Scottish Natural Heritage
Research Report No. 1000

Genetic analysis of horse mussel bed populations in Scotland

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RESEARCH REPORT

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Genetic analysis of horse mussel bed populations in Scotland

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RESEARCH REPORT

Summary

Genetic analysis of horse mussel bed populations in Scotland

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Keywords

Genetic connectivity; horse mussel; microsatellite marker; Marine Protected Area

Background

Horse mussels (*Modiolus modiolus*) form biogenic reefs or beds, which are a conservation priority under national and international legislation (UK BAP, 2008; Rees, 2009; Tyler-Walters *et al.,* 2016). Horse mussel beds are biodiversity hotspots and provide a number of ecosystem services including carbon sequestration and storage, and habitat provision for commercially important species (Rees, 2009; Burrows, 2014; Kent *et al.,* 2017a). However, a decline in the extent of beds has been noted at a number of places across the UK (e.g. Strain *et al*., 2012) and further loss of this habitat is predicted over the next 100 years due to increased seawater temperatures (Gormley *et al*., 2013).

The UK is committed to creating an ecologically coherent network of MPAs in accordance with the OSPAR Convention, which highlights connectivity as a key element to assist the interpretation of ecological coherence. Connectivity has also been considered a fundamental element in the development of the Scottish MPA network. Effective spatial management therefore relies on an understanding of the linkages between protected features. However, very little is known about connectivity of benthic features between MPAs and beyond MPA boundaries. Unlike mobile species, mussels remain fixed to the seabed and rely on ocean currents for larval dispersal. Consequently, certain populations may act as larvae sources for other populations and thus, are of substantial value to maintaining a viable network. Horse mussel aggregations acting as sink populations are important settlement sites for larvae. Clarification of such relationships will help to determine the extent to which MPAs act independently or are dependent on other MPAs or features falling outside protected areas.

Analyses of genetic connectivity and diversity can be used to determine how populations relate (i.e. are connected) to one another and shed light on gene flow between areas. This report provides the methodology, results and discussion for an in-depth analysis of genetic connectivity of horse mussel beds in three distinct marine areas (West Coast, North-east and Orkney, Shetland Isles) in Scottish waters. Horse mussels also occur as individuals and clumps across Scotland, however, aggregations defined as ‘beds’ are considered of conservation importance and key settlement and recruitment sites. Therefore a selection of horse mussel bed populations were sampled for the purposes of this study.

Genetic connectivity results are discussed in relation to hydrodynamics, demographics and coastline geography as well as the implications for the MPA network. Recommendations for future work on horse mussel connectivity are provided, and adaptive capacity of the horse mussel is also considered in the context of future climate change.

Main findings

- Horse mussels were collected by divers from nine beds across Scotland, with 50 replicate mussels collected from each site except at Noss Head where 50 juveniles and 50 adults were sampled.
- Populations were screened with 12 microsatellite markers to determine genetic differentiation, genetic structure, genetic migration rates and genetic diversity indices.
- Scottish horse mussel bed populations have moderate to high levels of genetic connectivity.
- Scottish horse mussel beds within MPAs are generally well connected to nearby non-protected beds at both local and regional scales.
- Genetic migration rates between populations are generally comparable across sites.
- Horse mussel bed populations from semi-enclosed water bodies (e.g. sea lochs and firths) appear to act as a source of migrants rather than a sink from adjacent populations.
- Port Appin (West Coast) and Karlsruhe (Scapa Flow, Orkney) horse mussel beds are part of a separate genetic grouping from all other sites sampled.
- The Noss Head horse mussel bed recruits from external beds with minimal degree of self-recruitment.
- Given the high connectivity of Scottish horse mussel beds, it is proposed that beds are genetically similar and therefore may have reduced capacity to adapt to climate change conditions that are site-specific (i.e. local stressors). However, further investigation into genetic diversity of horse mussel populations is required.
- Scottish horse mussel beds should be managed together rather than in isolation.

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Acknowledgements

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1. INTRODUCTION

1.1 Species description and distribution

The marine bivalve, *Modiolus modiolus* (horse mussel) is an Arctic-Boreal species with a distribution range that extends from the seas around Scandinavia and Iceland southward to the Bay of Biscay (Rees, 2009). Horse mussel aggregations, referred to here as 'beds' but can also be described as reefs[1], are more limited in their distribution compared to the species as whole, with current estimates placing the southern limit of such habitats in the southern Irish Sea (Rees, 2009; Morris, 2015). Across the UK, beds are known to occur in the Shetland and Orkney Isles as well as coastal areas of mainland Scotland, the Ards Peninsula and Strangford Lough in Northern Ireland and the Llŷn Peninsula in Wales (Figure 1.1).

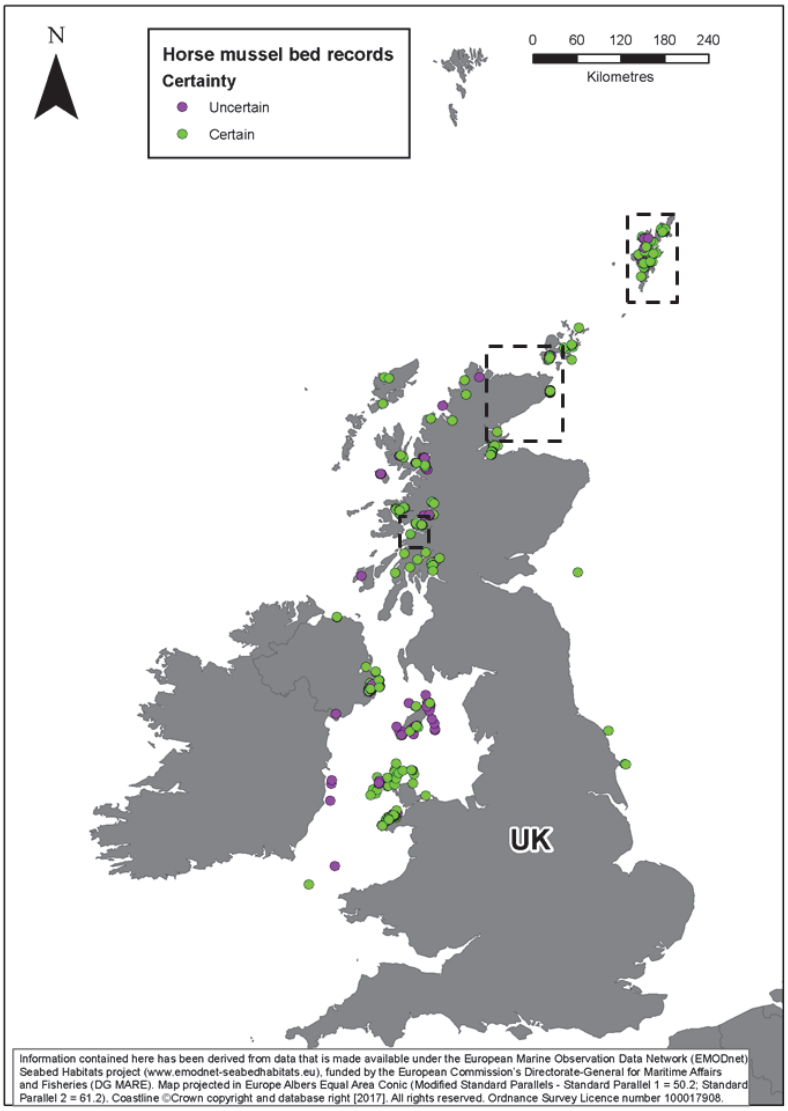

Figure 1.1. Distribution of known horse mussel beds in UK waters (OSPAR records "Modiolus modiolus horse mussel beds" from the European Marine Observation Data Network). Dashed boxes show sampling regions for the present study.

[1]characterised by clumped mussels and shell covering more than 30% of the substrate, which may be infaunal or embedded beds, semi-infaunal (with densities of greater than 5 live individuals per m^2) or form epifaunal mounds (standing clear of the substrate with more than 10 live individuals per clump), all of which support communities with high species richness (or diversity) compared to sediments of the surrounding area (Morris, 2015).

1.2 Horse mussel bed conservation value and sensitivity

Horse mussel beds are typically characterized by high species diversity (Sanderson *et al.*, 2008; Rees *et al.*, 2008; Ragnarsson & Burgos, 2012). Furthermore, habitat modification by horse mussels can have substantial effects on the composition and abundance of megafaunal benthic organisms in coastal waters (Ragnarsson & Burgos, 2012). In addition to being areas of high biodiversity, horse mussel beds also contribute a number of ecosystem services including carbon sequestration and storage (Burrows *et al.*, 2017), benthic-pelagic coupling (Kent *et al.*, 2017b), water filtration (Navarro & Thompson, 1996) and habitat provision for commercially important species (Rees, 2009; Kent *et al.*, 2017a).

Horse mussel beds are sensitive to physical impacts (Cook *et al.,* 2013) and a decline in the extent of beds has been noted at a number of sites across the species' European distribution. In the Irish Sea, historical fishing activity, namely scallop dredging and trawling, has caused widespread and long-term damage to beds including those situated around the Isle of Man and Northern Ireland (Rees, 2009; Strain *et al.*, 2012). Strong *et al.* (2016) describe a 12.6 km^2 loss of horse mussel beds in Strangford Lough since the 1980s with the habitat becoming restricted to deeper, sandier areas. Reasons for the decline include mobile fishing activity but also competition and predation by starfish following the physical damage and the resulting 'clumped' structure of remaining horse mussels. Further to this, Gormley *et al.* (2013) predict a loss of horse mussel beds over the next 50 years due to increased seawater temperatures. The combination of pressures on horse mussels and slow recovery of the habitat (Mazik *et al.,* 2015) make this feature increasingly vulnerable.

Scottish waters support more than 80% of all horse mussel beds in the British Isles and due to their functional importance, sensitivity and low recovery potential this habitat is considered a Priority Marine Feature (PMF) in Scotland (Tyler-Walters *et al.*, 2016). Horse mussel beds are also listed as a threatened and/or endangered habitat in all OSPAR regions and a UK Biodiversity Action Plan priority habitat (identified as most threatened and requiring conservation action) (UK BAP, 2008).

1.3 Spatial management

The UK is committed to creating an ecologically coherent network of MPAs in accordance with the OSPAR Convention, which highlights connectivity as a key element to assist with the interpretation of ecological coherence (Defra *et al.,* 2012). Connectivity has also been considered a fundamental element in the development of the Scottish MPA network (Marine Scotland, 2011). The concept of connectivity is evolving over time as our understanding of the linkages between marine species and processes across different geographic areas develops. Mobile species are able to move between different marine areas for feeding and reproduction, but horse mussels are sessile and rely on ocean currents for larvae dispersal. Very little is known about benthic habitats and how they are connected within and between MPAs in Scotland and the wider north-east Atlantic. However, Fox *et al.* (2016) used a hydronamic model to predict atmospheric circulation-related changes in *Lophelia pertusa* (a cold water coral) connectivity in the north-east Atlantic and Gormley *et al.* (2015) assessed the genetic structure of several horse mussel bed populations in the Irish Sea. Understanding the scale at which features such as horse mussel beds are connected is fundamental in managing discrete populations across different geographic areas.

The Scottish MPA network includes Special Areas of Conservation (SACs), Nature Conservation MPAs (NC MPAs), marine components of Sites of Special Scientific Interest (SSSIs), Special Protection Areas (SPAs), and Ramsar sites. Development of the MPA network has considered representation, replication, geographic range and variation, linkages and resilience of features (SNH & JNCC, 2012). Research on linkages has mostly focused on mobile species and large scale features (e.g. fronts). Gallego *et al.* (2013) used a

biophysical model to estimate larval transport of 18 benthic features (including the horse mussel) to determine connectivity between MPAs in Scotland. The results showed that the potential dispersal of horse mussel larvae was relatively wide but connectivity was not very strong. However, Gallego *et al.* (2013) did not consider small-scale sea loch populations but focused on exposed populations (e.g. Noss Head) and did not include populations outside MPAs. The findings of the study were also limited by a lack of ecological information for some of the species studied, such as reproductivity and larval behaviour.

Horse mussel beds are a protected feature of a number of NC MPAs, designated under the Marine (Scotland) Act 2010. For example, beds that occur in Hascosay Sound and Uyea Sound are protected features of the Fetlar to Haroldswick MPA. They are also protected as 'biogenic reefs' in a number of SACs, such as the horse mussel bed at Calback Ness in Sullom Voe SAC. For this study, NC MPAs and SACs were considered together as MPAs.

1.4 Genetic structure

In the marine environment, detailed knowledge about the genetic structure and status of species of conservation interest is required, as this information may be of use in informing the successful maintenance of these populations through breeding programmes (Jiale *et al.*, 2009) and/or restoration of habitats (Roberts *et al.*, 2011). In particular, understanding connectivity has also become a major factor in determining and defining the implications of threats to marine biodiversity and is crucial to the marine conservation and decision making management process (Schunter *et al.*, 2011; Weersing & Toonen, 2009). In the present study, connectivity is considered from a genetics perspective and defined as "the extent to which populations in different parts of a species' range are linked by the exchange of eggs, larvae recruits or other propagules, juveniles or adults" (Palumbi, 2003) (i.e. vectors of genetic material).

1.4.1 Genetic connectivity and diversity

When the distribution of a species is spread across a diverse landscape, spatial variation inevitably leads to spatially distinct populations inhabiting areas with a particular, and often unique, set of environmental parameters. Accordingly, intraspecific populations existing under different conditions may develop varying tolerances to environmental stressors. Variation in tolerance may be due to phenotypic plasticity, genetic effects (e.g. adaptation), or, as is most usual, a combination of both factors (Luttikhuizen *et al.,* 2003).

Genetic effects result when spatial variation in conditions exerts selective pressures upon a population. This leads to both local adaptation within the given population and increased genetic differentiation from other populations (Whitlock, 2008). While spatial heterogeneity can lead to genetic variation, gene flow driven by dispersal has the opposite effect. Gene flow refers to the movement of genetic material (via migration or larval movement) from one population to another, and results in populations with a shared genetic makeup and therefore reduced genetic differentiation. Consequently, gene flow has a large influence on both inter- and intra-population levels of genetic diversity and connectivity. Gene flow and adaptation are not exclusive, and populations are often influenced by both mechanisms. Inevitably, the interplay of these genetic drivers leads to varying levels of shared genetic material and shifting degrees of resistance to environmental stressors across populations (Whitlock, 2008).

Marine bivalves characteristically have high fecundity and release larvae with high dispersal potential, resulting in species with substantial geographic ranges, large population sizes and high rates of gene flow between populations (Luttikhuizen *et al.*, 2003). Previously, it has been largely assumed that the marine environment poses few absolute barriers to gene flow so that populations that are widely separated may be genetically very similar. Consequently,

it has been suggested that many marine species have little genetic population structure and rather, act as large panmictic units with low rates of allopatric speciation (Palumbi, 1994). However, oceanic processes have a large influence on connectivity and may promote gene flow between areas (e.g. via currents/tidal flows) or alternatively act as invisible barriers (e.g. via seasonal front systems) which restrict or hamper larval movement including imposing seasonal and directional limitations (Hohenlohe, 2004 Robins *et al.*, 2013). Additionally, such forces directly influence the availability and species composition of planktonic communities which, as a key food source, further shape the dispersal success of larvae (Palumbi, 1994).

1.4.2 Adaptive Capacity

As mentioned above, the determination of genetic connectivity of populations is fundamental in the development of an ecologically coherent MPA network, however, it is also an important step towards understanding vulnerability of that species to climate change. Low levels of connectivity (i.e. high genetic differentiation) drive local adaptation among populations and can create isolated populations with increased adaptive capacity. The degree to which populations are connected, therefore, has a large influence on the ability of populations to adapt to changing conditions at a local level (Harley *et al.*, 2006). This is particularly important for populations that experience increased stress conditions and, if pushed beyond tolerance limits, must adapt or perish. On the other hand, high levels of population connectivity allow genetic material to be shared with remote or sink populations and preserve high levels of genetic diversity across populations, thereby providing a diverse gene pool from which future adaptations may arise. In either case, determination of population connectivity has been advocated as a critical component towards effective protection and management of a species of high conservation importance such as horse mussel beds (Lowe & Allendorf, 2010).

1.4.3 Microsatellite Markers

Microsatellites refer to DNA sequences of variable length which are repeated in tandem through the genome and have a high rate of mutation. They have gained popular use for determination of genetic structure in eukaryotic nuclear genomes and hold the added benefits of being relatively simple and low-cost to run via PCR methodology (Hoshino *et al.*, 2012). Recent development of five microsatellite markers for horse mussels by Heriot-Watt University has allowed researchers to determine genetic connectivity and diversity of four horse mussel bed populations in the Irish Sea (Gormley *et al.,* 2015). Results show moderately significant genetic differentiation between two major groups: the Northern Ireland populations (group 1) and those around the Isle of Man and Wales (group 2), highlighting that connectivity is largely driven by oceanographic processes rather than simply a consequence of distance (Gormley *et al.,* 2015). Future analyses aim to take a broader approach and examine connectivity of populations from across the UK (Northern Ireland, Wales, Scotland) and Europe (Norway). In addition to the five microsatellite markers that have been previously used for assessing genetic connectivity of horse mussel beds, a further 12 have been recently developed to allow for a more robust analysis.

1.5 Overview of work

In Scotland, a number of horse mussel beds fall within MPA-designated waters, including populations in the Shetland Isles, Noss Head off mainland Scotland's north-east coast, Loch Creran on the west coast and Dornoch Firth on the east coast. Analyses of genetic connectivity and diversity will be used in this study to highlight how populations relate (i.e. are connected) to one another and shed light on the potential adaptive capacity of each population, respectively. Such information will be useful in a management context, particularly in light of current marine spatial planning developments and is also of potential value for restoration of these habitats. Well-managed MPAs can provide an ecologically

connected system that can potentially facilitate range shifts of populations in response to climate change. However, a greater understanding of the scale at which features are connected both within and outwith the current suite of protected areas in Scotland is required.

1.6 Objectives

- Use microsatellite screening to **examine genetic connectivity, genetic structure and genetic migration rates** in selected Scottish horse mussel bed populations in order to consider the role of Marine Protected Areas to inform regional horse mussel bed networks.

- Use microsatellite screening to examine **genetic diversity** of Scottish horse mussel bed populations in order to illustrate potential adaptive capacity.

2. METHODS

2.1 Site selection

Sites were selected based on known horse mussel bed records (see Figure 1.1) that were accessible (i.e. within scuba diving depths for hand collection) and covered a range of latitudes within Scotland as well as east and west coast populations. It was not possible to sample horse mussels from all horse mussel beds within the scope of this study, therefore, three geographic regions in Scotland were selected to allow for an investigation into the relationships between horse mussel populations at varying spatial scales (Figure 2.1, Table 2.1) and under varying oceanographic conditions (e.g. exposed coast vs enclosed sea lochs). The present study covered relevant spatial scales based on genetic analysis of horse mussels by Gormley *et al.* (2015) who found connectivity over a spatial scale of 150 km based on approximately 50 individuals per population. Within regions, horse mussel bed sites range from as little as 10 km apart (e.g. Loch Creran to Port Appin) to much greater distances (e.g. Karlsruhe to Dornoch Firth: 140 km). Sites across the entire study area are as much as much as 700 km apart (e.g. Loch Creran to Hascosay Sound). Sampling also targeted populations within and outwith MPAs with at least one protected site in each region to allow for some interpretation of the relative importance of protected areas. Individual horse mussels may also contribute towards genetic connectivity but were not included in the sampling strategy because the beds were considered key recruitment and settlement sites. In addition to examination of genetic connectivity, the Noss Head site was also selected for consideration of self-recruitment potential as it is the largest known horse mussel bed in Scotland and horse mussels are highly abundant here.

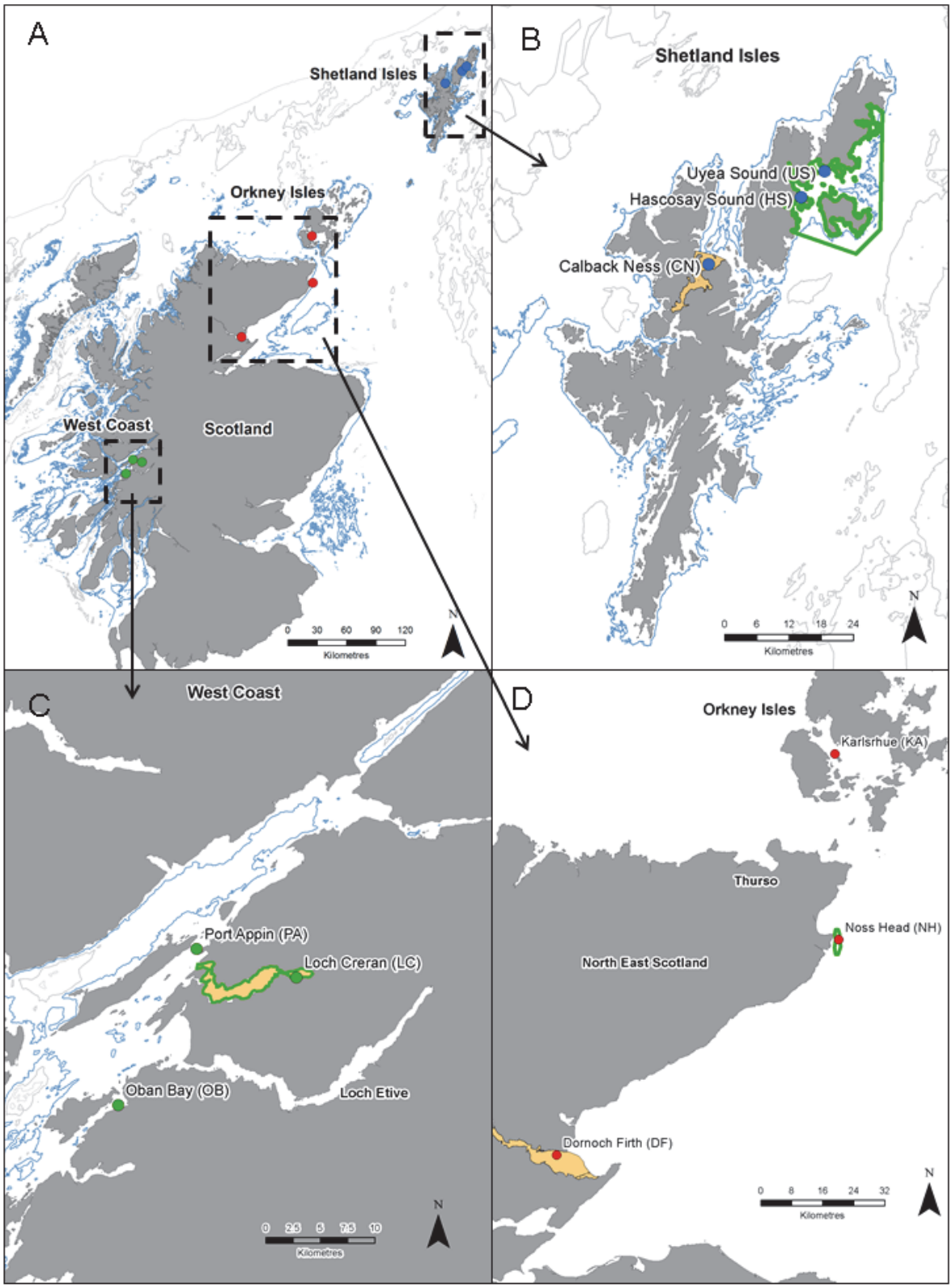

Figure 2.1. A) Horse mussel bed populations of Scotland to be included for genetic analysis. Dashed boxes indicate sampling regions to be examined. The three regions are: B) Shetland Isles, C) West Coast and D) North-east and Orkney. Protected areas are shown as solid orange polygons (SACs) and green polygons (NC MPAs). Coastline © Crown copyright and database right (2012). Ordnance Survey Licence number 100017908.

Table 2.1. Names and locations (latitude, longitude) of horse mussel beds included in genetic connectivity analyses for three areas in Scotland: B) Shetland Isles, C) West Coast and D) North-east and Orkney (lettering as per Figure 2.1). Collection dates are provided for all sites and designation type is also given for sites where horse mussel beds are a protected feature.

Region	Site Name	Lat	Long	Collection	Designation
(B) **Shetland Isles**	Hascosay Sound	60.618	-1.009	September	MPA
	Uyea Sound (US)	60.667	-0.944	September	MPA
	Calback Ness (CN)	60.483	-1.283	September	SAC
(C) **West Coast**	Loch Creran (LC)	56.546	-5.269	January 2016	MPA & SAC
	Port Appin (PA)	56.551	-5.424	November	*n/a*
	Oban Bay (OB)	56.412	-5.487	October 2011	*n/a*
(D) **North-east and Orkney**	Dornoch Firth (DF)	57.857	-4.056	June 2016	SAC
	Noss Head (NH)*	58.470	-3.019	July 2016	MPA
	Karlsrhue (KA)	58.889	-3.19	September	*n/a*

MPA=Nature Conservation Marine Protected Area; SAC=Special Area of Conservation; *juveniles and adults collected from this site

2.2 Sample collection

Adult (>80 mm shell length) horse mussels (n=50) were hand collected by the Heriot-Watt Scientific Dive Team from all selected sites between 2011-2016 (Table 2.1). Additionally, juvenile (<40 mm shell length, as per Anwar *et al.*, 1990) horse mussels (n=50) were collected from Noss Head. Adductor tissue was dissected from each sample, cut into several smaller pieces, and preserved in approximately 15-20 mL 96% ethanol solution in 25 mL specimen tubes (Figure 2.2). Samples were stored at 4°C prior to DNA extraction.

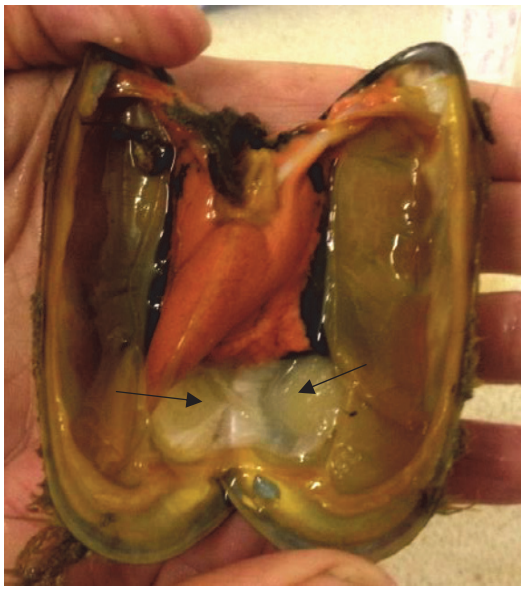

Figure 2.2. Horse mussel internal anatomy. Black arrows indicate the adductor muscle (bissected in image) which was dissected from each sample in order to carry out genetic screening of horse mussel populations. Image © Flora Kent.

2.3 Genetic screening

2.3.1 DNA extraction

DNA extractions were carried out with Qiagen DNeasy Blood & Tissue Kits (Qiagen, Manchester, UK) according to the Qiagen protocol (Qiagen, 2006). Approximately 2-3 g of preserved adductor tissue was removed from ethanol, and cut into small pieces on a tile surface using a sterile scalpel blade. All surfaces and utensils were cleaned with ethanol prior to the start of individual sample processing. Genetic material was transferred to a sterile 1.5 mL centrifuge tube. Next, 180 µL of ATL buffer (lysis buffer) and 20 µL of proteinase K were added and the sample was then vortexed before incubation at 56°C for one hour. Samples were re-vortexed after 30 minutes of incubation. Following the incubation period, 200 µL of AL buffer was added to each sample and samples were vortexed. Next 200 µL of ethanol was added to each sample and samples were vortexed again to allow for precipitation of DNA. All liquid was removed from each centrifuge tube and pipetted onto the filter of a centrifuge column (with collecting tube) before centrifugation for 1 min at 8000 rpm. Next, the column was placed into a new collection tube and the old tube and waste liquid discarded. 500 µL AW1 buffer was added to each column before centrifugation for one minute at 8000 rpm. Again, the column was placed into a new collection tube, and the old tube and waste liquid discarded. 500 µL of AW2 buffer was added to each column before centrifugation for 3 min at 14000 rpm. Finally, the column was placed in a new 1.5 mL centrifuge tube and the old tube and waste liquid discarded. 200 µL of elution buffer was added to the collection tube (onto the filter surface) and left for one minute at room temperature before a final centrifugation for one minute at 8000 rpm. Next, the column was disposed of and the centrifuge tube with DNA was labelled and stored at -20°C. Table 2.2 provides further details regarding the specific function of solutions used in the DNA extraction process.

Table 2.2. Function of solutions used in DNA extraction process.

Solution Name	Function
ATL Buffer AL Buffer	Lysis solutions that open tissues and dissolve membrane bound organelles including the nucleus and mitochondria.
Proteinase K	Rapidly digests protein, including enzymes that digest DNA.
Ethanol	Allows for precipitation of DNA.
AW Buffers	Wash solutions that wash away contaminants from DNA.
Elution Buffer	Elutes DNA from the membrane and allows stable storage of DNA.

2.3.2 DNA confirmation and quality check

Presence of DNA in DNA extraction samples was confirmed by gel electrophoresis. A 1% gel was created using 30 mL of 0.5M Tris/Borate/EDTA (TBE) buffer solution and 1.2 g agarose (high gel strength, Sigma Aldrich). TBE and agarose were measured, combined and microwaved at high power until reaching a clear liquid phase. Next, 7.2 µL of ethidium bromide (EtBr) stain was added to the solution. The gel was then poured into a gel tray (approximately 15cm x 12cm, with well comb) and left to set for approximately 20-30 minutes.

DNA samples were removed from the freezer and allowed to thaw at room temperature. Next, 5 µL of DNA sample, 5 µL of high performance liquid chromatography (HPLC) grade water and 3 µL of loading dye were added to a separate 1.5 mL centrifuge tube for each sample. Additionally, 1 µL of DNA ladder marker (λ Hind III, Thermo Fisher Scientific) and 5 µL of HPLC were added to a separate centrifuge tube. All tubes were pulsed in the centrifuge.

Once set, the gel was placed into an electrophoresis tank and 0.5M TBE buffer was added until the gel was covered and all wells filled. 5 µL of the DNA ladder marker solution were added to the first well and 10 µL of all DNA samples to subsequent wells. The electrophoresis tank was then turned on (100-150V) and the gel left to run for approximately 30-45 minutes (until samples run approximately ¾ way along gel). The gel was then removed from tank and imaged under UV light to check for presence and quality of DNA. The presence of a distinct single band indicated successful DNA extraction (Figure 2.3) while a smear indicated poorer quality extraction.

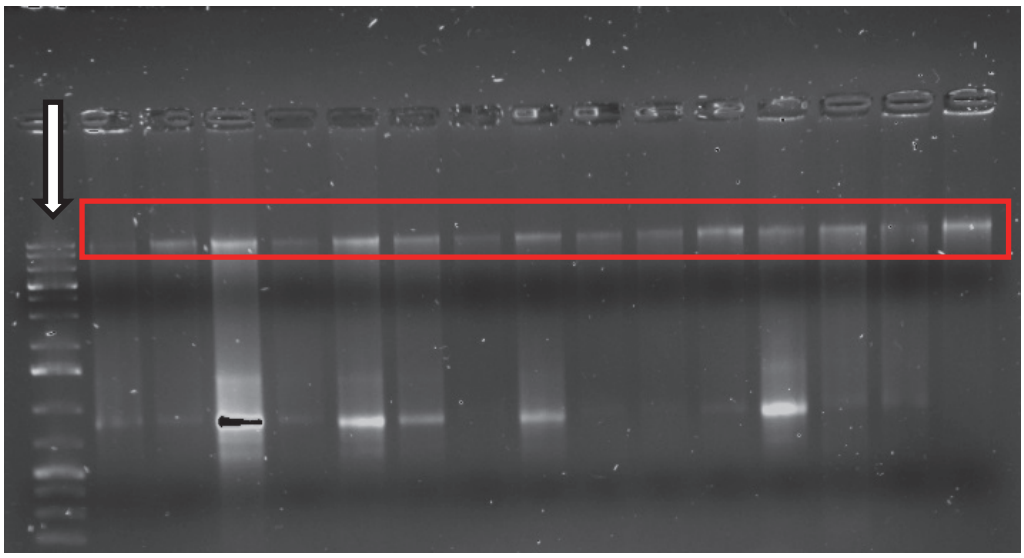

Figure 2.3. Electrophoresis gel confirmation of horse mussel DNA extractions. The top bands (indicated by red box) show that a reasonable yield of high molecular weight DNA is present. The λHind III DNA sizing ladder (indicated by white arrow) shows that DNA extractions are approximately 23130 base pairs in size.

2.3.3 DNA quantification and dilution

Following confirmation of DNA presence and quality, DNA was quantified for each sample. A Biophotometer (Eppendorf) programmed for double stranded DNA was used to quantify DNA samples. Samples were removed from the freezer and thawed at room temperature. 5 µL of elution buffer (as used for DNA extraction) and 45 µL of HPLC-grade water were added to a clean plastic cuvette and used as a blank. The cuvette was emptied and rinsed with HPLC grade water before a 5 µL sample of DNA extraction with 45 µL of HPLC-grade water were added and quantified. Readings were recorded at ng DNA µL-1. The cuvette was rinsed with HPLC grade water between samples. Following quantification, samples were diluted to 20 ng/µL with Tris-EDTA buffer solution (PH 8.0) for running in multiplex PCRs.

2.3.4 Microsatellite markers

Out of 17 microsatellite markers available, 12 were selected for screening of samples using Multiplex Manager (see 2.3.5 below). Markers included four previously developed by Gormley *et al.,* (2015) and eight newly developed at the University of Manchester (Annex 1: Table A1). All primer sets were ordered from Eurofins Genomics (0.05 µmol, salt-free, lyophilised) in February 2016. Primers were made to 100 µM in Tris-EDTA buffer solution (PH 8.0) and stored at -20°C until use.

2.3.5 Multiplex PCR

A multiplex PCR methodology was used to screen samples with the 12 selected microsatellite markers. This technique involves running PCR reactions that consist of multiple pairs of microsatellite markers per reaction. Locus-specific reverse (3'-5') primers and fluorescently labelled locus-specific forward (5'-3') primers for each marker set allow for multiple markers to be screened at one time. Multiplex Manager software (Holleley & Geerts, 2009) was used to design and optimise multiplex PCRs. Multiplex Manager combines markers to maximise spacing between markers in the same reaction and to minimise the variance of annealing temperature in each reaction. All 12 markers were inputted into the program and resulting multiplex groupings are shown in Annex 2. For the current investigation, three multiplex PCR reactions were designed with four sets of microsatellite markers used per reaction. Forward markers were labelled with unique fluorescent dyes (NED, HEX, FAM, and PET dyes) to distinguish markers (and product) within a given set (Annex 2: Table A2).

Prior to running PCR, a primer mix was created for each multiplex PCR reaction (1-3) per amounts shown in Annex 2: Table A3. Primer mixes were created in 0.2 mL capped tubes and stored in a light-proof box at 4°C until use. Multiple aliquots were created to avoid cross contamination.

Prior to screening large numbers of samples, all multiplex PCR reactions (1-3) were tested across a small number of samples (n=5) from multiple populations (n=3) to validate the protocol. Multiplex PCR reactions were carried out using Qiagen Type-it Microsatellite PCR Kit (Qiagen, Manchester, UK) and protocol, but prepared to a final reaction volume of 5 µL (to maximise consumables), comprising:

- 2.5 µL TypeIT MasterMix
- 1.5 µL H_2O (molecular grade)
- 0.5 µL primer mix (as prepared above)
- 0.5 µL normalised DNA template (diluted to 20 ng/µL)

Multiplex PCRs were run on a G-Storm (Kapa Biosystems, Wilmington, USA) thermocycler according to the following conditions:

- 95°C for 5 min.
- 35 cycles of: 95°C for 30 s; 60°C for 90 s; 72°C for 30 s.
- Final step at 60°C for 30 min.
- Held at 4°C until storage.

PCR of initial test samples was followed by gel electrophoresis to confirm correctly sized bands (Figure 2.4). Following confirmation of successful trial multiplexes, multiplex PCRs were carried out as described above for all remaining samples (13 populations x 50 samples). A sub-sample (~10%) of each set of PCR samples was run on PCR gel to confirm successful amplification.

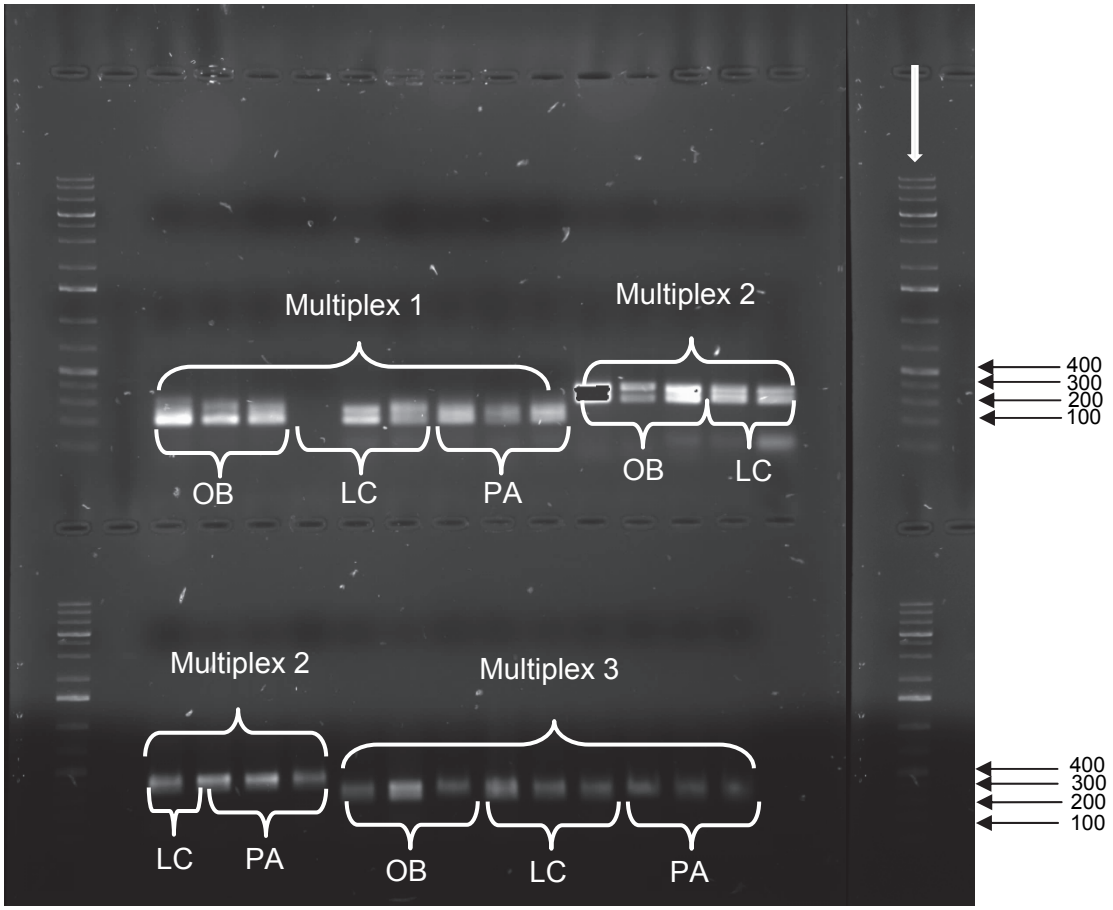

Figure 2.4. Gel electrophoresis results of trial multiplex (1-3) PCR of DNA samples (n=3) from three populations (OB: Oban; LC: Loch Creran; PA: Port Appin). 100 kb ladder (indicated by white arrow) to side shows approximate size (bp) of amplicons.

2.3.6 Fragment size analyses and peak scoring

Following gel confirmation of successful PCR, all product was transferred to a 96-well plate (10 µL per sample), sealed and sent on ice to University of Dundee for genotyping (via fragment size analyses). If there was a delay before sending samples, PCR product was kept at -20°C until delivery. PCR was carried out with 50 samples at a time and 150 samples were sent in one batch for genotyping at a given time. At University of Dundee, PCR fragments were run alongside a ROX500 size standard in an ABI 3130 Genetic Analyser. Output files were received in FAS file format and Peak Scanner (version 2) (Applied Biosystems, Foster City, USA) software was used to score peaks (Figure 2.5). Peaks scores represented the number of base pairs per allele (i.e. length of microsatellite region). All peak scores were entered in MS Excel software for subsequent analyses.

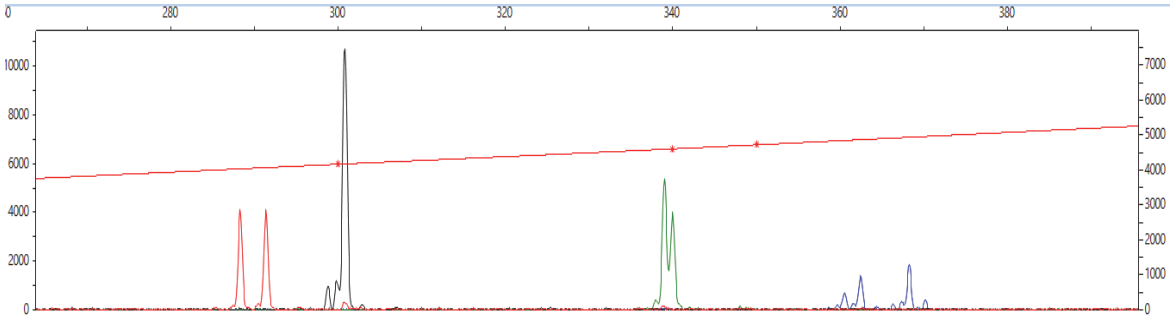

Figure 2.5. Example of peak data as visualised in Peak Scanner software. Different coloured peaks indicate different microsatellite markers. The position of the peak along the x axis indicates the size of the particular allele. The height of a peak is less important.

2.4 Data analyses

Microsoft (MS) Excel software with the MS Tools Add-in was used to generate files for genetics analyses. Peak data output files were then applied to (1) FreeNA software (Chapuis & Estoup, 1997) to determine genetic differentiation (F_{st} of Weir (1996)), allelic frequency and richness, and presence of null alleles; (2) Fstat software (V2.9.3) (Goudet, 1995) to determine inbreeding coefficient (F_{is}), allelic frequency and number of alleles, and carry out pairwise significance tests of differentiation; and (3) Arlequin software (Excoffier & Lischer, 2010) to determine deviations of genotype frequencies from the Hardy-Weinberg Equilibrium (HWE) via quantification of observed heterogeneity (Ho) and expected heterogeneity (He). Estimation of Null Allele (ENA) correction was performed (using FreeNA software) and pairwise F_{st} was calculated with and without ENA correction as described in Chapuis and Estoup (2007). The level of differentiation is reported according to F_{st} values with ENA correction.

Additionally, population structure was inferred using STRUCTURE software (version 2.3.1) (Pritchard *et al.,* 2000; Falush *et al.*, 2003; 2007). STRUCTURE assigns individuals to populations (i.e. groups, denoted *K*) and identifies distinct genetic populations. STRUCTURE parameters were set to allow for admixture and correlated allele frequencies with a burn-in period of 100,000 followed by 500,000 iterations. Outputs of five iterations of *K*=1-8 were run in STRUCTURE Harvester web v0.6.92 (Earl & vonHoldt, 2012) to determine the uppermost true (estimated) number of genetic units (*K*). Evaluation of the Delta*K* and L(*K)* plots with the Evanno Method (Evanno *et al.,* 2005) identified the uppermost *K* value.

Migration-N software (version 3.6) (Beerli & Palczewski, 2010) was used to determine genetic migration rates between populations. In brief, Migrate-N uses Fst values to calculate effective population sizes and mutation rates which are then used to determine theoretical migration rates (*m*) (Beerli, 2012). In this context, *m* is the fraction of immigrants in a population coming from another population over the most recent generation (i.e. immigrants per generation) (Figure 2.6). Such migration rates are theoretical estimations and thus it may be more useful to compare output values in relative rather than absolute terms. It may also be helpful to consider migration rates as a measure of the migration of genetic material (i.e. gene flow) between populations (Figure 2.7). Where the migration of juveniles to adult populations is considered (i.e. the Noss Head population), genetic migration rates can be thought of as the incorporation of juvenile genetic material to adult genetic structure.

MSAnalyser software (Dieringer & Schlotterer, 2003) was used to generate the Migrate-N input file. *m* values were inferred via Bayesian inference according to Fst values and using the Brownian motion model for microsatellites. A burn-in period of 1000000 was followed by 500000 iterations with constant mutation rates among loci assumed.

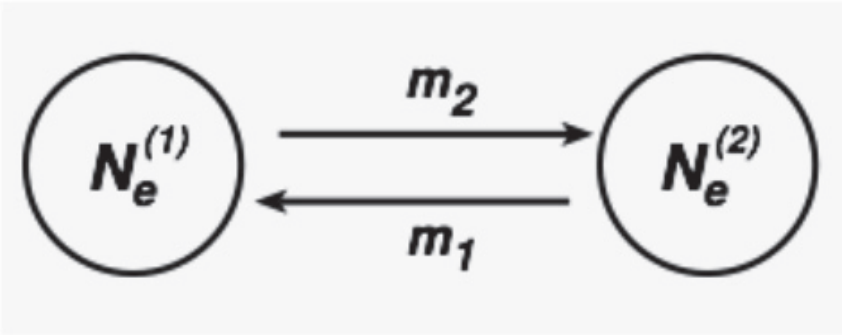

Figure 2.6. Migration rates (m) refer to the number of immigrants arriving over the last generation. For example, in the diagram above, m_1 refers to individuals moving from $N_e^{(2)}$ to $N_e^{(1)}$ while m_2 refers to individuals moving from to $N_e^{(1)}$ to $N_e^{(2)}$ (Image source: Beerli & Palczewski, 2010).

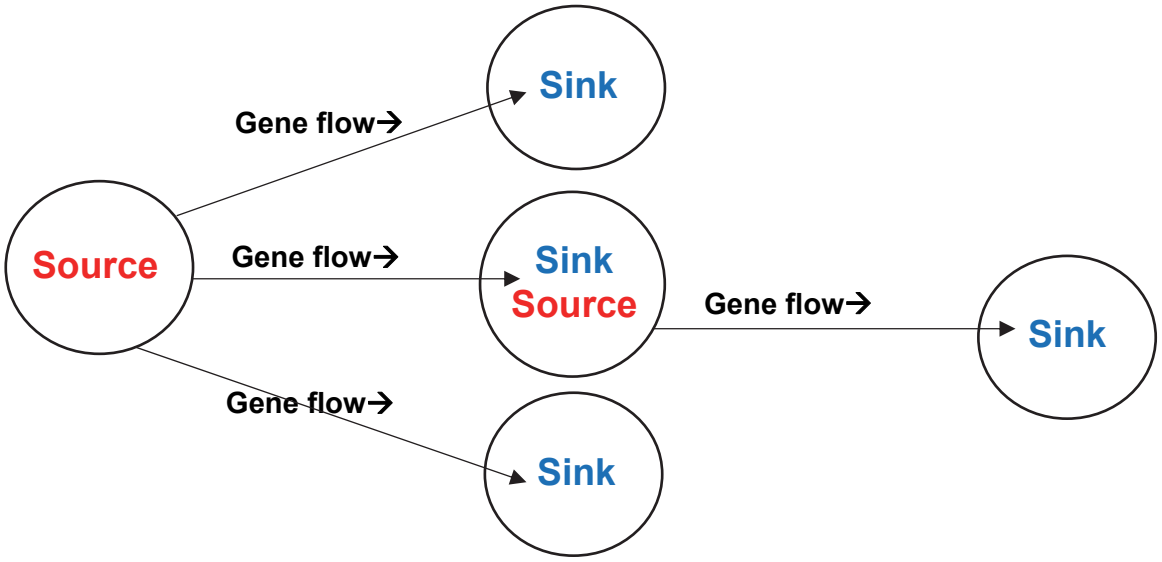

Figure 2.7. Migration, in the context of the current investigation, may also be thought of as gene flow from a source population to a sink population. A source population (indicated by red text) may act as a genetic source for one or multiple sink populations (indicated by blue text). A sink population may also act as a genetic source for other sink populations.

3. RESULTS

3.1 Genetic connectivity

Overall, F_{st} values indicated low-moderate differentiation[2] across Scottish horse mussel beds. None of the differentiation values between populations sampled were considered great or very great. However, comparison of intra-regional connectivity suggested higher levels of differentiation within populations of the West Coast and North-east and Orkney regions relative to those of the Shetland Isles region. Within the West Coast region, Port Appin showed significant ($p<0.05$) moderate differentiation from both Loch Creran ($F_{st}=0.069$) and Oban ($F_{st}=0.081$) populations. Conversely, Loch Creran and Oban populations showed low

[2] For the interpretation of Fst, a value lying in the range 0–0.05 indicates low genetic differentiation; a value between 0.05 and 0.15, moderate differentiation; a value between 0.15 and 0.25, great differentiation; and values above 0.25, very great genetic differentiation (Wright, 1978; Hartl & Clark, 1997).

differentiation (F_{st}=0.001), but this result was not significant. Within the North-east and Orkney region, Karlsruhe showed significant moderate differentiation from the Noss Head population (Noss Head: F_{st}=0.1064 (adults), F_{st}=0.066 (juveniles)). Results also suggest potentially low genetic differentiation between Noss Head adults and juveniles, but were not significant. Within the Shetland Isles region, all populations showed low differentiation but results were only significant between Hascosay Sound and Uyea Sound (F_{st}=0.012) (Tables 3.1 & 3.2).

Across all populations (i.e. inter-regionally), several key relationships were detected. Firstly, Port Appin and Karlsruhe populations showed significant moderate differentiation from all other populations except Dornoch Firth where moderate differentiation values were deemed not significant. Additionally, West Coast populations were generally moderately differentiated from North-east and Orkney populations while only low-moderately differentiated from Shetland Isles populations. In addition, North-east populations of the Scottish mainland (i.e. excluding Karlsruhe) also show low differentiation from Shetland Isles populations, though results were only found to be significant between Noss Head adults and the Shetland Isles' populations. Conversely, the Orkney population (Karlsruhe) was found to be significantly moderately differentiated from all Shetland Isles populations (Tables 3.1 & 3.2).

Genetic differentiation results (F_{st} values) provided here indicate genetic differences between analysed populations. The inverse of differentiation results can provide an indication of genetic connectivity. For example, where genetic differentiation is calculated to be low, one can assume genetic connectivity to be high. Likewise, it can be assumed that populations with high genetic differentiation have low levels of genetic connectivity. Table 3.2 presents results from both genetic differentiation and genetic connectivity points of view. Figure 3.1 is also provided as spatial representation of general patterns of genetic connectivity between populations so as to highlight key genetic relationships of populations. It should be noted that high genetic differentiation (i.e. low genetic connectivity) was not detected for any pair of populations.

3.2 Genetic diversity

Local inbreeding coefficients (F_{is} scores) of Scottish populations ranged from 0.176 (Uyea Sound) to 0.519 (Noss Head - adults), indicating a heterozygote deficit across all populations (Table 3.1)[3]. Likewise, all H_o values (ranging from 0.29373 in Noss Head adults to 0.56460 in Uyea Sound) were lower than H_e values (ranging from 0.6054 in Noss Head adults to 0.7203 in Noss Head juveniles), further indicating a heterozygote deficiency (Table 3.1). Therefore, it was concluded that all populations were not in Hardy-Weinberg Equilibrium (HWE) (see section 4.2 for discussion of HWE). Null alleles were detected across all markers and populations ($r > 0.05$).

Across all populations, Uyea Sound had the greatest number of alleles, highest H_o, lowest F_{is}, and a high allelic richness score (only lower than two other populations; Calback Ness and Noss Head juveniles), potentially suggesting increased genetic diversity in this population. Conversely, Noss Head (adults) had the lowest H_o and highest F_{is}. Regionally, the Shetland Isles appeared to have the greatest genetic diversity while genetic diversity values were similar between the other regions. Comparison of the difference between H_o and H_e values within each population showed greatest divergence of H_o from H_e in Loch Creran and Noss Head (both adults and juveniles) populations. Consequently, it can be concluded that these populations have the greatest deviation from HWE (refer to Section 4.2).

[3] For interpretation of Fis: Fis= 0 indicates no inbreeding; Fis>0 indicates a heterozygote deficit (low number of heterozygote individuals; indicative of inbreeding); Fis <0 indicates a heterozygote excess (high number of heterozygote individuals).

Table 3.1. Diversity indices and differentiation values for Scottish horse mussel beds. Regional groupings are indicated by coloured shading (blue=West Coast; red=North-east and Orkney; orange=Shetland Isles). Bold font F_{st} values indicate moderate differentiation ($0.05 \geq F_{st} > 0.15$). Non-bold F_{st} values indicate low differentiation ($F_{st} < 0.05$). F_{st} values above the grey shaded diagonal are before ENA correction. F_{st} values below the grey shaded diagonal are after ENA correction.

	Diversity Indices						Differentiation (F_{st}[2] before and after ENA correction)									
Population	N	H_e	H_o	N_A	A_R	F_{is}[3]	Oban	Loch Creran	Port Appin	Karlsruhe	Noss Head (A)	Noss Head (J)	Dornoch Firth	Calback Ness	Uyea Sound	Hascosay Sound
Oban	49	0.6406	0.35658	8.92	48.62 (±1.20)	0.447		0.004	**0.086**	**0.108**	**0.053**	**0.062**	0.040	0.015	0.029	0.013
Loch Creran	49	0.6413	0.3147	8.83	47.80 (±1.09)	0.518	0.001		**0.070**	**0.104**	**0.056**	**0.062**	0.038	0.016	0.028	0.013
Port Appin	41	0.6922	0.42998	10.58	53.37 (±1.79)	0.382	**0.081**	**0.069**		**0.059**	**0.142**	**0.097**	**0.138**	**0.081**	**0.080**	**0.077**
Karlsruhe	49	0.6672	0.4423	11.08	53.26 (±1.73)	0.340	**0.095**	**0.091**	**0.056**		**0.116**	**0.070**	**0.106**	**0.079**	**0.054**	**0.083**
Noss Head (A)	50	0.6054	0.29373	8.75	50.15 (±1.81)	0.519	0.044	**0.051**	**0.137**	**0.1064**		0.037	0.022	0.036	0.040	0.029
Noss Head (J)	50	0.7203	0.40919	8.83	55.77 (±1.24)	0.436	0.043	0.049	**0.085**	**0.066**	0.035		0.031	0.031	0.032	0.033
Dornoch Firth	50	0.6262	0.33113	7.92	48.67 (±1.58)	0.477	0.024	0.023	**0.119**	**0.090**	0.020	0.026		0.029	0.028	0.027
Calback Ness	45	0.6926	0.5254	10.58	55.81 (±1.48)	0.244	0.010	0.014	**0.079**	**0.070**	0.032	0.027	0.019		0.010	0.002
Uyea Sound	50	0.6839	0.56460	12.83	55.11 (±1.48)	0.176	0.020	0.022	**0.084**	**0.0539**	0.034	0.034	0.019	0.009		0.013
Hascosay Sound	50	0.6888	0.49496	11.75	55.05 (±1.69)	0.284	0.011	0.013	**0.080**	**0.0775**	0.025	0.033	0.018	0.003	0.012	

N = number of samples; H_e = Expected Heterozygosity; H_o = Observed Heterozygosity; N_A = number of alleles; A_R = Allelic Richness; F_{is} = inbreeding coefficient

Table 3.2. Level (low, moderate, high) and significance of genetic differentiation values for Scottish horse mussel beds. Comparison of populations within regional groups are indicated by bolded boxes. Level of genetic connectivity (high, moderate, low Fst scores after ENA correction) corresponding to each level of differentiation is also indicated in legend at bottom of the table.

Significance of Differentiation										
Population	Oban	Loch Creran	Port Appin	Karlsruhe	Noss Head (A)	Noss Head (J)	Dornoch Firth	Calback Ness	Uyea Sound	Hascosay Sound
Oban		NS	*	*	*	**NS**	NS	*	*	*
Loch Creran			*	*	**NS**	**NS**	NS	*	NS	NS
Port Appin				*	*	*	**NS**	*	*	*
Karlsruhe					*	*	**NS**	*	*	*
Noss Head (A)						NS	NS	*	*	*
Noss Head (J)							NS	NS	NS	NS
Dornoch Firth								NS	NS	NS
Calback Ness									NS	NS
Uyea Sound										*
Hascosay Sound										

Level of Differentiation	LOW	MODERATE	HIGH
Level of Connectivity	HIGH	MODERATE	LOW

*Significant; Indicative adjusted nominal level (5%) for multiple comparisons is: 0.000641 following Bonferroni correction, FStat; NS: non-significant

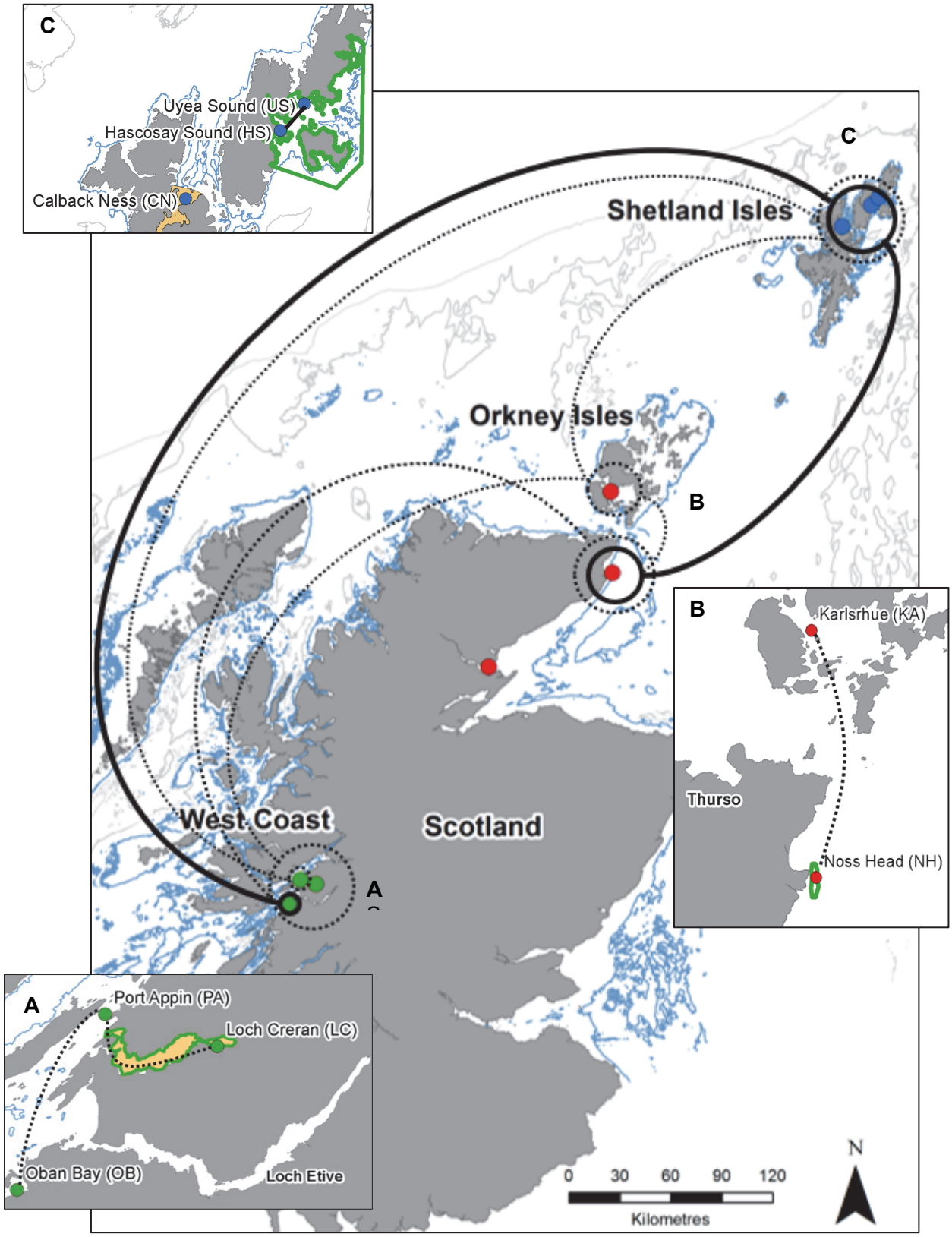

Figure 3.1. General patterns of genetic connectivity of selected horse mussel bed populations of Scotland. Level of connectivity is indicated by solid lines (high genetic connectivity, i.e. low differentiation) or dashed lines (moderate genetic connectivity, i.e. moderate differentiation). Circles contain any/all population(s) that is/are connected to adjoining population(s). Smaller inset maps (A-C) indicate levels of connectivity within regions and correspond to coding (A-C) on main map. Noss Head adults and juveniles are considered collectively. Please note: Low levels of connectivity were not detected and lines do not necessarily indicate direct connectivity or direction of movement. Coastline © Crown copyright and database right (2012). Ordnance Survey Licence number 100017908.

3.3 Genetic groups

STRUCTURE and STRUCTURE Harvester results found *K*=2 to be the most likely *K* value, indicating there are two main genetic groups across all currently sampled Scottish populations (Figures 3.2 & Annex 2: Figure A1). Group 1 included Loch Creran, Oban, Noss Head (adults), Dornoch Firth, Hascosay Sound, Uyea Sound and Calback Ness. Group 2 included only the Port Appin and Karlsruhe populations. Noss Head (juveniles) appeared to be part of both groups (though associated more strongly with Group 1) and thus potentially have a distinct structure as compared to Noss Head adults (therefore suggesting that the juvenile population receives recruits from a range of sources over time, i.e. not fully self-recruiting). STRUCTURE results for *K*=3 suggested a potential sub-group within Noss Head (juveniles) and *K*=4 suggested a potential sub-group within Port Appin (Figure 3.2).

3.4 Genetic migration rates

Genetic migration rates for all populations (across all regions) are presented in Annex 2: Table A4. Migrate-N analyses determined average migration rates (i.e. number of immigrants generation^{-1}) of 13.54 (SD±4.40) for the West Coast region; 15.73 (SD±8.01) for the North-east and Orkney region; and 14.46 (SD±4.22) for the Shetland Isles region. Migration rates ranged from 8.05-25.25 for the West Coast region; 5.62-54.34 for the North-east and Orkney region; and 5.56-23.55 for Shetland Isles region. The strongest and weakest migration rates were from Loch Creran to Dornoch Firth (54.3) and Noss Head Adults to both Noss Head Juveniles (5.6) and Uyea Sound (5.6), respectively.

A comparison of migration rates for populations within each region indicated directional bias (i.e. stronger unidirectional immigration from one population to another) to Port Appin (from Loch Creran) and to Oban (from Loch Creran and Port Appin) in West Coast populations; to Karlsruhe (from Noss Head and Dornoch Firth), to Noss Head (from Dornoch Firth) in North-east and Orkney populations; and to Uyea Sound (from Hascosay Sound and Calback Ness) and to Hascosay Sound (from Calback Ness) in Shetland Isles populations (Figure 3.3; Annex 2: Table A5). However, in many cases, migration rates were quite comparable between populations so directional bias may be negligible. Likewise, when mean migration rates from all other populations included in the study were calculated per population (Table 3.3), values and variation were generally similar across sites. Comparison of genetic migration rates of Noss Head adults and juveniles indicated a relatively weak contribution of genetic material from adult to juveniles mussels (5.6). When considered in combination with the STRUCTURE results above (juveniles having a distinct structure compared to the adults), this suggests that there is recruitment into the Noss Head population from external sources and only a small degree of self-recruitment.

Comparison of mean and sum migration rates (number of immigrants generation^{-1}) between regions showed directional bias from the West Coast region to the North-east and Orkney region, from the Shetland Isles region to the West Coast region, and from the Shetland Isles region to the North-east and Orkney region (see Annex 2: Table A6). Additionally, results indicate considerable levels of self-recruitment within regions, with highest levels calculated for the North-east and Orkney region (Table 3.4). Figure 3.3 provides an overview of directional bias of migration between and within regions.

Figure 3.2. STRUCTURE output with K=2-5 for horse mussel populations across Scottish regions. Population codes provided along x-axis. Populations showing the same colour are part of the same genetic group (K). As K is increased, structure becomes less clear but highlights potential genetic relationships across all populations including potential sub-groupings. Population codes as per Table 2.1.

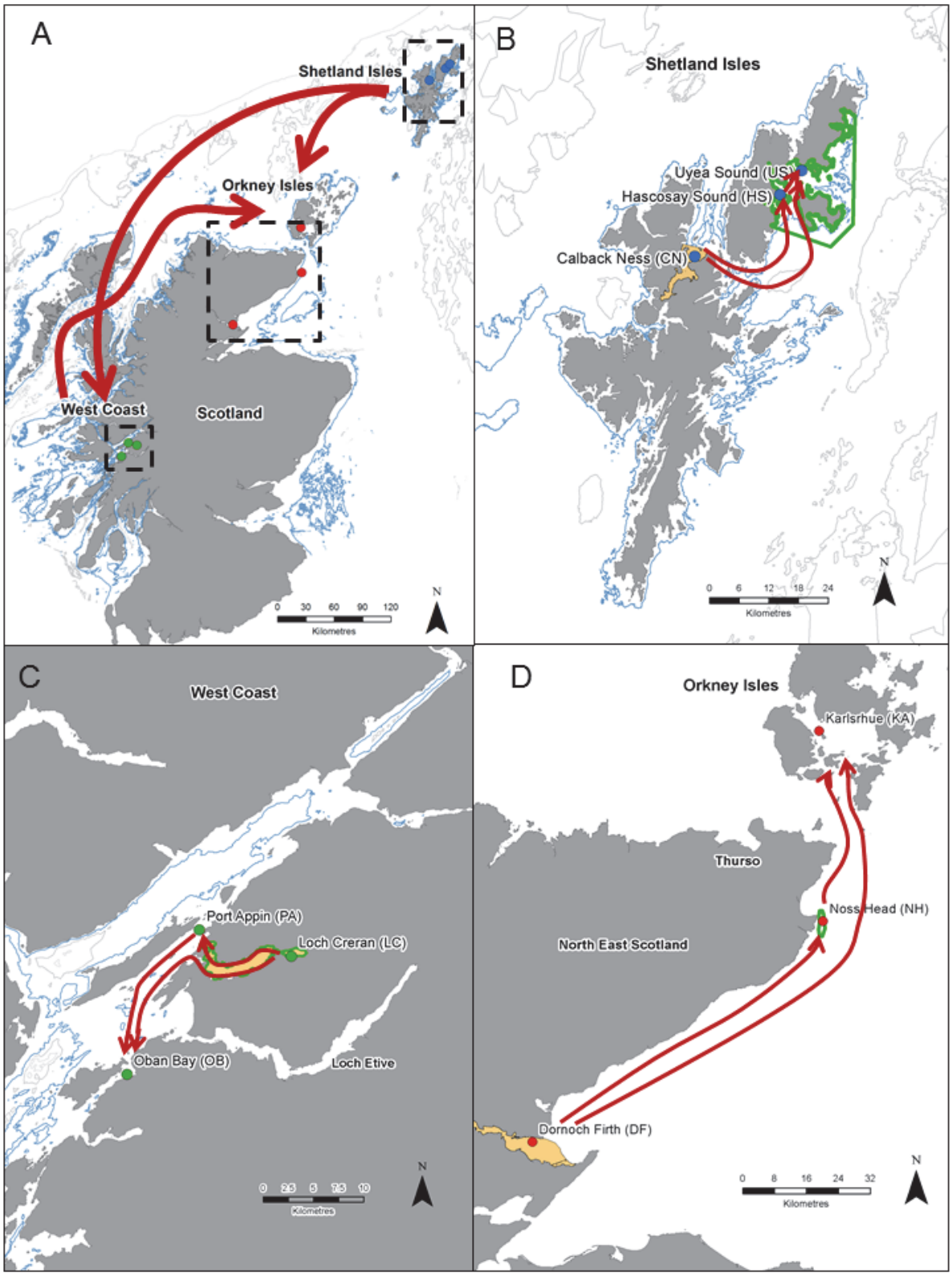

Figure 3.3. Directional bias of genetic migration between regions (A) and between sites within the Shetland Isles regions (B), the West Coast region (C), the North-east and Orkney region (D). Note: Results indicate the direction of higher migration rates only. They do not indicate uni-directional migration as migration was strong in both directions for most populations/regions.

Table 3.3. Mean migration rate (μm) (±SD) and sum migration rate (∑m) for each population from all other horse mussel beds, where m is the mean number of immigrants generation^{-1} from each source population into the sink population.

Sink population	μ*m*	±SD	∑*m*
Appin	13.56	4.86	188.00
Loch Creran	11.31	3.77	167.33
Oban Bay	13.25	4.16	184.00
Karlsruhe	14.79	4.00	197.33
Noss Head (adults)	14.42	5.57	198.00
Noss Head (juveniles)	10.72	3.51	162.67
Dornoch Firth	17.53	12.51	240.67
Hascosay Sound	13.10	4.01	181.33
Uyea Sound	14.94	5.41	202.00
Calback Ness	14.72	3.80	198.00

4. DISCUSSION

4.1 Genetic connectivity

In general, there appears to be moderate to strong connectivity of horse mussel beds across the sampled range of this Priority Marine Feature as shown by the low-moderate F_{st} scores (i.e. low-moderate differentiation). Furthermore, the results of the STRUCTURE analyses suggest only two genetic groups within the horse mussel populations sampled, with no clear spatial divide (i.e. minimal genetic structure). Additionally, mean migration values and variability in migration rates were generally comparable across sites and regions. However, the authors stress that migration metrics should be regarded as only preliminary indication of potential migration patterns, and strongly recommend that future work include larval dispersal modelling (see below) in order to validate/repudiate results.

Within the horse mussel beds sampled, distance does not appear to be a key driver of connectivity for Scottish horse mussels beds. The genetic connectivity analyses of Scottish populations showed both moderate differentiation across small spatial scales and minimal differentiation across larger spatial scales. For example, within the West Coast region, the Port Appin population was found to be moderately differentiated to both Loch Creran and Oban populations, despite being in close vicinity of one another. Conversely, populations of the West Coast region were less differentiated from Shetland Isles' populations than with closer North-east and Orkney populations. Similarly, migration analyses indicated the highest migration rate between relatively distant populations (Dornoch Firth and Loch Creran). However, for connectivity at this scale to be achieved it is likely that there are intermediate 'stepping-stone' populations (not sampled for the current analysis) rather than a result of direct movement of larvae.

Oceanic currents are largely responsible for controlling gene flow for marine bivalves (Palumbi, 1994) and therefore, likely to be a key driver of horse mussel bed population connectivity. For example, the results presented here show the genetic structure of the Karlsrhue population in Orkney to be largely different from surrounding sites (i.e. Noss Head) which may be due to the site being situated in an enclosed waterbody (Scapa Flow). Consequently, the site may be isolated and reliant upon self-recruitment or receive recruits from adjacent un-sampled populations. Complex oceanic processes can create isolated populations (reliant upon self-recruitment) or distinct source and sink populations (Palumbi, 1994). Additionally, knowledge of local demographics, larval biology and ecology, estimates of reproductive success of immigrants and residents, habitat suitability and geographical

influences can also improve understanding of the mechanisms driving genetic connectivity of horse mussel beds (Selkoe & Toonen, 2011). Such influences are discussed in the context of the genetic connectivity of Scottish horse mussels beds.

4.1.1 Hydrodynamics

Knowledge of local and regional hydrodynamics including current flows, tidal regimes, and front systems is vital for understanding the forces that promote or block larval dispersal and therefore directly advance or hamper gene flow between sites. Around the UK, these forces vary immensely and consequently timing, direction and success of larval dispersal from and recruitment to horse mussel populations is very much location dependent. Likewise, the influence of hydrodynamics is complex and the relationship between such forces and larval connectivity is not straightforward. For example, while beds situated in open areas of high flow that are exposed to large scale water movement might have improved dispersal and acquisition of larvae, long-range dispersal "success" may also lead to "dispersal dilution" and therefore low recruitment. Likewise, exposed sites might experience problems with retention of larvae as compared to enclosed bays (Adams *et al.*, 2014). Consequently, around the Noss Head population, where mean spring peak flows are relatively high (Figure 4.1; ABPmer, 2008), it is possible that larvae might be dispersed fairly quickly, thereby reducing potential for recruitment to nearby adjacent sites. This could help to explain the low genetic migration rate from Noss Head adults to Noss Head juvenile populations (i.e. low self-recruitment).

Exposed coastlines and open bays have typically been associated with greater dispersal potential of larvae (Robins *et al*., 2013). On the other hand, semi-enclosed water bodies have often been assumed to act as larval sinks due to factors such as the increased retention of seawater associated with sea loch environments and presence of local geographical barriers to dispersal (e.g.peninsulas, islands) (Roberts *et al*., 2011; Robins *et al.,* 2013). However, the connectivity results here show that beds within semi-enclosed water bodies such as Loch Creran are also capable of acting as source populations for other horse mussel beds. This suggests that horse mussel beds that occur in such locations are important for maintaining links between populations and loss of such beds could have a greater effect on surrounding beds, compared to beds that are largely self-recruiting, for example.The complex geography of Scotland's west coast coupled with high flow rates between islands and sea loch narrows may create conditions that aid in dispersal from more enclosed areas (Adams *et al.,* 2014).

Past studies have combined hydrodynamics modelling (i.e. particle transport models) with genetic techniques to provide improved understanding of actual connectivity for species with a planktonic larval stage (Schunter *et al.*, 2011; Selkoe & Toonen, 2011; Coscia *et al.*, 2012). Co-consideration of both aspects can also act as a means of validating either approach and therefore strengthen conclusions regarding actual levels of connectivity between sites. Gormley *et al.,* (2015), for example, combined genetic analyses and hydrodynamic modelling of larval dispersal from the same populations within the Irish Sea region to assess horse mussel connectivity. Simulations of larval dispersal over 30-day dispersal periods showed strong connectivity between Northern Ireland sites (Strangford Lough and Ards Peninsula) and confirmed potential for larval dispersal up to 150 km. Furthermore, dispersal modelling suggested that connectivity between Northern Ireland sites and Isle of Man and Wales sites was unlikely, confirming the results of the genetic analyses. However, while genetic analyses suggested genetic connectivity between North Wales and the Isle of Man sites, the dispersal model showed that direct recruitment of larvae from one population to another to be unlikely, and thus highlights the complex nature of population connectivity and potential limitations of either/both approach(es). Future work examining connectivity across a wider spatial scale (including the Irish Sea and Scottish territorial waters) as well as intermediate populations within regions, would be highly valuable towards clarifying genetic

structure and relationships across all UK populations, and could help to identify key source populations.

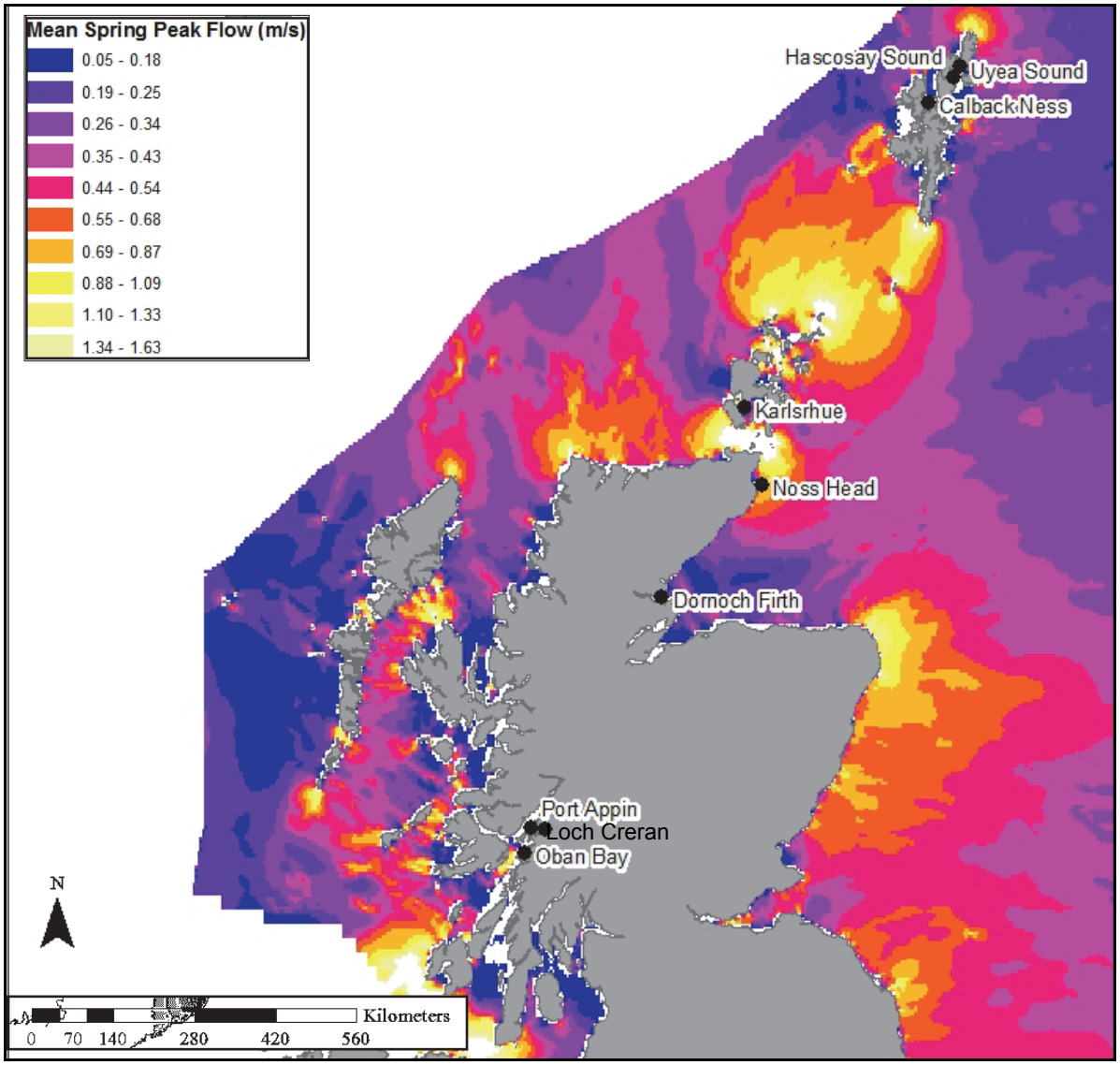

Figure 4.1. Mean spring peak flow (metres per second) across sampled Scottish horse mussel beds (Source: ABPmer website). Tidal data supplied by Proudman Oceanographic Laboratory (POL) on behalf of UK Department for Business, Enterprise and Regulatory Reform (BERR) for the Atlas of UK Marine Renewable Energy Resources project. The tidal current parameters have been computed for sigma levels closest to the depths of 50% of the surface.

Consideration of the interaction of spatial variation in hydrodynamics and larval behaviour is key to determining larval dispersal potential (Robins *et al.,* 2013). For example, marine larvae demonstrate various swimming behaviours (e.g. vertical migration) which allow them to benefit from or resist water currents, and thus have some influence over dispersal distance and settlement area (Adams *et al.*, 2014; Robins *et al.*, 2013). However, as current understanding of the larval biology and ecology of horse mussels is largely lacking (refer to Section 4.1.2), hydrodynamic modelling of the species' dispersal potential may be limited. Further, where previous hydrodynamic modelling of horse mussel bed connectivity has considered larval behaviour, specific model parameters have varied significantly with regards planktonic period and time of year of larval release, typically to reflect reproductive

characteristics of a particular population. For example, hydrodynamic modelling completed for Northern Ireland populations by Elsäßer *et al.* (2013) were run to simulate a dispersal period of 56 days and an autumn gamete release, as characteristic of Strangford Lough horse mussels. Conversely, modelling work by Gallego *et al.* (2013) assumed that horse mussels spawn all year round with a settlement window of 30-40 days. Such variation between populations highlights a significant issue of horse mussel larval dispersal modelling. Additionally, model outputs can be strongly influenced by the scale at which they are run, leading to results that may not correlate with genetic connectivity analyses. For example, the potential dispersal of offspring in the Gallego *et al.* (2013) model was widespread yet connectivity potential between MPAs was determined to be low, counter to results presented here. However, this model was run at a coarse scale, and therefore did not take into account nearshore beds (e.g. in sea lochs, where a number of beds are located) or habitat suitability, and used only a small (i.e. 3) number of release sites. It should be noted that neither of the fore-mentioned models was validated with genetic differentiation data, and thus it is suggested that future work consider completing such analyses.

4.1.2 Larval biology and ecology

In many marine invertebrate species, levels of self-recruitment and connectivity are highly dependent on the transport of larvae, particularly with regard to direction and magnitude of larval movement (Robins *et al.*, 2013). Consequently, understanding the influence of larval biology (including such aspects as growth and development, metabolism, mortality rates, health and disease, and life history traits) on larval transport is a key consideration when examining the role of larval dispersal in shaping genetic connectivity. The length of time that larvae remain in the plankton, for instance, is chiefly dependent on factors such as development mode (planktotrophic versus lecithotropic), accumulated energy reserves, and time required to become metamorphically competent (Jaeckle, 1994). For example, the blue mussel (*Mytilus edulis*) typically has a larval developmental period of approximately 30 days but can persist in the plankton beyond 2 months (Bayne, 1976). For many bivalve species, larvae development is correlated with sea temperature (e.g. His *et al.,* 1989) and Brown (1984) found that temperature also influences the timing of spawning in horse mussels. Therefore, changes in seawater temperature may impact reproduction and larvae development in horse mussels, which could in turn affect connectivity patterns.

Where larvae have a reduced developmental period, dispersal time (and therefore dispersal distance) may be reduced (Pineda *et al.*, 2007), reducing gene flow potential between populations that are situated over large distances (assuming there are no intermediate populations to sustain the connection). Alternatively these could be influenced by temporally occurring hydrodynamic processes acting as restrictions to movement. Conversely, where larvae are slow-developing and spend substantial time in the plankton (and a longer settlement window), greater dispersal distances may be achieved, potentially leading to greater recruitment success as larvae are able to reach a greater number of suitable sites (although predation risk should also be considered). Results here suggest that genetic connectivity of horse mussel populations is possible over sizable distances (i.e >500 km), however, it is likely that this is due to the presence of stepping-stone populations that support the transfer of genetic material over great distances rather than via a direct connection.

The role of larval ecology to dispersal and recruitment success is also an important consideration when assessing influences to genetic connectivity. Ecological aspects, such as the effects of temperature and algal concentrations (i.e. prey availability) to larval development and behaviour, could progress understanding of dispersal potential and recruitment success (Levin, 2006). Likewise, knowledge of larval traits that allow for survival to settlement and the roles of post-settlement competition and predator-prey interactions in recruitment success may be key to fully comprehending the mechanisms driving genetic

connectivity. This is particularly important over a wider spatial scale, as such ecological interactions are influenced by physical conditions occurring at source and sink population sites, as well as larval pelagic conditions (Cowen & Sponaugle, 2009). Consideration of ecological data could aid in explaining why distance appears to be a lesser influence to the genetic connectivity of horse mussel beds.

Current knowledge and understanding regarding the horse mussel reproduction, larval biology and ecology remains limited. Research that does exist suggests that the reproductive cycle of UK horse mussels lacks seasonal synchronicity with recruitment, spawning period, developmental period and gonadal maturation varying across seasons, year, depth and location (Holt *et al.,* 1998; Roberts *et al.,* 2011; Dinesen & Morton, 2014). Roberts *et al.* (2011) provide a detailed overview of larval development (including the effect of diet) for the species but investigation was confined to the Strangford Lough population. As larval growth and development is largely driven by local conditions such as prey availability and temperature (Bertram & Strathmann, 1998; Cowen & Sponaugle, 2009), results (e.g. developmental time) may not apply to other populations. This poses a considerable challenge to hydrodynamic modelling in that information based on a particular population or particular set of experimental conditions may not be relevant when applied across a wider spatial scale.

4.1.3 Local demographics

In addition to hydrodynamic influences, local demographics have a bearing on reproductive rates, dispersal, and recruitment success and therefore are likely to influence genetic connectivity of horse mussel bed populations. Brash *et al.* (2017), for example, examined juvenile abundance and bed density in relation to current flow in horse mussel beds across the UK distribution (including North Lleyn, Port Appin and Karlsruhe beds). The Karlsruhe bed was observed to have a lower abundance of juveniles and lower densities than other beds, with both variables associated with reduced tidal flow. Beds with greater densities tend to have higher structural complexity which can aid in recruitment by providing shelter to juveniles from predation (Comely, 1978; Holt *et al.*, 1998) and by increasing post-settlement survival (Gutierrez *et al.*, 2003; Nestlerode *et al.*, 2007). Consequently, the lower density, recruitment and current flow observed at the Karlsruhe bed suggest it could be relatively isolated from other beds and could partially explain the observed levels of moderate differentiation as compared to other Scottish horse mussel beds. Results from the present study also indicate that Karlsruhe is of a different genetic group than nearby northern sites on both the Scottish mainland and in the Shetland Isles, further supporting this argument.

The Port Appin population was also moderately differentiated from all other populations. Given that high levels of juvenile abundance have been recorded at this bed (Brash *et al*., 2017), results potentially indicate high levels of self-recruitment. However, given the high current flow associated with the site (Brash *et al.,* 2017), it is more likely that the population is recruiting from an unsampled or unknown site. For example, there are a number of other west coast horse mussel populations (e.g. Loch Alsh and around the Small Isles) that may act as larval sources. Likewise, there may be undiscovered beds that supply the Port Appin and Karlsruhe beds, thus accounting for their separation as a separate genetic group, as suggested by STRUCTURE results.

4.1.4 Coastline geography

The dispersal stage of a species with sessile adults, such as horse mussels, must select sites that are environmentally suitable for establishment and survival, but must also be able to reach such locations. Consequently, where local coastline features such as headlands, narrows and sea lochs directly influence accessibility (including dispersal and retention), such geographies may have a considerable influence on larval connectivity and therefore

genetic connectivity of sites (Adams *et al.,* 2014). For example, Adams *et al.* (2014) found that dispersal success was largely dependent on current velocity, as previously discussed, but also coastline geography (e.g. wave fetch, openness) and habitat availability. Particles released from regions of high current velocity, open coastline and low local habitat availability (e.g. headlands, islands) travelled furthest but were less likely to disperse successfully to other coastal sites as compared to those living along sheltered and enclosed coasts (Adams *et al.,* 2014). Additionally, nearshore processes such as flood-tide asymmetries (produced by coastal morphology) influence self-recruitment and connectivity (Robins *et al*., 2013). Man-made aspects of coastal geography should also be considered as potential influences to connectivity. For example, the construction of the Churchill Barriers (causeways) in Orkney is likely to have altered the genetic connectivity of horse mussel beds within Scapa Flow (e.g. Karlsruhe) to other external beds (e.g. those recorded off Copinsay but not yet tested).

Elsäßer *et al.* (2013) demonstrated the effect of local coastline to recruitment showing that reduced connectivity between Irish Sea horse mussel beds and Strangford Lough populations was a consequence of the larvae's inability to move beyond Strangford Narrows. While the processes influencing the Strangford Lough bed might be expected to occur for Scottish sea lochs, the presence of narrows (characteristic of loch environments) with high current flow may also aid in larval dispersal to other sites (Robins *et al*., 2013). Likewise, while high currents around headlands and other exposed sites might promote larval dispersal, the same conditions can lead to larval dilution (i.e. decreased density of larvae within the water column), particularly in species with extended larval duration, and thus reduce settlement and recruitment success (Adams *et al.*, 2014). Consequently, widespread dispersal from a source site may ultimately result in less larvae arriving at any sink sites and thus, could explain the moderate differentiation of the exposed Noss Head bed from all other Scottish beds. Furthermore, the differences in genetic structure of juveniles and migration results suggest low self-recruitment at the Noss Head bed and therefore this population is likely to be receiving larvae from external sources.

4.2 Genetic diversity

All horse mussel populations had lower H_o than H_e and positive F_{is} values indicating a heterozygote deficiency and therefore showing that all populations were not in HWE. HWE states that any genetic variation in a population will stay constant over generations in the absence of disturbing factors such as gene flow, natural selection or mutations (Lowe *et al.*, 2004). Consequently, deviation from HWE, where observed heterozygosity is different from expected heterozygosity, indicates that a factor such as inbreeding (if Ho<He, as seen here) or mixing of two previously isolated populations (if He<Ho) must be considered (Lowe *et al.*, 2004). Null alleles were detected across all markers and populations and may account for the observed heterozygote deficiency. Null alleles occur when a primer is prevented from annealing to template DNA during amplification of the microsatellite locus by PCR and can lead to under-estimation of genetic diversity (Chapuis & Estoup, 2007), and are a relatively common occurrence in mollusc species (Reece *et al.,* 2004; Mariani *et al.,* 2012; Coscia *et al.*, 2012).

Addison & Hart (2005) also found that species with planktonic reproduction (e.g. broadcast spawners with external fertilisation) tend to have greater heterozygote deficits and therefore increased departures from HWE. This may be due to factors such as increased mutation rates arising from the high number of cell cycles needed to produce excess gametes or the Wahlund effect (i.e. coexistence of genetically distinct cohorts within a sampling location) (Addison & Hart, 2005; Coscia *et al.*, 2012). Additionally, large variation in reproductive success may lead to effective population (i.e. that cohort of the population that reproduces) sizes that are much smaller than census numbers, and consequently lead to reduced heterozygosity across a population (Bierne *et al.,* 1998).

In their review of F_{is} values in marine species, Addison & Hart (2005) reported a mean F_{is} of 0.149 across 89 studies of species with planktonic larval dispersal. Comparatively, F_{is} scores calculated here for Scottish horse mussel beds seem high with a mean F_{is} of 0.382±0.118 (SD) across all populations. However, it has been reported that high F_{is} scores are typical of bivalve species (Addison & Hart, 2005). Further, while positive F_{is} values may indicate inbreeding (which could lead to adaptive decline in these populations) it is argued that this is unlikely to be occurring in horse mussels. Instead the high F_{is} scores are more likely to be a consequence of the heterozygote deficiency commonly observed in marine bivalves, as previously discussed.

While absolute values of diversity do not seem reliable here, for reasons discussed above, a relative comparison of metrics may at least illustrate variation in the genetic diversity between horse mussel populations. All populations sampled from the Shetland Isles had similarly high allelic richness scores and low F_{is} scores while northern (Noss Head and Dornoch Firth) and western (Loch Creran and Oban Bay) mainland populations showed similarly low H_o scores and increased F_{is} scores. Karlsruhe and Port Appin populations showed similar diversity indices, which may be a consequence of being part of the same genetic grouping.

Migration rates also indicate adequate gene flow between populations to prevent genetic differences between populations. For comparison, Palumbi (2003) reported that even one migrant per generation can have considerable influence on the genetic make-up of the sink population and as low as ten migrants per generation may be ample to prevent any gene-frequency differences between populations. Thus, it is suggested that migration rates are high between populations and sufficient to ensure genetic similarity (and diversity) across populations.

4.3 Adaptive capacity

Given that the population connectivity analyses presented here indicates moderate to high levels of gene flow across all horse mussel populations, one would expect that adaptive capacity is generally limited within populations at a site level (Sanford & Kelly, 2009) (i.e. reduced potential to adapt to site-specific stress conditions). On the other hand, it has also been suggested that gene flow may increase local adaptive potential by improving genetic diversity, particularly in populations that are exposed to temporal variation in stress levels (Baskett *et al*., 2010).

It is assumed that in bivalves, larval transport is driven by currents and the residual current direction on the west coast of Scotland is from south to north (Gallego, 2013). Therefore, southern populations could provide increasingly resilient larvae to northern populations and thereby improve adaptive capacity at northern sites. Conversely, northern (i.e. lower stress sites) populations may serve as both a demographic and adaptive source for higher stress locations, as has been suggested for coral reef populations (Baskett *et al*., 2010). More southern or shallower populations may struggle to adapt to warming conditions if they are sinks for larval supply from northern/deeper (i.e. cooler) environments.

Reduced adaptive capacity has historically been presumed for bivalve species due to the fact that they have planktonic larvae and therefore widespread dispersal. Conversely, local adaptation in marine species is typically observed in species with brooded or short-lived lecithotrophic larvae (i.e. non-feeding larvae that rely on energy stores of the yolk sac) (Levin, 2006) as opposed to species such as horse mussels that have a planktonic larval stage. However, differentiation and adaptation have been observed in other bivalve species, and over a range of spatial scales (i.e. metres to kilometres), so should not be ruled out (Kuo & Sanford, 2009).

Increased genetic connectivity may support higher levels of genetic diversity which may contribute to improved resilience by providing a diverse gene pool from which natural selection may occur (Sanford & Kelly, 2011). However, the relatively long generation length (i.e. years) of many bivalve species makes it is unlikely that these organisms will be able to undergo rapid evolution to keep up with current increasing rates of climate change predicted to occur by the next century (Kurihara, 2008; Harley *et al.,* 2006). This is particularly relevant to horse mussels which have an extended lifespan compared to many other bivalve species, with many individuals living in excess of 30 years (Halaynch *et al*., 2013; Farinas-Franco *et al.,* 2014). Regardless of whether adaptation is able to occur in horse mussels, the influence of genetic factors on resilience to climate change should not be disregarded. Horse mussels may be capable of adjusting gene expression (for example, by alteration of transcription or translation) to attain physiological plasticity in response to physical stressors in their environments (Hofmann & Todgman, 2010; Whitehead, 2012), particularly where populations have historically experienced variable or extreme conditions. Farinas-Franco *et al.* (2014), for example, observed phenotypic variation within horse mussel populations with changes in shell shape and size correlated with physical factors such as substrate type and tidal current.

5. CONCLUSIONS

Improved understanding of population connectivity and diversity will contribute to informing management discussions of Scottish MPAs that contain or are in proximity to horse mussel beds. Genetic structure indicated here should be considered for both local and regional scale protection of horse mussel beds within Scottish and UK frameworks. Results show that the populations sampled are generally well-connected at both local and regional spatial scales and thus, it is advised that horse mussel beds be considered as a network rather than isolated units. However, it would be useful to sample horse mussels from additional beds as well as more isolated individuals for genetic analyses to improve current understanding of genetic connectivity patterns. This is particularly noted for the Port Appin and Karlsruhe populations which were shown to be genetically separate from other Scottish beds; inclusion of other sites may help to identify beds that are critical for maintaining such populations or conversely, highlight sink populations that rely on Karlsruhe or Port Appin as source populations.

Protection of horse mussel bed populations across the MPA network may also help to maintain the cumulative genetic diversity that exists across populations. This may in turn promote resilience to climate change (Sanford & Kelly, 2011). Additionally, knowledge of connectivity may aid in understanding the recovery potential of other degraded populations (on a site by site basis) and inform management decisions. It is particularly useful to have an understanding of whether natural recovery might occur on its own (via dispersal from supporting populations) or whether human intervention (e.g. via transplantation) is necessary for recovery (Levin, 2006). Similarly, information on the genetic similarity of populations is important to consider in horse mussel translocation or restoration attempts. Improved information about the adaptive capacity of beds may guide the selection of source populations for restoration efforts, especially under the context of climate change (Sanford & Kelly, 2011). In future, it is advised that complementary connectivity analyses/approaches (e.g. larval transport modelling, demographic studies) be completed in parallel with genetic analyses as a means of validating results and providing a clearer understanding of those mechanisms that may be driving genetic structure. Adoption of a multi-disciplinary approach may also aid in reliably highlighting or identifying important source and stepping-stone populations, which should be considered as high priorities for conservation.

6. KEY RECOMMENDATIONS

In light of the key results and main conclusions of this report, the following recommendations for future horse mussel connectivity work are proposed:

- Carry out futher genetic connectivity analyses of Scottish horse mussel beds with additional horse mussel populations (as they are accessed/discovered) with both adults and juveniles to improve understanding of genetic relationships;

- Carry out UK-wide genetic analyses of horse mussel populations to examine connectivity between southern and northern populatlons. This could include investigation of whether southern populations can provide larvae with increased resilience to ocean warming to northern populations (and therefore contribute to adaptive capacity);

- Complete complementary larval dispersal modelling of horse mussel populations to validate genetic results and improve understanding of connectivity. Modelling could also account for differing physical scenarios (i.e.current conditions vs. future climate change conditions) in order to examine the influence of a changing physical environment to dispersal potential and population connectivity;

- Improve understanding of horse mussel larval biology and ecology (across multiple populations) in order to provide accurate/appropriate larval data for input parameters of larval dispersal models;

- Consider the role of other anthropogenic influences to genetic connectivity (e.g. Churchill Barriers in Orkney);

- Continue to collect complementary demographic information for horse mussel populations across the Atlantic range to investigate the influence of such aspects as sex ratios, size/age classes, growth rates, etc. to population connectivity.

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8. GLOSSARY

adaptive capacity: potential for an organism, population or species to evolve via natural selection (i.e. adapt) to suit a particular set of environmental conditions.

allelle: one form of a gene located at the same location (locus) on a particular chromosome. Two different alleles at a locus is referred to as heterozygous (=increased genetic diversity); two identical alleles at a locus is referred to as homozygous (=decreased genetic diversity).

allopatric speciation: speciation (evolution of a new species via natural selection) arising from geographical isolation.

connectivity: the movement of organisms between populations; linkage or interconnectness of populations across space and/or over time.

differentiation: the level of genetic variance existing between populations

differentiation coefficient (Fst): a measure of population genetic variance arising from changes in genetic structure between populations

expected heterozygosity (He): the level of genetic variation one would expect under HWE.

gene flow: movement of genetic material between populations via transfer of gametes, larvae or adult organisms

genetic connectivity: the extent to which populations in different parts of a species' range are linked by the exchange of eggs, larvae recruits or other propagules, juveniles or adults.

genetic diversity: variety in genetic structure arising from quantity and frequency of different alleles in a population.

genetic markers: specific DNA codes existing at known locations on a given chromosome that can be be used to identify a particular genetic group.

Hardy-Weinberg Equilibrium (HWE): the maintenance of stable genetic variation (i.e. unchanging allele frequencies) in a population over time due to the absence of disturbing factors such as gene flow, mutation, inbreeding, or other evolutionary forces; deviation from HWE occurs when Ho ≠ He.

heterozygosity: the level of genetic variation in a population (i.e. low heterozygosity associated with low genetic variation); organisms that are heterozygous have two forms of an allele occurring at a locus.

heterozygote deficiency: a lack of genetic diversity (i.e. increased frequency of single form of allele at a locus); indicates increased inbreeding within a population

heterozygote excess: increased genetic diversity (i.e. increased frequency of multiple forms of an allele at a particular locus); indicates increased gene flow between populations.

inbreeding coefficient (Fis): a measure of the level of intrapopulation inbreeding.

k-value: a metric that represents the number of genetic groupings existing across one or several populations of a given species.

microsatellite: a type of genetic marker comprising tandem repeats of di-, tri- or tetra-nucleotides; the structure of microsatellite markers leads to high mutation rates and thus they are highly effective in detecting genetic connectivity patterns.

null allele: a mutant copy of gene arising when a primer is prevented from annealing to template DNA during amplification of the microsatellite locus by PCR; inclusion of null alleles in analyses can lead to under-estimation of genetic diversity

observed heterozygosity (Ho): the level of genetic variation observed in a population.

panmictic: the genetic structure of a group of intra-specific populations where all individuals (regardless of population) have the same chance of mating with one another. Panmixia arises due to a lack of physical, behavioural, etc. barriers to reproduction across populations and consequently, panmictic populations show a lack of genetic differentiation from one another.

population: a group of organisms of the same species that exist within a defined geographical area and are capable of interbreeding.

sink population: a population into which larvae/adults immigrate from an external (or source) population of the same species

source population: a population from which larvae/adults emigrate to a sink population of the same species

ANNEX 1: METHODOLOGY - SUPPLEMENTARY MATERIALS

Table A1. Microsatellite markers used in genetic analyses including forward and reverse primer sequences, amplicon size range, repeat motif, annealing temperature and marker developer.

Locus	Forward Primer	Reverse Primer	Size range (bp)	Repeat motif	Annealing temp (°C)	Developed by
MM13	CACAGCCTCCTGGTCACAATA	TGGCGTGTTATTCTAGCAAATG	150-200	GAA	57	Gormley *et al.*, 2015
MM20	AATTGCTCACTTGGCGTAAAAC	TGGAAATGGAGAGACAGATCCT	180-248	TCA	57	Gormley *et al.*, 2015
MM2	CTCCGCTATGTT TGACCATGTA	TCCACACCGAGTAACAAATCAG	116-317	CA	57	Gormley *et al.*, 2015
MM30	CACACAAGACAGGCCAGATAGA	GAAGAATCCCCACAAACACATT	147-183	CA	57	Gormley *et al.*, 2015
MM_pp19	GGTCGTTCCCTTTGACATGAACCC	AAACATCTTTCGCACCCGTTTGCC	384-389	AT	60	Manchester University*
MM_pp15	TGAGGTTAGTGAAAACTAATTGAGCAACCC	CGTTTCAGATTCTCCTTACAATTTGCC	357-369	ATT	60	Manchester University*
MM_pp27	TTTACTGAGTTCACACTGTTTTGCC	GCATCATATGTTACCCGTTCCC	310-326	AT	60	Manchester University*
MM_pp07	TCCAGGTATTTTAGTTCCAGAGATAGGG	GATTATTCATCTTGGAGCCATTGCC	304-308	CGG	60	Manchester University*
MM_pp17	TCTTACAGATTCGGGATTGTGAACCG	TCAACTTCAATCTTTTGGCCTTATCGG	235-260	AC	60	Manchester University*
MM_pp37	CCGTTGTGGATTTGTGAGAATACGC	GCGACTTAGTTCCACGCTTTTATTACGG	227-272	AT	60	Manchester University*
MM_pp24	TTTTCCTTTCTCTCTCCGCATTCGC	TGCTACCAAGGTTGTAACGAGATTCCC	292-309	AT	60	Manchester University*
MM_pp05	ACACCAAATTTAGCCCCTTTAGGC	AAAACCAAATGTTCCACCTAACCC	266-292	TCG	60	Manchester University*

*Preziosi & Rowntree Labs, Faculty of Life Sciences, Manchester University

Table A2. Results of Multiplex Manager for design and optimisation of multiplex PCR reactions including multiplex group (1-3), marker name, and labelling dye (for forward primer only).

Multiplex Group	Marker	Dye*
1	MM13	FAM
1	MM20	VIC
1	MM2	NED
1	MM30	PET
2	MM_pp19	FAM
2	MM_pp15	VIC
2	MM_pp27	NED
2	MM_pp07	PET
3	MM_pp17	FAM
3	MM_pp37	VIC
3	MM_pp24	NED
3	MM_pp05	PET

*dye attached to forward primer

Table A3. Primer mixes prepared for multiplex PCR reactions (1-3) each consisting of four fluorescently labelled forward markers (denoted with _F), four reverse primers (denoted with _R) and made up to 50uL volume with molecular grade water.

MULTIPLEX PCR REACTIONS (1-3) (Markers listed vertically)			**Volume per multiplex**
Multiplex 1	***Multiplex 2***	***Multiplex 3***	
MM13_F	MM_pp19_F	MM_pp17_F	1.0 uL
MM20_F	MM_pp15_F	MM_pp37_F	1.0 uL
MM2_F	MM_pp27_F	MM_pp24_F	1.0 uL
MM30_F	MM_pp07_F	MM_pp05_F	1.0 uL
MM13_R	MM_pp19_R	MM_pp17_R	1.0 uL
MM20_R	MM_pp15_R	MM_pp37_R	1.0 uL
MM2_R	MM_pp27_R	MM_pp24_R	1.0 uL
MM30_R	MM_pp07_R	MM_pp05_R	1.0 uL
Molecular grade water			42 uL
TOTAL REACTION VOLUME			**50 uL**

ANNEX 2: RESULTS - SUPPLEMENTARY MATERIALS

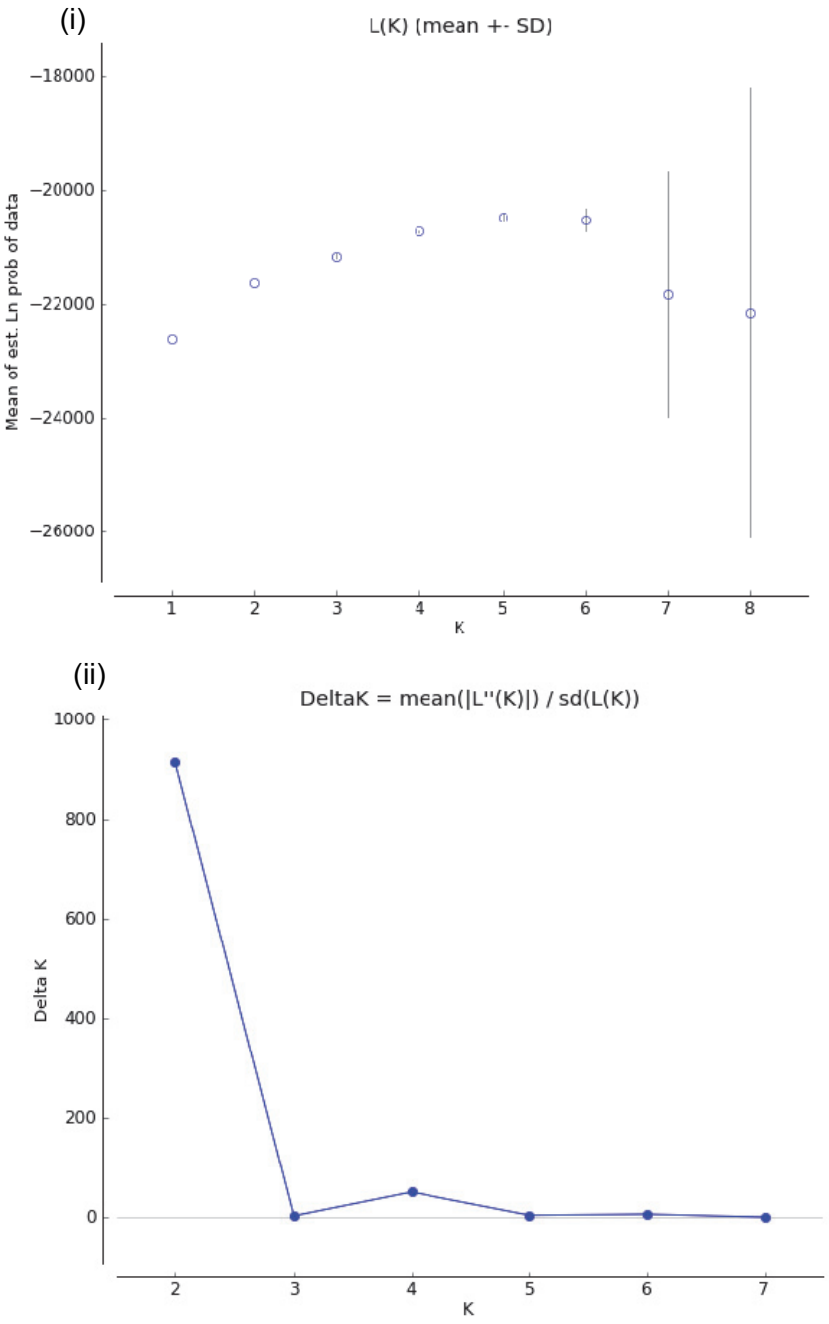

Figure A1. STRUCTURE Harvester (i) L(K) and (ii) DeltaK plots for determining best K value (plots generated by STRUCTURE Harvester).

Table A4. Pairwise estimates of migration (number of immigrants generation^{-1} into receiving population) between horse mussel bed populations within Scottish regions, as determined by Migrate-N software. Directional bias determined as the migration direction which resulted in a greater migration rate, though in most cases the difference is negligible. Population codes as per Table 2.1; for Noss Head (NH): J=juvenile, A=adult

	Number of immigrants generation^{-1} into receiving population			
Receiving Population (sink)	**Migration route (source-sink)**	**2.5% percentile**	**Mean**	**97.5% percentile**
Port Appin	mLC-AP	0.0	13.8	30.0
	mOB-AP	0.0	11.0	27.3
	mKA-AP	0.0	16.7	32.7
	mNHA-AP	0.0	12.8	28.7
	mNHJ-AP	0.0	8.9	25.3
	mDF-AP	0.0	9.1	26.0
	mHS-AP	0.0	9.8	26.0
	mUS-AP	2.0	19.8	37.3
	mCN-AP	7.3	25.3	42.0
Loch Creran	mAP-LC	0.0	11.3	28.0
	mOB-LC	0.0	9.9	26.7
	mKA-LC	0.7	17.1	33.3
	mNHA-LC	0.0	8.1	24.7
	mNHJ-LC	0.0	15.4	30.7
	mDF-LC	0.0	9.0	25.3
	mHS-LC	0.0	16.7	32.7
	mUS-LC	0.0	11.5	28.0
	mCN-LC	0.0	10.4	26.7
Oban	mAP--OB	0.0	12.6	28.7
	mLC-OB	0.0	13.1	29.3
	mKA-OB	0.7	17.7	34.0
	mNHA-OB	0.0	8.2	24.7
	mNHJ-OB	0.0	15.1	30.7
	mDF-OB	0.0	9.8	26.0
	mHS-OB	0.0	13.0	30.0
	mUS-OB	3.3	21.2	38.0
	mCN-OB	1.3	18.5	34.7
Karlsruhe	mAP-KA	0.0	16.4	32.7
	mLC-KA	0.0	15.8	31.3
	mOB-KA	0.0	10.6	26.7
	mNHA-KA	0.0	12.3	28.7
	mNHJ-KA	0.0	17.1	33.3
	mDF-KA	0.0	17.4	34.0
	mHS-KA	5.3	22.9	40.0
	mUS-KA	0.0	17.7	34.7
	mCN-KA	0.0	15.3	31.3
Noss Head Adults	mAP--NHA	0.0	15.6	31.3
	mLC-NHA	0.0	12.1	28.0
	mOB-NHA	0.0	16.6	32.7
	mKA-NHA	0.0	9.5	26.0
	mDF-NHA	2.0	19.7	36.7
	mHS-NHA	0.7	17.2	34.0
	mUS-NHA	3.3	22.1	39.3
	mCN-NHA	0.0	14.7	30.7

	Number of immigrants generation^{-1} into receiving population			
Receiving Population (sink)	**Migration route (source-sink)**	**2.5% percentile**	**Mean**	**97.5% percentile**
Noss Head Juveniles	mAP-NHJ	0.0	12.1	28.0
	mLC-NHJ	0.0	9.4	25.3
	mOB-NHJ	0.0	8.3	24.7
	mKA-NHJ	0.0	14.7	30.0
	mNHA-NHJ	0.0	5.6	22.0
	mDF-NHJ	0.0	12.9	28.7
	mHS-NHJ	0.0	8.9	26.0
	mUS-NHJ	0.0	15.0	30.7
	mCN-NHJ	0.0	14.7	30.7
Dornoch Firth	mAP-DF	0.0	9.1	25.3
	mLC-DF	42.0	54.3	84.7
	mOB-DF	0.0	8.7	25.3
	mKA-DF	0.0	16.5	32.7
	mNHA-DF	0.0	8.1	24.7
	mNHJ-DF	0.7	17.1	33.3
	mHS-DF	0.0	14.3	30.7
	mUS-DF	0.0	16.5	32.7
	mCN-DF	5.3	24.2	42.0
Hascosay Sound	mAP-HS	0.0	12.8	29.3
	mLC-HS	0.0	10.4	27.3
	mOB-HS	0.0	15.1	33.3
	mKA-HS	0.0	12.0	28.7
	mNHA-HS	0.0	12.7	28.7
	mNHJ-HS	0.0	16.5	32.7
	mDF-HS	0.0	18.7	27.3
	mUS-HS	0.0	11.8	29.3
	mCN-HS	0.0	13.6	29.3
Uyea Sound	mAP-US	0.0	14.8	32.7
	mLC-US	0.0	14.6	30.0
	mOB-US	0.0	15.2	30.7
	mKA-US	0.0	16.0	32.0
	mNHA-US	0.0	5.6	22.7
	mNHJ-US	5.3	23.5	40.7
	mDF-US	0.0	7.5	24.7
	mHS-US	4.0	23.5	43.3
	mCN-US	1.3	18.1	34.7
Calback Ness	mAP-CN	0.0	11.9	28.7
	mLC-CN	0.0	12.3	28.7
	mOB-CN	0.0	15.6	31.3
	mKA-CN	2.0	20.0	36.7
	mNHA-CN	0.7	17.6	34.0
	mNHJ-CN	0.0	15.5	31.3
	mDF-CN	0.0	9.5	26.7
	mHS-CN	0.0	11.8	30.0
	mUS-CN	0.0	11.3	27.3

Table A5. Pairwise estimates of migration (number of immigrants generation^{-1} into receiving population) between horse mussel bed populations within Scottish regions, as determined by Migrate-N software. Directional bias determined as the migration direction which resulted in a greater migration rate, though in most cases the difference is negligible. Population codes as per Table 2.1; for Noss Head (NH): J=juvenile, A=adult.

	Number of immigrants generation^{-1} into receiving population				
Region	**Migration direction (source-sink)**	**2.5% percentile**	**Mean**	**97.5% percentile**	**Directional bias**
West Coast	mLC-PA	6.7	13.8	30.0	LC to PA
	mAP-LC	4.0	11.3	28.0	
	mOB-PA	4.0	11.0	27.3	PA to OB
	mPA-OB	5.3	12.6	28.7	
	mLC-OB	6.0	13.1	29.3	LC to OB
	mOB-LC	3.3	9.9	26.7	
North-east and Orkney	mNHA-KA	5.3	12.3	28.7	NHA to K
	mKA-NHA	2.7	9.5	26.0	
	mNHA-NHJ	0.0	5.6	22.0	NHJ to NHA
	mNHJ-NHA	14.7	22.7	40.0	
	mDF-NHA	12.0	19.7	36.7	DF to NHA
	mNHA-DF	2.0	8.1	24.7	
	mKA-NHJ	7.3	14.7	30.0	NHJ to K
	mNHJ-KA	9.3	17.1	33.3	
	mDF-KA	10.0	17.4	34.0	DF to K
	mKA-DF	8.7	16.5	32.7	
	mDF-NHJ	5.3	12.9	28.7	NHJ to DF
	mNHJ-DF	9.3	17.1	33.3	
Shetland Isles	mUS-HS	0.0	11.8	29.3	HS to US
	mHS-US	4.0	23.5	43.3	
	mUS-CN	0.0	11.3	27.3	CN to US
	mCN-US	1.3	18.1	34.7	
	mHS-CN	0.0	11.8	30.0	CN to HS
	mCN-HS	0.0	13.6	29.3	

Table A6. Mean migration rate (μm) (±SD) and sum migration rate (∑m) between regions, where m is the mean number of immigrants generation^{-1} from each population within a given region into the sink region. Directional bias determined as the migration direction which resulted in a greater migration rate.

Source Region	Sink Region	μ*m*	±SD	∑*m*	Directional Bias
West Coast	West Coast	11.94	1.45	241.66	n/a
North-east and Orkney	North-east and Orkney	22.66	4.77	188.08	n/a
Shetland Isles	Shetland Isles	15.01	4.87	90.05	n/a
North-east and Orkney	West Coast	12.33	3.85	147.94	To North-east and Orkney
West Coast	North-east and Orkney	15.76	12.55	189.08	
Shetland Isles	West Coast	16.24	5.37	146.14	To West Coast
West Coast	Shetland Isles	13.63	1.83	122.69	
Shetland Isles	North-east and Orkney	17.19	4.44	189.10	To North-east and Orkney
North-east and Orkney	Shetland Isles	14.58	5.30	174.95	

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