex and stage of reproductive development.

Staging of gonadal development

Clam reproductive maturity was categorised into six stages using a modification of the maturity scale described for *E. siliqua* by Gaspar and Monteiro (1998) who designated these stages as 'inactive, early active gametogenesis, late active gametogenesis, ripe, partially spawned and spent'. In the present study, these stages were renamed to 'Indeterminate, Early Development, Late Development, Ripe, Spawning and Spent' respectively, to reflect the stages observed, though the definition of each stage remained the same. The term 'inactive' was amended as the sexes of *E. siliqua* are difficult to distinguish during the sexual rest period, and it was decided that the term 'Indeterminate' would better describe this stage of gametogenesis. When more than one stage was present in a single individual, the maturity was scored based on the condition of the majority of each section.

Environmental Parameters

The mean, minimum and maximum monthly surface seawater temperatures in the Skerries region, from January 2009 to December 2011, to cover the study period and the year prior to the study commencing were obtained from the Marine Institute (www.marine.ie).

3.3. Results

Wet Weight

Of the 450 individuals collected over 16 months from June 2010 to September 2011 the average wet weight of all *Ensis siliqua* individuals was 80.0±2g, with the lightest individual collected weighing 31.0g and the heaviest 153.0g. Over the study period, the mean monthly values of *E. siliqua* weight ranged from 40.68 ±0.9g in June 2011 to 117.2±2.4g in January 2011 (fig.1). Razor clams sampled during the sexual rest period of June to August in 2010 and 2011 were statistically significantly lighter than those collected during the months of December to February

2011, when the spawning period began (T-test, t=0.1124, *df* = 4, p<0.01). The average weight of female *E. siliqua* was 80.4±4.0g while the average weight of sampled male *E. siliqua* was 80.5±3.0g.

Length

The average length of all razor clams collected was 17.5±0.2cm. Individuals collected measured from 12.8cm to 21.4cm in length, with mean monthly lengths ranging from 14.5±0.1cm to 19.4±0.1cm (fig. 2). Unlike wet weight values, the average length of individuals did not significantly differ between seasons (t = 0.1124, *df* = 4, p>0.1). The average length of female *E. siliqua* was 17.5±0.3cm, and the average length of male clams was 17.6±0.2cm.

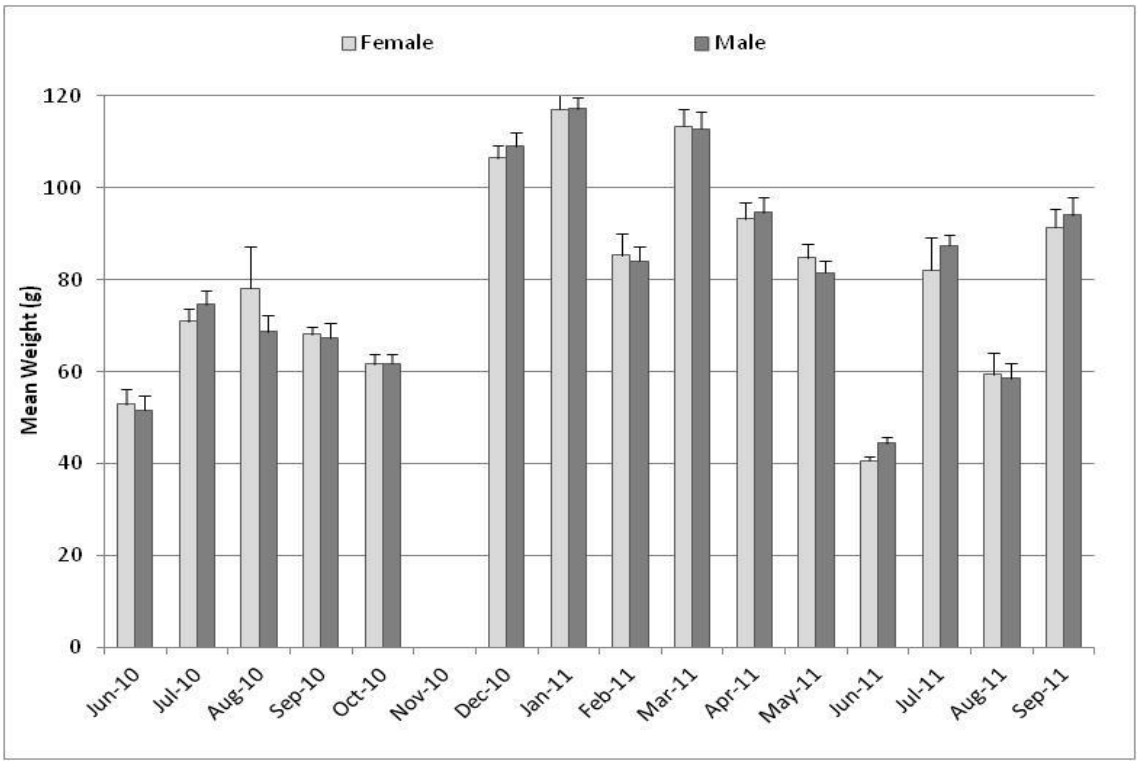


Figure 1: Mean weight (± S.E.) of female and male *Ensis siliqua* sampled at the Skerries region of the Irish Sea from June 2010 to September 2011.

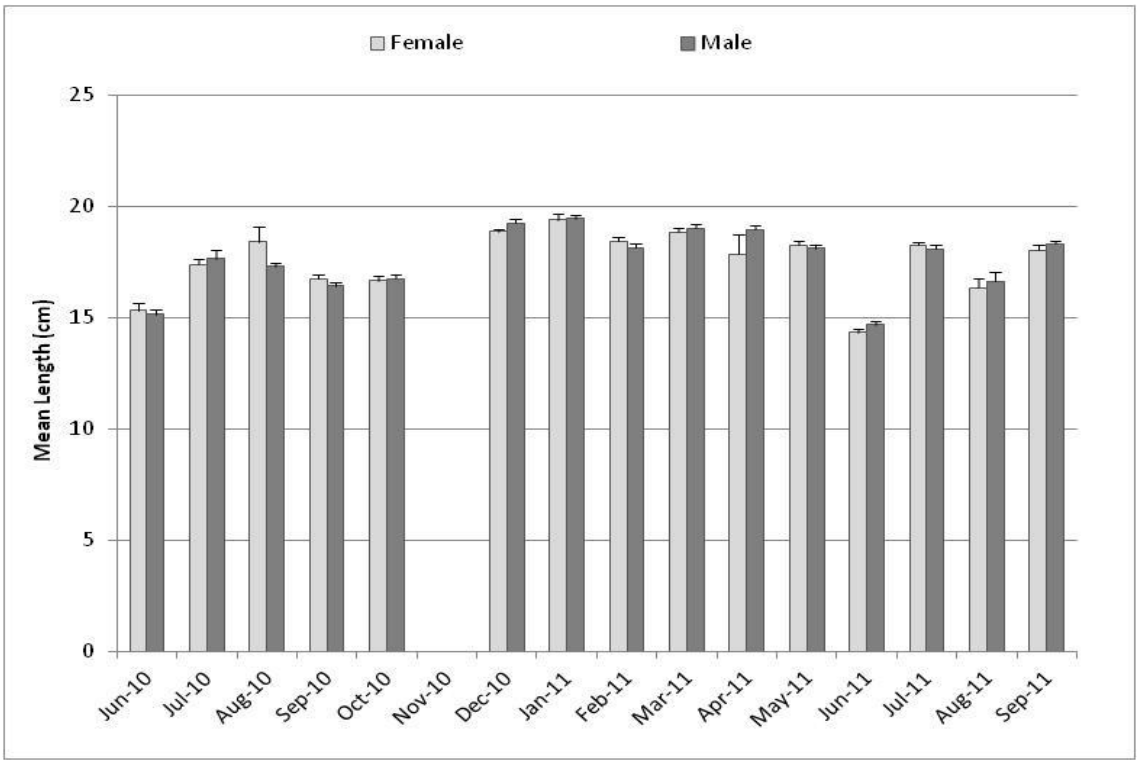


Figure 2: Mean length (± S.E.) of female and male *Ensis siliqua* sampled at the Skerries region from June 2010 to September 2011.

Sex Ratio

Of the 450 individuals sampled for histological analysis, 169 (38%) were female and 202 (44%) were male, with 79 (18%) termed "Inactive" (fig. 3). A Chi-squared (χ^2) with Yates correction was used to analyse sex ratios. The overall female: male sex ratio of 1:1.12 did not show a significant divergence from a 1:1 ratio (χ^2= 2.94, *df*= 1, *P* > 0.05), nor was there a statistically significant divergence within months, though the closest to showing a significant divergence was the May 2011 sample, with 10 female and 20 male *E. siliqua.* No hermaphroditic individuals were recorded in the sampled *E. siliqua*.

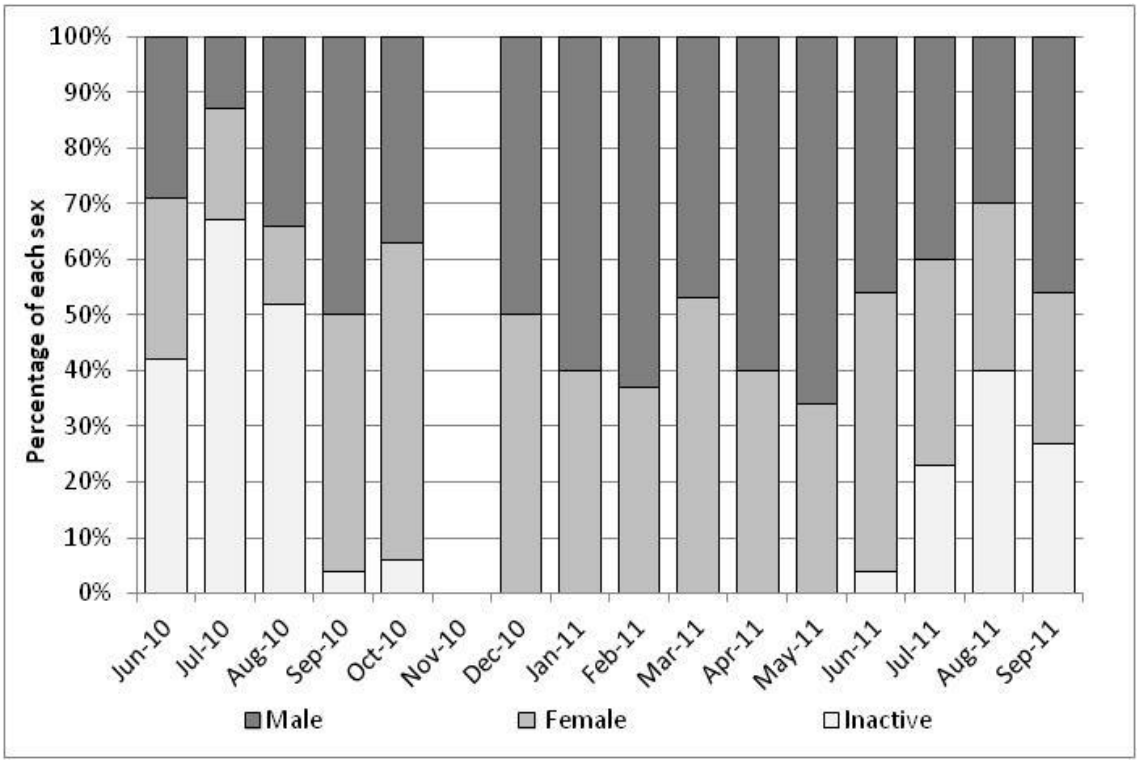


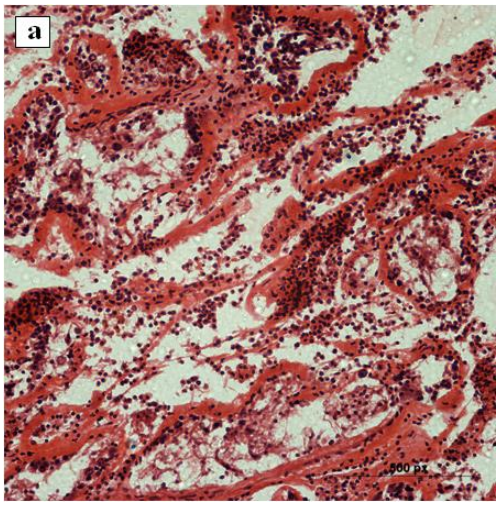
Figure 3: Sex ratio of *Ensis siliqua* sampled at The Skerries region over study period.

Sexual Cycle

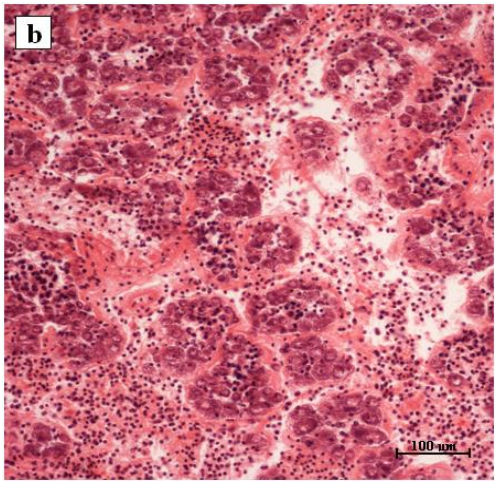
Females

During the course of the sampling period all six of the stages of gametogenesis described by Gasper and Monteiro (1998) were observed (fig. 4). In the first month of sampling, in June 2010, all of the female *Ensis siliqua* were spent. However, in July, 67% of females were spent and 33% were in the early developing stage. Early development rose to 75% in August, and all females sampled were identified as early developing by September 2010. The first late developing (41%) and ripe (35%) female individuals were recorded in October 2010, with most females (73%) in the late developing stage by December. The first spawning individuals (17%) were present in the January 2011 sample. This increased to 75% of female individuals spawning in March, and stayed at a high percentage (73%) until June 2011, when the first spent females were identified (20%). In July and August of 2011 female *Ensis siliqua* were mostly in the spent stage of gametogenesis at 82% and 100% respectively, with the final spawning individuals recorded as 18% of the sample in July 2011.

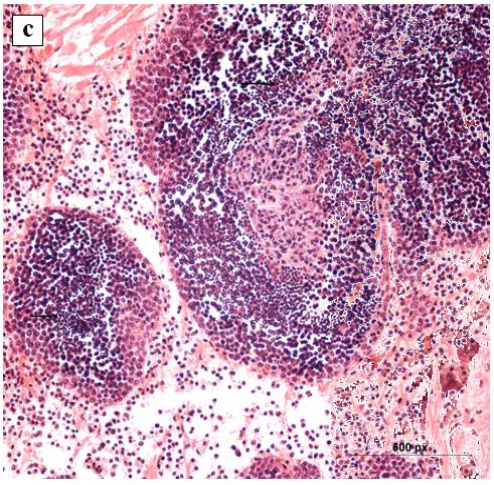
Indeterminate

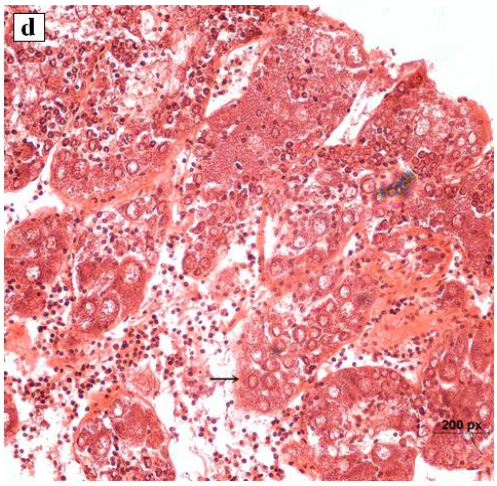


Female



Male





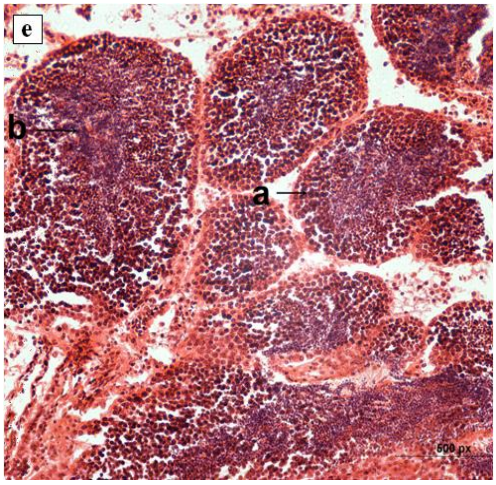


Figure 4. The different stages of gametogenesis of female and male *E. siliqua*, including the Indeterminate stage (a), female and male early development (b-c), late development (d-e), the ripe stage of both sexes. All photographs 20X.

Female Male

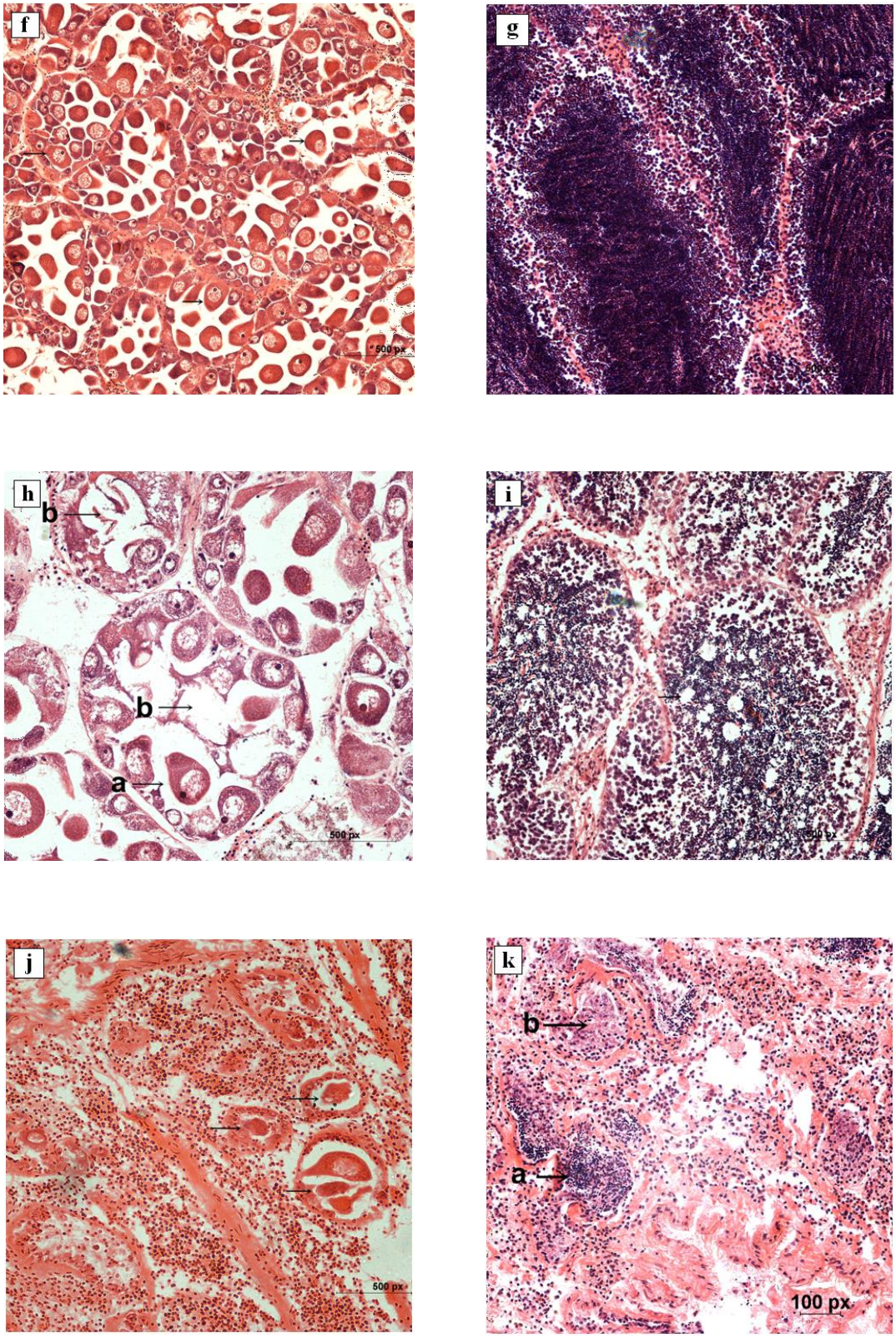


Figure 4 continued. The different stages of gametogenesis of female and male *E. siliqua*, including (f-g), spawning (h-i) and spent (j-k) stages. All photographs 20X.

Therefore, in 2011 spawning continued in June and July, while in the previous year all females were either spent or in early development by June 2010. All individuals sampled in September 2011 were in the early development stage (fig. 5).

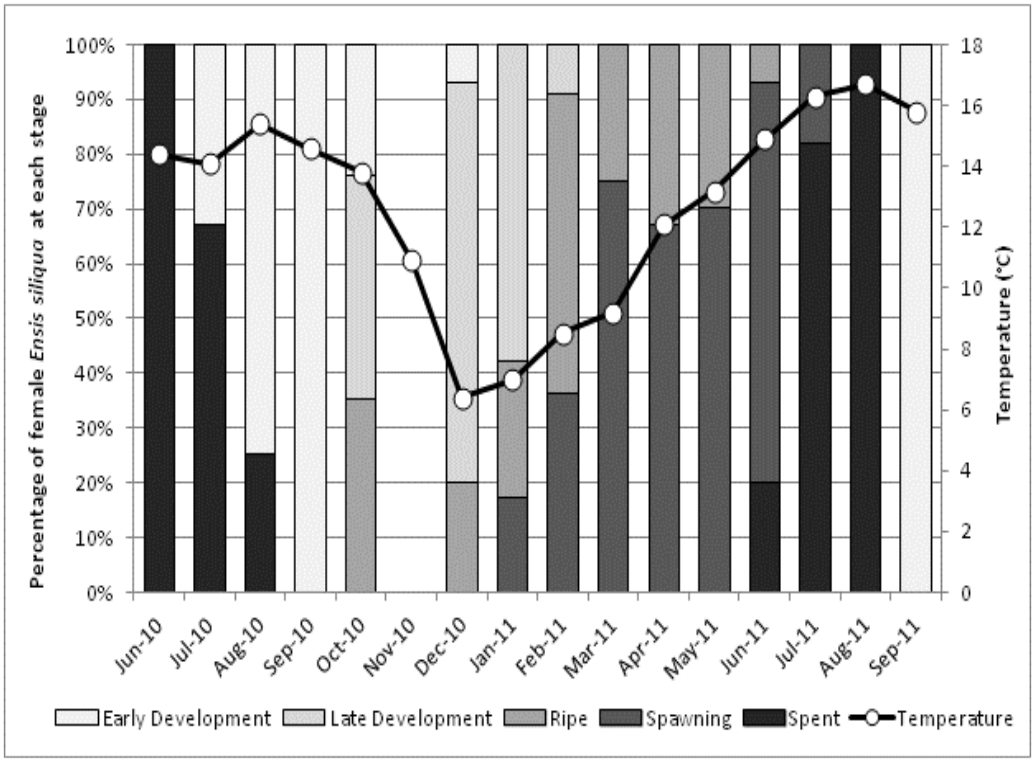


Figure 5: Stages of gametogenesis observed in female *Ensis siliqua* over the study period, with average monthly water temperatures shown.

Males

In the first samples collected in June 2010, only 11% of the male *Ensis siliqua* were still spawning, with 89% spent. The first male individuals in the early development stage were recorded in July (25%) with all males sampled in this stage in August and September 2010. 45% of male individuals were in the late development stage in October, when the first ripe male clams (10%) were recorded. By December 2010, 80% were in late development and 20% ripe, and the first spawning individuals were recorded in January 2011 (57%). All male *Ensis siliqua* sampled were spawning in March, with males either ripe or spawning until June 2011, when the first spent male clams were identified (8%). The majority of males were spent in July and August, with the final spawning males recorded in July 2011 (25%). As with female *E. siliqua*, 100% of male individuals were in the early development stage in September 2011 (fig. 6).

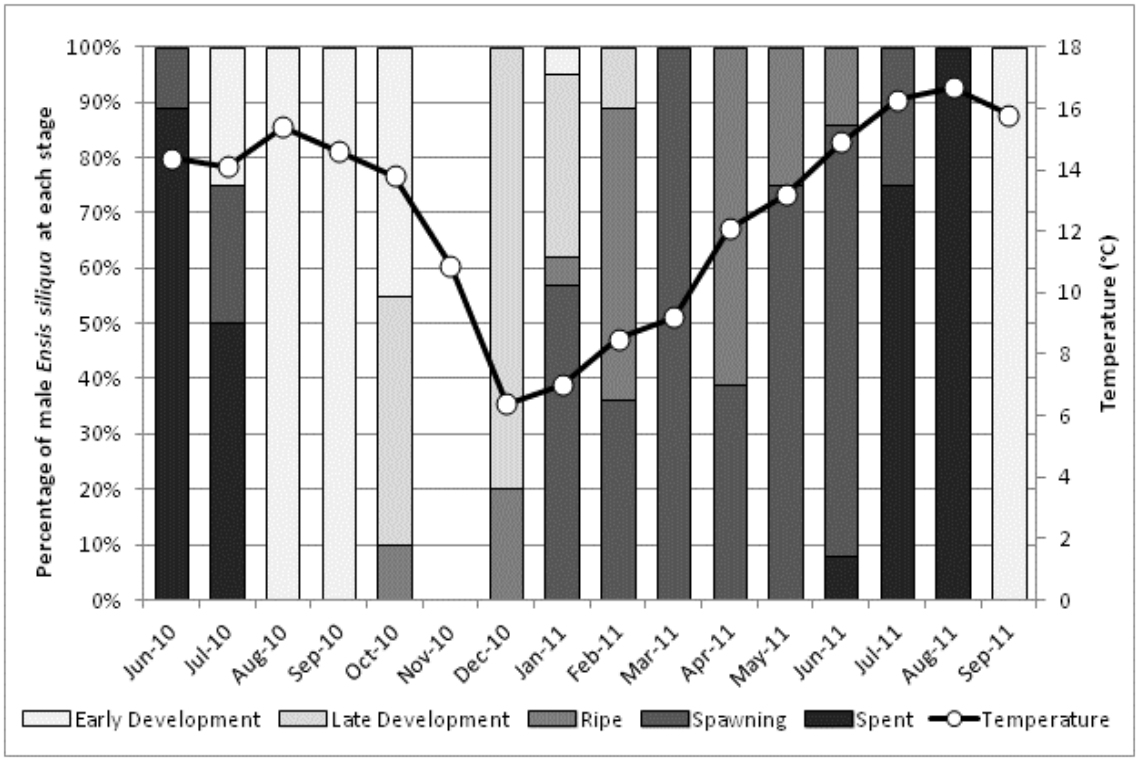


Figure 6: Stages of gametogenesis observed in male *Ensis siliqua* over the study period, with average monthly water temperatures shown.

Temperature

The mean monthly seawater temperature in the Skerries region was consistently higher in 2011 than 2009 and 2010, having the highest minimum and maximum temperatures recorded in the three years (fig. 7). The mean monthly water temperatures of 2011 were all 2°C warmer, on average, than those of 2010. The lowest mean monthly temperature reached during the sampling period was in December 2010, at 6°C, with a minimum temperature of 3.6°C recorded during that month. The highest mean temperature reached during the sampling period was in July 2011, at 16.7°C, with a maximum temperature of 17.9°C reached in July 2010, and in July and August 2011 (fig.7).

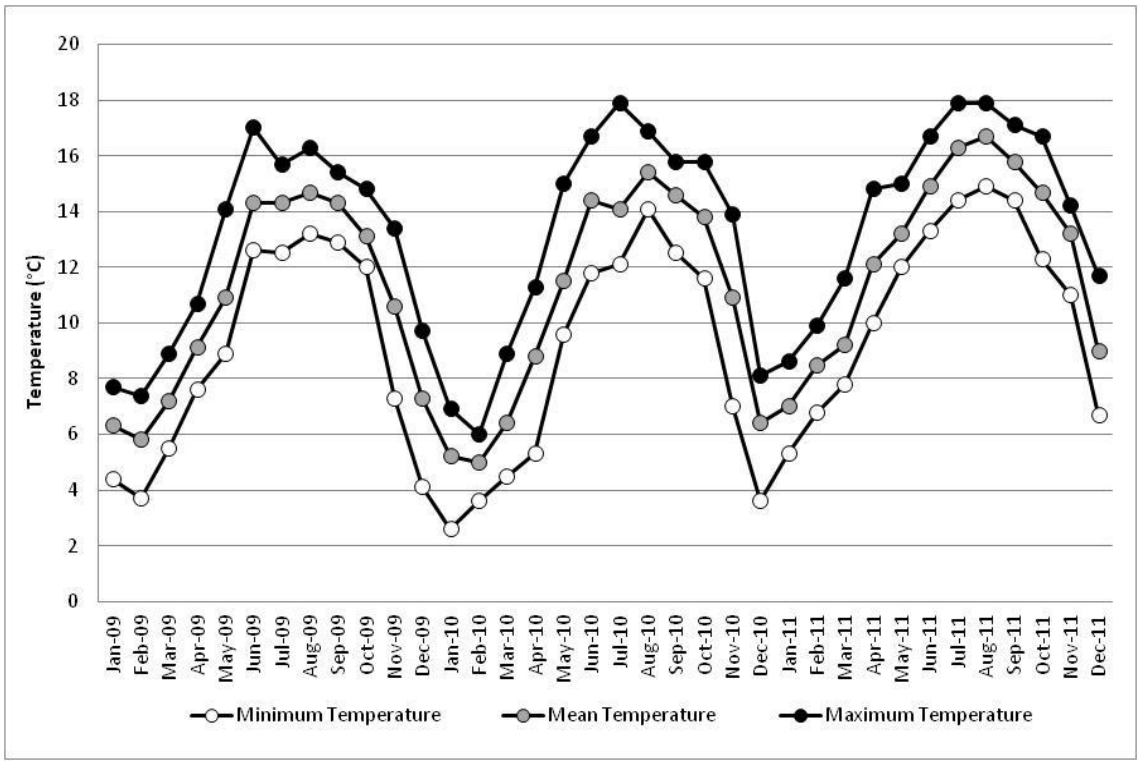


Figure 7: Water temperature readings for The Skerries region, from January 2009 to December 2011, showing mean, minimum and maximum temperatures recorded for each month.

3.4. Discussion

The *Ensis siliqua* sampled in the present study were both longer (average length: 17.5±0.2cm), and heavier (average wet weight: 80.0±2.0g), than in previous work in North-western Spain (Darriba *et al*., 2005), while similar to the mean length (17.19cm) of animals recorded in the Gormanstown bed in Ireland in 1999 (Fahy, 1999). The size and weight data of the present study could be affected by the fact that *E. siliqua* were obtained from a fishery, where only clams of a certain size are landed, while the Spanish clams were randomly collected from a clam bed by diving in the subtidal zone of Sardiñeiro Beach, Galicia. Past work on *E. siliqua* dredged in the Gormanstown fishing area in the Irish Sea (Fahy, 1999) revealed that male *E. siliqua* were both larger and more numerous than females. This contrasts with data in the present study, as the average length and weight of female *E. siliqua* are very similar and not significantly different to that of male individuals.

Ensis siliqua of the Skerries region were significantly lighter in weight in the summer months of June to August 2010 and 2011, than the winter months of December 2010 to February 2011, though the average length of individuals did not

significantly differ between the seasons. This is probably due to the clams spawning or being spent. Heavier weights outside this period are probably due to the increased presence of ripe gonads in both sexes. *Ensis siliqua* fisheries are carried out year-round in Ireland and other European countries. This differs from the fishing of other bivalve species (e.g. *Mytilus edulis* and *Ostrea edulis*), when fisheries are closed during the spawning period. As *Ensis siliqua* are currently sold by weight, and are significantly lighter in weight in the sexual rest period of the summer months, the economic benefits of fishing during the months of June to August should be considered. Decreased fishing during the sexual rest period of *E. siliqua* would potentially reduce the possibility of pathogenic load and diminished condition during this stressful time.

The female to male sex ratio of 1:1.12 in the present study was not significantly different from the 1:1 ratio previously described in southern Portugal (Gaspar and Monteiro, 1998) and northwest Spain (Darriba *et al*., 2005). There was no evidence of hermaphroditism in the present study, in keeping with low incidences of hermaphroditism in past studies (Gaspar & Monteiro, 1998; Fahy, 1999; South Wales Sea Fisheries Committee, 1999; Darriba *et al.*, 2005).

Six stages of maturation have previously been recognised in the gonadal cycle of *Ensis siliqua*, including a resting stage, during which the sexes are not distinguishable (Gaspar & Monteiro, 1998; Fahy, 1999). In the present study all of these six stages of gametogenesis were observed in Irish Sea *E. siliqua* at some time, and there was a distinct seasonal cycle in development. *Ensis siliqua* matured over the autumn and winter months of 2010, with all individuals either ripe or spawning from March to May 2011. This species has previously been described as gonochoric, with the sexes undergoing synchronous development and spawning (Gaspar & Monteiro, 1998; Fahy, 1999; Darriba *et al*., 2005). The data of the present study supports this theory, with female and male clams both beginning gametogenic development in July 2010, and the presence of spawning female and male individuals observed from January to July 2011.

Darriba *et al.* (2005) referred to the possibility of geographical differences in the reproductive pattern of *E. siliqua* caused by variations in environmental conditions. Brousseau (1995) stated that the duration of spawning in *Crassostrea virginica* populations along the Atlantic coast varies geographically, increasing as latitude decreases (Brousseau, 1995), while environmental conditions including food

availability and temperature have been shown to affect gametogenesis and spawning of *M. arenaria* on the western Atlantic coast (Brousseau, 1978). The first *E. siliqua* in a stage of early gametogenic development in both sexes were identified in the present study in July 2010, in comparison to November and December in Portugal and Spain (Gaspar & Monteiro, 1998; Darriba *et al.*, 2005). Average daily water temperatures for the Skerries region were recorded at 14.3°C in June and July of 2010, while seawater temperatures recorded in November and December in Portugal, and Spain average at 16.5°C and 14.5°C respectively (Sousa-Pinto & Araujo, 1998).

Ripe individuals were first present in October 2010 in the Skerries sample, when the monthly average water temperature was 13.8°C, with the majority of ripe shellfish present in February (8.5°C) and April (12.1°C) of 2011. These data correlate with the reproductive cycle of *Ensis siliqua* in Portugal and Spain, where ripe individuals were present in March and April (seawater temperature average of 16°C in Portugal, and 13.5°C in Spain). The spawning periods of the bivalve *Scrobicularia plana* has been described as lasting for a longer time in southern areas such as Portugal than in the northern regions of The Netherlands and Norway (Santos *et al*., 2011). In this case, water temperature was deemed the most important factor in shaping the reproductive pattern with more northern populations showing shorter reproductive periods, starting later in the year, and a lower reproductive output. The single spawning period observed in razor clams of the Skerries region, from March to June, is similar in timing to that of other European sites such as Portugal, Spain, Scotland and Plymouth (Lebour, 1938; Gaspar & Monteiro, 1998; Muir & Moore, 2003; Darriba *et al*., 2005), though all of the *E. siliqua* sampled in more southerly regions than the Skerries and Scotland produced spawning periods of a shorter duration than that of the Skerries razor clams. In contrast to *S. plana*, northern samples of *E. siliqua* would seem to have longer spawning periods than those of more southerly regions (Santos *et al*., 2011). *Ensis siliqua* of north Wales were reported to spawn later in the year, in July and August of 1999, (South Wales Sea Fisheries Committee, 1999), when the average monthly sea temperature reached 16.5°C (Norris, 2001), while Fahy (1999) observed a spawning period from mid-May to mid-August in the Gormanstown bed of *E. siliqua*, which is adjacent to the Skerries region of the Irish Sea. Water temperatures recorded from May to August at the Gormanstown bed in 1999 averaged 13.14°C, which is cooler than the average

water temperature of 15.27°C recorded in the present study in 2010. This variance in the timing of spawning periods could be due, in part, to differing environmental conditions such as water temperature and food availability, between sampling years and geographical sites.

Past work has indicated that annual seawater temperatures may rise in European, including Irish, waters in the future (Hiscock *et al*., 2004; Christensen *et al*., 2007). In the present study mean monthly data showed an increase in water temperature from 2009 and 2010 to 2011. The effect of environmental parameters on the reproductive process of bivalves is well documented in previous studies (Cano *et al*., 1997; Pazos *et al*., 1997; Gaspar & Monteiro, 1999; Ceballos-Vazquez *et al*., 2000; Luna-Gonzalez *et al*., 2000). Of these parameters, temperature is thought to be one of the most important (Darriba *et al*., 2004), as it is considered the main environmental cue for induction of gametogenesis and spawning in temperate regions (Harvey & Vincent, 1989; Grant & Creese, 1995). In July 2010, early development of *E. siliqua* began, and continued into August and September. In comparison, in 2011 the first *E. siliqua* in the early development stage of gametogenesis were only recorded in September, two months later than the previous year, and more similar in timing to *E. siliqua* in the warmer climes of Portugal and Spain (Gaspar & Monteiro, 1998; Darriba *et al*., 2005). Also, the spawning period extended for a longer time in the Skerries region in 2011, with 73% of individuals spawning in June, compared to 3% in June 2010. This extension of the spawning period would have resulted in a greater number of *Ensis siliqua* gametes released over a relatively longer period of time, and whether this would be beneficial to recruitment is unclear. Past work on *Macoma balthica* in the Wadden Sea has indicated that rising seawater temperatures could affect the stocks of this bivalve by lowering the reproductive output, with an earlier spawning period resulting in food availability during the pelagic phase being reduced (Philipart *et al*., 2003). If future climate change predictions come about, the effects of a longer spawning period on the health, growth, development and recruitment of *E. siliqua* will need to be considered in future management of this species in the Irish Sea.

In the present study, *E. siliqua* demonstrated some effects of short-term varying water temperatures on its general biology. With more dependence on aquaculture rather than fisheries in the future in Ireland (BIM, 2011), the need for

management of this and other commercial shellfish species under various climate change scenarios is necessary. Further studies examining the potential effects of climate variability are essential to allow for the long term fisheries management of this species.

Acknowledgements

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Chapter 4.The health status of two clam species in the Irish Sea.

Abstract

The soft shell clam, *Mya arenaria*, and the razor clam, *Ensis siliqua*, are widely distributed around the Irish Sea, where the latter is commercially exploited, and the former is a potential candidate for future exploitation. Though the reproductive biology of these two species has been previously investigated in Northern Europe, little or no data are currently available on their health status, and the possible effects of factors such as the gametogenic cycle or varying environmental temperatures on disease development. As this knowledge is essential for correct management of a species, live specimens of *M. arenaria* and *E. siliqua* were obtained monthly from March 2010 to September 2011, and examined for pathological conditions using histological methods. Additionally, to screen for as wide a group of pathogens as possible, extracted DNA of the gill tissue of sampled individuals was amplified using primers for Ostreid Herpes Virus (OHV), *Haplosporidium* spp. and generic bacterial 18s DNA, all of which have been found in this area in a variety of bivalve hosts. No pathogenic conditions were observed in *M. arenaria* using either histological or molecular methods. Prokaryote inclusions, trematode parasites, *Nematopsis* spp. and inflammatory pathologies were observed in the screened razor clams. This is the first time that these have been reported in *Ensis siliqua* in North European waters. The majority of pathogens detected in the present study were identified in individuals in an inactive or early development stage of their gametogenic cycle. The presence of pathological conditions of Irish *E. siliqua* is compared with razor clam species elsewhere in Europe, including the Iberian Peninsula.

Key words: *Ensis siliqua*, Irish Sea, *Mya arenaria*, *Nematopsis*, trematode.

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4.1. Introduction

The soft-shell clam, *Mya arenaria*, is widely distributed in coastal and intertidal soft substrates in European waters, including the Irish and British coasts (Eno *et al*., 1997; O'Riordan, 2002; Lasota *et al*., 2004). This clam is currently fished on North American shores (Beal, 2002; Connell *et al*., 2007), and is considered a species of interest for future commercial exploitation in the Irish Sea (S. Malham *pers. comm.*). *Ensis siliqua* is a native species in Europe, and is distributed along the European Atlantic coast from the Norwegian Sea south to the Mediterranean and along the Atlantic coast of Morocco (Darriba *et al*., 2005; Varela *et al*., 2007; DaCosta *et al*., 2010). It is abundant in the British Isles, and widely distributed along the east coast of Ireland (Fahy, 1999), where it is currently harvested by commercial fisheries. Because of the increasing global demand for shellfish, including *M. arenaria* and *E. siliqua*, natural beds of marine bivalve species are under pressure (Darriba *et al*., 2005; Hauton *et al*., 2007). The future commercial exploitation of these species will need careful management to ensure sustainability and avoid fishery over-depletion.

To manage an exploited species, knowledge of its biology, including the health status and the effects of environmental irregularities on this status, is essential (Bignell *et al*., 2008; Lopez *et al*., 2011). Pathogenic conditions can affect the fecundity, growth, condition, and morbidity and mortality rates of a population in the wild, leading to significant ecological implications, and can have negative consequences for the commercial value of an exploited species (Renault & Novoa, 2004; Villalba *et al*., 2004). Histopathology of aquatic organisms is a valuable tool for providing health assessments of individuals and populations as it reveals the range of pathogens that may affect morbidity and mortality in these populations (Bignell *et al*., 2008). Furthermore, advances in molecular techniques have allowed for the rapid detection of pathogenic factors present in marine species, supporting, and sometimes proving more sensitive, than histological detection methods (Kotob *et* al., 1999; Renault *et al*., 2000; Dungan *et al*., 2002; Ji *et al*., 2004; Villalba *et al*., 2004).

Presently, no data are available on the pathogens that may be present in European *M. arenaria*, though several diseases and parasites, including neoplasia, *Vibro parahaemolyticus* and *Perkinsus* sp. protozoans, have been documented in this

species on the east coast of North America, which are thought to influence the increasing mortalities of soft shell clams in that area (Earle & Crisley, 1975; Brown *et al*., 1977; Oprandy *et al*., 1981; Brousseau & Baglivo, 1991a; 1991b; Farley *et al*., 1991; McLaughlin & Faisal, 1999; Dungan *et al*., 2002). In Ireland, the *M. arenaria* used in this study are found in an area of oyster cultivation where herpes virus is present (Lynch *et al.*, 2010), and *Vibrio* species have been found associated with a range of invertebrates in this area (unpublished work, Emer Morgan, UCC.). A range of parasites and pathological alterations including protozoans, ciliates, trematodes and neoplastic disorders have recently been identified in *Ensis siliqua* on the Spanish coast, but to date there has been no report of these conditions present in razor clams further north in Europe (Perez, 2011; Ruiz *et al*., 2013).

Due to the lack of information currently available on the health status of *M. arenaria* and *E. siliqua* in Northern Europe, this study was undertaken to investigate the health status of these clams, and determine to what extent parasites and pathological conditions are present in these two species, using both histological and molecular methods. The effects of the reproductive cycle, and the environmental temperature on the health status, was also explored.

4.2. Materials and Methods

Sampling Areas of clams

Bannow Bay, Co. Wexford is an estuary on the southeast coast of Ireland (52° 27'N 006° 47'W). It covers an area of *ca* 1050 ha, is 8km long and between 1 and 3km wide (Malham *et al.*, 2009) (Figure 1). The intertidal habitat consists of mud and sand flats, and approximately 75% of the bay is uncovered at average low tide. There is oyster cultivation on the eastern side of the estuary, and the surrounding rural land mainly supports dairy and arable farming. Shellfish, including cockles, clams and mussels, are collected here by hand year-round, and the estuary is used for leisure activities during low tide. A large, wild bed of *M. arenaria* is known to have been present in this estuary for the last 10 years (A. Whittaker, UCC *pers. comm*.). Flaxfort Strand, Courtmacsherry Bay, County Cork, Ireland (51° 38' N, 8° 41' W) is a semi-exposed bay composed of sand flats on the southwest coast of Ireland. A small freshwater stream flows into the mouth of the bay, and extensive algal mats are present in the summer months, usually comprised of *Enteromorpha*

species (Morgan *et al*., 2012). The surrounding land is mainly used for farming and tourist activity including walking and horse-riding.

Figure 1: Map of the Irish Sea showing sampling sites for *Mya arenaria* and *Ensis siliqua* (modified from http://www.ferryports.net/ireland-ferries.cfm).

The Skerries is a fishing area on the east coast of Ireland, bounded by a line from Hampton Cove on the coast, east to a point at 06° W, 53°36.3’ N at sea, north to the point 06° W, 53°34.5’N, and west to Shenick Island on the coast (Dore & Nolan, 2008). The benthos in this part of the Irish Sea is mainly sand, with reef and rock around the five small islands off the coast. Commercial fishing currently takes place in the Skerries and surrounding fishing areas, with shellfish harvested by fluidised bed hydraulic dredging, though commercial vehicles now only fish to fill specific orders (Fahy, 1999; Fahy, 2011). Oxwich Bay, Swansea, Wales, is a broad, sandy beach located on the South Gower Peninsula in Wales (51.557 N, 04.155 W). The interior of Gower consists mainly of farmland and common land, and the landscape surrounding the beach features sand dunes, salt marshes and woodland. Oxwich Beach itself is backed by a wetland site at the rear of the dunes which forms Oxwich Burrows National Nature Reserve.

Soft-shell clam, *M. arenaria,* specimens (n=30) were collected by digging in the lower intertidal of Bannow Bay each month from March 2010 to June 2011 (fig. 1). Thirty one *M. arenaria* were also collected in Flaxfort Strand in June 2011, to compare individuals of this species from two areas in Ireland. Thirty live *Ensis siliqua*, which had been fished in the Skerries region of the Irish Sea were obtained monthly from June 2010 to September 2011, from a commercial shellfish wholesaler on the east coast of Ireland, as outlined in Chapter 2 (Figure 2). Thirty four *E. siliqua* were fished by hand at Oxwich Beach in September 2011, to compare razor clams from this region to ones collected in the Skerries region.Clam specimens were transported to a cold room (4°C) within four hours of collection and left overnight for dissection the next morning. Each clam was numbered and the total wet weight (g) and shell length from anterior to posterior end (cm) of each individual were recorded.

Histological Processing

A section of the body of each clam was cut out, which contained the gonad, renal gland and digestive tract, and sections of the gill and mantle. The tissue was fixed in Davidson's solution for 48 hours and stored at 4°C. Slides were prepared using standard histological techniques, where tissues were dehydrated in alcohol, cleared in xylene, embedded in paraffin wax, sectioned at 7µm, and stained with Harris' Hematoxylin and Eosin before being mounted (Porter, 1974). The prepared microscope slides were examined using 10X, 20X, and 40X magnifications, to determine sex and stage of reproductive development. Individuals were also screened for the presence of any parasites and abnormal conditions or pathologies. The process involved in determining the stage of gonadal development of each individual is outlined in Chapter 2 and Cross *et al.* (2012) for *M. arenaria* and Chapter 3 for *E. siliqua*.

DNA Extraction

All gill tissue samples of clams were preserved in 90% Ethanol at dissection. DNA extraction was carried out by Chelex-100 resin (Bio-Rad) extraction, using 10mg sections of gill tissue and 100µl of CHELEX, heated at 90°C for 70 minutes, and spun at 13,000rpm for 5 minutes. 100ul of supernatant was extracted and DNA was stored at 4°C, or -20°C for long-term storage.

PCR amplification

Screening for Ostreid Herpes Virus

The extracted DNA of 463 sampled *M. arenaria* was subjected to PCR amplification using Ostreid Herpes Virus (OSHV)-specific primers C2/C6 and OSHV-For/OSHV-Rev (Table 1) (Renault & Arzul, 2001). PCR reaction mixtures using the primers C2/C6 contained 10µl 5x Buffer, 0.25µl $MgCl_2$ (25mM), 5µl dNTPs (1.25mM), 1µl of each primer (100µM), 0.25µl Ampli-Taq (Invitrogen), 33.5µl dH_2O and 1µl template DNA in a total volume of 52µl. The C2/C6 reaction mixtures were cycled in a Techne TC-Plus thermocycler under the following conditions: 94°C for 2 min; 35x (94°C for 60 sec, 50°C for 60 sec, 72°C for 60 sec); 72°C for 5 minutes (Renault & Arzul, 2001). The OHV-For/OHV-Rev primers were used in 25ul reactions of 5µl 5x Buffer, 0.25µl $MgCl_2$ (25mM), 2.5µl dNTPs (1.25mM), 0.5µl of each primer (100µM), 0.25µl Ampli-Taq (Invitrogen), 15µl dH_2O and 1µl template DNA. The thermocycling conditions were as follows: 95°C for 1 min; 35x (94°C for 20 sec, 56°C for 30 sec, 72°C for 30 sec); 72°C for 7 minutes (Renault & Arzul, 2001).

Screening for bacterial DNA

Mya arenaria (n=463) and *E. siliqua* (n=484) samples were screened for the presence of bacterial DNA using the generic forward primer 18Scom-F and reverse primer 18Scom-R (Zhang & Lin, 2005). PCR amplification was carried out in 25µl volumes, containing 5µl 5x Buffer, 0.5µl $MgCl_2$ (25mM), 5µl dNTPs (1.25mM), 0.25µl of each primer (100µM), 0.1µl Ampli-Taq (0.625units) (Invitrogen), 12.9µl dH_2O and 1µl template DNA. Amplifications were performed on a Techne TC-Plus thermocycler, and were carried out under the following conditions: 95°C for 1 min; 35x (94°C for 20 sec, 56°C for 30 sec, 72°C for 30 sec); 72°C for 7 minutes.

Screening for haplosporidia

Mya arenaria (n=463) and *E. siliqua* (n=484) samples were screened for the presence of haplosporidian species using the forward primer (Hap-F1) and reverse primer (Hap-R3) in a PCR amplification, as these primers amplify regions of the small subunit ribosomal DNA (SSU rDNA) of most haplosporidian species (Renault *et al*., 2000). PCR reactions consisted of 50µl volumes, containing 10µl 5x Buffer,

1.6µl $MgCl_2$ (25mM), 8µl dNTPs (1.25mM), 0.5µl of each primer (100µM), 0.11µl Ampli-Taq (0.625units) (Invitrogen), 28.29µl dH_2O and 1µl template DNA of *M. arenaria* and *E. siliqua* samples. Amplifications were performed on a Techne TC-Plus thermocycler, and were carried out under the following conditions: 95°C for 1 min; 35x (94°C for 30 sec, 48°C for 30 sec, 72°C for 60 sec); 72°C for 5 minutes. PCR products were viewed on a 2% Agarose gel.

Table 1. Details of the primers used in PCR reactions investigating the health status of *Mya arenaria* and *Ensis siliqua*.

DNA Amplified	Primers	Resulting fragment size
Ostreid Herpes Virus (A)	C2 (5'-CTC TTT ACC ATG AAG ATA CCC ACC-3') C6 (5'-GTG CAC GGC TTA CCA TTT TT-3')	800bp
Ostreid Herpes Virus (B)	OHV-For (5'-GGA GCT GCG GCG CTA TGG AT-3') OHV-Rev (5'-GGA GGT TCA GGT CTT TGC GTT CCG-3')	350bp
18Scom Bacterial	18ScomF (5'-GCT TGT CTC AAA GAT TAA GCC ATG-3') 18ScomR (5'-CAC CTA CGG AAA CCT TGT TAC GAC-3')	300-400bp
Haplosporidian spp.	HAP-F1 (5'-GTT CTT TCW TGA TTC TAT GMA-3') HAP-R3 (5'-AKR HRT TCC TWG TTC AAG AYG A-3')	300-400bp

4.3. Results

Histological Results

Pathology of Mya arenaria

Screening of histological sections of the visceral mass, gill and mantle of the 494 *Mya arenaria* revealed no parasites or pathological indications of disease in any of the individuals sampled in Bannow Bay over the 16 month study period of March 2010 to June 2011, nor those collected in Flaxfort Strand in June 2011.

Pathology of Ensis siliqua

Of the 484 *Ensis siliqua* screened in the current study, only six individuals were identified with potential infections, all of which were sampled in the Skerries region of the Irish Sea. No pathogens were identified in the 34 individuals sampled in Oxwich Bay, Swansea.

Of the six *E. siliqua* sampled in the Skerries region of the Irish Sea that showed the presence of some parasites, the following were observed; Prokaryote

inclusions (n=2), Trematode metacercaria (n=1), *Nematopsis* spp. oocysts or sporozoites (n=3), and eosinophilic bodies (n=2). Prokaryote inclusions were identified in one early developing female individual sampled in September 2010 (Figure 2b) and in a spawning female *E. siliqua* sampled in March 2011 (Figure 2a).

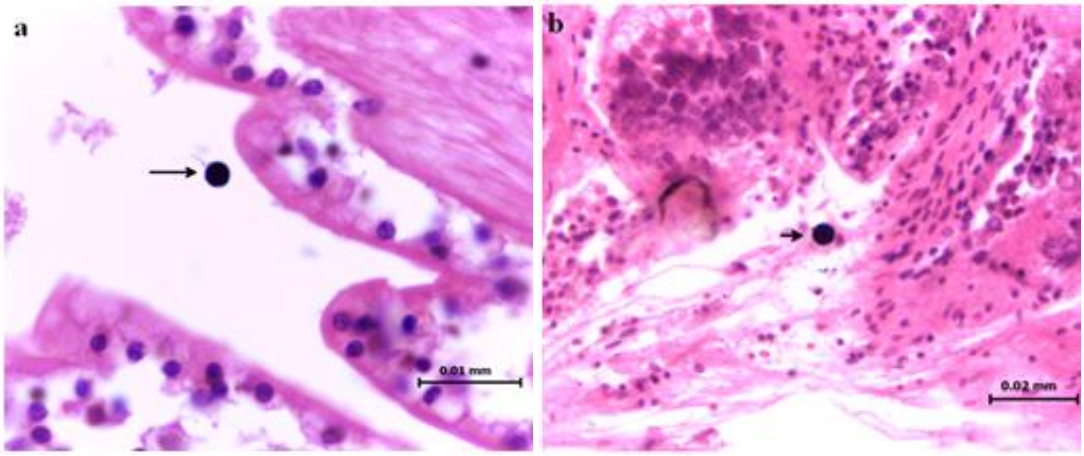


Figure 2 a-b. Histological sections showing prokaryote inclusion in *E. siliqua* (H & E stain). Arrows indicate prokaryote inclusion (scale bar = 10µm and 20µm respectively).

A trematode metacercarium was identified in one early developing female individual sampled in September 2010 (Figure 3).

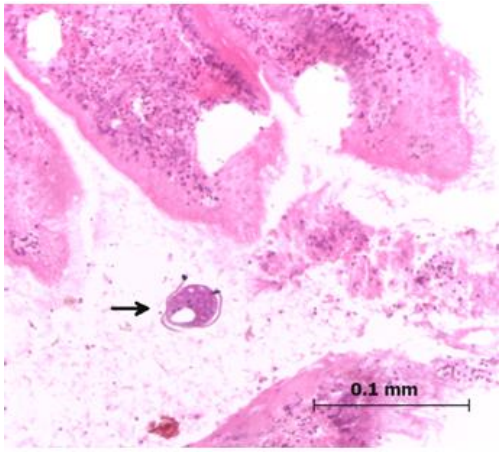


Figure 3. Histological section showing Trematode metacercaria in *E. siliqua* (H & E stain). (scale bar = 100µm)

Three *Nematopsis* species oocysts were observed in a single spawning female *E. siliqua* sampled in March 2011 (Figure 4a), two in a female from September 2011, and four in a single early developing male clam sampled in August 2011 (Figure 4b).

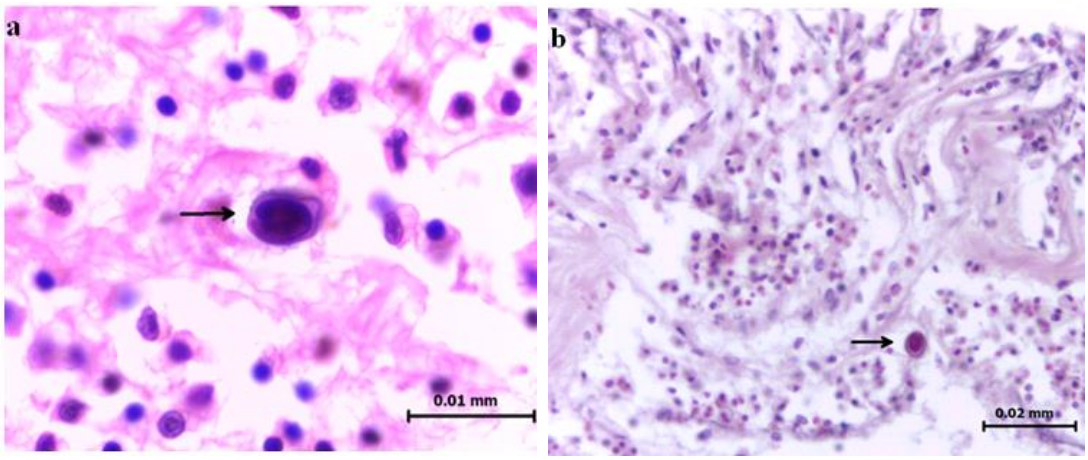


Figure 4 a-b. Histological sections showing *Nematopsis* spp. in *Ensis siliqua* (H & E stain). Arrows indicate *Nematopsis*. (a) *Nematopsis* oocyst or sporozoite. (b) *Nematopsis* spp. in the gill tissue of *E. siliqua* (scale bar = 10µm and 20µm respectively).

The same male individual sampled in August 2011 also displayed the presence of eosinophilic bodies (Figure 5a), which may be indicative of stress (Bignell *et al*., 2008), as did an individual of indeterminate sex in July 2010 (Figure 5b).

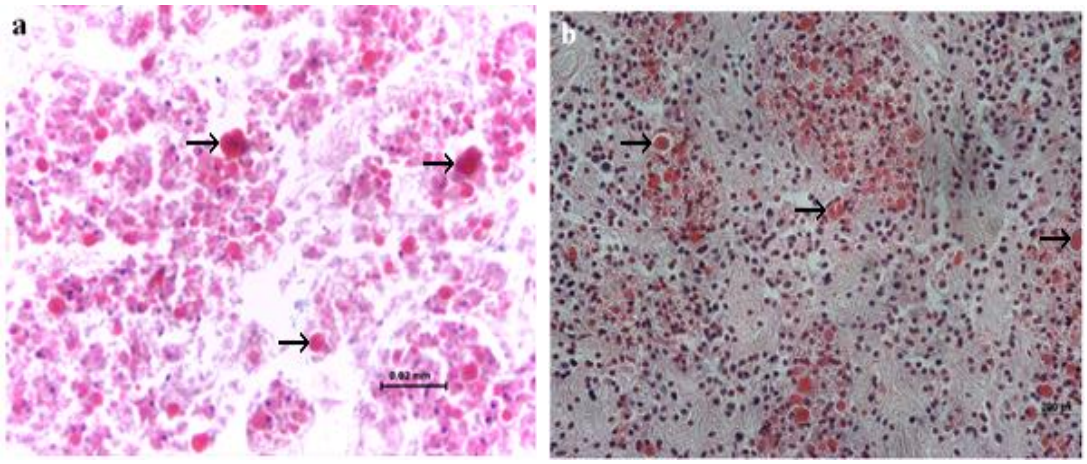
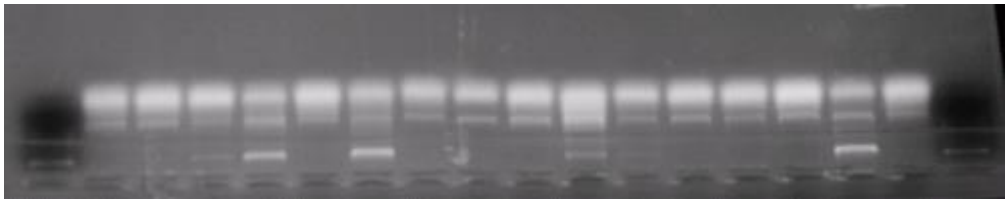


Figure 5 a-b. Histological sections showing unidentified eosinophilic bodies present in *E. siliqua* tissue (H & E stain). Scale bar = 20µm.

Molecular Results

Mya arenaria

Polymerase chain reaction (PCR) amplification of *M. arenaria* samples using both sets of primers specific to Ostreid Herpes Virus (OSHV) resulted in different sized fragments of DNA for all individuals, when the resulting amplicons were run on a 2% agarose gel with one positive control per gel. However, subsequent sequencing of 10 amplicons completed by a commercial company (Eurofins) detected only clam DNA. Amplicons were also present in all individuals after PCR amplification using the generic bacterial 18Scom primers (Figure 6). Ten individuals were confirmed as *M. arenaria* DNA. No amplification took place following use of the Haplosporidian species SSU rDNA primers.

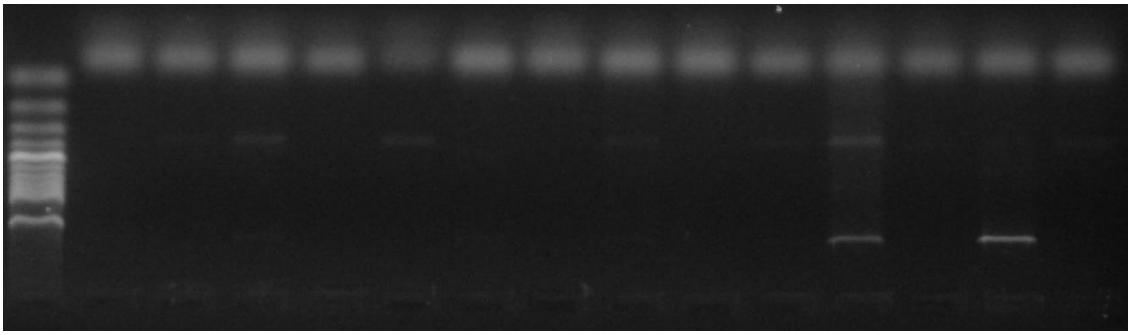


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 6. Photograph of 2% agarose gel showing amplification of *M. arenaria* DNA in all individuals following use of 18Scom generic bacterial primers. Lanes 1+ 18: DNA ladder; lanes 2-17: *M. arenaria* samples.

Ensis siliqua

Following screening of 484 *E. siliqua* samples for the presence of bacterial DNA, 10 resulting amplicons of the PCR using 18Scom primers were sequenced but only clam DNA was detected (Figure 7).



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 7. Photograph of 2% agarose gel showing amplification of generic bacterial 18Scom in samples of *E. siliqua* (number 4-17). Lane 1: DNA ladder; lane 2: negative control; lanes 3-15: *E. siliqua* samples number 4-16.

No fragments of DNA were amplified when using the Hap-F1 and Hap-R3 primers, implying a lack of *Haplosporidium* species in sampled *E. siliqua* from the Skerries region of the Irish Sea.

4.4. Discussion

To date, little or no disease or pathogens have been reported in *Mya arenaria* on European shores (Strasser *et al.,* 1999), despite a number of diseases and parasites being present in the *M. arenaria* populations from North America. No parasites, pathogenic states or disease was found in any of the 463 soft shell clams sampled in two sites in the current study. Histological and molecular screening revealed a healthy population of *M. arenaria* that undergoes a yearly cycle of gametogenesis, which has little or no effect on the weight and condition of the clams (Cross *et al*., 2012). While varying environmental temperatures would seem to have affected the timing of gametogenesis in this population (Cross *et al*., 2012), neither energy used for spawning, nor the stress of oscillating environmental temperatures resulted in a disease state (see Chapter 2), as has been recorded in *M. arenaria* of other areas (Brousseau & Baglivo, 1991a), and other bivalve species (Cheng & Combes, 1990; Chu & La Peyre, 1993; Goulletquer *et al*., 1998; Harvell *et al*., 1999; Gagnaire *et al*., 2006; Malham *et al*., 2009).

Within two of the same sites that were examined in the present study, Bannow Bay and Flaxfort, sampled bivalves of other species such as *Cerastoderma edule* have contained a heavy prevalence of trematodes, neoplasia and *Nematopsis* spp. (Morgan *et al.*, 2012), while Ostreid Herpes Virus has been identified in *Crassostrea gigas* in Bannow Bay (Lynch *et al.*, 2012). OsHV1 has also been identified in other molluscan species such as French scallops, *Pecten maximus*, and *Ruditapes decussatus* in the past (Renault & Novoa, 2004). PCR amplification of *M. arenaria* using primers to identify OsHV1 and bacterial 18Scom resulted in amplicons that when sequenced revealed only host DNA.

In the present study no pathogens were found in *E. siliqua* sampled in Oxwich, Swansea, and only 1.2% of *E. siliqua* clams screened from the Skerries showed the presence of parasites or pathologies, and the majority of the symbionts and conditions observed did not cause host damage. Little was known about parasites and diseases associated with *Ensis* species until relatively recently (Lopez *et al*., 2011). Previous work has described parasites and pathological alterations such

as protozoans, ciliates, trematodes, germinoma, disseminated neoplasia, basophilic inclusions, intracellular prokaryote-like organisms, viral inclusions, and bacterial proliferations including *Vibrio,* in razor clam species in the important *Ensis* fisheries region of Galicia in Spain (Conchas *et al*., 2005; Fernandez-Tajes *et al*., 2010; Perez, 2011; Darriba *et al.*, 2012; Ruiz *et al*., 2013).

Though gender and stage of gametogenesis were noted for all individuals, a valid relationship between these factors and the presence of parasites could not be investigated due to the low incidence of parasites. Prokaryote inclusions were detected in two female individuals of *E. siliqua* from the Skerries sample but at low prevalence and intensity. Therefore, at this prevalence, they are not considered a pathological problem as observed in *Ensis* species from Galicia, Spain (Ruiz *et al.*, 2013). Trematodes in large numbers may cause total castration of *E. siliqua*, as observed in one study in Galicia (Perez, 2011), while low prevalence without host inflammatory reaction was observed in another (Ruiz *et al*., 2013). In the present study only two *E. siliqua* individuals were infected with trematode metacercaria. *Nematopsis* was observed in three clams in the present study, while its presence has previously been recorded in *E. arcuatus* clams sampled in Cill Chiarain Bay, Co. Galway, Western Ireland (Fahy *et al.*, 2002). This genus has previously been described in several molluscs, including the cockle *C. edule* and clam *R. decussatus* in Portugal (Azevedo and Cachola, 1992), and *Ruditapes decussatus* and the razor clam species *E. arcuatus* and *Ensis siliqua* in Spain (Navas *et al*., 1992; Conchas *et al*., 2005) without detrimental health effects (Ruiz *et al.*, 2013), though there are references about associated mortalities in cockles (Azevedo and Cachola, 1992). In the present study eosinophilic bodies were observed in two individuals of indeterminate sex post-spawning. The highest prevalence of these eosinophilic bodies, which may be indicative of environmental stress, was also recorded in mussels during and after spawning in the UK (Bignell *et al*., 2008).

Prokaryote inclusions were detected only in female individuals of *E. siliqua*, at different stages of their gametogenic cycle. Both females additionally contained *Nematopsis* oocytes, and one revealed the single example of a trematode metacercarium. *Nematopsis* oocytes were also identified in an early developing male. The prevalence and severity of pathogenic parameters showed fluctuations throughout the seasons in previous work with mussel species (Bignell *et al*., 2008). 1950Parasites and pathologies were observed in *E. siliqua* from the Skerries during

the summer months but numbers were too low to examine any potential relationship with season, environment or gametogenesis.

The lack of any disease or parasites found in the sampled *M. arenaria* would indicate that this species is very healthy in its European range, especially in the wild population of softshell clams of Bannow Bay. This is in contrast to North American populations, where high density aquaculture takes place. A low level of parasites and pathogens were observed in *E. siliqua* samples from the Irish Sea, leading us to conclude that razor clam populations are also healthy in this area. The production of *E. siliqua* in the Galicia region of Spain has increased dramatically since 2005 due to the development of specific plans for its exploitation, while *Mya arenaria* has been harvested commercially year - round since the mid-1800s in North America (Wallace, 1997). The intensive culture of clams in these areas may have resulted in *E. siliqua* and *M. arenaria* populations being under stress due to over-crowding, which could make them more susceptible to infections and disease (Renault & Novoa, 2004). Knowledge of the pathological aspects of a commercial species is also essential for correct management of fisheries, and to ensure future sustainable aquaculture of the species. Future exploitation in the Irish Sea with increased pressures on stock may influence the health status of bivalve species. Data from the present study indicates healthy populations of *M. arenaria* and *E. siliqua* in the Irish Sea, with little or no prevalence of disease.

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Chapter 5

Mya arenaria population genetics throughout its contemporary range.

Abstract

Mya arenaria (L.), the softshell clam, is currently widespread on the east and west coasts of North America. This bivalve also occurs on western European shores where the post Pleistocene origin of the species, whether introduced or relict, has been debated in the past. In this study 320 *M. arenaria* from eight sites in Europe, and North America were collected and at least ten clams from each of seven of the locations were examined for mitochondrial DNA variation by sequencing a section of the cytochrome oxidase I (*CO*I) gene, while 34 individuals from all eight locations were sequenced to assess variation in the 16S ribosomal RNA gene. Finally, 10 microsatellite loci were investigated in all 320 clams. Both mitochondrial *CO*1 and all nuclear markers showed reduced levels of variation in certain European samples. The same common haplotypes and alleles were present throughout the range of *M. arenaria*. However, significant differences were identified in haplotype and allelic composition between most samples, particularly those from the two continents of Europe and North America. These findings support the hypothesis of post Pleistocene recolonisation of European shores from eastern North America (and the recorded transfer of clams from the east to the west coast of North America in the 19C). However, the presence of some endemic mitochondrial haplotypes and microsatellite alleles in European samples suggest southern European refugia, from which clams might have spread north and mixed with those introduced from eastern North America.

Keywords: COI, Europe, Irish Sea, microsatellite loci, mitochondrial DNA, *Mya arenaria*, North America.

5.1. Introduction

Marine organisms live in a constantly changing environment, and the genetic patterns that are observed in their populations today are the result of both current and historical factors (Avise, 2000). Marine species have been classically described as showing less geographical differentiation than terrestrial species due to the lack of physical barriers to genetic exchange in the ocean, and the large population sizes of most marine species (Launey *et al.*, 2002; Kenchington *et al.*, 2006). Additionally, the existence of a pelagic larval phase in the life cycle of most marine invertebrates is thought to ensure high levels of effective dispersal, enhancing population connectivity over large distances (Luttikhuizen *et al.*, 2003; Dupont *et al.*, 2007; Arias-Perez *et al.*, 2012). The idea that marine invertebrate populations tend to be "open" was reinforced by early genetic studies which indicated a lack of genetic differentiation in bivalves such as *Mytilus* spp., and geoduck clams (*Panopea generosa)* across wide geographic scales (Levinton & Koehn, 1976; Skibinski *et al.*, 1983; Benzie & Williams, 1992; Palumbi, 1996; Vadopalas *et al.*, 2004). This generalization, however, would seem to be dependent on the species studied, and particularly the markers used, as an increasing number of new studies have described distinct genetic structuring for invertebrate and marine fish species at both large and small geographical scales (Beaumont, 1982; Beaumont & Zouros, 1991; Arnaud *et al.*, 2000; Launey *et al.*, 2002; Luttikhuizen *et al.*, 2003; Jorgensen *et al.*, 2005; Kenchington *et al.*, 2006; Banks *et al.*, 2007; Hemmer Hansen *et al.*, 2007; Arias-Perez *et al.*, 2012).

The softshell clam, *Mya arenaria* (L.), is a soft sediment bivalve, found most abundantly in intertidal and shallow sub tidal areas (Strasser, 1999). This clam species occurs over a wide geographical range on western European shores, from northern Norway to the Mediterranean Sea (Strasser, 1999; Conde *et al.*, 2012), and on the west and east coasts of North America, where it is of commercial importance (Beal, 2002; Connell *et al.*, 2007; Weston & Buttner, 2010). *Mya arenaria* possesses those traits commonly associated with a weak population genetic structure, such as wide environmental tolerance, high fecundity, and a relatively long planktonic larval stage of up to 35 days, compared with other molluscan species including *Crepidula fornicata*, *Rapana venosa* and *Adalaria proxima*, which are also broadcast spawners (Strasser, 1999; Kenchington *et al.*, 2006; Shanks, 2009).

An interesting aspect of the phylogeographic history of *M. arenaria* is that the species is thought to have been native to Europe up to the Pleistocene Epoch, and became extinct in this area when the North Atlantic passed through a series of ice ages (Foster, 1946). The species survival on eastern North American shores (Reise *et al.*, 1999) allowed for a re-introduction during the 16th or 17th century, either deliberately introduced for food or bait, or as larvae in the bilges of ships (as postulated by Eno *et al.*, 1997), though Petersen *et al.* (1992) suggests a pre-Columbian introduction in the late 13th Century (Petersen *et al.*, 1992).

Previous work investigating the genetic variability of *Mya arenaria* across the geographic range of the species has involved the use of allozymes and the sequencing of different sections of mitochondrial DNA (Levinton, 1973; Morgan *et al.*, 1978; Caporale *et al.*, 1997; Sparagano *et al.*, 2002; Lasota *et al.*, 2004). These investigations mainly analysed the North American range of the species, and all revealed a relatively low level of genetic variability compared with other marine species such as *Semibalanus balanoides* (Brown *et al.*, 2001), *Idotea balthica* (Wares, 2001), and *Placopecten magellanicus* (Kenchington *et al.*, 2006), and a lack of genetic differentiation among the populations studied. As *M. arenaria* is thought to be a successful colonising species, it has been suggested that the genetic homogeneity among soft-shell clam populations reflects rapid range expansion, allele neutrality and high gene flow (Zaitsev & Mamaev, 1997; Lasota *et al.*, 2004).

Strasser & Barber (2009) investigated the genetic structure in *M. arenaria* populations by sequencing 661bp of the mitochondrial cytochrome oxidase I (COI) region in samples of softshell clams from across their distributional range. Softshell clams were sampled from sites on both coasts of North America and in one site in Western Europe, concentrating mostly on Eastern North American *M. arenaria*. In support of re-introduction from eastern North America this genetic work revealed a reduced level of haplotype diversity in the European population investigated, compared to those from eastern North America (Strasser & Barber, 2009). It was concluded that populations exhibited relatively low genetic variation compared to other bivalve species, with one haplotype dominating (65–100%) at all sites sampled (Strasser & Barber, 2009). However, the presence of unique haplotypes in the European North Sea and Western North American samples in the Strasser & Barber (2009) study suggests the need for further investigation into the history and origin of

M. arenaria in these areas. Records suggest that the recent origin of the species in western North America, where *M. arenaria* were thought to have been reintroduced in the late nineteenth century (Strasser & Barber, 2009), is more clear-cut than in Europe. More recently, the development and use of polymorphic microsatellite markers has revealed a much greater genetic structuring of *M. arenaria* on North American shores than was previously reported (St-Onge *et al.*, 2011; Krapal *et al.*, 2012; St-Onge *et al.*, 2013). It was subsequently proposed that a latitudinal cline in allelic richness provided evidence for a northward post-glacial expansion range in eastern North American *M. arenaria* (St-Onge *et al.*, 2013).

As population genetics has proved a useful approach for reconstructing routes of the introduction of species (Estoup & Guillemaud, 2010: Geller *et al.*, 2010), the aim of the present study was to examine the genetic variation in samples of *M. arenaria* from both sides of the North Atlantic, since post Pleistocene recolonization of Europe from eastern North American populations has been suggested in the past (Petersen *et al.*, 1992; Eno *et al.*, 1997; Strasser, 1999). Compared with previous work, which used a small European sample size of *M. arenaria* from the North Sea, the variable COI region and the 16S region of a considerably higher number of soft shell clams from four European sites were sequenced in the present study. In addition, to further investigate the genetic structure and phylogeography of this species, these samples were screened at ten polymorphic microsatellite loci, derived from the 17 primer sets described by St-Onge *et al.* (2011) and Krapal *et al.* (2012) (the multi-allelic nature and rapid mutation rate of microsatellites making them ideal for further investigations of the origins of the European softshell clam populations).

The present work is based on the hypothesis that one or more small (post-Glacial) introductions of *M. arenaria* from eastern North America to north-western Europe, either natural or anthropomorphic, would result in the presence of the same haplotypes and alleles as the donor populations, but with a reduction in variability at each type of marker. Furthermore, the extent of loss of haplotypes and alleles would correlate negatively with the size of the introduction/s. Therefore, the presence of new haplotypes or alleles would not be expected in recently introduced animals, apart from those caused by the relatively rare occurrence of novel mutations.

Therefore, the objectives of the present work were:

1. To explore, for the first time, the genetic variation in *Mya arenaria* from a number of European sites, using sequence analysis of both the COI and 16S regions of mtDNA and also ten polymorphic microsatellite markers.

2. To compare the results with those from *Mya arenaria* sampled on the east and west coasts of North America.

5.2. Materials and Methods

Sample Acquisition

Specimens of *Mya arenaria* (n=320 in total) were obtained from eight intertidal locations throughout the contemporary species range, in Ireland (Bannow Bay, Wexford, and Flaxfort, Cork), North Wales (Menai Bridge), Holland (Hinderplaat, The Netherlands), eastern Canada (Prince Edward Island) and three sites in the United States (two locations in Maryland on the east coast, and one in Oregon, on the Pacific North West coast) (Figure 1) (Table 1).

Table 1. Sampling locations of *Mya arenaria* across their distributional range.

Sample Name (Code)	(n)	Sample Area	Latitude	Longitude
Bannow Bay (BB)	48	Wexford, Ireland, Europe	52.251 N	06.763 W
Flaxfort (FF)	31	Cork, Ireland, Europe	51.648 N	08.698 W
Welsh (W)	25	Menai Bridge, Wales, Europe	53.238 N	04.091 W
Netherlands (N)	30	Hinderplaat, Holland, Europe	51.843 N	-04.009 W
Canadian (C)	30	Prince Edward Island, Gulf of St. Lawrence, Eastern Canada	46.446 N	63.767 W
Maryland A (MLEB)	43	Romancoke, East Bay, Maryland, USA, Eastern North America	38.886 N	76.340 W
Maryland B (MLMR)	53	Miles River, Maryland, USA, Eastern North America	38.816 N	76.237 W
Oregon (O)	60	Alsea Bay, Oregon, USA, Western North America	44.584 N	123.964 W

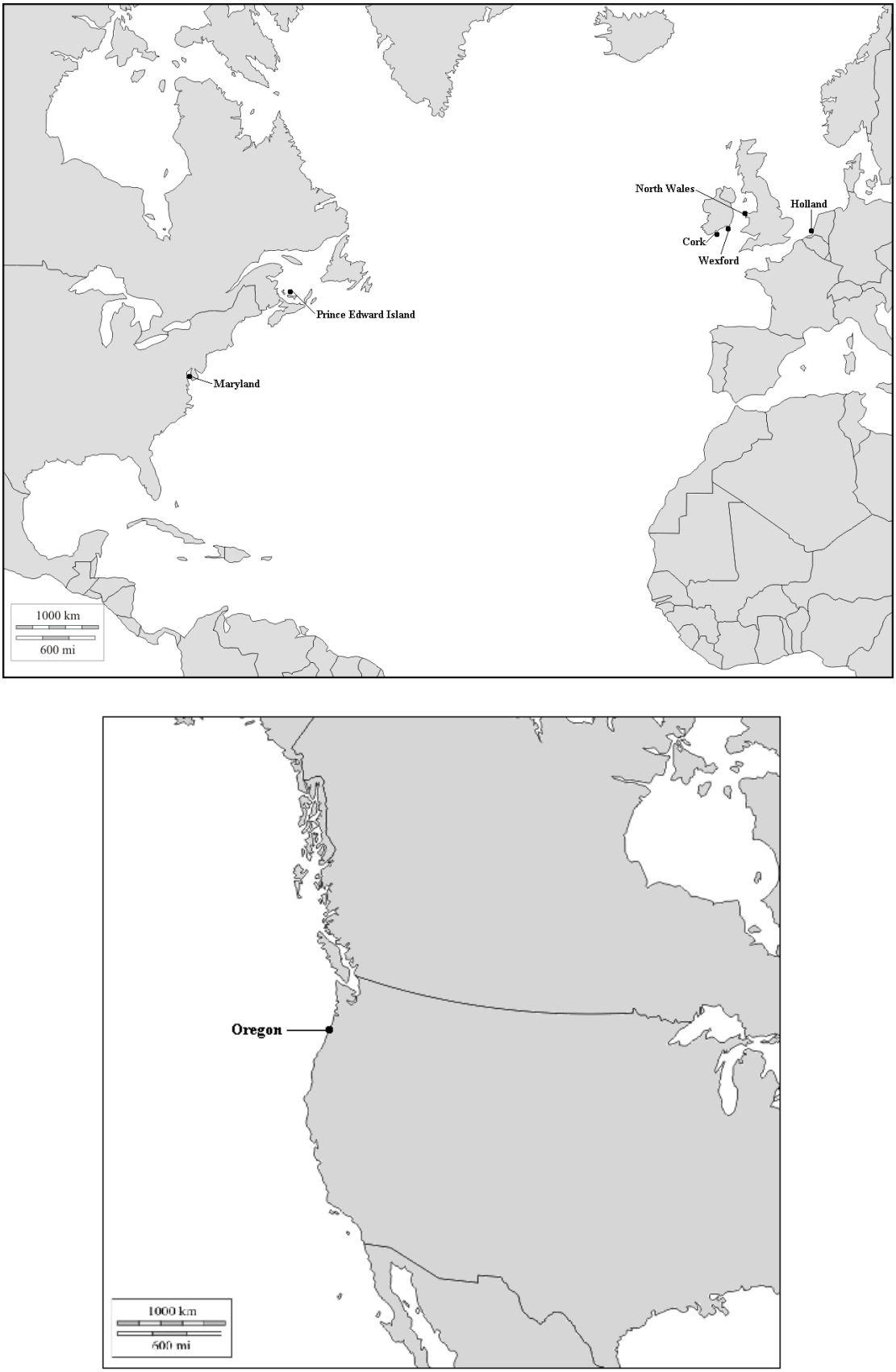

Figure 1. Sampling locations of *Mya arenaria* in the North Atlantic region and on the Pacific coast of North America.

Two levels of geographical scale were present in these samples; on a large scale between European and North American individuals, and on a smaller scale, between the two Maryland samples, which were collected eight kilometres apart, and the Flaxfort and Bannow Bay samples, which are 200 kilometres apart on the southern Irish Coast. All specimens were collected as outlined in Chapter 2, whereby *M. arenaria* specimens were collected by digging in the lower intertidal at each site, and gill tissue samples from each individual were preserved in 90% ethanol for use in genetic analysis. A subsample of the collected individuals, consisting of the first five (16S) or 16 (*CO*I) clams sampled at each location, were used for mitochondrial sequencing, whereas all individuals were used for the microsatellite investigation.

Laboratory analysis

Mitochondrial DNA

Sixteen samples of soft shell clam DNA from each area (Table 2) were purified using a QIAamp DNA mini Kit (Qiagen), and stored at -20°C. For the 533 bp section of the *CO*I gene, using the primers HCO-2198 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and LCO-1490 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer *et al.*, 1994), PCR amplification was carried out in 50µl volumes, containing 5µl 10x Invitrogen Buffer, 3.5µl $MgCl_2$ (50mM), 2µl dNTPs (5mM), 1µl of each primer, 0.5µl BSA, 0.8µl Ampli-Taq (Invitrogen), 34.2µl dH_2O and 2µl template DNA.

Table 2. Numbers of *Mya arenaria* successfully sequenced at the *CO*I mtDNA site.

Sample	Individuals chosen for sequencing	Individuals successfully sequenced for *CO*I
BB	16	10
FF	16	10
W	16	12
N	16	12
C	16	16
MLEB	16	12
MLMR	16	12
O	16	0
Total	128	84

Amplification of the 16S region of five individuals from each site, except MLEB (Table 3), was carried out using the primers 16Sar-L (5'-CGC CTG TTT AAC AAA AAC AT-3') and 16Sbr-H (5'-CCG GTC TGA ACT CAG ATC ACG T-'3) (Palumbi *et al.*, 1991) in 25µl volumes, containing 2.5µl 10x Buffer, 0.75µl $MgCl_2$ (50mM), 1µl dNTPs (5mM), 0.5µl of each primer, 0.25µl BSA, 0.4µl Ampli-Taq (Invitrogen), 18.1µl dH_2O and 1µl template DNA.

Table 3. Numbers of *Mya arenaria* successfully sequenced at the 16S mtDNA site.

Sample	Individuals chosen for sequencing	Individuals successfully sequenced for 16S
BB	5	5
FF	5	5
W	5	5
N	5	5
C	5	5
MLMR	5	5
O	5	4
Total	35	34

Amplifications, using both sets of primers, were performed on a Techne TC-Plus thermocycler, and were carried out under the following conditions: 95°C for 15min; 5x (95°C for 60sec, 50°C for 60sec, 72°C for 60sec); 30x (95°C for 60sec, 52°C for 60sec, 72°C for 60sec), and 72°C for 5minutes.

PCR products were viewed on a 0.8% agarose gel and purified using EXOSAP-IT (Affymetrix, USB Products). The sequencing reaction was performed on a Techne TC-Plus thermocycler, and was carried out under the following conditions: 94°C for 3min; 25x (94°C for 10sec, 50°C for 5sec, 60°C for 4min), and 8°C for 10minutes. The resulting product was purified using 125mM EDTA and 100% ethanol and sequenced in a 96-well plate format on an ABI 3730XL.

Microsatellite DNA

DNA extraction of all 320 individuals was carried out by Chelex-100 resin (Bio-Rad) extraction, using 10mg sections of gill tissue and 100µl of CHELEX, heated at 90°C for 70 minutes, and spun at 13,000rpm for 5 minutes. 100µl of supernatant was extracted and DNA was stored at 4°C (or at -20°C for long-term storage).

Primers were designed and optimized using previously published microsatellite primer data (Krapal *et al.*, 2011; St-Onge *et al.*, 2011). All primers are listed in Table 4, but only 10 of the original 16 gave results of satisfactory quality for use; i.e. resulting in sharp, strong bands, unambiguous reading of alleles, and repeatability. Rejected loci are indicated by an additional superscript in Table 2. Multiplex PCR amplification was carried out in 5µl of reaction volume, consisting of 2.5µl 2x Qiagen Multiplex mix, 0.5µl 10X Primer mix, 1µl DNA and 1µl dH_2O. Amplifications were performed on a Techne TC-Plus thermocycler, and were carried out under the following conditions: 15min at 95°C; 30 cycles of 30s at 94°C, 90 s at 56°C, and 60 s at 72°C; 8 cycles of 30s at 94°C, 90 s at 53°C, and 60 s at 72°C, with a final step of 30 min at 60°C. PCR amplification of each individual was repeated at least five times in the current study. PCR products were visualised using an ABI 3720 16 capillary Prism machine. Allele sizes were determined by means of an internal GENESCAN 500-LIZ size standard and genotypes were obtained using GENEMARKER v 1.6 (Applied Biosystems).

Table 4. Characteristics of the ten microsatellite markers in the softshell clam *Mya arenaria* used in the present study, and including six previously published loci, which yielded results of insufficient quality *(1= st. Onge et al., 2011, 2= Krappal et al., 2011, 3= rejected).*

Primer Name	Product Size	Primer Sequence (5'-3')
Mar-01[1]	213-277	F: TGACCGGCAGCAAAATTGAAGCCACGTCTCAAGCCTTA
		R: GTTTCTTATGCGTTCGTCCGTATGTG
Mar-02[1,3]	167-239	F: AGGTGAGATGACAGGAGATCATATGTGGGTAAATGGTTGGC
		R: GTTTCTTAATTTCCTGCTATTACTGAGG
Mar-03[1,3]	143-187	F: TGACCGGCAGCAAAATTGAGAAATGTAAGAGGAGATGC
		R: GTTTCTTCCGTAACATTTCACGTCCA
Mar-04[1]	181-269	F: AGGTGAGATGACAGGAGATCTCAATGCCAAAACATTGGTTA
		R: GTTTCTTACCCCCAAGCCTTTACTAGC
Mar-05[1,3]	170-214	F: TGACCGGCAGCAAAATTGTTGGGTCAAAACGTTCACAA
		R: GTTTCTTCATGGCCACTGGAAGTGTTA
Mar-06[1]	243-247	F: AGGTGAGATGACAGGAGATCCAATGCCCAACCCACTAAAC
		R: GTTTCTTGCGATGTTGGTTGTGTTGAC
Mar-07[1]	183-215	F: GGATAACAATTTCACACAGGTTGCAGGCGATGTTTGTATC
		R: GTTTCTTATACGGCATTCTTGGTCAGG
Mar-08[1]	180-248	F: AATTAACCCTCACTAAAGGGCATGTGTGACGAAATGTTGATG
		R: GTTTCTTCAGAGTCATACACTGTCCATTGC
Ma–02[2]	270-370	F: GGATAACAATTTCACACAGGGGGCCCTATATACCCAGCAC
		R: GTTTCTTAAAACGGGTCACAAATAGGC
Ma–06[2]	203-386	F: AATTAACCCTCACTAAAGGGCCTGACGGGAATAAAAACCA
		R: GTTTCTTCAGATGTAAATTGAGGCTTTGG
Ma-10[2,3]	203-386	F: AATTAACCCTCACTAAAGGGCCTGACGGGAATAAAAACCA
		R: GTTTCTTCAGATGTAAATTGAGGCTTTGG
Ma–11[2]	99-199	F: GGATAACAATTTCACACAGGTGGCTCAGACCATGTCAAAA
		R: GTTTCTTCGGCGAGCACACTGTACTAT
Ma-12[2,3]	93-193	F:AATTAACCCTCACTAAAGGGGACATGGCTGTAGAAGAAATTAGAA
		R: GTTTAAGGCGAACATAACACTGCAT
Ma–14[2]	99-199	F: TGACCGGCAGCAAAATTGCGCCTGAAAGGTAGTTTGACA
		R: GTTTCTTGAAAGTCCAGAGCAAGTATGAA
Ma–15[2]	253-353	F: TGACCGGCAGCAAAATTGAAATTGCCAAGGGAGGGAGT
		R: GTTTCTTATTAAAAGAGAAGCAAAAGCCACT
Ma–23[2,3]	93-293	F: GGATAACAATTTCACACAGGGTGCTTGGTTATGGCGAGTT
		R: GTTTCTTGCACACATTTTATTACGAGTGTATGA

Data analysis

Mitochondrial sequences

Quality of sequencing results was manually checked with CHROMASPRO 150 (Technelysium Pty Ltd.), and sequences were aligned using "CLUSTAL W" as implemented in BIOEDIT (Hall, 1999). Haplotype (h) and nucleotide diversity (π)

analyses, and neutrality tests (Tajima's D value, Fu's F_s statistic, and its associated *P* value) were performed with the software package DNASP v5.1 (Rozas *et al.*, 2003), and values for theta (θ), and average number of nucleotide differences (*k*) estimated. Pairwise F_{ST}, and associated *P* values were calculated in ARLEQUIN 3.01 using uncorrected pairwise distances. ARLEQUIN 3.01 was also used to conduct hierarchal analysis of molecular variance (AMOVA) based on mtDNA pairwise uncorrected genetic distance for all *Mya arenaria* samples, divided into European and North American groups.

Microsatellites

Within-sample statistics, including total number of alleles and mean number of alleles, expected and observed heterozygosity, and deviations from Hardy-Weinberg equilibrium (HWE), were calculated using GENEPOP 3.4 (Raymond & Rousset, 1995). Significance levels for multiple comparisons were adjusted using standard Bonferroni correction (Rice, 1989). Allelic richness was estimated using FSTAT 2.9.3.2 (Goudet, 2001). Because null alleles can result in an underestimation of within-population genetic variation (Chapuis & Estoup 2007), loci that deviated from HWE, with heterozygote deficiencies, were evaluated for the existence of null alleles in MICRO-CHECKER 2.2.3 (van Oosterhout *et al.*, 2004), and null allele frequency was determined by means of FREENA 1.0 (Chapuis & Estoup, 2007).

Genetic divergence among samples was compared using uncorrected F_{ST} estimates calculated in GENEPOP 3.4 (Raymond & Rousset, 1995), and unbiased F_{ST} estimates computed, accounting for null alleles, in FREENA (Chapuis & Estoup 2007). Significance of F_{ST} values was assessed by bootstrapping (95% CI, 5000 replications over loci, where significance is indicated by the lower CI not overlapping with zero) as implemented in FREENA. The partitioning of genetic variance among samples was examined with a hierarchical multilocus analysis of molecular variance (AMOVA) using the locus-by-locus option in Arlequin 3.01 (Schneider *et al*., 2000) with 10,000 permutations. A Factorial Component analysis was carried out using GENETIX 4.05 (Belkhir *et al.*, 1996-2004), and resulting data were illustrated in graphs drawn using MATLAB 2.04 (The Mathworks Inc.). As another way of visualising relationships among samples, an unrooted neighbour joining tree was created based on genetic distances from Nei *et al*. (1983). Distances and bootstrap values were calculated using Populations 1.2.3.1 analysis.

5.3. Results

Mt DNA

The *CO*I sequences obtained here (n = 84, Table 2) in general matched the previously identified properties of the *M. arenaria CO*I gene as described by Strasser & Barber (2009), (sharing of haplotypes between North America and Europe, a novel haplotype in the single European sample-data not shown), though different sequence lengths were used in the two studies so direct comparison was not attempted. For each *M. arenaria* sample a 533bp fragment of the mtDNA *CO*I gene and a 384bp fragment of the 16S ribosomal RNA gene that were common to all individuals sequenced were analysed. Only reliable sequence configurations were used in analysis of the resulting data.

CO*I Region*

The alignment of all *CO*I gene sequences revealed a total of 15 haplotypes within the 84 soft shell clam individuals where sequences were analysed in the present study (Table 2). Not all individuals sampled produced reliable sequence data. The dominant or commonest haplotype (A-in all but the Irish samples) was found at all of the locations sampled, ranging in frequency from 0.3 to 1.0, for individual samples, with an overall frequency of 0.65. Haplotype B was present in European and Canadian samples of *M. arenaria* only, at frequencies of 0.06 to 0.40, with an overall frequency of 0.12, while Haplotype C was present in European samples exclusively, with an overall frequency of 0.07, and frequencies in different areas ranging from 0.08 in the Netherlands to 0.30 in Flaxfort, Ireland. Haplotype B and C both differed from the common haplotype by a transition mutation each. Of the remaining 12 haplotypes (haplotypes D - O), each was unique to the area of sampling. One unique haplotype was found in each of the Bannow Bay (haplotype D) and Netherlands (haplotype E) samples, with three unique haplotypes present in the Maryland Eastern Bay area, and seven in the Miles River sample. One private haplotype, defined as a haplotype that occurs more than one time in only one site (Slatkin, 1985), was found in the Miles River site of Maryland (haplotype K), at a frequency of 0.17 (Table 5). Of all haplotypes, 13% were found only once overall.

Table 5. Haplotype distribution of the *CO*I region of *M. arenaria*

Haplotype	Location							
	BB	FF	W	N	C	MLEB	MLMR	Sum
A	0.3 (3)	0.4 (4)	1.0 (12)	0.67 (8)	0.94 (15)	0.76 (9)	0.33 (4)	0.6548 (55)
B	0.3 (3)	0.4 (4)		0.17 (2)	0.06 (1)			0.1190 (10)
C	0.3 (3)	0.2 (2)		0.08 (1)				0.0714 (6)
D	0.1 (1)							0.0119 (1)
E				0.08 (1)				0.0119 (1)
F						0.08 (1)		0.0119 (1)
G						0.08 (1)		0.0119 (1)
H						0.08 (1)		0.0119 (1)
I							0.08 (1)	0.0119 (1)
J							0.08 (1)	0.0119 (1)
K							0.17 (2)	0.0238 (2)
L							0.08 (1)	0.0119 (1)
M							0.08 (1)	0.0119 (1)
N							0.08 (1)	0.0119 (1)
O							0.08 (1)	0.0119 (1)
Total	10	10	12	12	16	12	12	84

Site abbreviations are given in Table 1. Haplotype frequencies are given for each area sampled, with the number of individuals per haplotype in brackets. Sum = sum of all sites sampled. Total = number of individuals sampled per area.

Haplotype E involved a deletion mutation; haplotypes F and H were due to a transversion mutation each, while all other unique haplotypes were due to single transition mutations (Table 6).

Table 6. *Mya arenaria CO*I mtDNA sequence details

Haplotype	Base Pair													
	63	78	102	132	216	246	351	345	405	417	494	507	519	531
A	T	G	G	T	G	G	G	A	G	G	A	T	A	A
B	-	-	-	-	-	-	-	-	-	-	G	-	-	-
C	-	-	-	-	-	-	-	-	-	A	-	-	-	-
D	C	-	-	-	-	-	-	-	-	-	-	-	-	-
E	-	-	-	-	-	-	-	-	-	-	G	-	Indel	-
F	-	-	-	-	-	-	-	-	-	-	-	A	-	-
G	-	-	-	-	A	-	-	-	-	-	-	-	-	-
H	-	-	-	G	-	-	-	-	-	-	-	-	-	-
I	-	-	-	-	-	-	-	-	-	-	-	-	-	G
J	-	-	-	-	-	-	-	-	A	-	-	-	-	-
K	-	-	-	-	-	-	A	-	-	-	-	-	-	-
L	-	-	-	-	-	-	-	G	-	-	-	-	-	-
M	-	-	-	-	-	A	-	-	-	-	-	-	-	-
N	-	-	A	-	-	-	-	-	-	-	-	-	-	-
O	-	A	-	-	-	-	-	-	-	-	-	-	-	-

Within sample variability

Haplotype diversity (*h*) ranged from a zero value in Wales, where only the common haplotype occurred and the Canadian sample (0.125), to high values such as 0.800 and 0.711 in the Irish samples (Bannow Bay and Flaxfort) and 0.894 in the Miles River sample (Table 7). Nucleotide diversity (π) was relatively low for all localities, ranging from 0.00023 in the Canadian sample to 0.00245 in the Miles River group. Theta (θ) ranged from 0.00057 in the Canadian sample to 0.00435 in the Miles River, with an average value of 0.00162 across all localities sampled, while values for the average number of nucleotide differences (*k*) ranged from zero in Wales and 0.125 in Canada to 1.303 in the Miles River sample (Table 7).

Table 7. Mitochondrial DNA within sample variability parameters

Population	N	nh	*h*	π	θ	*k*
BB	10	4	0.800 ±0.08	0.226 ±0.0	0.199 ±0.13	1.200
FF	10	3	0.711 ±0.09	0.167 ±0.0	0.133 ±0.10	0.889
W	12	1	0.000	0.000	0.000	0.000
N	12	3	0.530 ±0.14	0.108 ±0.0	0.124 ±0.09	0.576
C	16	2	0.125 ±0.11	0.023 ±0.0	0.057 ±0.06	0.125
MLEB	12	4	0.455 ±0.17	0.094 ±0.0	0.186 ±0.12	0.500
MLMR	12	8	0.894 ±0.08	0.245 ±0.0	0.435 ±0.23	1.303
Average	12	3.6	0.502 ±0.09	0.123	0.162 ±0.10	0.670
Total	84	14	0.554 ±0.06	0.126 ±0.0	0.489 ±0.18	

Showing: number of individuals (N), number of haplotypes (nh), haplotype diversity (h), nucleotide diversity (π) 10^{-2}, theta (θ) 10^{-2}, and average number of nucleotide differences (k). Site abbreviations are given in Table 1

Neutrality tests revealed that Tajima's *D* statistics were negative for all populations except the Welsh and Irish samples (Table 8). All values were revealed to be non-significant, meaning the populations sampled were in equilibrium with respect to genetic drift and mutation, except the value for total populations (-2.051), which was significant at $p<0.05$. Fu's F*s* statistics were all negative, excepting the Welsh and Flaxfort sample, and the only significant value was recorded in the Maryland Miles River sample, indicating an excess of alleles at this site (Table 8). It is interesting to note that while there is agreement between these two neutrality tests with regards to characteristics of most *M. arenaria* samples, the exception is the Miles River sample value which was indicated as significant using Fu's *Fs* statistics methodology, though not when using Tajima's *D* statistics method. Though not significant, the positive Fu's *Fs* value of 0.253 observed in the Flaxfort sample suggests a deficiency of alleles, as would be expected from a recent bottleneck or the presence of over-dominant selection for certain haplotypes.

Table 8. Neutrality tests on mtDNA CO1 sequences

Population	Tajima's *D*	Fu's F*s*
BB	0.437 n.s.	-0.497 (0.237)
FF	0.830 n.s.	0.253 (0.317)
W	0.000	0.000 (0.000)
N	-0.382 n.s.	-0.362 (0.270)
C	-1.162 n.s.	-0.700 (0.277)
MLEB	-1.629 n.s.	-2.124 (0.085)
MLMR	-1.713 n.s.	-5.837 (0.003)
All populations	-2.051 *	-13.641 (0.000)

Showing: Tajima's D value (*= significant, p<0.05), Fu's F_s statistic and its associated *P* value. Site abbreviations are given in Table 1

Among sample variability

After standard Bonferroni correction, pair wise inter-population F_{ST} comparisons revealed certain samples of *M. arenaria* to be statistically significantly different from each other (Table 9), including the Irish samples with Welsh and Maryland *M. arenaria*, and the Canadian sample with the Flaxfort and Miles River, Maryland individuals. The Dutch sample was not deemed statistically significantly different from any other areas using this method.

Table 9. Pairwise population comparisons, F_{ST}, for mtDNA sequence data.

	BB	FF	W	N	C	MLEB	MLMR
BB	-	0.5742	**0.0000**	0.2773	*0.0088*	**0.0000**	**0.0000**
FF	-0.0444	-	**0.0029**	0.5996	**0.0068**	**0.0010**	**0.0039**
W	0.2500*	0.2593*	-	0.1982	0.9990	0.9990	0.4745
N	0.0314	-0.0462	0.1364	-	0.1670	0.0918	0.0879
C	0.1969	0.1723*	0.0000	0.0389	-	0.1211	**0.0029**
MLEB	0.1905*	0.1830*	0.0000	0.0779	0.0000	-	0.4746
MLMR	0.1467*	0.1348*	0.0227	0.0606	0.0208*	0.1653	-

Showing population pairwise F_{ST} values below the diagonal and associated *P* values above. Bold values remained significant after standard Bonferroni correction; italicised values became non-significant after correction. *The value is significant after standard Bonferroni correction. Site abbreviations are given in Table 1

In the hierarchical AMOVA looking between the two major geographical regions (Europe and North America), within these regions and within each sample of the seven areas sampled, 87% of mtDNA CO1 variation was found within populations, whereas 6.8% and 6.2% of total haplotype frequency variation was represented by variation between European and North American samples and among populations within regions respectively (Table 10).

Table 10. Hierarchical analysis of molecular variance (AMOVA) for mtDNA variation.

	d. f.	Variance components	Percentage of Variation	*P* value
Among regions	1	0.0239	06.80	0.0293
Among populations Within regions	5	0.0218	06.22	<0.0010
Within populations	77	0.3052	86.98	0.0987
Total	83	0.3509	100	

Fixation Indices: F_{SC} = 0.06673, F_{ST} = 0.13020, F_{CT} = 0.06801

16S Region

Of 35 *Mya arenaria* sampled, 34 individuals were satisfactorily sequenced at the 16S ribosomal RNA region (Table 3), resulting in a 600bp fragment. The entire segment of 600bp was not amplified in all individuals, and 384bp of this segment was usable, as it was common to all the individuals analysed. The common haplotype was present in all areas sampled, with a unique haplotype present in one Maryland Miles River sample due to a single transition mutation in one individual (Table 11). As genetic variation at the 16S RNA region was negligible, sequence analysis focused exclusively on the *CO*I mtDNA data.

Table 11. Haplotype distribution of the 16S mtDNA region of *M. arenaria.*

Haplotype	Location							
	BB	FF	W	D	Can	MLMR	O	Sum
A	1.0 (5)	1.0 (5)	1.0 (5)	1.0 (5)	1.0 (5)	0.8 (4)	1.0 (4)	0.97 (33)
B						0.2 (1)		0.03 (1)
Total	5	5	5	5	5	5	4	34

Site abbreviations are given in Table 1. Haplotype frequencies are given for each area sampled, with the number of individuals per haplotype in brackets. Sum = sum of all sites sampled. Total = number of individuals sampled per area.

Microsatellite Results

Intra sample parameters

In total, 320 *M. arenaria* from eight geographical sites across the species range were genotyped at ten microsatellite loci. No linkage disequilibrium was observed between loci pairs and all ten loci were polymorphic. The three loci that deviated from HWE with heterozygote deficiencies (Ma 02, Ma 06 and Ma 15), were positively identified as possessing null alleles in MICRO-CHECKER 2.2.3 (van Oosterhout *et al.*, 2004). Using the same method, there was no evidence of technical problems associated with large allele dropout or stuttering patterns. Null allele frequency was determined by means of FREENA 1.0 (Chapuis & Estoup, 2007), and it was observed that the apparent presence of null alleles at these three loci had a negligible effect on estimation of overall F_{ST}, with an uncorrected value of 0.058, and an F_{ST} value of 0.057 when using data corrected for the presence of null alleles. Therefore, the significant level of population structuring observed using these ten loci was judged to be genuine, despite the presence of null alleles as determined by MICRO-CHECKER.

The number of alleles per locus within samples varied from four at locus Ma-15 in the Welsh sample, to 36 at Ma-06 in both Maryland samples (Table 12), with the total average number of alleles per locus valued at 15. The average number of alleles revealed using different loci across all samples ranged from 8.3 (Mar-06) to 21.0 (Mar-04) (Figure 2), with a total of 355 alleles being detected overall. Across all loci sampled, alleles unique to an area were present in small numbers in all sites except the Welsh sample (Table 12).

Table 12. Summary statistics for ten microsatellite loci in *Mya arenaria* samples.

Sample	**Mar-01**	**Mar-04**	**Mar-06**	**Mar-07**	**Mar-08**	**Ma 02**	**Ma 06**	**Ma 11**	**Ma 14**	**Ma 15**
Bannow Bay										
n	39	41	44	45	43	19	19	44	46	23
a	9	11	7	9	11	5	9	11	9	8
Rs	6.448	6.146	4.608	5.066	6.487	4.806	7.640	7.140	5.093	6.540
as	247-269	198-274	269-297	220-252	221-265	341-367	331-395	160-206	149-195	330-382
He	0.835	0.744	0.687	0.692	0.795	0.702	0.841	0.842	0.584	0.802
Ho	0.821	0.854	0.727	0.889	0.884	0.368	0.368	0.909	0.652	0.304
HW	0.010	0.404	0.058	0.259	0.559	0.002	0.000	0.885	0.419	0.000
Flaxfort										
n	30	27	31	31	31	21	17	31	31	12
a	10	11	6	6	11	9	11	10	8	7
Rs	7.158	6.921	4.224	4.307	7.356	6.683	8.221	7.019	3.945	6.239
as	243-269	212-274	263-289	220-254	137-265	331-367	349-413	160-192	137-209	330-388
He	0.821	0.790	0.545	0.632	0.833	0.781	0.822	0.830	0.495	0.778
Ho	0.667	0.963	0.677	0.774	0.807	0.714	0.412	1.000	0.516	0.333
HW	0.001	0.628	0.930	0.646	0.234	0.069	0.000	0.801	0.585	0.000
Wales										
n	24	19	22	24	24	12	18	25	23	17
a	10	10	5	7	6	6	8	10	6	4
Rs	7.353	7.686	3.725	5.088	5.454	5.685	5.892	7.532	4.688	3.397
as	241-269	212-286	253-273	220-250	221-265	339-353	331-395	156-192	149-195	334-364
He	0.831	0.835	0.471	0.760	0.777	0.778	0.711	0.844	0.655	0.393
Ho	0.833	1.000	0.364	0.792	0.917	0.917	0.667	0.960	0.783	0.353
HW	0.145	0.811	0.002	0.758	0.169	0.008	0.667	0.008	0.446	0.453
Netherlands										
n	29	28	30	29	29	19	22	29	29	20
a	11	13	10	11	16	11	10	14	14	10
Rs	6.872	8.502	5.977	7.001	9.478	8.680	7.905	9.198	8.742	8.006
as	243-303	212-286	243-299	222-258	137-273	331-367	343-413	162-206	149-223	330-368
He	0.805	0.865	0.697	0.791	0.883	0.875	0.853	0.888	0.881	0.831
Ho	0.828	0.821	0.667	0.586	1.000	0.895	0.546	0.897	0.828	0.300
HW	0.512	0.422	0.798	0.007	0.805	0.000	0.000	0.318	0.043	0.000

n = number of individuals that amplified, a = number of alleles, Rs = allelic richness, as = allelic size range, He = expected heterozygosity, Ho = observed heterozygosity, HW = Hardy Weinberg test p-value.

	Locus									
Sample	**Mar-01**	**Mar-04**	**Mar-06**	**Mar-07**	**Mar-08**	**Ma 02**	**Ma 06**	**Ma 11**	**Ma 14**	**Ma 15**
Canadian										
n	30	30	30	30	28	11	27	30	25	9
a	16	27	7	10	12	8	21	19	18	8
Rs	10.324	12.443	4.622	6.702	7.367	7.556	12.003	10.863	10.251	8.000
as	227-309	206-292	247-289	222-250	229-267	343-361	335-401	136-206	149-215	328-368
He	0.911	0.926	0.655	0.791	0.747	0.826	0.931	0.901	0.892	0.858
Ho	0.900	0.967	0.800	0.900	0.821	0.455	0.704	0.933	0.880	0.111
HW	0.659	0.204	0.055	0.027	0.883	0.000	0.000	0.736	0.123	0.000
ML – EB										
n	41	41	43	42	42	31	36	42	42	27
a	19	29	10	14	24	18	33	28	26	21
Rs	10.686	13.084	5.381	8.048	11.254	10.816	14.484	13.123	12.185	12.373
as	227-303	212-302	243-277	220-258	215-269	325-361	335-421	148-201	137-221	322-390
He	0.917	0.946	0.682	0.865	0.907	0.915	0.960	0.948	0.937	0.934
Ho	0.902	0.927	0.837	0.714	0.976	0.839	0.694	0.905	0.857	0.333
HW	0.285	0.305	0.389	0.009	0.786	0.000	0.002	0.264	0.004	0.000
ML-MR										
n	51	51	52	52	52	37	47	52	51	31
a	17	39	10	19	28	21	36	25	27	26
Rs	9.756	14.160	5.110	8.768	11.270	11.642	14.022	11.827	12.008	12.934
as	235-309	212-320	225-275	212-262	167-283	311-369	329-425	140-200	137-219	316-402
He	0.905	0.959	0.662	0.878	0.910	0.929	0.958	0.933	0.933	0.939
Ho	0.863	0.980	0.827	0.885	0.962	0.892	0.745	0.923	0.882	0.355
HW	0.177	0.173	0.009	0.424	0.694	0.000	0.000	0.024	0.021	0.003
Oregon										
n	55	58	58	58	57	51	54	56	56	43
a	14	28	11	17	25	18	27	24	25	30
Rs	8.427	11.433	5.754	8.784	10.236	10.555	11.387	10.764	11.504	12.154
as	237-285	190-294	251-289	204-266	197-277	329-373	319-441	146-208	147-227	302-390
He	0.876	0.926	0.763	0.872	0.890	0.912	0.923	0.912	0.929	0.934
Ho	0.891	0.983	0.931	0.828	0.947	0.922	0.944	0.911	0.964	0.814
HW	0.131	0.834	0.106	0.054	0.319	0.000	0.548	0.333	0.609	0.014

n = number of individuals that amplified, a = number of alleles, Rs = allelic richness, as = allelic size range, He = expected heterozygosity, Ho = observed heterozygosity, HW = Hardy Weinberg test p-value.

Table 13. Unique microsatellite alleles specific to sampling areas.

	Percentage of unique alleles	Number of unique alleles
Oregon	34.1	31
MLMR	28.6	26
MLEB	20.9	19
Bannow Bay	3.3	3
Netherlands	6.6	6
Canada	5.5	5
Flaxfort	1.1	1
Wales	0.0	0
Total	100	91

Excluding Ma 02, Ma 06 and Ma 15 because of the likely presence of null alleles, observed and expected within-sample heterozygosity values varied from 0.36 (Mar-06 in Wales) to 1.00 (Ma 11 in Flaxfort, Mar-04 in Wales, and Mar-08 in the Netherlands), and from 0.47 (Mar-06 in Wales) to 0.96 (Mar-04 in the Miles River Maryland sample) (Table 12). The highest percentage of unique alleles present in an area was found in the Oregon sample of *M. arenaria*, and the lowest in the Welsh sample (Table 13).

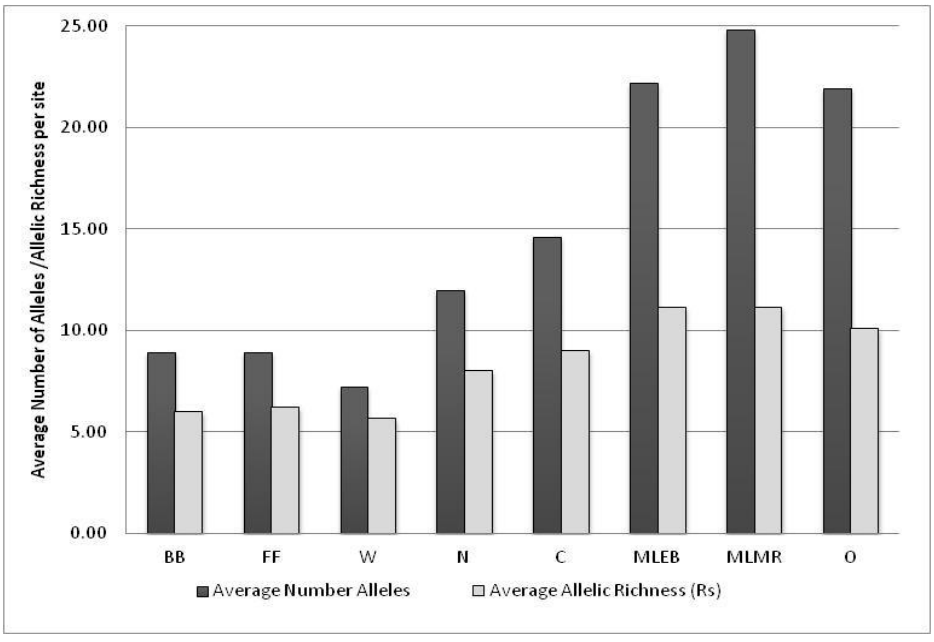

Figure 2. Showing average number of alleles and allelic richness (Rs) per sampling area of *Mya arenaria*.

Genetic variability as measured by allelic richness (Rs) ranged from 11.1 in the two Maryland sites, to 5.6 in the Welsh sample (Figure 2), with a mean allelic richness of 8.41.

Inter sample comparisons

Genetic differentiation over the eight softshell clam samples was significant at an overall value of 0.057 (95% CI). Pairwise F_{ST} values ranged from the single not statistically significant 0.0017 between the two Maryland sites, to 0.113 and 0.101 between Welsh and Canadian, and Welsh and Flaxfort *M. arenaria* respectively (Table 15). All samples of *M. arenaria* were significantly statistically different from each other excepting those from the two Maryland sites. Correction for the presence of null alleles did not significantly affect these data (Tables 14 and 15).

Table 14: Summary of F_{ST} tests for uncorrected pair wise comparisons of sample differentiation based on allelic frequency differences at 10 microsatellite loci.

	BB	FF	W	N	C	MLEB	MLMR
FF	0.0176						
W	0.0906	0.1041					
N	0.0547	0.0557	0.1012				
C	0.0965	0.102	0.1152	0.0678			
MLEB	0.0728	0.0755	0.0899	0.0337	0.0386		
MLMR	0.0772	0.0783	0.0838	0.0396	0.0377	0.0014*	
Or	0.0836	0.0849	0.1007	0.0394	0.0499	0.0173	0.0181

Pairwise F_{ST} values are shown. *The value is not statistically significantly different after standard Bonferroni correction.

Table 15: F_{ST} estimates for ten microsatellite loci, after correction for the presence of null alleles.

	BB	FF	W	N	C	MLEB	MLMR
FF	0.0165						
W	0.0909	0.1009					
N	0.0513	0.0549	0.0975				
C	0.0923	0.0996	0.1131	0.0648			
MLEB	0.0701	0.0763	0.0910	0.0316	0.0381		
MLMR	0.0742	0.0771	0.0832	0.0377	0.0379	0.0017*	
Or	0.0773	0.0816	0.0980	0.0355	0.0486	0.0174	0.0182

Pairwise F_{ST} values are shown. *The value is not statistically significantly different after standard Bonferroni correction.

The significant level of population structuring observed with pairwise F_{ST} values was supported by the results of Factorial Component Analysis (Figures 3 and 4). It can be seen that *M. arenaria* samples from European and North American sites are grouped separately (Figure 3).

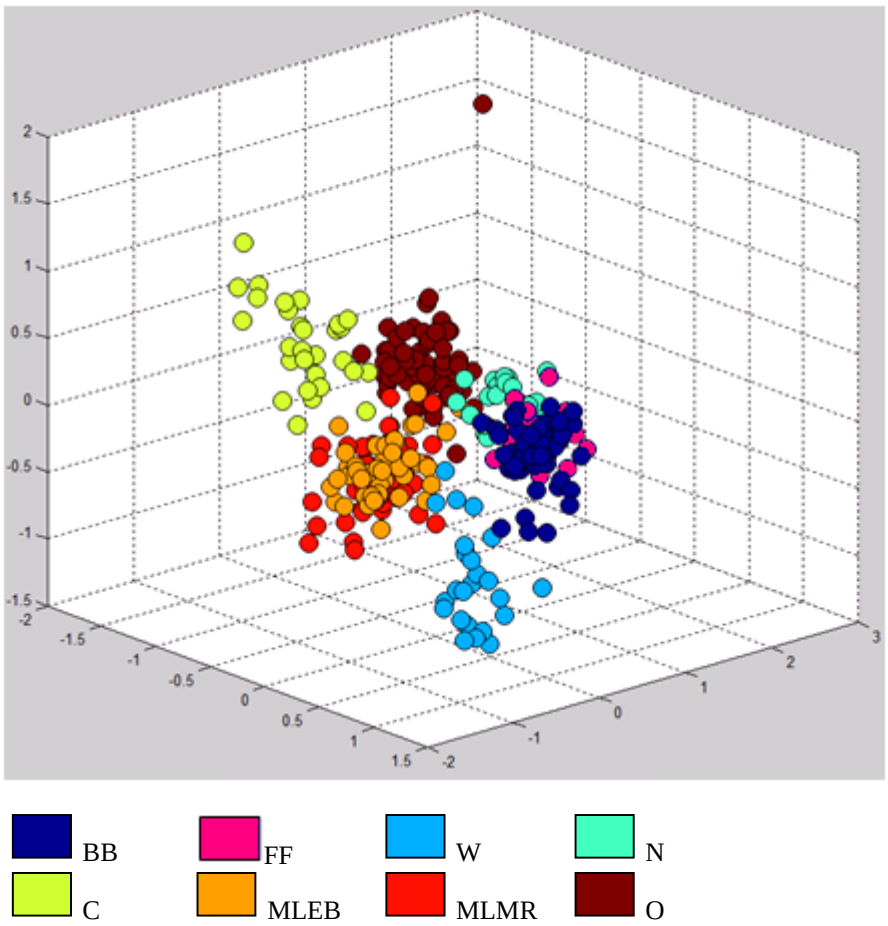

Figure 3. Factorial Component Analysis of *M. arenaria* microsatellite data, showing population structure (All populations).

Genetic differentiation over the eight softshell clam samples was significant at an overall value of 0.057 (95% CI). Pairwise F_{ST} values ranged from the single not statistically significant 0.0017 between the two Maryland sites, to 0.113 and 0.101 between Welsh and Canadian, and Welsh and Flaxfort *M. arenaria* respectively (Table 15). All samples of *M. arenaria* were significantly statistically different from each other excepting those from the two Maryland sites. Correction for the presence of null alleles did not significantly affect these data (Tables 14 and 15).

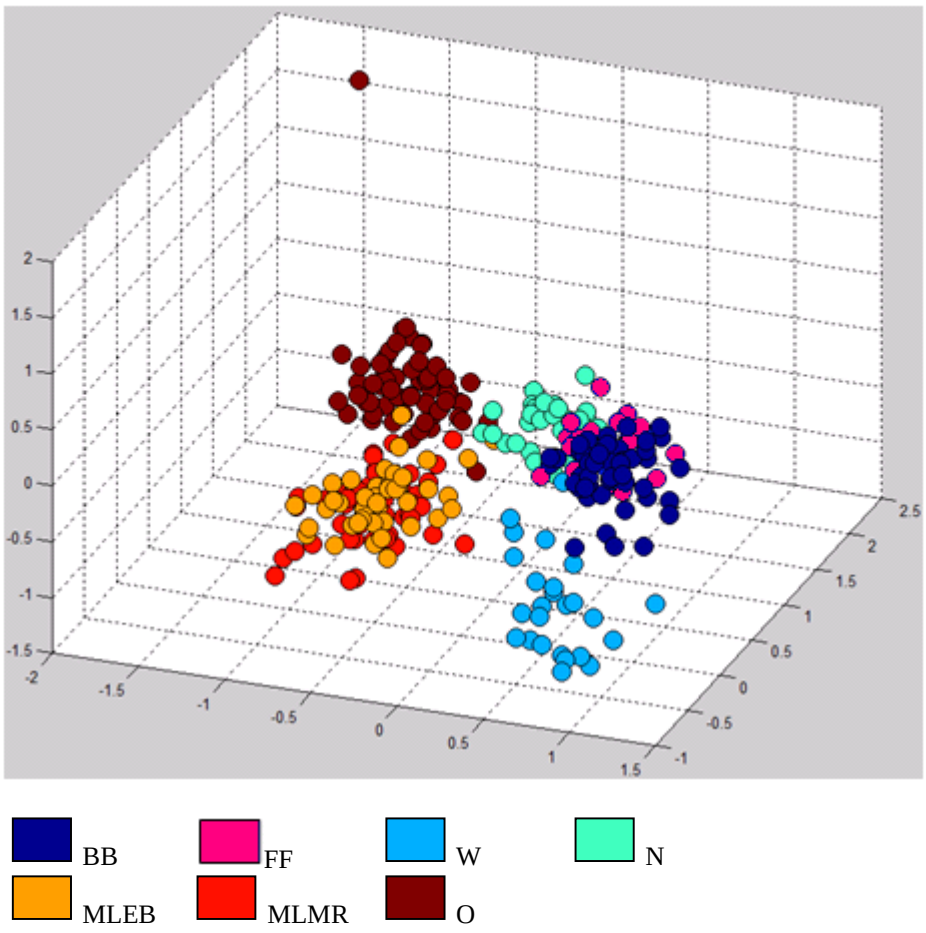

Figure 4. Factorial Component Analysis of *M. arenaria* microsatellite data, showing population structure (Canadian sample removed for enriched observation).

Samples of *M. arenaria* from the two Irish sites, Bannow Bay and Flaxfort, clustered together on the neighbour-joining tree, as did the two samples from Maryland, North America. The population structuring suggested by the factorial component analysis is supported in the un-rooted neighbour-joining tree based on inter sample D_a values, with the European and North American samples clearly separated, and the Netherlands sample present taking an intermediate position (Figure 5).

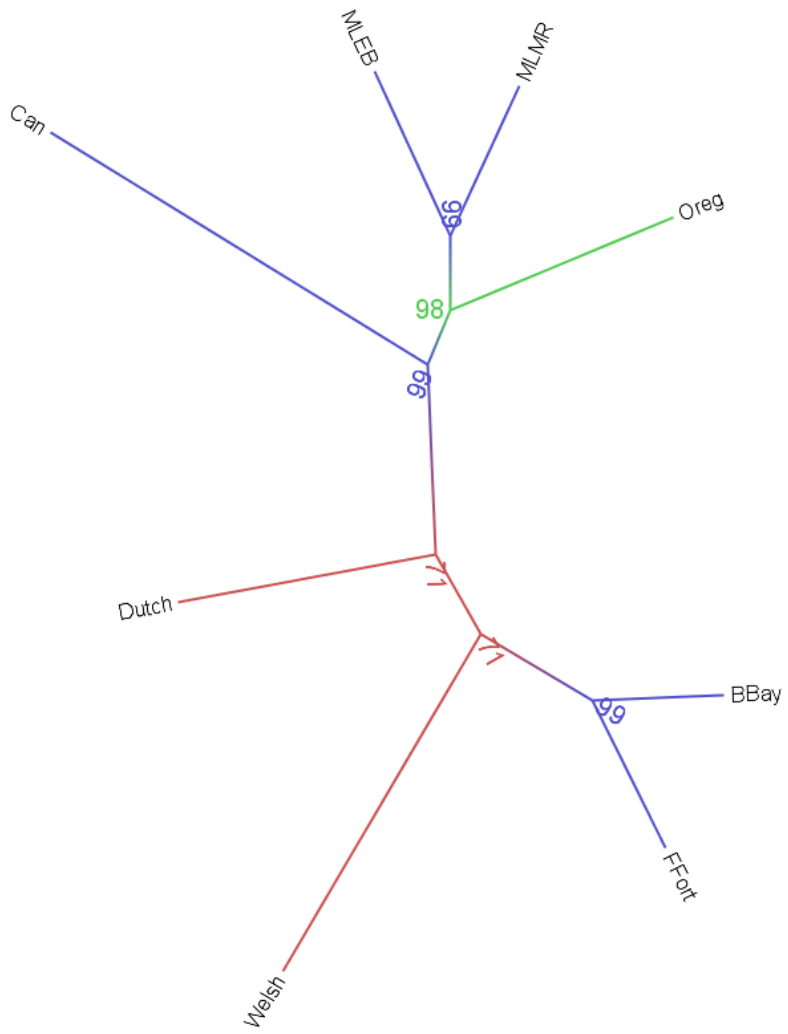

Figure 5. Unrooted neighbour-joining tree based on Nei *et al*. (1983).Values on nodes represent bootstrap support for groupings (out of 10,000 bootstrap).

Hierarchical AMOVA analyses revealed significant differences in genetic partitioning of *M. arenaria* at all levels of geographic organisation (as used above for *CO*I data), between the two regions, Europe and North America; among populations within regions; and within populations (Table 16). As with mtDNA *CO*1 AMOVA results, most of the percentage of variation was due to differentiation within populations (88.29%), while 5.48% and 6.23 % of total variation was represented by variation among Europe and North American samples, and among populations within regions respectively.

Table 16. Hierarchical analysis of molecular variance (AMOVA) for microsatellite variation.

	d. f.	Variance components	Percentage of Variation	*P* value
Among regions	1	0.02448	5.4820	< 0.001
Among populations within regions	6	0.2782	6.2310	< 0.001
Within populations	282	3.9416	88.2870	< 0.001

Fixation Indices: F_{SC} = 0.0659, F_{ST} = 0.1171, F_{CT} = 0.0548.

Discussion

The present study was undertaken to examine the multi-spatial scale genetic structure of *Mya arenaria* across the species range assuming a post glacial recolonization of Western Europe from eastern North America, since understanding the scale of marine population connectivity is critical for the conservation and sustainable management of marine resources (Banks *et al.*, 2007). The hypothesis was that a small recent introduction of *M. arenaria* to Europe from North America would result in the presence of the same haplotypes and alleles as the donor population, but with a reduction in genetic variability in both marker types. Novel haplotypes and alleles would not be expected to be present in recently introduced animals. The influence of genetic drift could affect the number of both, but mutations creating new haplotypes and alleles are less likely, because incorporation of new mutations is a relatively slow process. In contrast, the presence of novel mitochondrial haplotypes and microsatellite alleles in European *M. arenaria* would suggest the persistence of one or more relict populations of the clam in the south of Europe (Atlantic coast of the Iberian peninsula or Mediterranean coasts) in addition to assuming a post glacial recolonization of western Europe from eastern North America coasts after the Pleistocene era. Further study of variation in southern European *M. arenaria* using the genetic markers specified could help to clarify the post-glacial movement of this species in European waters.

Genetic variability and composition in Mya arenaria

Polymorphic microsatellites are considered more powerful for resolving population structure than mtDNA markers, particularly for recently diverged lineages or geographically proximate populations (Lukoschek *et al.*, 2008). However, microsatellites are often less appropriate for inferring historical purposes because of their extremely high variability and mutation rate (Gysels *et al.*, 2004). In the present study relatively high genetic variability was found at microsatellite loci in softshell clams sampled across the region known as the temperate North Atlantic biogeographic realm, as defined by Spalding *et al.* (2007). When assessing genetic variability in terms of haplotype diversity (*h*) for mtDNA and allelic richness (R_s) for microsatellite loci, the results from the two marker types are similar for some sampling areas, but not for others. While the Miles River sample from Maryland *M. arenaria* is the most variable at both of these markers, with the Netherlands sample usually in the middle of the range, the Welsh sample was consistently the least variable. The Irish clams are more variable at the *CO*I region of mtDNA compared to the Canadian and Netherlands samples, whereas at the microsatellite loci, the Oregon, Canadian and Netherlands sample each have a greater value of average allelic richness than the Irish samples.

A number of reasons could account for these differences in levels of variability. In the present study, a much smaller number of clams were analysed for mtDNA compared with microsatellite loci. Since mitochondrial DNA, because of largely or exclusively maternal inheritance, reveals the characteristics of female variation only, while microsatellite data encompasses the genetic information of both sexes, the effective population size (Ne) in the former is four times less. Also, only one mitochondrial locus was investigated here, while ten microsatellite loci were screened for variation in nuclear DNA. However, the reduced occurrence of recombination in mtDNA can lead to a less complicated phylogeny. The higher mutation rate of nuclear versus mitochondrial DNA may naturally lead to greater variability in the former, but these data may be complicated by recombination and the possibility of homoplasy. Higher genetic variability was revealed in softshell clams across the ten microsatellite loci investigated, as opposed to the *CO*I region of mtDNA, with a significant global F_{ST} value of 0.057 in the former. All samples of

M. arenaria were found to be significantly statistically different from each other excepting those from the two Maryland sites, which are located just 8 km from each other. The results obtained using both mitochondrial and microsatellite markers are similar in that significant genetic variation was revealed in *M. arenaria* from the eastern Atlantic range, previously dismissed as a panmictic grouping with little or no variation (Sparagano *et al.*, 2002; Lasota *et al.*, 2004).

Large scale: Variation between North American and European Mya arenaria

The coastlines of North America and Europe exhibit similar but not identical gradients of environmental factors associated with latitude. Therefore, when the distribution of the species includes both coastlines, the North American and European populations may be genetically distinct (Schmidt *et al.*, 2008), even in the absence of the complications caused by likely colonisation of western Europe from eastern North America. In the present study, hierarchical AMOVA analyses of microsatellite data revealed significant differences in genetic partitioning of *M. arenaria* at all levels of geographic organisation, while neighbour-joining tree and Factorial Component Analysis provide strong support for grouping Northern American and European samples of *M. arenaria* separately. This distinction could be due to historical post-glacial movement of the species, as the last glacial maxima seem to have affected other biota living on either side of the North Atlantic differently (Jolly *et al.*, 2006). This phenomenon is thought to be a result of the differences in temperature regimes and positions of old shorelines in the glacial histories of these two coasts (Maggs *et al.*, 2008). Present-day environmental and climatic factors can also influence gene flow for existing populations (Wares, 2002; Baker *et al.*, 2008).

The climatic cycles with subsequent glacial and inter-glacial periods have had a great impact on the colonization, re-colonization, distribution and evolution of species in the Northern Atlantic (Schmitt, 2007). The Pleistocene glaciations (1.8 million - 18,000 years ago), and in particular the last glacial maximum (LGM: 20,000 - 18,000 years ago) have been the most important events shaping the evolution, population genetic structure and distribution of most present-day species, as repeated episodes of global warming and cooling have resulted in water temperature fluctuations, alterations in upwelling patterns, and sea level changes,

which had profound impacts on coastal marine species (Bernatchez & Wilson 1998; Hewitt, 2000; Luttikhuizen *et al.*, 2003; Baker *et al*., 2008; Kenchington *et al.*, 2009). During the advances of Northern Hemisphere ice sheets, temperate species are thought to have become restricted to southern or periglacial refugia (Maggs *et al.*, 2008). Isolation into refugia reduced geographical ranges and numbers of populations (Wares, 2002). Traditionally, this was thought to have resulted in southern populations possessing high diversity including private alleles, and a loss of allele diversity and genetic homogeneity in the northern areas recolonized by range expansion (Hewitt, 2000; Maggs *et al.*, 2008; Kenchington *et al.*, 2009). While true for some marine species, including the broadcast-spawning northern quahog *Mercenaria mercenaria* (Baker *et al.*, 2008), and American lobster *Homarus americanus* (Kenchington *et al.*, 2009), marine gastropod *Acanthinucella spirata* (Hellberg *et al.*, 2001) the red algae *Bostrychia* (Zuccarello *et al.*, 2006), and the dog whelk *Nucella ostrina* (Marko, 2004), all of which have different types of lifecycle, this pattern of post glacial colonization has been challenged by others. These include the broadcast-spawning ocean quahog *Arctica islandica* (Dahlgren *et al*., 2000), *Macoma balthica* (Luttikhuizen *et al.*, 2003), *Cerastoderma edule* (Krakau *et al.*, 2012), and *Pagurus longicarpus* (Young *et al.*, 2002) and *Littorina saxatilis* (Panova *et al.*, 2011).

Smaller Scale: Latitudinal clines in genetic variability

The recent work by St-Onge *et al*. (2013) using seven microsatellite loci revealed a latitudinal cline in allelic richness from south to north in eastern North American *M. arenaria*. This pattern has also been observed in eastern North American samples in the present study, with values for haplotype and nucleotide diversity being higher in Maryland sites compared with the Canadian location. Average allelic richness and observed heterozygosity values were also higher in Maryland (11.15) than Canadian (9.01) samples. Thirty four percent of all unique microsatellite alleles were identified in the presumed human-introduced Oregon sample of *M. arenaria,* and an average allelic richness value similar to those in Maryland was observed, all of which were higher than in the Canadian sample. This could, in part, be explained by reports that a large number of clams were introduced to the Pacific coast of North America coast during the last 400 years from western

North Atlantic stocks (Powers *et al.*, 2006; Strasser & Barber, 2009; St-Onge *et al.*, 2013), ensuring that a great deal of the genetic variability present in the donor population was preserved in the introduced strain. The Oregon sample of soft shell clams has some alleles that do not appear in contemporary eastern North American samples, but most of these occur at low frequency, so they may be the result of new mutations or sampling error, the latter which can occur when a large number of alleles are present at a locus, and it is difficult to distinguish certain alleles from others.

The pattern of a latitudinal cline in allelic diversity has not been observed in European soft shell clams in the present study but note that the latitudinal range investigated in Europe (≈915km) was smaller than in eastern North America (≈1700km). However this might also be because other forces, such as the location and size of introductions, have influenced genetic composition in European stocks of *M. arenaria*. A similar pattern of allele frequency variation on both sides of the North Atlantic was revealed in the acorn barnacle *Semibalanus balanoides*, where shallow clines across a large latitudinal gradient were observed in the western North Atlantic but no detectable latitudinal pattern was evident in the eastern North Atlantic (Flowerdew, 1983). While the differing patterns of variation in *S. balanoides* were thought to be due to differences in marine temperature on the two coasts (Schmidt *et al.*, 2008), the genetic structure of European *M. arenaria* is, as noted above, more likely to be a result of complex post-glacial recolonisation of this species.

Mya arenaria in Europe

As outlined above, the history of *M. arenaria* in Europe is not as clear-cut as in North America. According to fossil dating, this species was present in eastern Atlantic waters in the late Pliocene (Strauch, 1972; Eno *et al.*, 1997), having arrived by an Arctic or Central America route (Powers *et al.*, 2006). It is thought to have become extinct in Europe during the Pleistocene Epoch, when the continent passed through a series of Ice Ages (Foster, 1946), and re-introduced by anthropogenic or natural means. *Mya arenaria* in both Danish and Baltic Sea waters have been dated to precede the time of Columbus, leading the two separate groups of authors to suggest a Viking introduction for this species to Northern Europe after the LGM,

around 1310 AD±70 years (Petersen *et al.*, 1992; Behrends *et al.*, 2005). While this theory is possible, it is more probable that the soft shell clam recolonised European waters by natural means, in a similar fashion to this species first European colonisation (Strauch, 1972), or that periglacial refugia of individuals persisted in Europe, as has been recorded for the cockle *Cerastoderma edule* (Krakau *et al.*, 2011), and the clam *Macoma balthica* (Luttikhuizen *et al.*, 2003), both of which inhabit a similar ecological niche to *M. arenaria*, as intertidal and shallow sublittoral soft sediment infaunal bivalve species.

The historical processes which have led to the present-day patterns of genetic structure in the marine coastal fauna of the Northeast Atlantic are still poorly understood (Jolly *et al.*, 2006). In the present study, variability at the microsatellite loci suggests initial colonisation of *M. arenaria* into Europe through the North Sea. There is no way of determining whether this introduction was natural or human mediated (e.g. larvae in the ballast water of boats, or as food). Adding to the data on microsatellite composition, both the un-rooted neighbour-joining tree and factorial component analysis placed the Netherlands sample of *M. arenaria* as intermediate between the North American and European individuals, identifying this area as a mixture zone for the two significantly different regions. Pairwise F_{ST} values of the *CO*I region also demonstrate that the Netherlands sample of soft shell clams is not statistically significantly different from any other areas sampled. Six percent of microsatellite alleles were unique to European sites (see Appendix, Table 16), with 2% endemic to the European and the Pacific coasts (arguably the result of new mutations). The identification of a common *CO*I mtDNA haplotype at all locations sampled also suggests a North American source for current *M. arenaria* populations.

The presence of private haplotypes in the Bannow Bay and Netherlands sample, and haplotype C being exclusive to European locations infers an older split from the "original" North American group than previously thought. This is reinforced by high haplotype diversity values for all European samples, except the Welsh sample (where the common haplotype prevailed), and non-significant values for both Fu's F*s* and Tajima's D statistics, which imply the European samples of *M. arenaria* are in equilibrium with regards to genetic drift and mutation, and are not the result of a recent population expansion. Differential distributions of haplotypes

between isolated geographical areas are also thought to represent long-term isolation (Maggs *et al.*, 2008). Of the *M. arenaria* individuals sampled at the Bannow Bay site, 30% of individuals possessed the common haplotype (Haplotype A), 30% possessed a European and Canadian shared haplotype (Haplotype B), and 30% of clams comprised of a European-only haplotype (Haplotype C). Also a haplotype occurred in one individual that was unique to the Bannow Bay sample (Haplotype D). A similar distribution of common and endemic haplotypes was identified in the Flaxfort sample of softshell clams, with individuals from both southern Irish sites possessing a higher haplotype and nucleotide diversity than those from the Netherlands. These data suggest that *M. arenaria* could have originally spread from the North Sea area to Irish waters, and then to Wales by a small introduction, leading to the presence of only the common haplotype and low nuclear genetic variability in this area.

The overall strong signature of subdivision among European populations of *M. arenaria* is not consistent with divergence solely since the last glacial maximum, and the more complex patterns of genetic structure in European softshell clams may be an indication of survival in cryptic refugia and periglacial zones, similar to observations in other benthic organisms such as the green crab, *Carcinus maenas* (Roman & Palumbi, 2004), seaweed species (Hoarau *et al.*, 2007; Olsen *et al.*, 2010; Coyer *et al.*, 2011), the common shrimp, *Crangon crangon* (L) (Luttikhuizen *et al.*, 2008), the genus *Patella* (Sa-Pinto *et al.*, 2005) and tubiferous polychaetes (Jolly *et al.*, 2006). It is currently unknown whether an ice sheet extended fully to the south of Ireland during the last glacial maximum, or whether this area was partially unglaciated (Maggs *et al.*, 2008). The possibility of past southern European refugium/a of *M. arenaria*, is indicated by the presence of novel European haplotypes and microsatellite alleles, the former of which are at relatively high frequencies at southern Ireland sites. The alternative explanation for the presence of these novel haplotypes is a chance founder effect or allele surfing (Waters *et al.*, 2012). However, it is most likely that a common haplotype would prevail, as has been demonstrated in the Welsh sample of softshell clams.

Conclusions

While differing with regards to some aspects, the results obtained using both mitochondrial and microsatellite markers in the present study are similar to each other, in that significant genetic variation was revealed in *M. arenaria* from the eastern Atlantic range for the first time. Based on present data a model of panmixia is rejected for the population structure of this species in both the North American and European range. Similar to the phylogenetic history of *Littorina littorea,* where the reverse situation occurs in that the species is native to Europe and there is argument as to whether small refugia persisted in eastern North America or Post Pleistocene human introduction (possibly by Vikings in Anse aux Meadows) was involved (Chapman *et al.*, 2008), the origin and survival of *M. arenaria* in its European range is not categorically determined. Although it is difficult to identify potential refugia from genetic data, the high genetic variability, presence of novel haplotypes and microsatellite alleles, and complex genetic structure revealed in European samples of *M. arenaria* suggest the presence of cryptic southern European refugium/a of the clam during the Pleistocene era. To clarify the phylogenetic history of soft shell clams in Europe, future work should include investigating genetic variation at these markers in *M. arenaria* from southern Europe, and the use of finer resolution markers including SNPs and next generation sequencing.

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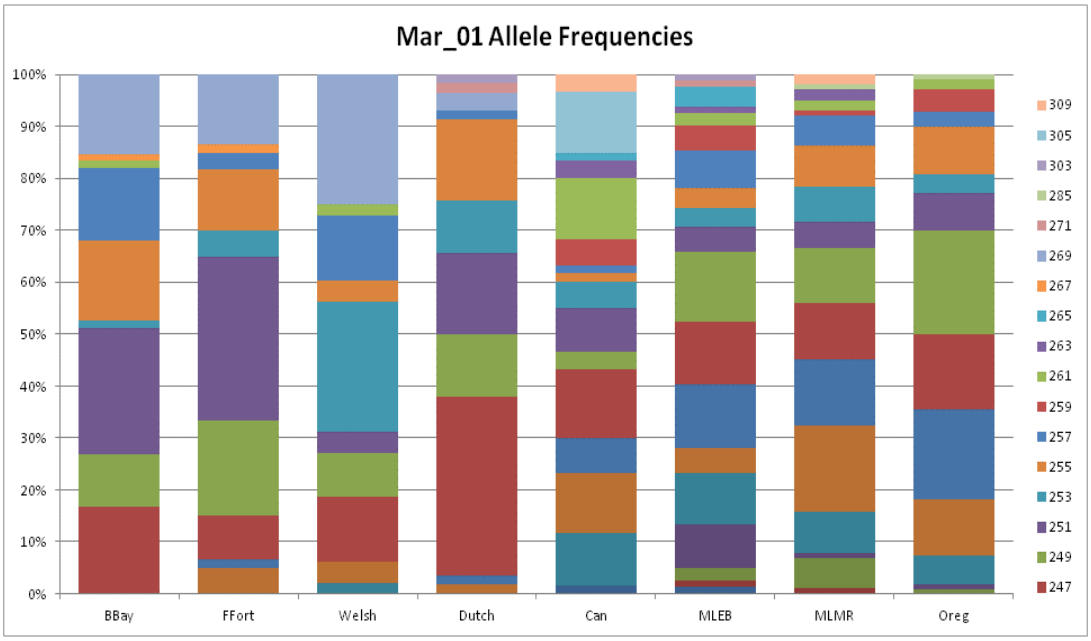
Appendix

Allele frequencies for all populations by locus

Each table depicts the characteristics of alleles identified in each population. Colour coding illustrates the frequency of alleles, ranging from low frequency (red) to higher (green). The Table for each locus is followed by stacked histograms depicting the allelic composition of each sample

Locus: Mar_01

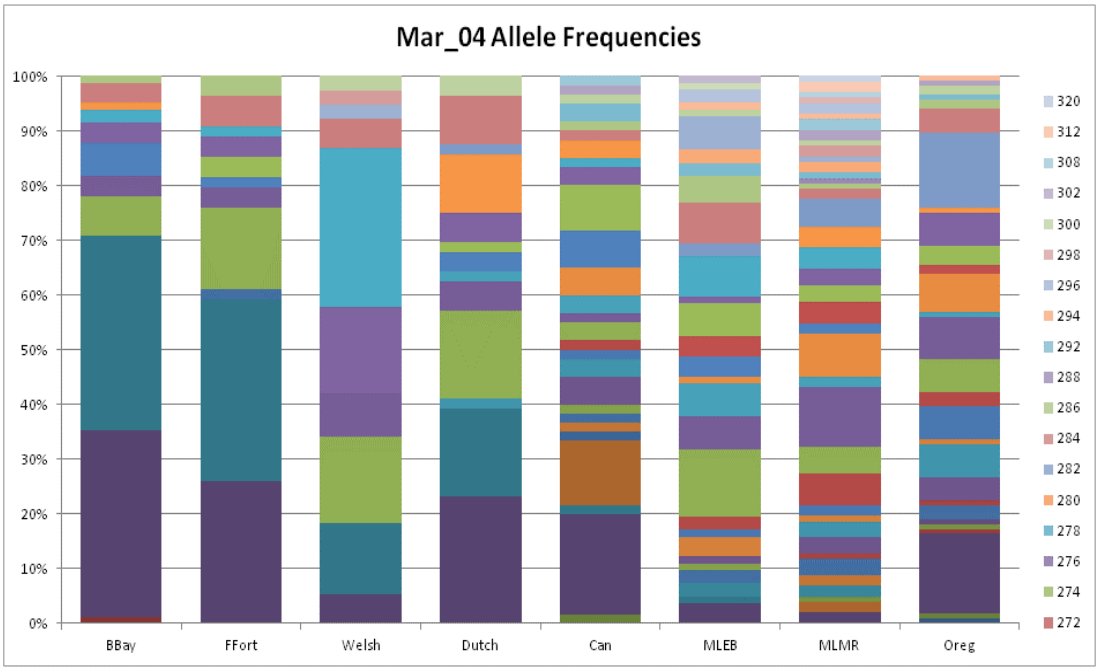
BP Shift	Allele	BBay	FFort	Welsh	Dutch	Can	MLEB	MLMR	Oreg
	227					1.67	1.22		
8	235						1.22	0.98	
2	237						2.44	5.88	0.91
2	239						8.54	0.98	0.91
2	241			2.08		10.00	9.76	7.84	5.45
2	243		5.00	4.17	1.72	11.67	4.88	16.67	10.91
2	245		1.67		1.72	6.67	12.20	12.75	17.27
2	247	16.67	8.33	12.50	34.48	13.33	12.20	10.78	14.55
2	249	10.26	18.33	8.33	12.07	3.33	13.41	10.78	20.00
2	251	24.36	31.67	4.17	15.52	8.33	4.88	4.90	7.27
2	253	1.28	5.00	25.00	10.34	5.00	3.66	6.86	3.64
2	255	15.38	11.67	4.17	15.52	1.67	3.66	7.84	9.09
2	257	14.10	3.33	12.50	1.72	1.67	7.32	5.88	2.73
2	259					5.00	4.88	0.98	4.55
2	261	1.28		2.08		11.67	2.44	1.96	1.82
2	263					3.33	1.22	1.96	
2	265					1.67	3.66		
2	267	1.28	1.67						
2	269	15.38	13.33	25.00	3.45				
2	271				1.72		1.22		
14	285							0.98	0.91
18	303				1.72		1.22		
2	305					11.67			
4	309					3.33		1.96	



Locus: Mar_04

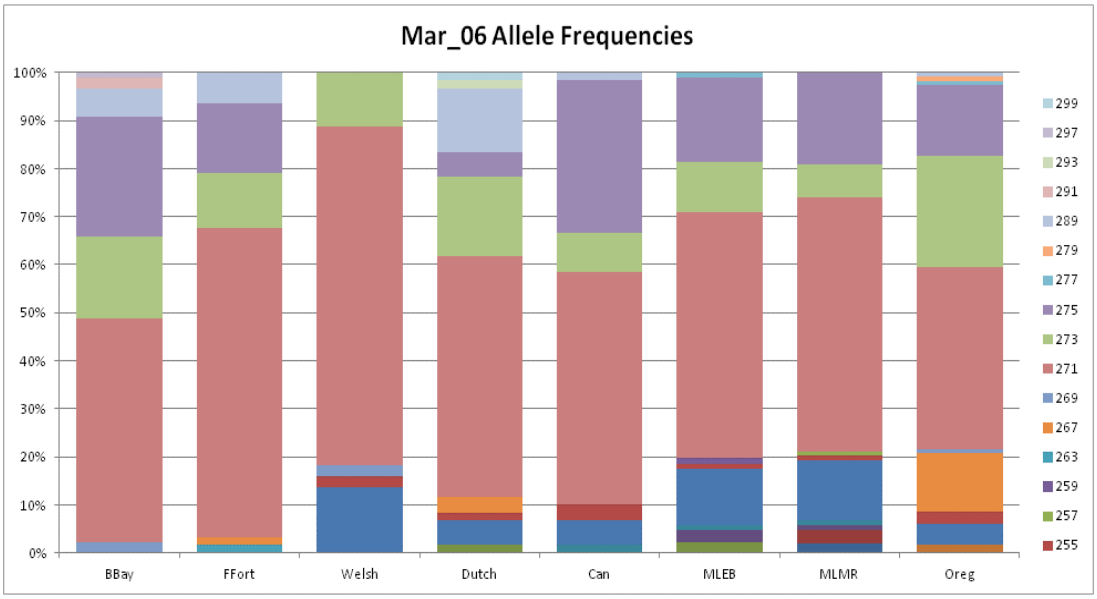
BP Shift	Allele	BBay	FFort	Welsh	Dutch	Can	MLEB	MLMR	Oreg
	190								0.86
8	198	1.22							
8	206					1.67			0.86
6	212	34.15	25.93	5.26	23.21	18.33	3.66	1.96	14.66
4	216	35.37	33.33	13.16	16.07	1.67	1.22		
2	218					11.67		1.96	
2	220					1.67			
2	222								0.86
4	226							0.98	0.86
2	228								0.86
2	230						2.44	1.96	
2	232					1.67		1.96	
2	234		1.85			1.67	2.44	2.94	2.59
2	236							0.98	0.86
2	238					1.67	1.22		
2	240					5.00	1.22	2.94	4.31
2	242				1.79	3.33		2.94	6.03
2	244						3.66	0.98	0.86
2	246					1.67	1.22	1.96	6.03
2	248					1.67	2.44	5.88	2.59
2	250	7.32	14.81	15.79	16.07	3.33	12.20	4.90	6.03
2	252	3.66	3.70	7.89	5.36	1.67	6.10	10.78	7.76
2	254				1.79	3.33	6.10	1.96	0.86
2	256					5.00	1.22	7.84	6.90

2	258	6.10	1.85		3.57	6.67	3.66	1.96	
2	260						3.66	3.92	1.72
2	262		3.70		1.79	8.33	6.10	2.94	3.45
2	264	3.66	3.70	15.79	5.36	3.33	1.22	2.94	6.03
2	266	2.44	1.85	28.95		1.67	7.32	3.92	
2	268	1.22			10.71	3.33		3.92	0.86
2	270				1.79		2.44	4.90	13.79
2	272	3.66	5.56	5.26	8.93	1.67	7.32	1.96	4.31
2	274	1.22	3.70			1.67	4.88	0.98	1.72
2	276							0.98	
2	278					3.33	2.44	0.98	0.86
2	280						2.44	1.96	
2	282			2.63			6.10	0.98	
2	284			2.63				1.96	
2	286			2.63	3.57	1.67	1.22	0.98	1.72
2	288					1.67		1.96	0.86
4	292					1.67		1.96	
2	294						1.22	0.98	0.86
2	296						2.44	1.96	
2	298							0.98	
2	300						1.22		
2	302						1.22		
6	308							0.98	
4	312							1.96	
8	320							0.98	



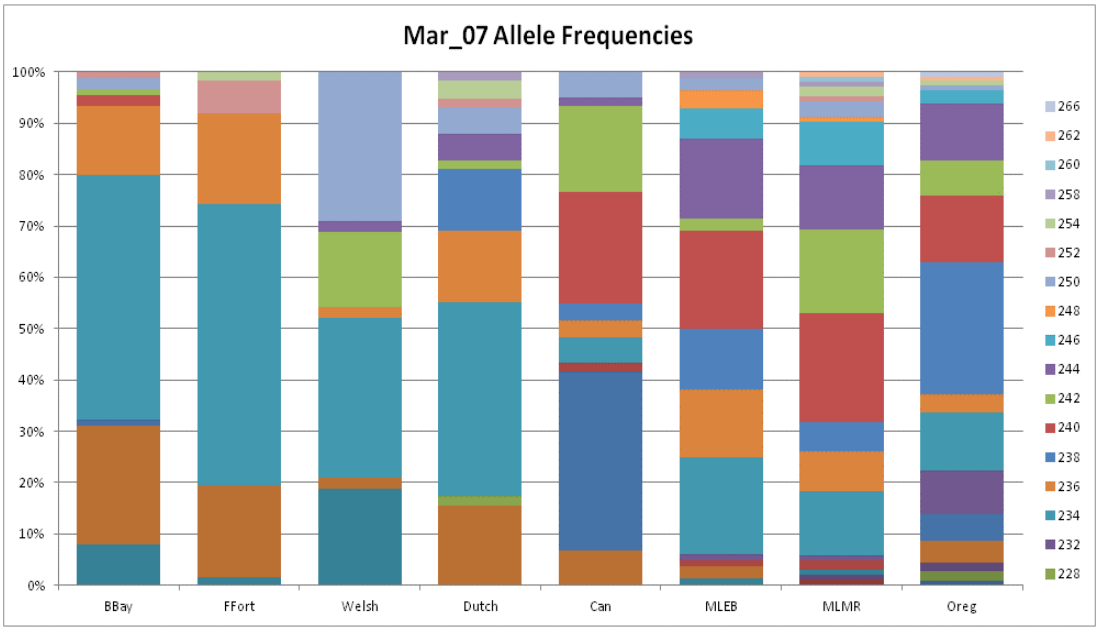
Locus: Mar_06

BP Shift	Allele	BBay	FFort	Welsh	Dutch	Can	MLEB	MLMR	Oreg
	225							1.92	
16	241							2.88	
2	243				1.67		2.33		
2	245						2.33	0.96	
2	247					1.67	1.16	0.96	
4	251								1.72
2	253			13.64	5.00	5.00	11.63	12.50	4.31
2	255			2.27	1.67	3.33	1.16	0.96	2.59
2	257							0.96	
2	259						1.16		
4	263		1.61						
4	267		1.61		3.33				12.07
2	269	2.27		2.27					0.86
2	271	46.59	64.52	70.45	50.00	48.33	51.16	52.88	37.93
2	273	17.05	11.29	11.36	16.67	8.33	10.47	6.73	23.28
2	275	25.00	14.52		5.00	31.67	17.44	19.23	14.66
2	277						1.16		0.86
2	279								0.86
10	289	5.68	6.45		13.33	1.67			0.86
2	291	2.27							
2	293				1.67				
4	297	1.14							
2	299				1.67				



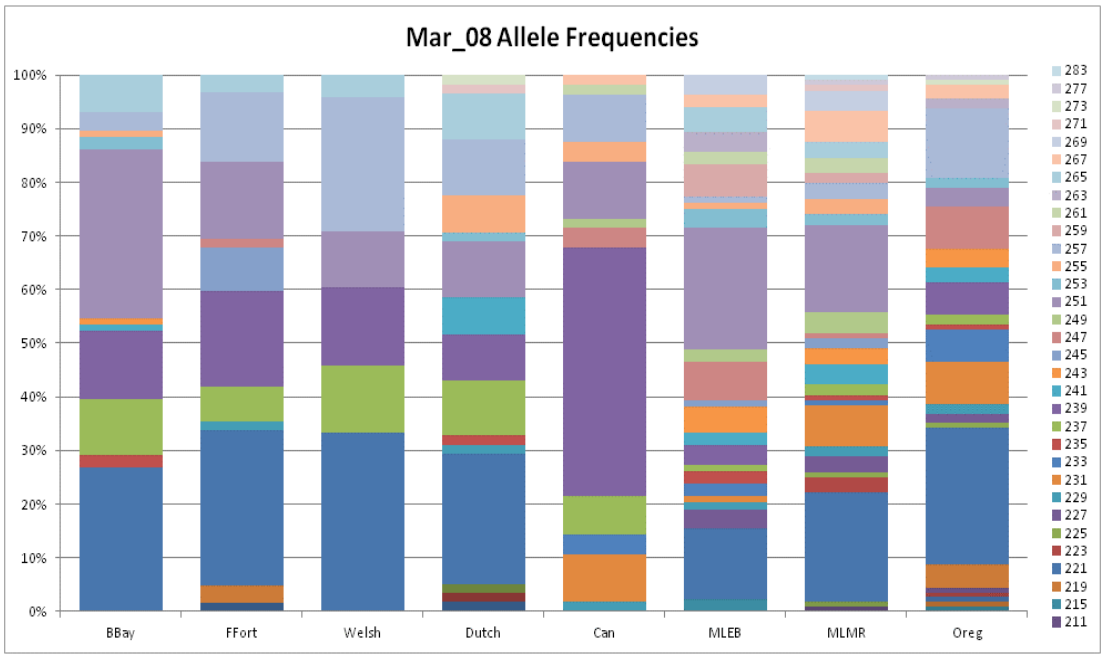
Locus: Mar_07

BP Shift	Allele	BBay	FFort	Welsh	Dutch	Can	MLEB	MLMR	Oreg
	204								0.86
8	212							0.96	
4	216								1.72
2	218							0.96	1.72
2	220	7.78	1.61	18.75			1.19	0.96	
2	222	23.33	17.74	2.08	15.52	6.67	2.38		4.31
2	224	1.11				35.00			5.17
2	226					1.67	1.19	1.92	
2	228				1.72				
4	232						1.19	0.96	8.62
2	234	47.78	54.84	31.25	37.93	5.00	19.05	12.50	11.21
2	236	13.33	17.74	2.08	13.79	3.33	13.10	7.69	3.45
2	238				12.07	3.33	11.90	5.77	25.86
2	240	2.22				21.67	19.05	21.15	12.93
2	242	1.11		14.58	1.72	16.67	2.38	16.35	6.90
2	244			2.08	5.17	1.67	15.48	12.50	11.21
2	246						5.95	8.65	2.59
2	248						3.57	0.96	
2	250	2.22		29.17	5.17	5.00	2.38	2.88	0.86
2	252	1.11	6.45		1.72			0.96	
2	254		1.61		3.45			1.92	0.86
4	258				1.72		1.19	0.96	
2	260							0.96	
2	262							0.96	0.86
4	266								0.86



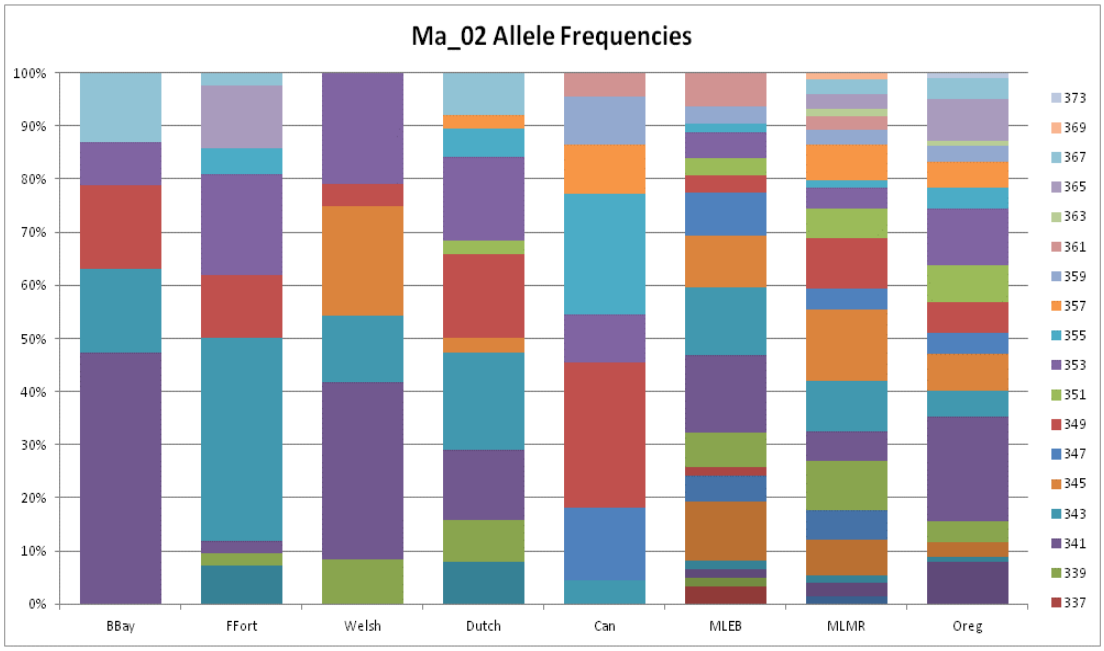
Locus: Mar_08

BP Shift	Allele	BBay	FFort	Welsh	Dutch	Can	MLEB	MLMR	Oreg
	137		1.61		1.72				
2	139				1.72				
8	147				1.72				
20	167							0.96	
30	197								0.88
4	201								0.88
2	203								0.88
4	207								0.88
2	209							0.96	
2	211								0.88
4	215						2.38		
4	219		3.23						4.39
2	221	26.74	29.03	33.33	24.14		13.10	20.19	25.44
2	223							2.88	
2	225							0.96	0.88
2	227						3.57	2.88	1.75
2	229		1.61		1.72	1.79	1.19	1.92	1.75
2	231					8.93	1.19	7.69	7.89
2	233					3.57	2.38	0.96	6.14
2	235	2.33			1.72		2.38	0.96	0.88
2	237	10.47	6.45	12.50	10.34	7.14	1.19	1.92	1.75
2	239	12.79	17.74	14.58	8.62	46.43	3.57		6.14
2	241	1.16			6.90		2.38	3.85	2.63
2	243	1.16					4.76	2.88	3.51
2	245		8.06				1.19	1.92	
2	247		1.61			3.57	7.14	0.96	7.89
2	249					1.79	2.38	3.85	
2	251	31.40	14.52	10.42	10.34	10.71	22.62	16.35	3.51
2	253	2.33			1.72		3.57	1.92	1.75
2	255	1.16			6.90	3.57	1.19	2.88	
2	257	3.49	12.90	25.00	10.34	8.93	1.19	2.88	13.16
2	259						5.95	1.92	
2	261					1.79	2.38	2.88	
2	263						3.57		1.75
2	265	6.98	3.23	4.17	8.62		4.76	2.88	
2	267					1.79	2.38	5.77	2.63
2	269						3.57	3.85	
2	271				1.72			0.96	
2	273				1.72				0.88
4	277							0.96	0.88
6	283							0.96	



Locus: Ma_02

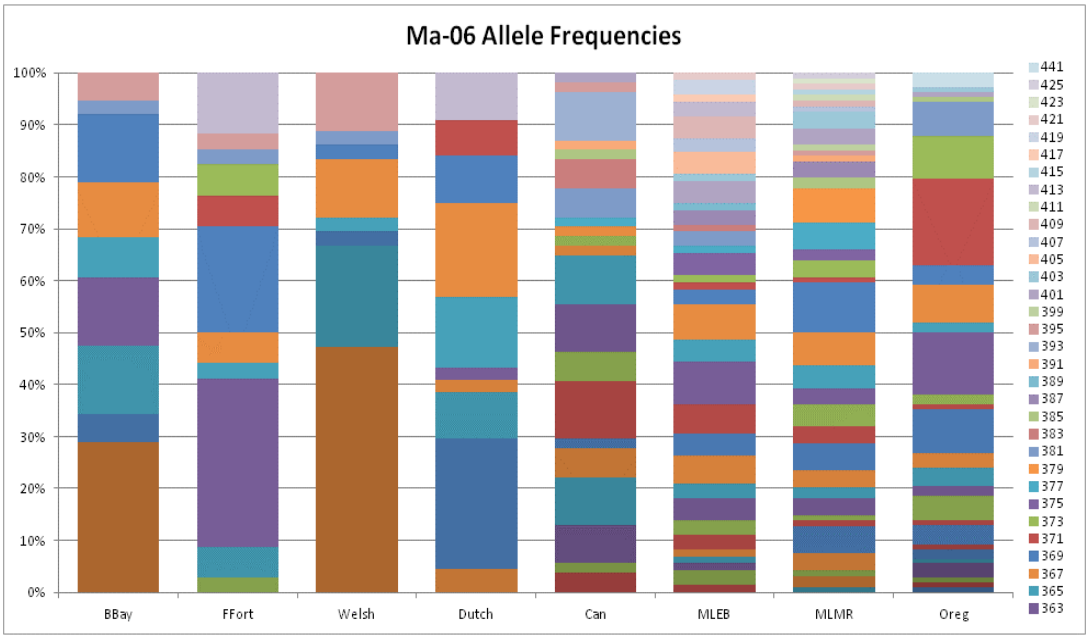
BP Shift	Allele	BBay	FFort	Welsh	Dutch	Can	MLEB	MLMR	Oreg
	311							1.35	
14	325						3.23		
2	327						1.61		
2	329						1.61	2.70	7.84
2	331		7.14		7.89		1.61	1.35	0.98
2	333						11.29	6.76	2.94
2	335						4.84	5.41	
2	337						1.61		
2	339		2.38	8.33	7.89		6.45	9.46	3.92
2	341	47.37	2.38	33.33	13.16		14.52	5.41	19.61
2	343	15.79	38.10	12.50	18.42	4.55	12.90	9.46	4.90
2	345			20.83	2.63		9.68	13.51	6.86
2	347					13.64	8.06	4.05	3.92
2	349	15.79	11.90	4.17	15.79	27.27	3.23	9.46	5.88
2	351				2.63		3.23	5.41	6.86
2	353	7.89	19.05	20.83	15.79	9.09	4.84	4.05	10.78
2	355		4.76		5.26	22.73	1.61	1.35	3.92
2	357				2.63	9.09		6.76	4.90
2	359					9.09	3.23	2.70	2.94
2	361					4.55	6.45	2.70	
2	363							1.35	0.98
2	365		11.90					2.70	7.84
2	367	13.16	2.38		7.89			2.70	3.92
2	369							1.35	
4	373								0.98



Locus: Ma_06

BP Shift	Allele	BBay	FFort	Welsh	Dutch	Can	MLEB	MLMR	Oreg
	319								0.93
2	321								0.93
2	323								0.93
2	325								2.78
4	329							1.06	0.93
2	331	28.95		47.22				2.13	
2	333								1.85
2	335					3.70	1.39		0.93
2	337					1.85	2.78	1.06	
2	339					7.41	1.39		
2	341			19.44		9.26	1.39		
2	343				4.55	5.56	1.39	3.19	
2	345	5.26		2.78	25.00	1.85		5.32	3.70
2	347					11.11	2.78	1.06	0.93
2	349		2.94			5.56	2.78	1.06	4.63
2	351					9.26	4.17	3.19	1.85
2	353	13.16	5.88		9.09	9.26	2.78	2.13	3.70
2	355				2.27	1.85	5.56	3.19	2.78
2	357						4.17	5.32	8.33
2	359						5.56	3.19	0.93
2	361					1.85		4.26	1.85
2	363	13.16	32.35		2.27		8.33	3.19	12.04
2	365	7.89	2.94	2.78	13.64		4.17	4.26	1.85
2	367	10.53	5.88	11.11	18.18	1.85	6.94	6.38	7.41
2	369	13.16	20.59	2.78	9.09		2.78	9.57	3.70

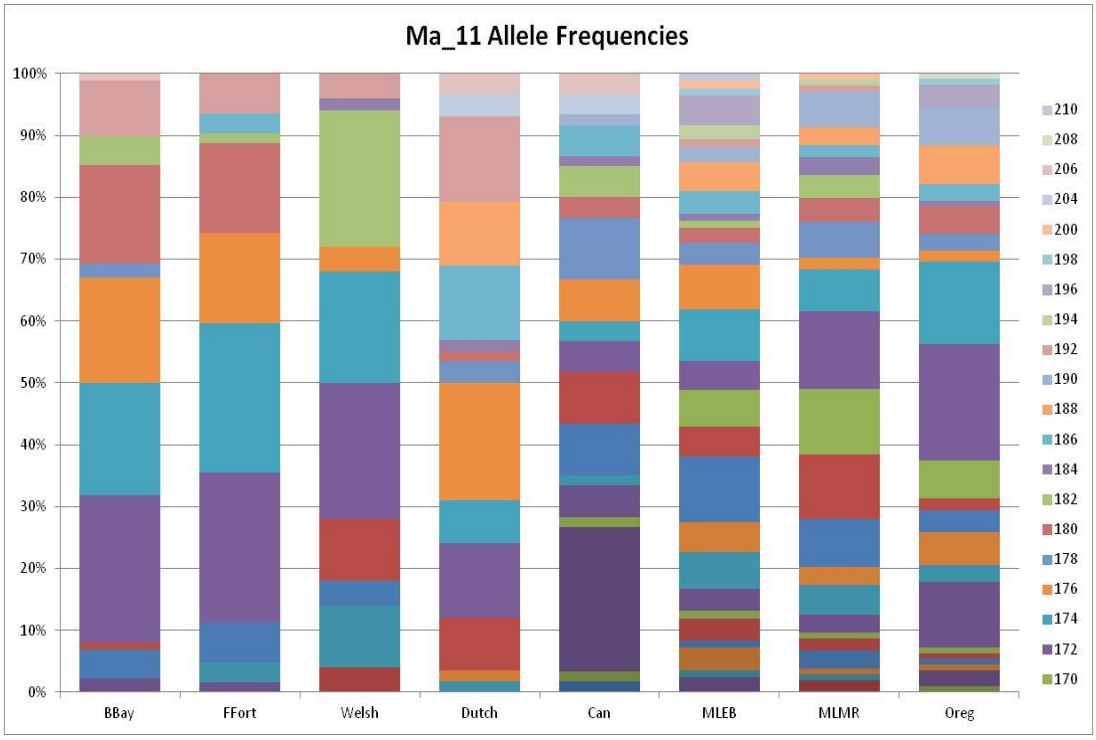
2	371		5.88		6.82		1.39	1.06	16.67
2	373		5.88				1.39	3.19	8.33
2	375						4.17	2.13	
2	377					1.85	1.39	5.32	
2	379							6.38	
2	381	2.63	2.94	2.78		5.56	2.78		6.48
2	383					5.56	1.39		
2	385					1.85		2.13	0.93
2	387						2.78	3.19	
2	389						1.39		
2	391					1.85		1.06	
2	393					9.26			
2	395	5.26	2.94	11.11		1.85		1.06	
4	399							1.06	
2	401					1.85	4.17	3.19	0.93
2	403						1.39	3.19	0.93
2	405						4.17		
2	407						2.78	1.06	
2	409						4.17	1.06	
2	411							1.06	
2	413		11.76		9.09		2.78		
2	415							1.06	
2	417						1.39		
2	419						2.78		
2	421						1.39	1.06	
2	423							1.06	
2	425							1.06	
16	441								2.78



Locus: Ma_11

BP Shift	Allele	BBay	FFort	Welsh	Dutch	Can	MLEB	MLMR	Oreg
	136					1.67			
4	140							1.92	
6	146					1.67			0.89
2	148					23.33	2.38		2.68
2	150						1.19	0.96	
2	152						3.57	0.96	0.89
2	154						1.19	2.88	0.89
2	156			4.00			3.57	1.92	0.89
2	158					1.67	1.19	0.96	0.89
2	160	2.27	1.61			5.00	3.57	2.88	10.71
2	162		3.23	10.00	1.72	1.67	5.95	4.81	2.68
2	164				1.72		4.76	2.88	5.36
2	166	4.55	6.45	4.00		8.33	10.71	7.69	3.57
2	168	1.14		10.00	8.62	8.33	4.76	10.58	1.79
2	170						5.95	10.58	6.25
2	172	23.86	24.19	22.00	12.07	5.00	4.76	12.50	18.75
2	174	18.18	24.19	18.00	6.90	3.33	8.33	6.73	13.39
2	176	17.05	14.52	4.00	18.97	6.67	7.14	1.92	1.79
2	178	2.27			3.45	10.00	3.57	5.77	2.68
2	180	15.91	14.52		1.72	3.33	2.38	3.85	4.46
2	182	4.55	1.61	22.00		5.00	1.19	3.85	
2	184			2.00	1.72	1.67	1.19	2.88	0.89
2	186		3.23		12.07	5.00	3.57	1.92	2.68
2	188				10.34		4.76	2.88	6.25
2	190					1.67	2.38	5.77	6.25
2	192	9.09	6.45	4.00	13.79		1.19	0.96	

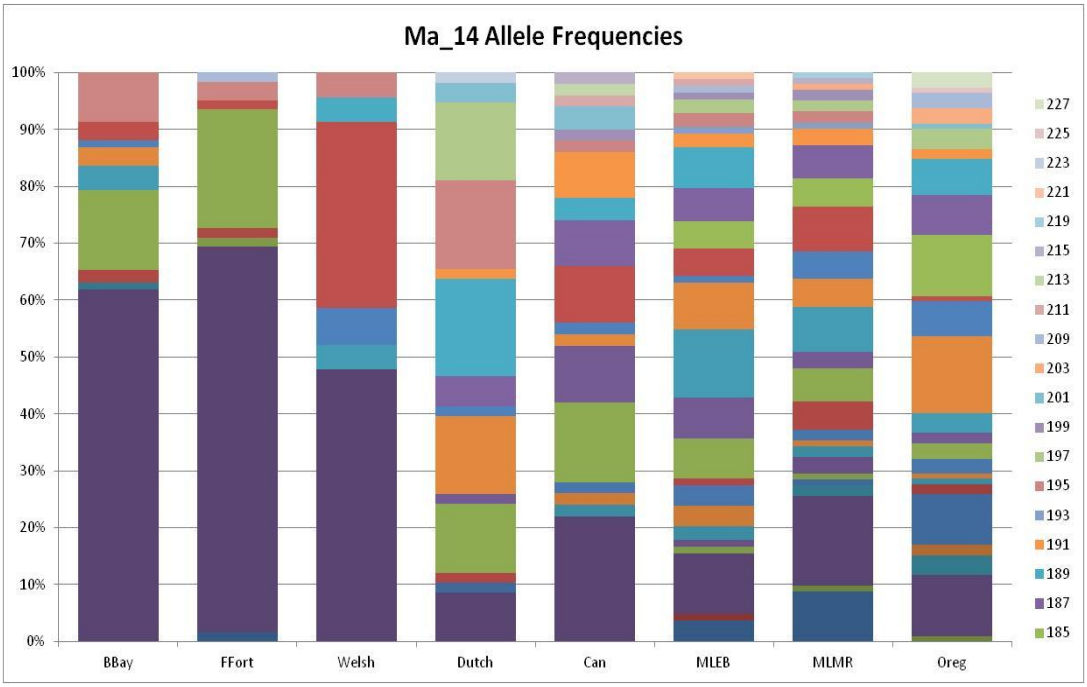
2	194						2.38	0.96	
2	196						4.76		3.57
2	198						1.19		0.89
2	200						1.19	0.96	
4	204				3.45	3.33			
2	206	1.14			3.45	3.33			
2	208								0.89
2	210						1.19		



Locus: Ma_14

BP Shift	Allele	BBay	FFort	Welsh	Dutch	Can	MLEB	MLMR	Oreg
	137		1.61				3.57	8.82	
2	139						1.19		
8	147							0.98	0.89
2	149	61.96	67.74	47.83	8.62	22.00	10.71	15.69	10.71
2	151	1.09						1.96	3.57
4	155								1.79
2	157				1.72			0.98	8.93
2	159								1.79
2	161		1.61				1.19	0.98	
2	163						1.19	2.94	
2	165					2.00	2.38	1.96	0.89
2	167					2.00	3.57	0.98	0.89
2	169					2.00	3.57	1.96	2.68

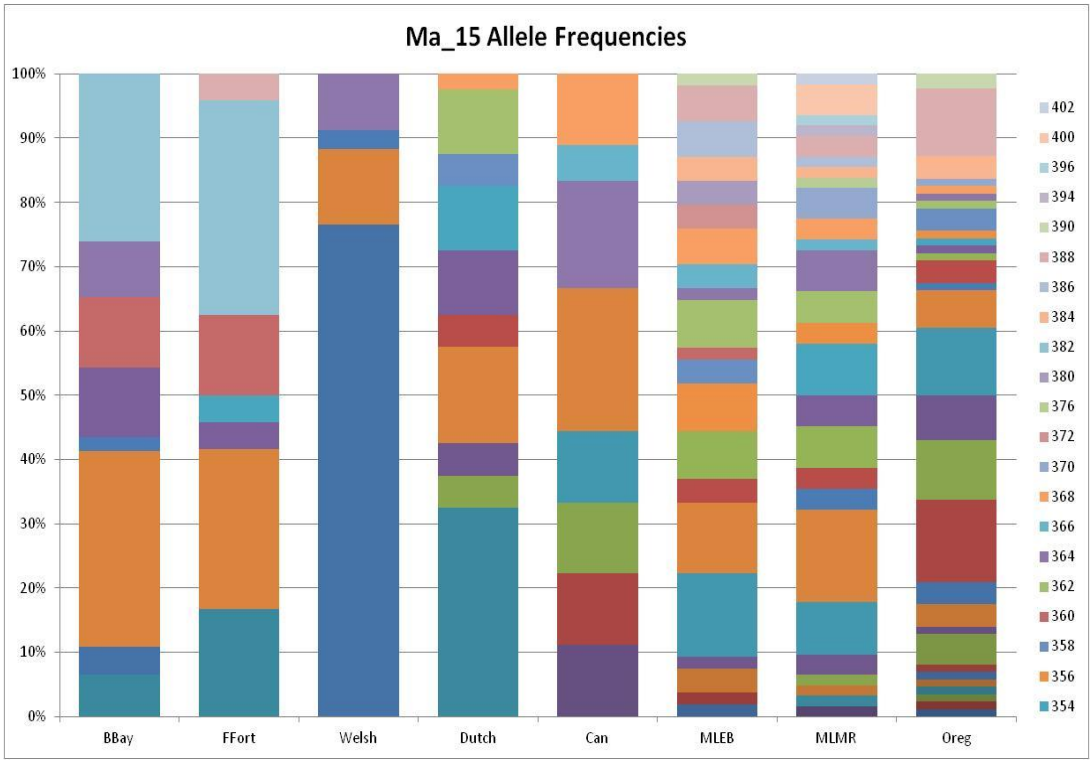
2	171	2.17	1.61		1.72		1.19	4.90	
2	173	14.13	20.97		12.07	14.00	7.14	5.88	2.68
2	175				1.72	10.00	7.14	2.94	1.79
2	177	4.35		4.35			11.90	7.84	3.57
2	179	3.26			13.79	2.00	8.33	4.90	13.39
2	181	1.09		6.52	1.72	2.00	1.19	4.90	6.25
2	183	3.26	1.61	32.61		10.00	4.76	7.84	0.89
2	185						4.76	4.90	10.71
2	187				5.17	8.00	5.95	5.88	7.14
2	189			4.35	17.24	4.00	7.14		6.25
2	191				1.72	8.00	2.38	2.94	1.79
2	193						1.19	0.98	
2	195	8.70	3.23	4.35	15.52	2.00	2.38	1.96	
2	197				13.79		2.38	1.96	3.57
2	199					2.00	1.19	1.96	
2	201				3.45	4.00			0.89
2	203							0.98	2.68
6	209		1.61				1.19		2.68
2	211					2.00	1.19		
2	213					2.00			
2	215					2.00		0.98	
4	219							0.98	
2	221						1.19		
2	223				1.72				
2	225								0.89
2	227								2.68



Locus: Ma_15

BP Shift	Allele	BBay	FFort	Welsh	Dutch	Can	MLEB	MLMR	Oreg
	302								1.16
6	308								1.16
4	312								1.16
4	316							1.61	
2	318								1.16
2	320								1.16
2	322						1.85		1.16
2	324						1.85		1.16
2	326								4.65
2	328					11.11			1.16
2	330	6.52	16.67		32.50			1.61	
2	332						3.70	1.61	3.49
2	334	4.35		76.47					3.49
2	336					11.11			12.79
2	338				5.00	11.11		1.61	9.30
2	340				5.00		1.85	3.23	6.98
2	342					11.11	12.96	8.06	10.47
2	344	30.43	25.00	11.76	15.00	22.22	11.11	14.52	5.81
2	346	2.17		2.94				3.23	1.16
2	348				5.00		3.70	3.23	3.49
2	350						7.41	6.45	1.16
2	352	10.87	4.17		10.00			4.84	1.16
2	354		4.17		10.00			8.06	1.16
2	356						7.41	3.23	1.16
2	358				5.00		3.70		3.49
2	360	10.87	12.50				1.85		
2	362				10.00		7.41	4.84	1.16
2	364	8.70		8.82		16.67	1.85	6.45	1.16
2	366					5.56	3.70	1.61	
2	368				2.50	11.11	5.56	3.23	1.16
2	370							4.84	1.16
2	372						3.70		
4	376							1.61	
4	380						3.70		
2	382	26.09	33.33						
2	384						3.70	1.61	3.49
2	386						5.56	1.61	
2	388		4.17				5.56	3.23	10.47
2	390						1.85		2.33
4	394							1.61	
2	396							1.61	
4	400							4.84	

2	402							1.61	


Ma_15 Allele Frequencies
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
BBay
FFort
Welsh
Dutch
Can
MLEB
MLMR
Oreg
402
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Chapter 6.

A genetic investigation to identify which *Ensis* species are present in the Irish Sea and adjacent areas.

Abstract

The razor clam species *Ensis siliqua*, and *Ensis arcuatus* and the introduced *Ensis macha* and *Ensis directus* are widely distributed in European coastal areas. Past work on *Ensis* species in the Irish Sea and around Ireland used morphology of shell to confirm three species, *Ensis siliqua, E. arcuatus* and *E. directus* as present, but a molecular investigation of the *Ensis* spp. present in Irish waters has yet to be carried out. Specimens of *Ensis* spp. (n=134) were obtained from five coastal areas around Ireland and Wales in 2012. Extracted DNA from gill tissue was amplified using species-specific primers for the internal transcribed spacer region 1 (ITSI), which identifies individuals as *E. siliqua/E. arcuatus*, *E. macha* or *E. directus*. To distinguish between *E. siliqua* and *E. arcuatus* a further amplification and enzyme digestion using *Ksp*I was performed. Species-specific primers were also used to amplify the mitochondrial DNA cytochrome oxidase 1 region (CO1), and sequencing of resulting amplicons was attempted to identify to species level. All but 15 of the sampled razor clams were identified as *Ensis siliqua*. Of the latter, the 10 specimens from Derrynane (SW Ireland) all failed to amplify for ITSI but were indicated to be *E. siliqua* for the mitochondrial COI gene. Two possible reasons for this result are that, either another non target species (*E. ensis* or *E. minor*) are present in Derrynane and the CO1 primers are not completely species specific, or extensive hybridisation is taking place between native species such that the individuals typed have *E. siliqua* mtDNA but nuclear DNA of another native species. Sequencing of COI from these and other individuals might help to resolve this issue but was repeatedly unsuccessful when attempted by a commercial company in Queens UniversityBelfast.

Keywords: CO1, *Ensis*, ITS, Irish Sea, *Ksp*1, PCR-RFLP, razor clam.

6.1. Introduction

Razor clams, or pod razors, (genus *Ensis*) of the family Solenidae are widely distributed in European waters (Fernandez-Tajes & Mendez, 2007). Past observations have shown the distribution of European *Ensis* species to be extremely patchy, with the highest numbers found in areas with favourable sediments and flow of water, from the lower intertidal to depths of 40metres or lower (South Wales Sea Fisheries Committee, 1999). Among native species, *Ensis siliqua* and *Ensis arcuatus* (formerly known as *Ensis magnus* Schumacher (Von Cosel, 2009)) are the most common, and are distributed from Norway to the Mediterranean Sea (Van Urk, 1964; Hayward & Ryland, 1998; Freire *et al.*, 2008), while *Ensis ensis* and *Ensis minor* are found less frequently (Von Cosel, 2009). *Ensis directus* is native to the Atlantic coast of North America, and is thought to have been introduced to European shores in the late 1970s as larvae in the ballast water of ships (Luczak *et al.*, 1993; Vierna *et al.*, 2010), while *Ensis macha* is a native species of South America, and was recently introduced to European markets (Fernandez-Tajes *et al.*, 2010).

Four species of *Ensis* have been previously identified around Ireland and the British Isles, those being *E. siliqua*, *E. arcuatus*, *E. minor* and *E. ensis* (Holme, 1951; Henderson & Richardson, 1994; Robinson & Richardson, 1998; South Wales Sea Fisheries Committee, 1999; Von Cosel, 2009; www.marine.ie, 2010). These species are thought to inhabit different types of sediment, and have been rarely reported as living in the same area (Van Urk, 1964; Fahy & Carroll, 2007). In Irish waters, *E. siliqua* is thought to be the most common on the east coast and *E. ensis* the least, with only two out of 2,500 razor clam specimens morphologically identified as *E. ensis* in a 1998 study (Fahy, 1999). *Ensis arcuatus* is mainly found in coarse, sandy sediments on the west coast of Ireland (www.marine.ie, 2010), while *E. minor* is usually located subtidally in fine sand in the northern part of its range (Von Cosel, 2009).

The demand for *Ensis* species in European markets has increased in the last 10 years, resulting in a harvest of approximately 1539 tons in 2012 (Fernandez-Tajes *et al.*, 2012), making it a very commercially important bivalve. Within European markets, *E. siliqua*, *E. arcuatus* and the introduced *E. directus* are the most commercially important (Fernandez-Tajes & Mendez, 2007), with *E. siliqua* used

mainly in the canning industry, and *E. arcuatus* being preferred for fresh fish markets (Freire *et al*., 2008). *Ensis arcuatus* is the most important species of Solenidae in Spain, and its commercial value increased considerably in the 2000s (Darriba *et al.*, 2004), while *E. siliqua* and *E. arcuatus* make up the bulk of the live market trade in the British Isles, while smaller razor species such as *E. ensis* and *E. directus* are collected for cooking and sale in prepared form (South Wales Sea Fisheries Committee, 1999). Within Irish waters *Ensis siliqua* makes up the vast bulk of landings, with most commercial fishing taking place in the Irish Sea (Fahy, 1999; Fahy & Carroll, 2007; www.marine.ie, 2010).

Past classification of the different species of *Ensis* has relied on morphological identification involving a detailed analysis of the shell curvature, colouration, size of teeth and muscle impressions (Bloomer, 1901-1902), though this has proven to be unreliable in most cases (Holme, 1951; Van Urk, 1964; Rufino *et al.*, 2012). The length the shell in each species and the type of sediment in which they are found are also inaccurate means of identifying the different species, as these factors can be influenced by geographical location (Holme, 1951; Fahy, 1999; Muir & Moore, 2003; Fahy & Carroll, 2007; www.marine.ie, 2010). The misidentification of commercial marine species, including *Ensis* can be problematic when estimating official landings (Rasmussen & Morrissey, 2009; Arias-Perez *et al.*, 2012; Fernandez-Tajes *et al.*, 2012). Also there can be issues, because of the different economic value of each razor clam species and the fact that, under European legislation (Regulation No. 104/2000 of Dec. 17, 1999), canned products must be correctly labelled with the commercial name of the species before retail distribution (Council Regulation, 2000). As the shells of *Ensis* are removed when canned and only small fragments of the animal may exist in a can, morphological identification can be impossible (Fernandez-Tajes *et al.*, 2010). Therefore, a more accurate and efficient method of species identification is required to avoid commercial fraud and protect consumer rights (Fernandez-Tajes & Mendez, 2007; Freire *et al.*, 2008).

Genetic approaches based on molecular ecological techniques have recently been employed to resolve this matter (Fernandez-Tajes & Mendez, 2007; Fernandez-Tajes *et al.*, 2010). These molecular procedures allow relatively easy *Ensis* species identification independently of the size of sample and condition of the animal (Freire

et al., 2008). Data on the extent of molecular genetic structure and genetic variability in different populations of *E. siliqua* throughout the geographical range are also essential to preserve natural populations of this species in Europe (Fernandez-Tajes *et al.*, 2007; Arias *et al.*, 2011). The polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) method was employed to investigate the internal transcribed spacer region 1 (ITS1) in ribosomal DNA of four *Ensis* species (*E. arcuatus, E. siliqua, E. directus,* and *E. macha*) by Freire *et al.* (2008). A species-specific restriction endonuclease pattern was found using the enzyme *Ksp*I, allowing for accurate identification of *E. arcuatus* and *E. siliqua* (Freire *et al.*, 2008). Furthermore, a multiplex PCR method was developed, using species-specific reverse primers to differentiate between the four razor clam species *Ensis arcuatus, Ensis siliqua, Ensis directus,* and *Ensis macha* as mentioned above (Fernandez-Tajes *et al.*, 2010). In conjunction with the previous method described by Freire *et al.* (2008), it was reported that fast, accurate identification of these four razor clam species was now possible.

While past molecular studies have focused on the genetic variation of *E. siliqua* in Ireland and other European areas, it is still not known with certainty what species exist in Irish waters (Fernandez-Tajes *et al.*, 2007; Arias *et al.*, 2011; Arias-Perez *et al.*, 2012; Fernandez-Tajes *et al.*, 2012; Varela *et al.*, 2012). To date, there has been no identification of razor clam species around Ireland and in the Irish Sea using molecular methods. Therefore, the aim of the current study was to identify the different species of *Ensis* present in this area, using molecular methods involving PCR-RFLP of the ITS1 region of ribosomal DNA, and the COI region of mitochondrial DNA.

6.2. Materials and Methods

Sample Acquisition

Specimens of *Ensis spp.* (n=134) were obtained from five coastal areas (fig. 1) around Ireland and in the Irish Sea (Table 1). All gill tissue samples were preserved in 90% Ethanol before use.

Table 1. *Ensis* spp. Sampling Sites:

Area	**Country**	**N**	**Latitude**	**Longitude**
Howth	Ireland	30	N 52° 13’ 07”	W 06° 47’ 38”
Ringaskiddy	Ireland	12	N 51° 49' 48"	W 08° 19' 28"
Derrynane	Ireland	10	N 51° 45' 46"	W 10° 07' 14"
Menai Bridge	Wales	48	N 53° 13' 00"	W 04° 09' 00"
Oxwich Bay, Swansea	Wales	34	N 53° 17' 39"	W 03° 45' 34"

DNA extraction was carried out using Chelex-100 resin (Bio-Rad), using 10mg sections of gill tissue in 100µl of CHELEX, heated at 90°C for 70 minutes, and spun at 13,000 rpm for 5 minutes. 100ul of supernatant was extracted and DNA was stored at 4°C, or -20°C for long-term storage.

Figure 1. Sampling sites of *Ensis* species around Ireland and Wales. Adapted from google maps.

ITS-I PCR amplification

Since the multiplex method outlined by Fernández-Tajes *et al.* was not repeatable in the laboratory (Fernandez-Tajes *et al.*, 2010), as the primers specified did not amplify DNA correctly, the single PCR method outlined by Freire *et al.* was used instead (Freire *et al.*, 2008). Using the forward primer ITSIN-F (5’-CGG ATG GAT CAT TAC CAA AG-3’) (Freire *et al.*, 2008), and each of the species-specific reverse primers ITSArSil-R (5’-CTTCGCGTCGGCAATA-3’), ITSDir-R (5’-

GACGGAGTGCATAGTGTATAAC-3'), and ITSMa-R (5'-CGTTGTTTGTGGTAATAAGGC-3') (Fernandez-Tajes *et al.*, 2010), PCR amplification was carried out in 25µl volumes, containing 2.5µl 5x Buffer, 2µl $MgCl_2$ (25mM), 4.8µl dNTPs (1.25mM), 0.25µl of each primer (100µM), 0.25µl Ampli-Taq (0.625units)(Invitrogen), 13.95µl dH_2O and 1µl template DNA. Amplifications were performed on a "Techne" thermocycler, and were carried out under the following conditions: 95°C for 5min; 35x (94°C for 20sec, 56°C for 20sec, 72°C for 60sec); 72°C for 5minutes. PCR products were viewed on a 2% Agarose gel. Each PCR reaction was repeated several times.

KspI digestion

To distinguish between the two razor clam species *Ensis siliqua* and *Ensis arcuatus* (formerly *E. magnus* Schumacher), the primers ITSIN-F (5'-CGG ATG GAT CAT TAC CAA AG-3') and ITSIN-R (5'-GAGTGATCCACCGCATAGAG-3') (Freire *et al.*, 2008) were used in a 25µl PCR amplification containing 2.5µl 5x Buffer, 2µl $MgCl_2$ (25mM), 4µl dNTPs (1.25mM), 0.25µl of each primer (100µM), 0.25µl Ampli-Taq (0.625units)(Invitrogen), 14.75µl dH_2O and 1µl template DNA. Amplifications were performed on a "Techne" thermocycler, and were carried out under the following conditions: 95°C for 5min; 35x (94°C for 20sec, 56°C for 20sec, 72°C for 60sec); 72°C for 5minutes. PCR products were viewed on a 2% Agarose gel. Digestion of amplification products were performed in 20µl volumes at 37°C overnight, containing 5µl of PCR product, 2µl of Buffer (provided with *Ksp*I), 0.2µl (1 unit) of *Ksp*I restriction enzyme, and 12.8µl dH_2O per well. Two drops of mineral oil were added to each well, to prevent evaporation of product overnight. The reactions were stopped by the addition of a loading buffer, and the restriction fragments and uncut products were visualised on a 2% Agarose gel.

COI *amplification*

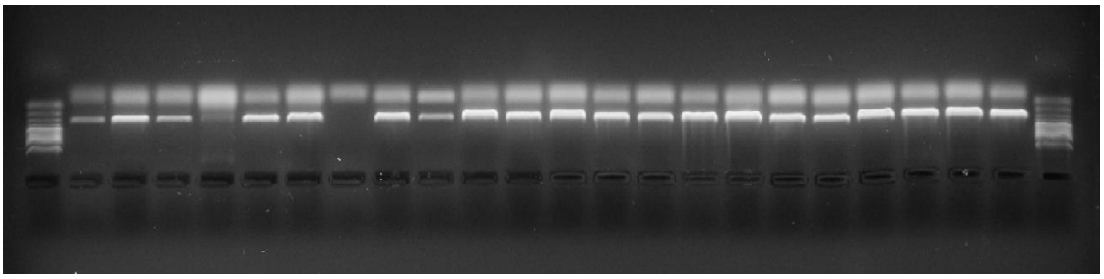
In order to amplify the *Cox*I region of *Ensis* spp. the species-specific primers COI-siliqua-F (5'-GGGATTAGTTGGGACTAGG-3'), COI-siliqua-R (5'-GTTAAAGCCCCTGCCAA-3'), COI-arcuatus-F (5'-TAGAGTTAGCTCGTCCT-3'), COI-arcuatus-R (5'-AAATAGGGTCACCACCA-3'), COI-directus-F (5'-CAGGTTTAGTTGGAACTAGG-3'), COI-directus(a)-R (5'-

GATCTCCACCACCTCT-3'), and COI-directus(b)-R (5'-GATCTCCGCCACCTCT-3') were used (Jaoquín Vierna, Evolutionary Biology Group, University of Coruna, Spain, pers. comm.). PCR amplifications were carried out in 25µl volumes, containing 2.5µl 5x Buffer, 3µl $MgCl_2$ (25mM), 2µl dNTPs (8mM), 0.5µl of each primer (100µM), 0.4µl Ampli-Taq (Invitrogen), 15.1µl dH_2O and 1µl template DNA. Amplifications were performed on a "Techne" thermocycler, and were carried out under the following conditions: 95°C for 3min; 35x (94°C for 20sec, 48°C for 20sec, 72°C for 30sec); 72°C for 5minutes. Resulting products were viewed on a 2% Agarose gel. Purified amplicons were sent to Eurofins MWG Operon for DNA sequencing.

6.3. Results

ITS-I PCR amplification

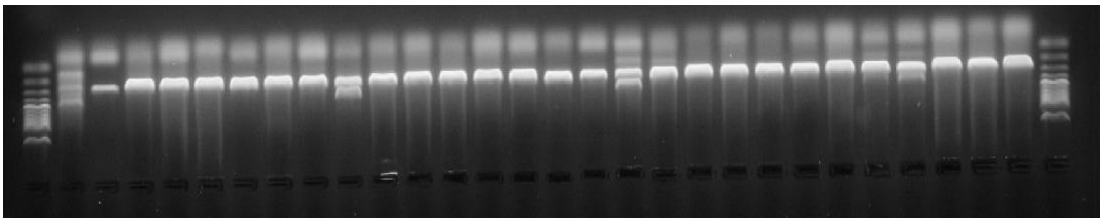
The 134 razor clams sampled around Ireland and in the Irish Sea were amplified in PCR reactions using species-specific primers for the identification of *Ensis siliqua* and *Ensis arcuatus* (*arcuatus*), *Ensis directus* and *Ensis macha*, resulting in varying band patterns being present for each individual when run out on a 2% Agarose gel (Figures 2–5). When re-tested it was confirmed that all 30 of the Howth individuals, all 12 of the Ringaskiddy, 47 of the 48 Menai Bridge (97%) and 30 of the 34 Swansea (88%) razor clam individuals were identified as either *E. siliqua* or *E. arcuatus*, using amplification of the internal transcribed spacer region 1 (ITS-I). No amplifications were present in the 10 individuals sampled in Derrynane, from the west coast of Ireland.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Figure 2. Photograph of 2% agarose gel showing the identification of Howth razor clam samples (number 1 to 22) as either *Ensis siliqua* or *E. arcuatus*. Lanes 1 and 24: DNA Ladder; lanes 2-23: Howth *Ensis* samples number 1-22. Note lack of DNA in Lane 8.

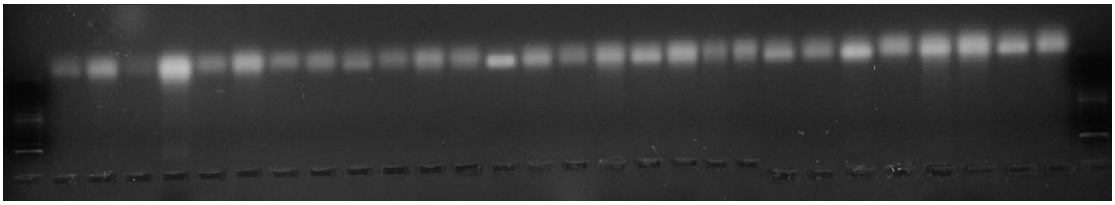
One individual of the Menai Bridge collection (data not shown), and four of the Swansea collection could not be positively identified as either *E. siliqua* or *arcuatus*, due to absent or unusual amplifications (Figure 3).



1 9 17 25

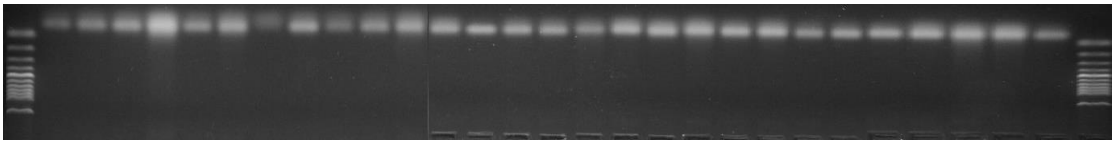
Figure 3. Photograph of 2% agarose gel showing the identification of Swansea razor clam samples (number 1 to 28) as either *Ensis siliqua* or *E. arcuatus*, excluding individuals number 1, 9, 17 and 25. Lanes 1 and 30; DNA ladders; lanes 2-29; Swansea *Ensis* samples number 1-28, with lanes 1, 9, 17 and 25 showing unusual amplification.

On repeated trials, all 134 of the razor clam individuals sampled showed non-amplification when primers specific to *E. directus* and *E. macha* were used in PCR reactions (Figures 4 and 5, are examples of analysis of individuals from Howth), further confirming the identification of 119 individuals (89%) as either *E. siliqua* or *E. arcuatus*. Note that reference samples of known *E. directus* or *E. macha* to act as positive controls could not be sourced, but repeated trials using similar analytical conditions to those applied in Figures 2 and 3 yielded no specific bands



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Figure 4. Photograph of 2% agarose gel showing non-amplification of Howth razor clam samples (number 1 to 28), using primers specific to *Ensis directus*. Lanes 1 and 30: DNA ladder; lanes 2-29: Howth *Ensis* samples number 1-28.

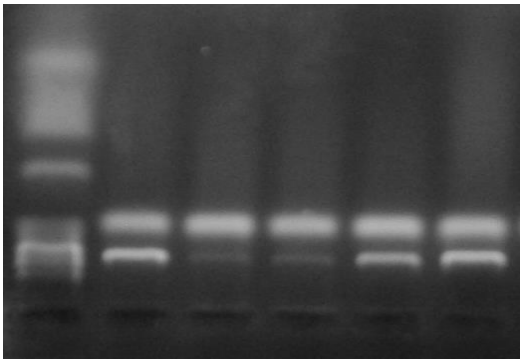


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Figure 5. Photograph of 2% agarose gel showing non-amplification of Howth razor clam samples (number 1 to 28), using primers specific to *Ensis macha*. Lanes 1 and 30: DNA ladder; lanes 2-29: Howth *Ensis* samples number 1-28.

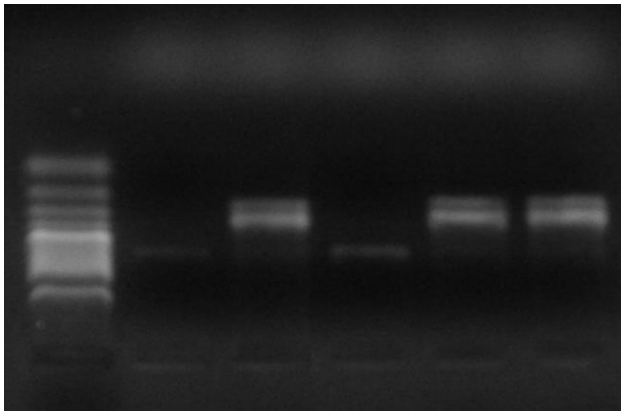
KspI Digestion

Following PCR amplification (Figure 6) and enzyme digestion using K*sp*I, all 119 individuals ascertained as either *E. siliqua* or *E. arcuatus* in previous amplifications were identified as *Ensis siliqua* (Figure 7).



1 2 3 4 5 6

Figure 6. Photograph of 2% agarose gel showing amplification of Menai Bridge *Ensis* individuals before K*sp*I digestion, resulting in fragments of ~580 bp in length. Lane 1: DNA ladder; lanes 2-6: Menai Bridge individuals number 1-5.



1 2 3 4 5 6

Figure 7. Photograph of 2% agarose gel showing resulting bands of K*sp*I digestion of Menai Bridge *Ensis* individuals, resulting in two fragments of 257 and 332 bp in length in three individuals. Lane 1: DNA ladder; lanes 2 - 6: Menai Bridge individuals number 1-5. Lanes 2 and 4 individuals incompletely digested in the gel shown, but present in other assays.

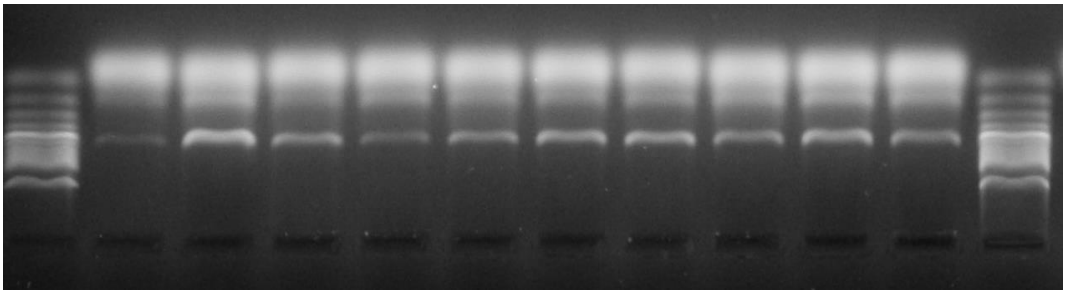
COI *PCR amplification*

Razor clam individuals from Howth (n=8), Ringaskiddy (n=6), Menai Bridge (n=7) and Swansea (n=8) were amplified using primers specific to the *CO*I region of *Ensis siliqua, E. arcuatus*, and two varieties of *E. directus*. All amplifications resulted in single or multiple bands present for each individual using the four

different primers for the three species of *Ensis*, a confusing result (data not shown). Purified amplicons of these specimens and those from Derrynane (see below) were sent to Eurofins MWG Operon and subsequently to Queen's University Belfast (QUB) for DNA sequencing, but results were inconclusive.

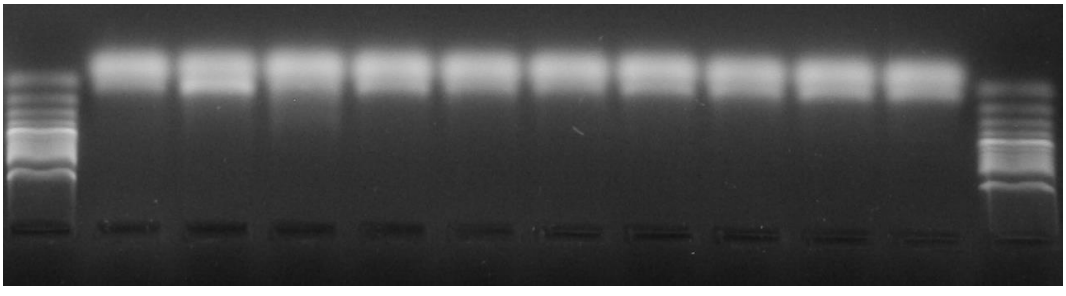
Derrynane Individuals

As noted above, when using *Ensis* species-specific primers in amplifications of the ITS-1 region of DNA, no amplifications were observed in the 10 individuals sampled in Derrynane, from the west coast of Ireland. PCR amplifications using primers specific to the COI region of *E. siliqua* however, resulted in fragments of DNA amplified (figure 8), with very weak or no bands present after amplification using primers specific to the COI region of *E. arcuatus*, and *E. directus* (figures 9-11). As stated above, purified amplicons were sent to Eurofins MWG Operon and QUB for DNA sequencing, but results were inconclusive.



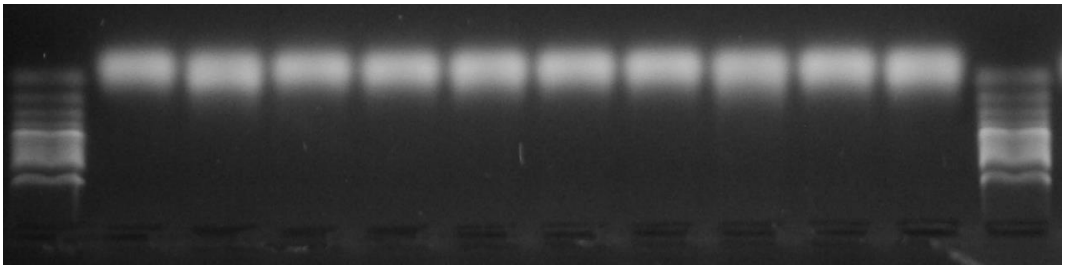
1 2 3 4 5 6 7 8 9 10 11 12

Figure 8. Photograph of 2% agarose gel showing resulting bands of Derrynane individuals when using primers specific to the COI region of *E. siliqua.* Lanes 1 and 12: DNA ladders; lanes 2-11: Derrynane individuals number 1-10.



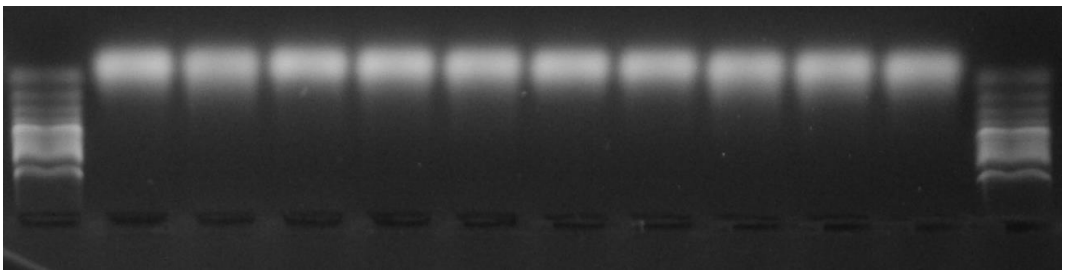
1 2 3 4 5 6 7 8 9 10 11 12

Figure 9. Photograph of 2% agarose gel showing resulting bands of Derrynane individuals when using primers specific to the COI region of *E. arcuatus.* Lanes 1 and 12: DNA ladders; lanes 2-11: Derrynane individuals number 1-10.



1 2 3 4 5 6 7 8 9 10 11 12

Figure 10. Photograph of 2% agarose gel showing resulting bands of Derrynane individuals when using primers specific to the COI region of *E. directus* (A). Lanes 1 and 12: DNA ladders; lanes 2-11: Derrynane individuals number 1-10.



1 2 3 4 5 6 7 8 9 10 11 12

Figure 11. Photograph of 2% agarose gel showing resulting bands of Derrynane individuals when using primers specific to the COI region of *E. directus* (B). Lanes 1 and 12: DNA ladders; lanes 2-11: Derrynane individuals number 1-10.

Discussion

Twenty three species of razor shells and jack knife clams (Solenoidea) have been previously morphologically identified in the eastern Atlantic (Von Cosel, 2009), with four of these species, *E. siliqua*, *E. arcuatus* (*arcuatus*), *E. minor* and *E. ensis*, being present in Irish waters (Holme, 1951; Henderson & Richardson, 1994; Robinson & Richardson, 1998; South Wales Sea Fisheries Committee, 1999; Von Cosel, 2009; www.marine.ie, 2010). The present study has identified *Ensis siliqua* as the abundant species in five regions around Ireland and on the east coast of Wales, using molecular methods. Twelve percent of the individuals sampled have not been positively identified using these methods, including all 10 individuals sampled from the west coast of Ireland in Derrynane Bay.

As the correct classification of *Ensis* species in Europe is important for both commercial and ecological reasons, previous work has been carried out to identify razor clams using both morphological and molecular means (Fernandez-Tajes & Mendez, 2007; Freire *et al.*, 2008; Von Cosel *et al.*, 2009; Fernandez-Tajes *et al.*, 2010; Vierna *et al.*, 2010; Fernandez-Tajes *et al.*, 2012). The method of PCR-RFLP has previously been used in the identification of bivalve species such as mussels, clams, oysters and scallops (Heath *et al.*, 1995; Fernandez *et al.*, 2000; Regoe *et al.*, 2002; Cross *et al.*, 2006) and fish species (Cespedes *et al.*, 1999; Carrera *et al.*, 2000), while species-specific variation at the internal transcribed spacer region (ITS) has been successfully used to identify marine species such as clam species including *Ruditapes decussates*, *Venerupis pullastra*, and *Ruditapes philippinarum*, (Fernandez *et al.*, 2000), scallop species (Lopez-Pinon *et al.*, 2002), mussel species (Santaclare *et al.*, 2006), common oyster species (Wang & Guo, 2008), the cockle species *Cerastoderma edule* and *C. glaucum* (Freire *et al.*, 2011), the great white shark *Carcharodon carcharias* (Chapman *et al.*, 2003), and the marine algae Raphidophyceae (Connell, 2002).

The rapid evolution of the variable internal transcribed spacer (ITS) region compared to their flanking coding regions and other conserved regions, allow for comparisons between closely related species (Presa *et al.*, 2002; Freire *et al.*, 2008), and have been recognized as informative phylogenetic markers within the genus *Ensis* in the past (Vierna *et al.*, 2010). In the present study, the method outlined by Fernandez-Tajes *et al.* (2010) successfully identified 88% of the sampled razor clams, though the multiplex PCR amplification, described in the previous work by Fernandez-Tajes *et al.* (2010), was not repeatable within the laboratory. Therefore, PCR amplifications had to be performed individually using each of the *Ensis* species-specific primers.

While variation at the *CO*I region has been successfully used to distinguish between different species of fish and bivalves (Boudry *et al.*, 1998; Hare *et al.*, 2000; Marshall *et al.*, 2007; Jerome *et al.*, 2008; Wong & Hanner, 2008), the results of the present study are inconclusive, as sequencing of the amplicons was not successful. However, the presence of amplified fragments of DNA in the Derrynane samples of

Ensis (figure 8) are intriguing, as these individuals were not identified as *Ensis siliqua* when using the ITS-I primer method.

The presence of razor clam species hybrids has been previously suggested by Von Cosel (2009), based on morphological identification of *Ensis* individuals sampled throughout Europe. These intergrades were thought to be present between *E. minor* and *E. siliqua* on the French Atlantic coast, *E. ensis* and *E. arcuatus* on the Netherlands coast, and between *E. minor* and *E. arcuatus* (*arcuatus*) on the west coast of Ireland. It was suggested that these intermediate forms may occur in areas where two species of *Ensis* are equally common (Von Cosel, 2009). In this way, *Ensis* could be similar to mussel species present in Irish waters, where hybridisation and introgression between *M. edulis* and *M. galloprovincialis* generate large numbers of intermediate forms (Seed, 1974; Gosling *et al.*, 2008). Where hybridisation is extensive and progresses for many generations, the result of strong selective mortality can be that the hybrid swarm is dominated by individuals that have the nuclear DNA of one parent species and the mtDNA of the other. This situation has been observed by Wilson & Bernatchez (1998) for charr (*Salvelinus fontinalis* x *S. alpinus)* hybrids in Labrador and Quebec. Thus, if extensive hybridisation was occurring between *E. siliqua/arcuatus* and *E. ensis* or *minor* it is conceivable that *CO*I, as a mitochondrial gene would be positive for *E. siliqua/arcuatus* but that no signal would be apparent at ITS1 which was fixed for the nuclear DNA of the other parent species.

Another possibility would be the presence of alternative razor clam species in the Derrynane sample to *E. siliqua*, *E. arcuatus*, *E. directus* or *E. macha*. *Ensis ensis* and *E. minor* have been previously documented in Irish and British waters (Fahy, 1999: Fahy & Carroll, 2007; Von Cosel, 2009), though no molecular method of identifying these species has been detailed to date. Thus it is possible that the ITS primers are more species specific than *CO*1 primers, so the latter amplify *E. ensis* or *minor* products as well as those from *E. siliqua/arcuatus*. Further study and sequencing of the PCR amplicons produced using species-specific *CO*I primers in the present study, would help to resolve this issue, and it is recommended that this be carried out.

The clam species *E. directus* and *E. macha* have been reported as being present in eastern Atlantic waters (Luczak *et al.*, 1993; Baron *et al.*, 2004; Dauvin *et al.*, 2007; Fernandez-Tajes *et al.*, 2010; Vierna *et al.*, 2010; Arias & Anadon, 2012). As the current range of *E. directus* is thought to stretch from the North Sea to the Iberian Peninsula, it is surprising that no clams sampled in the present study were identified as this species, though this could be due to the relatively small number of individuals sampled at each site, and the location of the sites sampled.

In conclusion, the present study has identified the razor clam *Ensis siliqua* as present in two sites on the East and south coast of Ireland, and in two sites on the east coast of Wales.

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Chapter 7: Concluding Discussion

To manage an exploited species, knowledge of biological characteristics such as the reproductive cycle, and health status of the species is essential, as it provides valuable data for recruitment, age and growth studies, leading to a greater understanding of the species life history in a specific area, and the effects of environmental irregularities on these characteristics. Data on the extent of molecular genetic structure and levels of genetic variability in different populations of shellfish species throughout their geographical range are also crucial to identify and preserve natural populations, and to develop sustainable aquaculture programmes. The current thesis was undertaken to gain knowledge of the general biology, health status and genetic makeup of the two clam species *Mya arenaria* and *Ensis siliqua*, in Irish waters. This knowledge, and the data gathered in this study can shed light on how these two clam species are being affected by current environmental and anthropogenic factors in the marine habitat, and the potential effect of fishing, aquaculture and a changing climate on these bivalves in Irish waters in the future. Here, the initial aims of the thesis as listed at the end of the Introduction are revisited and the advances made in each area are highlighted.

Aim 1 To investigate the biology, including the reproductive cycles of *Mya arenaria* and *Ensis siliqua* in the wild in the Irish Sea.

The reproductive cycle of *Mya arenaria* over a 16 month period was described for the first time in Ireland in the present study. *Mya arenaria* sampled in Bannow Bay, Wexford, Ireland displayed similar reproductive characteristics, such as the size at first gametogenesis, sex ratio of individuals, low prevalence of hermaphrodites, all previously described stages of gametogenic development, and timing of the single spawning event, to soft shell clams of other European and some eastern North American sites. Interestingly, the highly variable environmental temperatures of 2010 and 2011 appear to have affected *M. arenaria* gametogenesis in Bannow Bay, leading to a discrepancy between spawning events of the two sexes, which could potentially affect fertilisation success and recruitment of this species, if unusual weather events continue to occur.

Similarly, the reproductive cycle of *Ensis siliqua* was described in the present study. *Ensis siliqua* of the Skerries region in the Irish Sea, where this species is

commercially fished, also displayed similar reproductive characteristics to razor clams previously investigated in Spain and Portugal. However, the clams sampled in the present study were both longer and heavier than those screened in North-western Spain, while similar sized to animals recorded in the nearby Gormanstown bed in 1999, suggesting that these physical characteristics may be dependent on location, with a trend for slower growth and maturation of this species at more northerly latitudes (Muir & Moore, 2003). In the present study, *E. siliqua* also appeared to demonstrate some effects of short-term varying water temperatures on its general biology, which could potentially affect the gametogenic development of razor clams in the Irish Sea. For the first time, it was noted that wet weight dropped significantly in the summer months of both years, immediately after spawning, which may impact on the economic viability of fishing for this species during this period, since razor clams are sold by wet weight in Ireland.

Aim 2 To look at the health status of these clam species and to identify any pathogens present, using both histological and molecular methods.

No pathogenic conditions were observed in Irish *M. arenaria* using either histological or molecular methods. For the first time in North European, the health status of *E. siliqua* was investigated and prokaryote inclusions, trematode parasites, *Nematopsis* spp. and inflammatory pathologies were observed at very low frequencies in razor clams from the Skerries region of the Irish Sea, indicating that the overall health status of these two clam species is currently good.

Aim 3 To study the population genetics of Irish Sea soft shell clams and to determine how they fit into their entire distributional range.

European *M. arenaria* have classically been considered a successful colonising species, recently introduced from eastern North America, and displaying genetic homogeneity at neutral markers, due to rapid range expansion and high gene flow. However, genetic data resulting from the present study have suggested an alternative, more complicated, colonisation history for the sampled European stocks of the soft shell clam. The lack of a latitudinal cline in allelic diversity, the significant inter-sample genetic variability revealed using both mitochondrial and nuclear markers, the presence of novel European haplotypes and microsatellite

alleles, albeit at low frequencies, especially in the Irish sites sampled, and complex genetic structure revealed in European samples of *M. arenaria* suggest that soft shell clams currently inhabiting European waters have a more intricate phylogeographic history than North American stocks. They are more likely to be a result of complex post-glacial recolonisation of this species, possibly involving cryptic southern European refugia of the clam during the Pleistocene era, in addition to recolonisation from eastern North America.

Aim 4 To identify the *Ensis* spp. sampled from different areas around the Irish Sea.

The present study identified that the dominant razor clam species present in five sites investigated around Ireland and on the east coast of Wales is *Ensis siliqua*. However, twelve percent of the individuals sampled were not positively identified as *E. siliqua, E. arcuatus, E. macha* or *E. directus*, using molecular methods, including all 10 individuals sampled from the west coast of Ireland in Derrynane Bay, which were indicated to be *E. siliqua* using one molecular marker, but not another. Explanations include possible introgressive hybridisation of different *Ensis* species, or the presence of alternative razor clam species in the Derrynane sample, such as *Ensis ensis* or *E. minor*. Interestingly, the American jack knife clam, *E. directus*, was not identified at any of the sites sampled in the present study, despite the fact that the range of this "invasive species" is currently thought to stretch from the North Sea to the Iberian Peninsula in eastern Atlantic waters.

Fisheries in the Irish Sea

Total shellfish exports from Ireland are increasing each year (Marine Institute, 2006), and the OECD / FAO 2010-2011 outlook forecasts continuous positive long term growth in the global seafood market. Current and future fishing ventures around Ireland and in the Irish Sea will need careful management to preserve natural populations and avoid depletion of wild stocks (Arias *et al*., 2011). As outlined in Chapter 3, *Ensis siliqua* fisheries are carried out year-round in Ireland and other European countries, without closures during the spawning period, as happens with fisheries of other bivalve species such as *Mytilus edulis* and *Ostrea edulis*. Investigations of the reproductive cycle of razor clams in the Irish Sea have

highlighted the post-spawning decrease in wet weight of this species. For both economic and ecological reasons, the closure of razor clam fishing for a few months each year should be considered in the future. Current distribution, density, and the health status of the soft shell clam, *Mya arenaria*, on other Irish coasts is unknown, but future work investigating these would allow for consideration of fisheries and the species potential as a commercial species in the future.

On eastern North America coastlines, soft shell clam fishing is regulated by individual municipalities in each county, which are responsible for maintaining the clam flats by monitoring stocks and transplanting softshell clams from dense beds to lower density areas, that may be more productive (Marsh, 2004). While trawls and dredges have the potential to affect both the physical and biological components of bottom habitat, by disturbing the sediment and benthic structure, the hand-held implements used by 93% of fishers are believed to have a lesser impact, and there is currently no evidence of food web disruption or ecosystem change using these tools (Newell & Hidu, 1986; Peterson *et al.*, 1987; Brown and Wilson 1997; Hall and Harding 1997; Kaiser *et al*., 2001; Marsh, 2004). Knowledge of the impact of shellfish harvesting in North America will be of great benefit if *M. arenaria* was to become a commercially important species in Irish waters, as this species is currently found in relatively undisturbed habitats here.

Over-fishing of natural resources in the marine environment is increasingly becoming a problem in Europe and around the world (Baird *et al*., 1991; Brand *et al*., 1991; Hill *et al.*, 1999; South Wales Sea Fisheries Committee, 1999; Beukers-Stewart *et al*., 2003; Darriba *et al*., 2005; Marine, 2010; Fahy, 2012). To maintain and enhance current stocks of shellfish, restocking of natural populations and aquaculture of species in Irish waters may be considered in the future. When considering aquaculture or stock enhancement of either *Mya arenaria* or *Ensis siliqua*, certain factors need to be taken into account, such as the potential for infection and disease, and the genetic make-up of stocks.

Aquaculture - Disease

While disease seems currently non-existent or at very low prevalence in Irish stocks of *M. arenaria* and *E. siliqua*, previous work has shown the presence of a variety of pathogenic states in these species on the eastern seaboard of the United

States and the coast of Galicia, Spain, respectively (Oprandy *et al*., 1981; Brousseau & Baglivo, 1991a; 1991b; Farley *et al*., 1991; McLaughlin & Faisal, 1999; Dungan *et al*., 2002; Perez, 2011; Ruiz *et al*., 2013). In these regions, clams are present at much higher densities and experience much warmer summer temperatures, both factors which could, in part, contribute to the increased likelihood of disease states. High density can lead to easier transmission of pathogens (Renault and Novoa, 2004), and warmer waters are thought to cause physiological stress to bivalves not adapted to such temperatures, which may compromise host resistance, and increase frequency of opportunistic disease, consequently increasing bivalve morbidity (Harvell *et al.*, 1999; Beldomenico & Begon, 2010). Some bivalve pathogens are also sensitive to water temperature (Harvell *et al.*, 2002). For example, high environmental temperatures have been directly and indirectly linked to *Crassostrea gigas* mortalities in the past (Lipovsky & Chew, 1972; Soletchnik *et al*., 2003; Malham *et al*., 2009)

The current study has demonstrated the very low prevalence of pathogenic states in *M. arenaria* and *E. siliqua* in the Irish Sea and around Ireland, particularly in comparison to other bivalve species fished in this area. Examples include *Ostrea edulis* stocks affected by *Bonamia ostreae* infection (Culloty & Mulcahy, 1996; Culloty *et al*., 2001), brown ring disease in the Manila clam, *Ruditapes philippinarum* (Drummond *et al.*, 2007), summer mortality of the Pacific oyster, *Crassostrea gigas* (Malham *et al.*, 2009), and mass mortalities of the cockle species *Cerastoderma edule*, with haplosporidians, trematode infections and the presence of disseminated neoplasia thought to be the causative factors in different areas (Morgan *et al.*, 2012). The increased intervention in these bivalves stocks, due to aquaculture and fisheries, is in itself a large stressor, and may lead to an increase in pathogenic states and mortality levels. However, both soft shell and razor clams could potentially be good candidates for aquaculture in Ireland, if regular monitoring of their health status is upheld, and summer water temperatures remain comparatively cool.

Aquaculture - Molecular Ecology

Restocking and aquaculture of bivalve species in the eastern North Atlantic may be considered in the future to combat the reduction of bivalve stocks across

Europe, due to declining numbers of breeding adult specimens leading to reduced genetic variation, that may occur due to over-fishing of currently-viable populations (Linnane *et al.*, 2000; Hauton *et al.*, 2007; BIM, 2011b). Data obtained in the current study suggest that certain Irish populations of soft shell clams are genetically distinct from those in Wales, the Netherlands and North America. If aquaculture of this species was to be undertaken in Ireland, local clams, despite their probable introduced origin, may be more suitable as broodstock than imported individuals, in order to maintain current genetic composition and levels of genetic variation, which may be indicative of local adaptation. In contrast, since low genetic variability within bivalve or any other populations is considered undesirable for long term survival, it might be beneficial to either introduce similar, but more diverse individuals (for example, from the Netherlands), instead of maintaining current stocks. If this second option were to be adopted, care would have to be taken when selecting sufficient broodstock, as using too few individuals, particularly close sibs, can result in genetic bottlenecks and ultimately in fixing of alleles at particular loci. Such inbreeding can also decrease the potential for local adaptation to specific future conditions. It is probable that local adaptation may already have occurred in Irish stocks of *M. arenaria*. Recent work has shown evidence for selection for resistance to paralytic shellfish toxins (caused by blooms of paralytic shellfish poisoning toxin-producing *Alexandrium* spp.) in North American *M. arenaria* (Bricelj *et al.*, 2002; Bricelj *et al.*, 2005; Bricelj *et al.*, 2010), and local adaptation may be at play with regard to different environmental cues in the Irish Sea and adjacent areas. A similar situation has been recorded in the oyster species *Ostrea edulis*, where susceptibility to being infected by the protistan parasite *Bonamia ostreae* in European populations of oysters may be a locally adaptive trait (Culloty *et al.*, 2004).

Also at a molecular level, future work could clarify which of the different species of razor clams are present in Irish waters, and in particular, the population genetics of *E. siliqua*, in order to provide insight into the population relationships, migration and evolution of this species, so as to maintain stock structures for future exploitation. Microsatellite analysis, such as that outlined by Arias-Perez *et al.* (2012) would be useful in this context. However, there has recently been a move from population genetics, where a small number of genes are screened, using for example microsatellites, towards population genomics, where direct analysis of

sequence variation allows investigation of numerous genes throughout the genome (Helyar *et al.*, 2011). Work investigating the benefits of genetic analysis using single nucleotide polymorphisms (SNPs) and next generation sequencing have revealed that SNPs are showing promise as highly informative markers, with even a small fraction of SNPs analysed demonstrating very high information content for population structure analysis (Helyar *et al.*, 2011; Seeb *et al.*, 2011). With the ability in genome wide SNP studies, to examine both neutral genetic variation, as has traditionally been the case in population genetics (enabling investigation of phenomena such as population size, migration and gene flow), and regions under selection, (which will, for example, be involved in local adaptations), analysis of single nucleotide polymorphisms allows expansive screening of genomes (potentially at large sample sizes) in natural populations (Helyar *et al.*, 2010). Understanding of local adaptation in *M. arenaria* and *E. siliqua* would allow for a greater precision in future selection of broodstock and in planning transplantation exercises. Further, to illustrate the phylogenetic history of soft shell clams in Europe (and particularly the idea that *M. arenaria* survived to Pleistocene in one or more southern European refugia), future work should include investigating genetic variation in *M. arenaria* from southern Europe, both at the mitochondrial and nuclear markers used in the current study, and at finer resolution markers including SNPs and other sources of genetic variability detected by next generation sequencing (such as indels). The latter marker types would be particularly appropriate to apply throughout the species range to investigate the genetic anatomy of recolonisation and invasion.

Aquaculture of Mya arenaria and Ensis siliqua

Aquaculture and stock enhancement of both *M. arenaria* and *E. siliqua* might be contemplated in Irish waters in the near future, but it is recommended that more information is gathered on their population genetics, among other aspects of their biology. As outlined in the Introduction, successful culture of *M. arenaria* has been achieved in North America and of *E. siliqua* and *E. directus* in Europe (Da Costa *et al*, 2010). Using experience from previous culture of these two clam species as models, and knowledge gained in the current culture of commercially important species such as *R. philippinarum* and *C. gigas* in Ireland, culture of these two clam species should certainly be possible. As mentioned above, it would also be

recommended to use locally sourced broodstock, and to introduce regular monitoring for immunological stress, pathogenic state and level of genetic variability. Marine aquaculture systems, such as land-based recirculating systems, which are engineered to completely isolate livestock from the surrounding environment, are anticipated to increase in the future to enable continued supplies of established aquaculture species, irrespective of changing environmental conditions (Callaway *et al.*, 2012). While systems of this type might prove ideal for spat production, they are very expensive, and factors such as water quality, pathogenic load and the presence of natural predators would need to be considered in areas where restocking is planned. The dominance of *E. siliqua* in Irish waters, compared with other razor clam species, as demonstrated in the present and past work leads to the presumption that future aquaculture ventures concentrating on *E. siliqua* might be successful in this area, depending on the correct management procedures, and the aforementioned further investigation of intra-species genetic variation.

The assumed invasive nature of *M. arenaria* in Europe (by post Pleistocene re-introduction from eastern North America) would need to be considered in management of this species in European culture. While it was found to be one of the species with the highest biomass in studies on the south-west coast of England and the Wadden Sea (Warwick & Price, 1975; Beukema, 1992), and one of the dominant species on the west coast of Sweden and in the Baltic Sea (Evans & Tallmark, 1977; Brey, 1991; Zaitsev & Mamaev, 1997), no negative effects on other species have been reported to date in the presence of *M. arenaria* (Reise et al., 1999). However, this clam has been deemed invasive by many authors (Lasota *et al.*, 2004; Powers *et al.*, 2006; Conde *et al.*, 2011; Conde *et al.*, 2012; Krapal *et al.*, 2012), and its recent introduction in the Black Sea has been blamed for a 50 to 60% reduction of invertebrate species number since the 1960s (Bologa *et al.*, 1995).

The effects of a changing climate on Fisheries and Aquaculture, with particular reference to bivalve species

The consequences of climate change are increasingly being felt in Europe and worldwide, as the average global temperature, currently around 0.8°C above pre-industrial levels, continues to rise (EEA, 2012; COM, 2013a). Some natural processes are already being altered, with precipitation patterns changing, extreme

weather events occurring more frequently, glaciers melting, and sea levels rising (COM, 2013a). Coastal zones are particularly vulnerable to climate change, which has already had an impact on sea temperature, sea levels, and ocean acidification (COM, 2013b). These changes have had subsequently-detected effects on ocean circulation, coastal erosion, biodiversity and ecosystem processes. All of these changes will affect coastal populations and the marine and maritime economy. Climate change is an additional pressure on top of the many challenges that marine stocks already experience, including loss of habitat, pollution, disturbance, and introduced species (Brander, 2010). Recent and anticipated future changes in the temperature and chemistry of marine waters around the UK and Ireland are predicted to have effects on the phenology, productivity and distribution of marine fish and shellfish in the future (Heath *et al.*, 2012). The present study has illustrated that two unusually cold winters, followed by a warmer than average spring may have affected *M. arenaria* and *E. siliqua* gametogenesis in Bannow Bay and the Skerries region of the Irish Sea respectively, potentially affecting the time of spawning, fertilisation success, and recruitment of these species.

The environmental impacts of climate change are likely to affect future fish and shellfish production, and may lead to increasing challenges in ensuring long-term sustainable fisheries management (Cheung *et al.*, 2012). Consequently, changes in supply of fisheries products because of climate change are expected to affect their market prices, with the price of high-value crustaceans and molluscs expected to increase substantially (Delgado *et al.*, 2003). Coastal and estuarine systems, the habitats of most bivalves fished in Ireland, are expected to experience effects of ocean acidification earlier and more severely than other systems (Callaway *et al.*, 2012), with changing sea levels, rainfall patterns, and increased extreme weather events also affecting these ecosystems severely.

Climate-induced changes in the environment affect the health and productivity of marine ecosystems (Harvell *et al.*, 1999). It is currently thought that bivalves may be immune-compromised as a result of the changes in marine salinity and pH, and increase in water temperature with associated decreases in available oxygen for physiological processes, (Cheng *et al*., 2003; Matozzo & Marin, 2011). Many pathogens of marine taxa are also sensitive to temperature, rainfall and humidity, all of which can be potentially affected by climate change. Aquatic

parasites show temperature optima for the successful completion of their life-cycle, and an extension of the season will provide more time for the proliferation of parasites to occur, and a longer time for host infection (Brander, 2010; Callaway *et al.*, 2012). In this way, climate warming, as well as increasing host susceptibility, can increase pathogen development and survival rates, so increasing disease incidence (Harvell *et al.*, 2002). The introduction of marine species to a new area, due to the change in optimal environmental parameters, could also allow the introduction of novel disease. Climate change is one of the factors which may have accelerated global movement of species, and thus novel disease, to previously unexposed host populations (Harvell *et al.*, 1999). The breakdown of synchrony between a species and its prey can also pose a problem for survival. For example, if bivalve species larvae are released into the water column earlier than their plankton prey there will be obviously a major nutritional problem.

Past work has revealed evidence which suggests that the intensity of precipitation events will increase in the winter months, while summers may become drier (Callaway *et al.*, 2012; Frost *et al.*, 2012). These small variations in water temperature between seasons can effect marine organisms detrimentally (Brander, 2010), and the variance between summer and winter temperatures may be more important than a total increase or decrease in average temperature by a few degrees, an example of which has been illustrated in the present study.

Bivalve farming, which often depends on wild spat, phytoplankton for food and water quality for health, is highly susceptible to various effects of climate change. Furthermore, aquaculture facilities are often located in areas which are likely to bear the brunt of climate change impacts, such as changes in water temperature, storm intensity or frequency and sea level. However, there is currently very little evidence to indicate that climate change is affecting aquaculture in the UK and Ireland. However, it is believed that future ventures in aquaculture and management of fisheries in the Irish Sea and adjacent areas will be affected in a major way by climate change or increased variability in this area (Callaway *et al.*, 2012).

While rising marine temperatures may create the opportunity to rear warmer water species in the UK and Ireland (Callaway *et al.*, 2012), species currently present may be detrimentally affected by changing environmental temperatures. Mitigation efforts, in the form of yearly checks of health status, marine environmental

monitoring, and the prioritisation of flexible and participatory approaches, will allow for planned adaptation actions in the future of shellfish fishing and culture (Com-2013-216). By improving habitat quality for marine organisms, rebuilding over-exploited populations and maintaining ecosystem structure, using measures such as maintenance of marine protected areas, no-take zones, and yearly prohibition of fishing post-spawning, the fishing industry could gain from more productive shellfish stocks, higher biodiversity and higher resilience and adaptive capacity to climate change (Cheung *et al.*, 2011; Cheung *et al.*, 2012).

The current thesis has provided data valuable to both short term and long term study of two clam species and their survival in Irish waters. Knowledge of the reproductive cycle and health status of these two species allows for optimisation of their current management, and also provides data useful for longer term climate change studies in European waters. Genetic data revealed in study of *M. arenaria* indicates the history of this bivalve in European waters as possibly more complicated than previously thought, while investigation of *Ensis* species shows a dominance of *Ensis siliqua* in most areas sampled. While both of these species would seem to be well established in Irish waters, the impact of changing stressors such as environmental variability, intensive culture or overfishing, is currently unknown.

Future work, to establish the impact of stressors, could include repeated monitoring of the reproductive biology and health status of both clam species with regards to climatic conditions in northern Europe. Experimental harvesting of *M. arenaria*, using knowledge gleaned from previous work in North America, should be possible under controlled conditions. Also, participation in European studies on *Ensis* culture would be of benefit on both an ecological and economical level, providing for the increasing worldwide demand for shellfish protein, while minimising the risk of overfishing natural resources.

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Appendix I

The Reproductive Biology of the Softshell Clam, Mya arenaria, in Ireland, and the Possible Impacts of Climate Variability

M. E.Cross, S. Lynch,A.Whitaker, R. M. O' Riordan, and S.C.Culloty

Appendix II

The reproductive biology of the exploited razor clam, Ensis siliqua, in the Irish Sea.

M. E.Cross, R. M. O' Riordan, and S.C.Culloty

Appendix III

A health status survey of clams, Mya arenaria and Ensis siliqua, in the Irish Sea.

M. E.Cross, S. Lynch, R. M. O' Riordan, and S.C.Culloty