A Nordic network for the stock identification and increased value of Northeast Atlantic herring (HerMix)

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ABBREVIATIONS

MRI	Marine Research Institute, Iceland
IMR	Institute of Marine Research, Norway
FAMRI	Faroe Marine Research Institute, Faroe Islands
SVN	Síldarvinnslan, Iceland
MSS	Marine Scotland Science
ISSH	Icelandic summer spawning herring
NSSH	Norwegian spring spawning herring
FASH	Faroese autumn spawning herring
FSSH	Faroese spring spawning herring
NLSSH	Local fjord autumn and spring spawning herring
NASH	Late summer-early autumn spawning population in northern Norway
NSAH	North Sea autumn spawning herring
PCR	Polymerase chain reaction
NGS	Next generation sequencing
HWE	Hardy-Weinberg expectations
H_e	Expected heterozygosity
H_o	Observed heterozygosity
SNP	Single nucleotide polymorphisms
NIR	Near infrared reflectance
CH	Condition factor
WHC	Water holding capacity
GSI	Gonadosomatic index

1. INTRODUCTION

The basic information for management of a fish stock derives from an analytical assessment of the stock that is carried out by data on catch statistics and often survey indices (ICES 2008a, b). Thus, uncertainty in the recruitment and assessment is influenced by how well the different stocks can be separated in a mixed fishery (Frank and Brickman 2000, 2001). Different methods exist to separate between the stocks of the same species in mixed fisheries. The separation can be based on morphological form, life history traits (e.g. fecundity, egg size, growth, longevity, maturity), population parameters (recruitment, age structure) or spatial and temporal consistency in spawning associated with each population (McQuinn 1997). All of the proceeding parameters are based on the definition that individuals that breed together form a stock that is biologically, spatially or temporally isolated from other stocks, although some degree of interbreeding between stocks can exist even if the stock is by definition genetically discrete from other stocks. Importantly the assessment of these morphological parameters is complex, and the ability to assign fish to their stock of origin based on genetic differences could improve the stock assessments and hence the stock management.

Atlantic herring (*Clupea harengus* L.) is considered a population rich species (Sinclair and Iles 1988) with a number of populations both in the NW and NE Atlantic (Blaxter and Hunter 1982). In addition to the stock complexes in the North Sea, west of the British Isles and Baltic Sea, there are at least three herring stocks within the north eastern Atlantic, which are identified on the basis of spawning time and spawning area, which are managed separately (Figure 1). They are: Icelandic summer spawning herring (ISSH); Norwegian spring spawning herring (NSSH) and Faroese autumn spawning herring (FASH). In addition, there are number of local autumn and spring spawning herring stocks (NLSSH) in Norwegian fjords (Jørstad et al. 1994), and a late summer/early autumn spawning population in northern Norway (NASH) (Aril Slotte, pers. obs.). Although there is temporal and spatial separation during the spawning of these stocks, they are known to aggregate at the same feeding grounds during other periods of the year. This results in a mixed stock fishery on these grounds, for which separation between the stocks can be difficult. A fourth herring stock that is likely to mix with both the NSSH and FASH stocks during the summer feeding phase is the northern part of the autumn spawning North Sea herring stock (NSAH), which spawns west of Shetland (Figure 1).

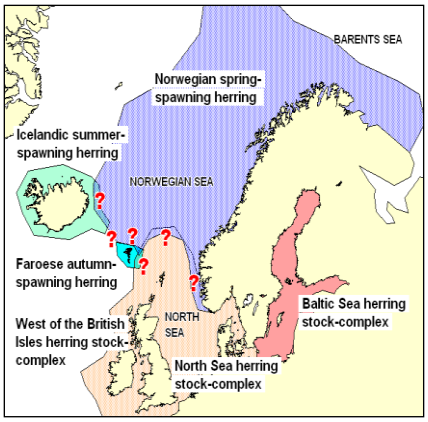


Figure 1. Map of the Northeast Atlantic herring stocks and possible areas of mixture between the different stocks (Toresen and Østvedt 2000). Herring stock-complexes in the southern areas and Baltic are indicated.

The NSSH is the largest of all the herring stocks, but its stock size has showed huge fluctuations in the last 50 years (ICES 2008b). Prior to the collapse of NSSH in the late 1960s, a part of this stock spawned on the banks east of the Faroes, the majority, however spawned along the west coast of Norway (Figure 2a). After the collapse, the stock was confined to the coastal areas along the western coast of Norway. With the appearance of the strong 1983 year class in to the stock, its recovery started (Toresen and Østvedt 2000). Since then, the spawning stock size has been increasing and reached a maximum level in 2010 of around 10 million tons, but has since then decreased due to poor recruitment since 2004 (ICES 2012b). Following the increase in stock size, it reappeared on its previous feeding grounds in the Norwegian Sea and adjacent waters between Norway, Faroe Islands and Iceland (Figure 2b) (Dragesund et al. 1997). The pattern of the feeding migration has therefore changed simultaneously to the recovery of the stock. The locations of the overwintering areas have also taken changes. When the 2002 year class entered the fishable stock, large part of it behaved different from the older year classes, where it overwintered in the open waters off the Troms, northern Norway, as opposed to the wintering areas in the inner fjords of Lofoten after the stock collapsed (Figure 2b)(ICES 2004). This behaviour of the 2002 year class was more in line with the herring life-cycle observed in the late 1960's, when the overwintering area was northwest of Troms in open waters (Figure 2a). All the year classes after the 2002 year class has followed the same migration pattern meaning that almost the whole stock overwinter now in the open ocean (ICES 2012b). Thus, there is anticipation that the herring might be returning to their original migration patterns, i.e. as in 1950-1967 (Figure 2a) in the future.

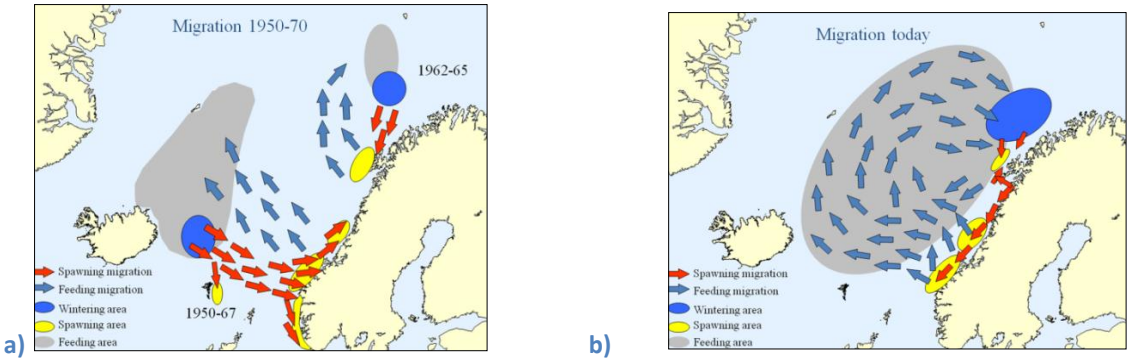


Figure 2. Map of the Northeast Atlantic illustrating the a) former, i.e. prior to 1970, and b) present Norwegian spring spawning herring (NSSH) migration cycles in the Norwegian Sea.

The two Icelandic local herring stocks, ISSH and Icelandic spring spawning herring (ISPH) used to be found in relatively equal portions before they also both collapsed in the late 1960s (Jakobsson 1978). Until then, the herring fishery in Icelandic waters was directed at ISSH, ISPH and NSSH. Except around their spawning season (ISSH in July and ISPH and NSSH in March), the stocks were mixed most of the year (Jakobsson et al. 1969). Accordingly, it was often a mixed fishery in Icelandic waters, where the stocks were separated on the basis of the maturity stage as this reflects the spawning time (ISSH vs. the others) (Jakobsson et al. 1969) and the growth type that was reflected in their scales (ISPH vs. NSSH) (Jakobsson 1973). Separation between the immature herring, or juveniles, of ISSH and ISPH was however, not possible (Jakobsson 1973), while juveniles of NSSH were not found in Icelandic waters.

ISSH recovered relatively quickly after its collapse in the end of the 1960s and the spawning stock size has been more or less continuously increasing since then and reached 800 thousand tons in 2008 (ICES 2012a). Since 2008, the spawning stock has declined to 440 thousand tons in 2012 due to mortality imposed by the protozoa *Ichthyophonus hoferi* (ICES 2012a). Conversely, ISPH has not recovered since the collapse in the late 1960s, and it has been suggested (Jakob Jakobsson pers. comm.) that it will probably not do so until the NSSH resume their pre-collapse migration route and some of the individuals join the ISPH and start to spawn in Icelandic waters. In other words, ISPH and NSSH are considered closely related stocks. As the NSSH seems to be resuming its pre-collapse migration route, there is a strong suggestion that the fishery in Icelandic waters is increasingly likely to return to a mixed stock fishery in coming years, including the recovered ISPH.

NSSH is primarily an oceanic stock, but is also interacts with several coastal and fjord stocks of herring, which are mainly named after the area or fjord in which they are found. Some of these stocks in Norway have been described; the Balsfjord herring (Jørstad and Pedersen 1986), the Østerbø herring (Aasen 1953), the Lusterfjord herring (Aasen 1952), the Borge Poll herring (Rasmussen 1942), the Lake Rossfjord herring (Hognestad 1994), the Lindåspollene herring (Dahl et al. 1973; Johannessen et al. 2009; Lie et al. 1978), the Trondheimsfjord herring (Runnstrøm 1941; Silva et al. 2012) and the Landvik lake herring (Silva et al. 2012). The geographical distribution, migration behavior, migration pattern, meristic and morphological characters are important factors in separation of these stock units. Tronheimfjord herring and Balsfjord herring have been demonstrated to differentiate from NSSH and other populations with allozymes and mtDNA markers (Jørstad et al. 1994; Turan et al. 1998), and another study of the Balsfjord herring has shown differentiation with four microsatellites (Shaw et al. 1999).No genetic studies have been conducted on Lindås herring and Landvik lake herring.

Up to four principal herring stocks have been observed either sequentially or simultaneously within the areas bordering the Faroes. These include: 1) FASH (also called Faroese fjord herring) found in fjords as well as further off-shore on the Faroese plateau and on some of the banks east of the isles, 2) NSSH entering the northern part of the Faroese Exclusive Economic Zone (EEZ) during summer, 3) the (northern) NSAH occasionally entering the banks east of the Faroe Islands, and 4) the local ISSH suspected to have entered the north-western part of the Faroese EEZ on some occasions. Recent findings suggest that herring stocks, other than the local Faroese herring, inhabit the banks east of the Faroes during part of their life cycle. Examinations of samples taken in November 2004 and in May 2005 on “Sandoyarbanka” indicates a mixture of spring and autumn spawning herring suggesting that the NSSH stock might be about to re-enter a former spawning area on the banks east of the Faroes. In 2011, it was possible to obtain a sample of spawning herring in early April in a Faroese fjord (Skálafjørður), thus confirming the recently observed presence of spring spawning herring around the Faroes (FSSH). A further indication of this presence is the samples taken from the herring fishery northeast of the Faroes in the last two years of young (2 and 3 year old) herring of the spring spawning type (ICES 2011). It is believed that these are offspring from recent spawning of spring spawning herring at Faroes.

Further, NSAH herring have also been observed on the banks east of the Faroes in the early 1990s (Corten 2001; Jacobsen 1990, 1991) and recently the northernmost components of herring from the North Sea are migrating relatively far north during late spring, actually mixing with NSSH in the south-eastern part of the Norwegian Sea (Jacobsen et al. 2005). In the Faroe Islands, there is a need

to provide information on the stock structure of the herring on the plateau in order to decide which population units should be assessed and managed individually, i.e. to be able to protect a possible re-establishment of NSSH herring on the banks east of the Faroes, amongst herring of other origin.

Identification of the population of origin of an individual or group of individuals poses several challenges compared to species identification, where individuals can be assigned based on diagnostic genetic differences, e.g. by COI barcoding (Ward 2009; Ward et al. 2005) or by Forensically Identifiable Nucleotides (FINS) (Bartlett and Davidson 1992; Gil 2007; Jérôme et al. 2003). Fixed genetic differences among populations within species are rare, since most populations are, to some extent, connected by migration and gene flow. Instead, different genetic marker variants have varying frequencies among populations, and the identification of the origin of fish relies on probabilistic methods using a combination of genetic markers to provide sufficient statistical support (Hansen et al. 2001; Manel et al. 2005).

In the recent years, genetic markers such as mtDNA (Carr and Crutcher 1998; Carr et al. 1995) and microsatellite loci (Beacham et al. 2002; Carlsson et al. 2006; Hoarau et al. 2002; Pampoulie et al. 2006; Pampoulie et al. 2008) have assumed a more significant role in the discrimination of exploited marine species stocks, both between and within management areas. Microsatellite loci, which are short repeated sequence of nucleotides in the DNA have been proven to be particularly useful for this purpose and are currently one of the most frequently used. Some studies of Atlantic herring population structure, using microsatellite markers have previously been carried out. However, these studies have generally used low numbers of loci and individuals, and the overall conclusion has been that the observed levels of differentiation are low.

When this project started, there were about 40-50 published microsatellites for Atlantic and Pacific herring. Some of these markers have been widely used, where others have been used less (McPherson et al. 2001; Miller et al. 2001; O'Connell et al. 1998; Olsen et al. 2002). Very recently, 61 new microsatellite markers were published for Atlantic herring (Teacher et al. 2012). However, well-developed multiplex systems with 20-25 markers are not available. Studies using microsatellite DNA and allozyme markers have found minor differentiation among spatially discrete populations of Atlantic herring. Previous publications focus on the genetic differences among Atlantic herring in the North Sea (Mariani et al. 2005), the Baltic Sea (Jørgensen et al. 2005), and on the comparison of Atlantic herring in the North and Baltic Seas (Andre et al. 2011; Bekkevold et al. 2005; Gaggiotti et al. 2009) and adjacent waters (Ruzzante et al. 2006). These studies have found significant differentiation between populations in the North and the Baltic Seas (Bekkevold et al. 2005) as well as correlated genetic and life-history patterns across these regions (Ruzzante et al. 2006). In addition, isolation by distance has been observed among populations in the North Sea, determined predominantly by the divergence of the English Channel herring and NSSH (Mariani et al. 2005). Only a small number of ISSH have been analysed with microsatellite markers. One study using four microsatellite loci, showed a low but significant genetic difference between ISSH, NSSH and NLSSH (Shaw et al. 1999). Another study using nine highly polymorphic tetra nucleotide microsatellites, showed a significant difference between north-eastern and north-western Atlantic herring and among north-western Atlantic spawning groups (McPherson et al. 2004). However the number of ISSH samples included in these studies has been low.

The primary objective of the present study was to develop a genetic approach using 20-25 microsatellite loci to reliably discriminate herring stocks in the north east Atlantic. Other objectives were to apply biological and otolith microstructure analyses for the stock identification as the basis for a larger study on stock structure of the Northeast Atlantic herring and management of mixed-stock fisheries.

To separate between the ISSH and NASH stocks using the maturity stage alone can be problematic in the early summer if they are aggregated. In the same way, separation between ISSH and NSSH can be problematic in the late summer, especially when the research personnel are working with catch samples that have been frozen on board the fishing vessels at sea, which is a common sampling method. There is currently an urgent need within the fishing sector and among fish processors to analyse the origin and quality of fish. Information on physicochemical properties (processing properties) of herring, post mortem influence, water content and fat content/fat composition, are of importance for the production of increased value for human consumption (Rodríguez et al. 2005). Pelagic fish are more exposed to both spoilage and damage than most whitefish species (Doyle 1995; Whittle et al. 1990). Rapid spoilage of this group is exacerbated especially by oxidative rancidity which occurs due to high lipid content (Anon 1991). Damage can readily occur throughout the supply chain particularly as this group is generally processed as a bulk resource and the associated problems of crush damage and poor chill chain management from handling and storing of large volumes of fish can have a negative impact if good management practices are not implemented.

This pilot study aimed to apply a multidisciplinary approach (involving various fields of science, e.g. basic biology, morphometric, genetic, physicochemical properties) to study the stock structure of herring in the Northeast Atlantic and their conservation of biodiversity, sustainable fisheries management, product quality for increased human safety and consumption.

2. MATERIALS & METHODS

2.1 Sampling and biological data collection

Sampling was done in Iceland, Faroe Islands, Norway and Scotland in the years 2008, 2009, 2010, 2011 and 2012. Samples were collected from the following stocks: ISSH, NSSH, NSAH, NASH, FASH, FSSH and NLSSH from different fjords in Norway. Not all stocks were sampled in all years. Sampling on spawning grounds (baseline samples) was carried out as a part of institutional surveys of the Marine Research Institute in Iceland (MRI), the Institute of Marine Research in Norway (IMR) and the Marine Scotland Science (MSS). Sampling in the Faroese waters was performed by the Faroe Marine Research Institute in the Faroe Islands (FAMRI) by the help of the fisheries. Sampling for the physicochemical characteristics analyses were done by Síldarvinnslan (SVN) on ISSH and NSSH in the years 2010 and 2011 but whole fish was frozen individually in plastic bags on board. Then the fish was sent to MRI and defrosted for biological factor measurements and sampling of genetic samples).

All fish was characterized according to location, time of catch, weight, length, sex, gonad weight, age and reproductive status. Genetic samples of mussel tissues, gills or fins were sampled in 2 ml tubes containing 96% ethanol. Otoliths were taken for part of the samples. For the Faroese samples, the otolith type (nucleus) was also recorded, i.e. whether it was a presumed autumn spawning type (hyaline) or spring spawning type (opaque). If the date of sampling combined with the maturation stage was not sufficient to uniquely identify the samples as being from a “baseline” stock, the “otolith type” method (described in more detail in the results section 3.2), was used to separate spawning types of herring.

2.2 Biological analysis of samples

This work package has been changed in some detail from the original plan. Initially, it was planned to analyse morphometric and meristic characters of the herring samples, after the genetic analysis had allocated the various samples to putative stocks. Thus, it was planned to use morphometrics as an additional variable to strengthen the power of the genetic discriminations. However, as shown in the results chapter (section 3), the present genetic methods were not able to distinguish between the herring stocks in the oceanic parts of the Northeast Atlantic, therefore no statistical exploration of this part of the WP could be carried out. However, full otolith microstructure analyses were initiated during the project. The methodology of otolith microstructure analyses and their results will be published later in the PhD thesis of Lisa Anne Libungan (University of Iceland).

Age reading of all samples was carried out on either scales (MRI and IMR) or otoliths (FAMRI and MSS) by counting yearly growth rings (Anon 1962). The infection status of *Ichthyophonus hoferi* was evaluated for some of the ISSH samples. Sex and phenotypic traits relevant to the study were quantitatively measured and recorded in all individual fish. These include total length, whole body weight, gonad weight, and maturity stage (Bowers and Holliday 1961). For some of the samples information about stomach content and mesenteric fat was also recorded.

2.3 Microsatellite testing and multiplex development

2.3.1 Testing of published microsatellite markers

An extensive search for published microsatellite markers for Pacific and Atlantic herring was done at the beginning of the project (2008 - 9). Published information was used to support the selection of markers to test on samples from ISSSH and NSSH. DNA was extracted from either muscle tissue or gills by AGOWA mag Midi DNA Isolation Kit (AGOWA Gmbh). All selected markers were tested by using variable annealing temperatures and primer concentrations. The forward primers of each microsatellite pair was labelled with one fluorescent dye (6-FAM, VIC, NED or PET). Polymerase chain reactions (PCR) were performed in a 10 µl volume containing 2-3 µl DNA (10-100 ng/µl), 0.80 µl of dNTP (10mM), 0.6-1.2 U Teg polymerase (Matís Ltd., Taq comparable, (Olafsson et al. 2010)), 1 µl of 10x buffer (Matís Ltd.), 0.03-0.25 µl of a 50:50 ratio of labelled forward (100 µM) and reverse (100 µM) primer tagged on the 5´-end with a GTTTCTT PIG-tail (Brownstein et al. 1996) adding 1 µl betaine (5 M) when improvements of DNA amplification were needed. Samples were analysed on an ABI PRISM 3730 sequencer using the GeneScan-500 LIZ size standard and genotyping scorings were done by using GeneMapper v4.0 and v4.1 (Applied Biosystems). Markers that were polymorphic and gave clear, readable peaks with high signals were used for further testing and multiplex developments. Some of the published markers were redesigned for the project by using the software CloneMarg Suite 7, Primer3 (http://frodo.wi.mit.edu/primer3/) and GenBank sequence information.

2.3.2 Development of new microsatellite markers

New microsatellite markers were developed at Matís by next generation sequencing of a single herring. The methodology of the developmental work has been published elsewhere in detail (Libungan et al. 2012) (see Appendix 1).

2.3.3 Multiplex development of published and Matís developed microsatellite markers

The microsatellite markers (both those previously published and Matís developed) that gave the best test results were mixed in multiplexes according to their annealing temperature and allelic range. Multiplex developments were mainly done by varying primer concentrations and by testing different markers together. PCR reactions, genotyping runs and scoring were performed as described above (section 2.3.1).

2.4 Genotyping with neutral and non-neutral microsatellite markers

Baseline and physicochemical samples (ISSH, NSSH, NSAH, NASH, FASH, FSSH and NLSSH herring stocks) were genotyped with markers included in the multiplex systems developed in section 2.3.3. DNA was extracted from either muscle, fin or gill tissue by AGOWA mag Midi DNA Isolation Kit (AGOWA Gmbh) or HotShot DNA extraction method (Montero-Pau et al. 2008). PCR reactions, genotyping runs and scoring were performed as described above (section 2.3.1).

2.5 Statistical analysis

2.5.1 Statistical analyses of microsatellite markers

Initial analyses for the genetic data were performed to check for scoring errors and statistical power of each locus. Observed (H_o) and expected heterozygosity (H_e) were estimated in GENEPOP'007 (Rousset 2008) as well as genetic linkage disequilibrium statistics and Hardy-Weinberg expectations (HWE) for all loci. MICRO-CHECKER (van Oosterhout et al. 2004) was used to check for null alleles. The F_{ST} outlier methods implemented in LOSITAN was used to determine if markers were under selection (Antao et al. 2008; Beaumont and Nichols 1996); e.g. to detect positive and balancing selection on a specific locus.

2.5.2 Population structure analysis of herring in the North Atlantic Ocean

Genetic differentiation was estimated for the geographically separated regions using theta estimates (θ) (Weir and Cockerham 1984) implemented in GENEPOP'007 (Rousset 2008), and significance was assessed using allelic and genotypic frequency homogeneity tests (5000 permutations). The significance levels were adjusted by a simple Bonferroni correction when multiple tests were applied (Rice 1989)

STRUCTURE 2.3.2 (Pritchard et al. 2000) was used to assess the potential number of populations within our samples without a priori designation of the sampling location or any assumption about population structure. The admixture model was used with a "burn-in" period of 300 000 iterations and 600 000 MCMC iterations. The potential number of populations (K) varied from 1 to 13, and was tested with 5 independent analyses for each K.

To infer the spatial distribution of subpopulations (K) in the samples sets, the Bayesian approach implemented in GENELAND version 0.3 (Guillot et al. 2005) was used, which incorporates geographical coordinates in the model development to detect genetic discontinuities among populations with possible uncertainty in spatial coordinates using a Markov chain Monte Carlo algorithm (Guillot et al. 2005). The potential number of subpopulations contained in the samples was first estimated for a range of values of K (1 - 8; equivalent to the number of geographical regions), using 100 000 MCMC iterations and 200 thinning. Allele frequencies were subsequently drawn from independent Dirichlet distributions (Pritchard et al. 2000) which have been shown to perform better than the alternative model (F-model; Guillot et al. 2005). Five runs with fixed K (K detected) were finally performed for the spatially explicit model (uncertainty 50 km). For each of the 5 runs with fixed K, the posterior probability of subpopulation membership was computed for each pixel of the spatial domain (150 x 150 pixels), using a burn-in of 200 iterations.

Finally, the statistical power of the microsatellite loci was estimated using the program POWSIM (Ryman and Palm 2006), which assesses the α (type I) error (the probability of rejecting *H*o when it is true) and the power of the genetic design performed using information on sample sizes, number of samples, number of loci, and allele frequencies for any hypothetical degree of true differentiation quantified as F_{ST} (Ryman and Palm 2006). The significance of the tests is assessed by Fisher's exact tests as well as by χ^2 tests. The objective of this analysis is to assess the sampling scheme and the microsatellite loci used to determine what level of differentiation could be detected in the data.

2.6 Physicochemical (processing) properties

The goal of this part of the project was to measure the differences in physicochemical characteristics between herring from the NSSH and ISSH stocks, fishing seasons and catching area.

2.6.1 Sampling - Trial 1 and Trial 2

Individuals of ISSH and NSSH stocks for the physicochemical analyses were sampled at different seasons and catching areas in Icelandic waters. The measurements were done in two trials (Trial 1 and Trial 2). All the herring were frozen individually in plastic bags at MRI and kept frozen for six months until measured. Before physicochemical measurements, the herring was thawed at 10 °C over night.

2.6.2 Physicochemical measurements - Trial 1 and Trial 2

Physicochemical analyses of water content, water holding capacity (WHC) and fat content were used as reference for determination of these parameters with Near Infrared Reflectance (NIR) spectroscopy. Analyses were performed at the Matís laboratory 6 months after sampling. The water content was measured by drying 5 g of minced muscle mixed with sand in a ceramic bowl for 4 h at 103 ± 2 °C. The water content was based on the weight differences before and after drying of three replicates for each sample (ISO standard 6496, 1999). The fat content was determined by using Soxtec method with minor changes according to application notes Tecator no AN 301 (Patricia Cunniff 1999). WHC was determined with the centrifugal method described by Eide and others (Eide et al. 1982). Approximately 2 g of the samples were weighed precisely into vials and centrifuged (Sorvall RC-5B, Dupoint Company, USA) at 210 *g* rotation and temperatures in the range of 2 to 5°C for 5 min. The WHC (%) was calculated as the ratio of water in the sample after centrifugation to water in the sample before centrifugation.

Gaping was evaluated after filleting. Classification was carried out according to the scale presented in Table 1 (Andersen et al. 1994).

Table 1. Description of the gaping scores 0-5, according to Andersen *et al*. 1994.

Score	Description
0	No gaping
1	Few small[1)] slits (less than 5)
2	Some small slits (less than 10)
3	Many slits (more than 10 small or a few large[2)])
4	Severe gaping (many large slits)
5	Extreme gaping (the fillet falls apart)

[1)] < 2 cm
[2)] > 2 cm

Condition factor (CF) (Fulton 1903) was calculated from the length and weight of each herring as followed: ***CF = weight of ungutted fish (g)/length(cm)^3 *100***

The gonadosomatic index (GSI) was calculated as followed: ***GSI = weight of gonads (g)/weight of ungutted fish (g)*100***

The GSI describes the maturity of the herring. It is a tool for measuring the sexual maturity of the fish in correlation to gonad development. The GSI is particularly helpful in identifying days and seasons of

spawning, as the ovaries of gravid females swiftly increase in size just prior to spawning. These measurements were performed for Trial 1 and Trial 2.

2.6.3 NIR measurements - Trial 1 and Trial 2

Water content, fat content and WHC results were determined by NIR based on calibration with methods mentioned in section 2.6.2. NIR reflectance measurements were performed over the wavelength range from 800 to 2500 nm using a Bruker Multi Purpose Analyzer (MPA) system with a filter probe (Bruker Optics, Rheinstetten, Germany). Measurements were done in the middle part on whole fillets. Five scans were used to build each absorption spectrum and all samples were measured in triplicate. These measurements were performed for Trial 1 and Trial 2.

2.6.4 Colour and pH measurements - Trial 2

The intensity of the flesh colour was measured by using a Minolta type CR-300 colorimeter (Konica Minolta; Tokyo, Japan), using the D_{65} light source. The instrument recorded the *L* (lightness-intensity of white colour), *a* (redness-intensity of red colour), and *b* (yellowness-intensity of yellow colour) values.

The pH of the herring muscle was measured by inserting a combined electrode (SE 104 – Mettler Toledo, Knick, Berlin, Germany), connected to a portable pH meter (Portames 913, Knick, Berlin, Germany) into the intact muscle sample. The intensity of the flesh colour and the pH of the herring muscle were measured in each fillet at the frontal part, at the middle and at the tail part. These measurements were only performed in Trial 2.

2.6.5 Data handling and analysis - Trial 1 and Trial 2

Statistical analysis and plotting of figures was performed in Microsoft Excel 2007 (Microsoft Corporation, U.S.). Data sets were compared by multiple comparisons ANOVA, using all pair wise comparisons by Sigmastat 3.5 (Jandel Scientific Software, Ontario, Canada). Significance levels were set at $p<0.05$).

NIR calibrations for prediction of water content, fat content and WHC were made in the Bruker Opus software. The whole NIR spectral range (800 to 2500 nm) was used for calibrations without any further data processing and compared to measurements of water content, fat content and WHC as measured by the drying-, Soxtec- and centrifugal methods, respectively, as described in the physicochemical measurements methods (section 2.6.2).

Multivariate analysis on weighted principal components (PCA) was performed on all data using Unscrambler® (version 10.0.1, CAMO ASA, Trondheim, Norway) to identify similarities and differences between samples. The main objective was to detect structure in the relationship between measured parameters and experimental factors. It has been used to transform a number of possibly correlated variables in to a (smaller) number of uncorrelated variables (principal components). The 1^{st} component explains as much of the variability in the data as possible, then the 2^{nd} component will account for as much of the remaining variability as possible, and so forth. All variables were weighted with the inverse of the standard deviation to correct for the different scales of the variables and the data were centred prior to analysis. All models were fully cross validated and all significant levels were set to $p<0.05$.

2.7 Multidisciplinary analyses

All data obtained in the project was gathered into a project database, including all sampling information, genetic (microsatellite genotypes), biological, functional and chemical properties data. A combined analysis of genetic, biological and physicochemical characters of the herring samples was planned based on the traditional statistical and above genetic statistical analysis methods. While these analyses were carried out for the physicochemical and biological properties (see sections 3.6), it was not possible to also include the genetic data in this analysis, as shown in sections 3.5.2 and 3.5.3, that there is insufficient differentiation between the herring stocks with these markers to distinguish between the different stocks in the North East Atlantic. For this reason it was not possible to combine all the datasets produced in this project for the allocation of fish in mixed fisheries to their spawning components.

3. RESULTS

3.1 Sampling and biological data collection

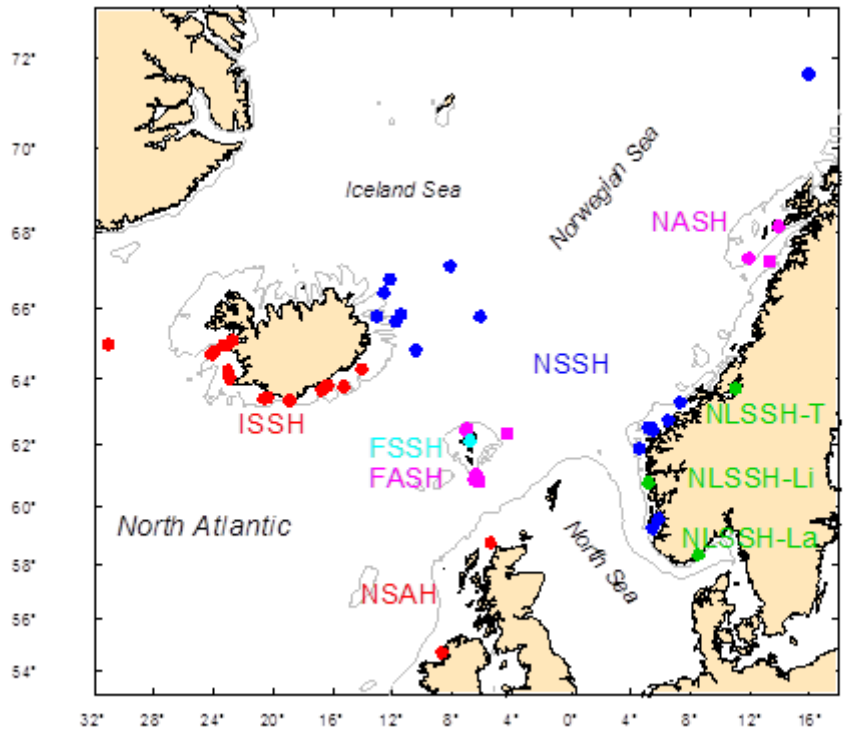
Somatic tissues and whole fish samples from 4593 individuals and 59 sampling sites were collected by MRI, IMR, FAMRI, MSS and SVN in the years 2008 to 2012 (Figure 3a). Of these samples, 1130 baseline individuals from 21 samples and 369 physicochemical samples from six sampling sites were genotyped with neutral and non-neutral microsatellite markers (Table 2 and Figure 3b).

Table 2. Sample number and sampling information for all collected samples (baseline and physicochemical) in the HERMIX project.

Acronym	Spawning time	Sampling site	Year	Spawning	Feeding/ overwintering	Otoliths	Geno-typed	Physicochemical analyses*	Total
FASH	Autumn	The Faroe plateau	2009	0	214	214	116	0	214
-	-	-	2011	0	289	289	0	0	289
FSSH	Spring	Skálafjørður FO	2009	0	54	54	3	0	54
-	-	-	2011	258	0	258	40	0	258
ISSH	Summer	IS coastal waters	2009	631	150	627	132	0	781
-	-	-	2010	400	0	400	163	0	400
-	-	-	2011	100	131	50	0	131	231
NASH	Autumn	Lofoten NO	2010	233	0	233	88	0	233
NLSSH-La	Spring	Landvikvannet NO	2010	200	0	200	149	0	200
NLSSH-Li	Spring	Lindås NO	2010	383	0	383	64	0	383
NLSSH-T	Spring	Trondheimsfjorden	2010	122	0	122	111	0	122
NSAH	Autumn	North of Scotland	2010	300	0	300	105	0	300
NSSH	Spring	Spawning and	2008	0	76	0	0	0	76
-	-	feeding grounds in	2009	0	100	0	0	0	100
-	-	the Nordic Seas	2010	199	381	371	72	144	580
-	-	-	2011	0	100	99	0	94	100
-	-	-	2012	272	0	272	87	0	272
Grand Total				**3098**	1446	3872	1130	**369**	**4593**

* All physicochemical samples were genotyped but genotyping results were obtained for 232 samples.

a)



b)

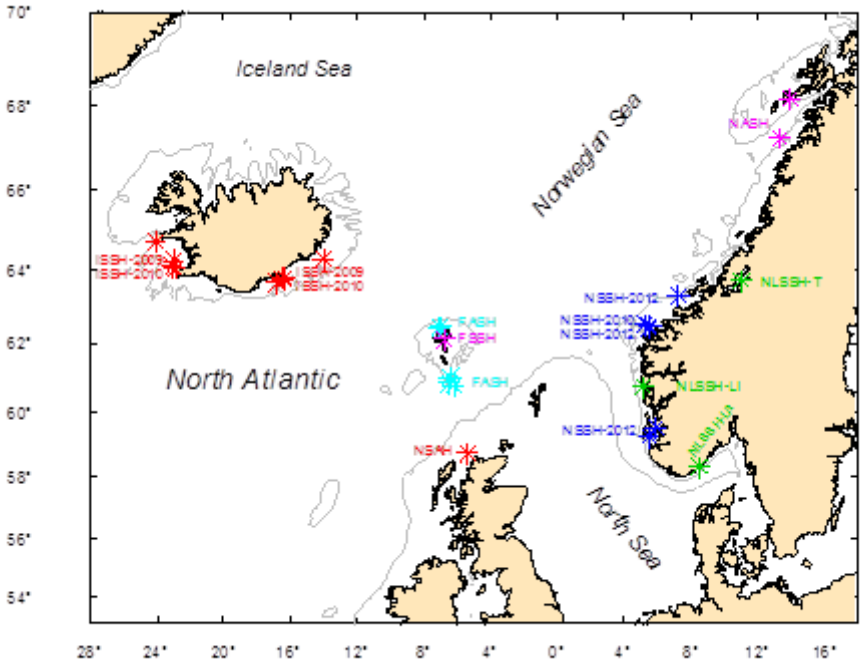


Figure 3. Sampling locations of *C. harengus* in the Northeast Atlantic Ocean (a) and the location of samples taken as baseline samples in the project (b; each sample identified). Abbreviations: Icelandic summer-spawning herring (ISSH); Norwegian spring spawning herring (NSSH); Faroese autumn spawning herring (FASH); Faroese spring spawning herring (FSSH); Norwegian local fjord spring spawning herring in Trondheimfjorden (NLSSH-T), in Landvikvannet (NLSSH-La) and in Lindås (NLSSH-Li); Norwegian autumn spawning herring (NASH); and North Sea autumn spawning herring (NSAH).

3.2 Biological analysis of samples

The biological characteristic of the 1130 baseline herring individuals genotyped in the project was verified and compared among the 12 groups analysed below. The proportion of females in the groups varied from 40 to 59% but was overall near 50% (Figure 4).

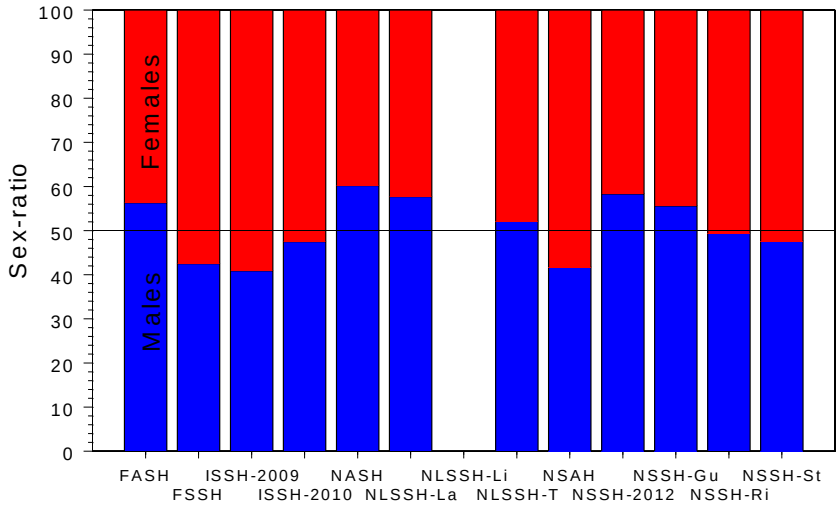


Figure 4. Sex ratio of the different herring groups genotyped in the project (the sex of NLSSH-Li was not available).

The median length, weight, age, and GSI varied also within and between groups (Figure 5). The four samples of NSSH had all similar length and age distributions as did the two samples of ISSH, thus they were combined for each stock before analysing the mean length-at-age. FASH had the highest mean length-at-age, the Trondheim stock (NLSSH-T) had the lowest, while the Landvikvannet (NLSSH-La) and the North Sea (NSAH) stocks were also low (Figure 6). The other four stocks had all similar mean length-at-age (Figure 6). The significantly higher growth rates of the Faroese autumn spawning herring (filled diamonds in Figure 6) could be explained by continuous growth during winter on the Faroe Plateau due to relatively warm waters on the shelf as compared to most of the other stocks that inhabit in cold waters during winter. In the lower end, the local Norwegian herring stocks (NLSSH-T and NLSSH-La) and the NSAH, the low growth might be due to low ambient temperatures and short feeding season for some of the stocks. A further interesting feature is the observation that the presumed FSSH that might be about to re-established at the Faroes has the same growth characteristics as the NSSH, but their presumed phylogenetic relationships are unknown.

All these analysed samples were considered to represent baseline samples, meaning that they were fully mature spawners (maturity stage 5), spawning (stage 6) or just spawned (stage 7) collected on or near the spawning grounds. These conditions were fulfilled for all the stocks (Figure 5d), except for FASH, which spawning grounds and spawning period have not been defined in detail and it is therefore hard to get appropriate samples. The maturity stage and GSI of the FASH reflect that they were sampled during the resting period (stage 8) and consequently with low gonad weights (Figure 5d and e). For the FASH, the maturity stage together with the otolith type (hyaline for the FASH) was considered to be a sufficient discriminating factor to group the sample as being a native baseline sample of the FASH, although it was not obtained from spawning fish. The significantly higher growth rates of the Faroese autumn spawning herring reported below (Figure 6), presumably due to

extended winter growth on the Faroe Plateau, is also the plausible explanation to the observed difference in the growth signatures in the otoliths between the FASH and the NSSH described below.

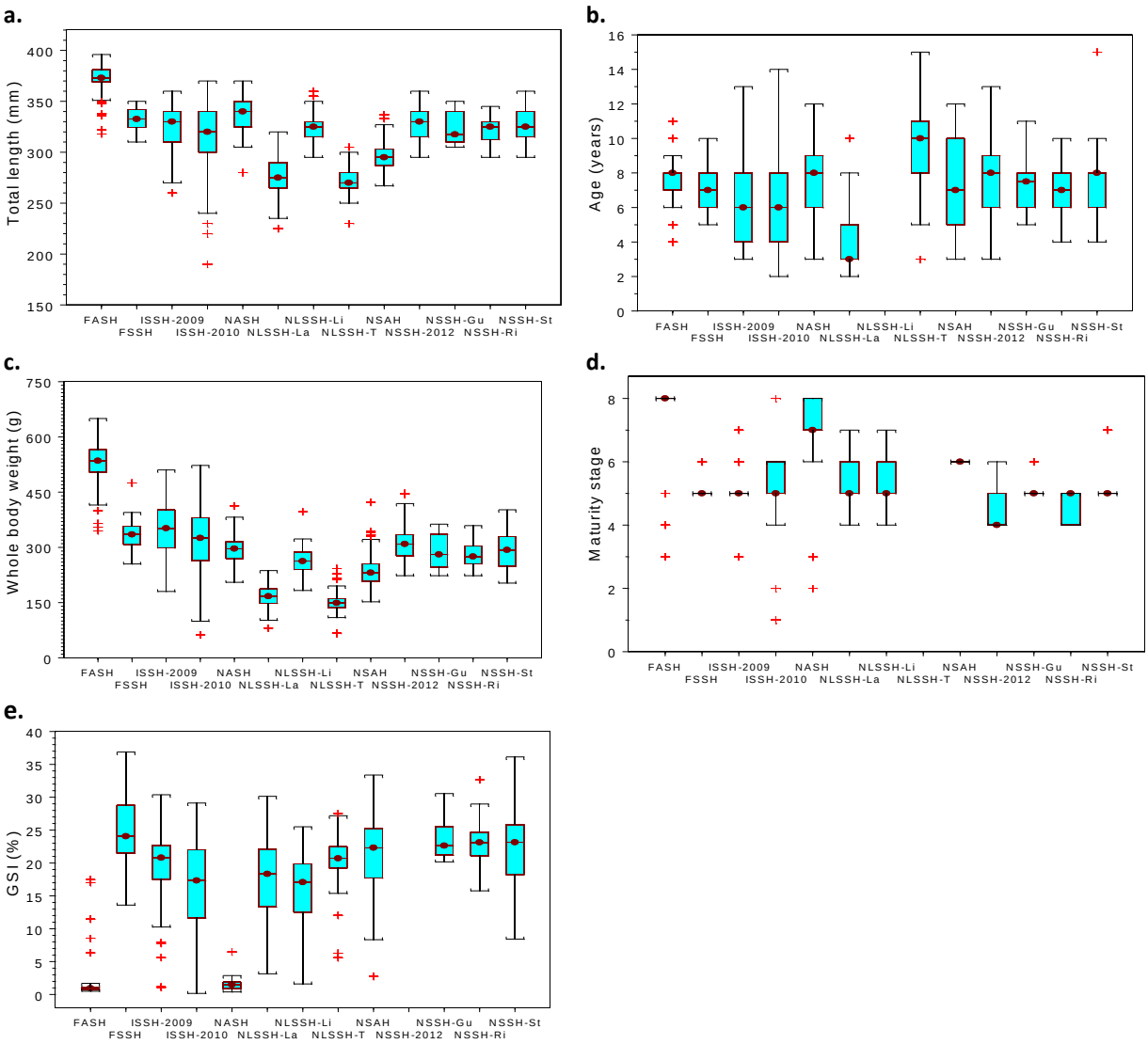


Figure 5. Box and whisker (median, +; 1st and 3rd quartiles, bar; non-outlier min. and max., the whisker; extreme outliers, +, i.e. beyond 1.5·inter-quartile range) representation of (a) total length, (b) age, (c) whole body weight, (d) maturity stage, and (e) gonad somatic index (GSI) for the different groups genotyped in the project.

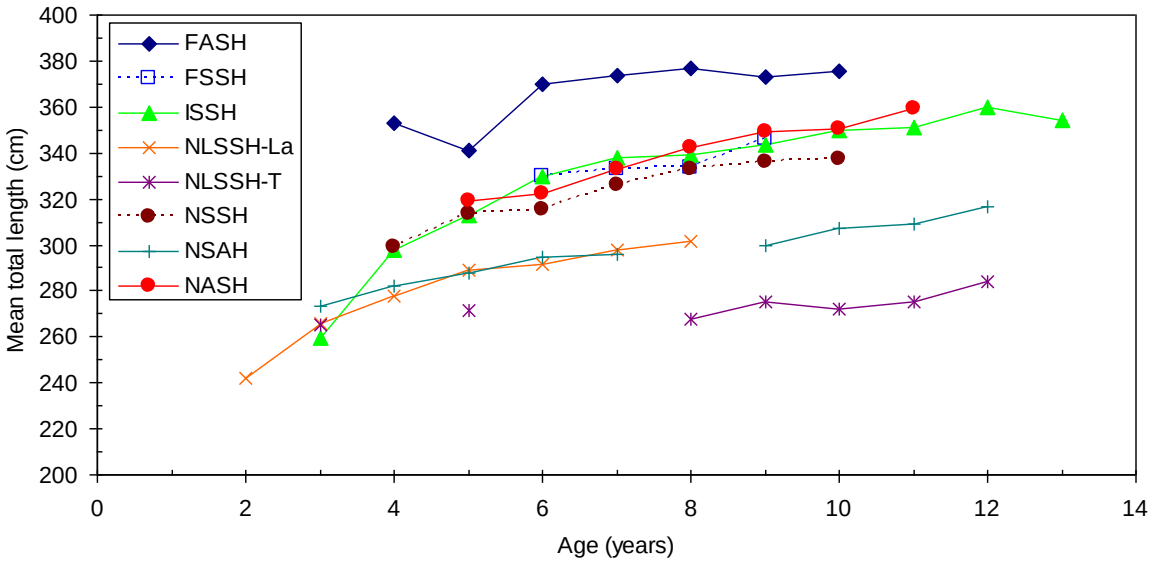


Figure 6. Mean length-at-age for the eight herring stocks, based on the samples taken for the genotyping (age of NLSSH-Li was not available).

Results of otolith microstructure analyses will be published as a part of the PhD work of Lisa Anne Libungan at the University of Iceland (to be published later, with due reference to the HerMix project). Otoliths are calcified structures found in all teleost fishes. There are three pairs of otoliths (Sagitta, Lapillus and Asteriscus) located in chambers in the inner ear beside and behind the brain. They play a role in hearing and sense of equilibrium. The major element (96%) of otoliths is calcium carbonate ($CaCO_3$). Otoliths acquire yearly growth rings, or annuli, much like trees do (concentric rings around year 1 at the centre).

The otolith (nucleus) type was noted for all otoliths for the Faroese samples. This is routinely done in all herring samples analysed at FAMRI. The method is done visually based on the nucleus type; hyaline (H type) or opaque (O type) and of the width of the first growth ring (year) in the otolith, measured as the diameter (D_1) of or the radius (L_1) from centre to the first annual ring (Figure 7a and 7b). The herring spawn during autumn and is probably able to feed to some extent during the first winter as indicated by its wider growth ring in the first year (Fig 7b) compared to the lesser first year growth rings in the NSSH, which inhabit colder environments in the Barents Sea or northern Norwegian waters (Figure 7a). Alternatively, the difference in the first growth ring might be due to the difference in the length of the first feeding season, which is significantly shorter for the NSSH larvae than in the south. Experienced scientists are able to distinguish the otolith type by visual inspection only with high probability of classifying the otolith as the correct type (designated O or H types at FAMRI).

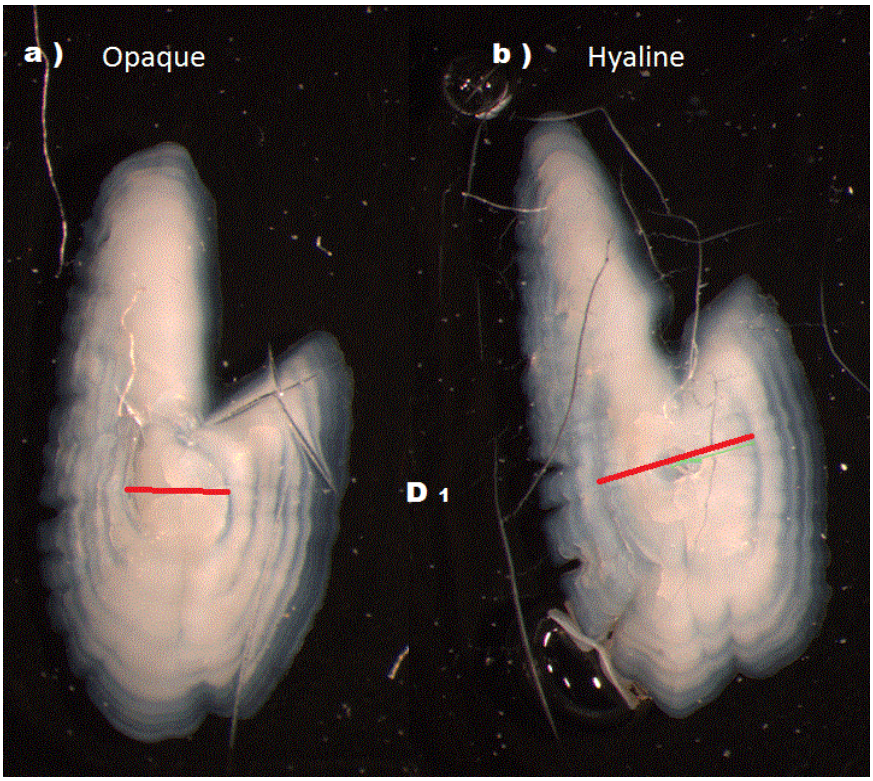


Figure 7. Examples of herring otolith (nucleus) types: a) opaque type and b) hyaline type. The type is determined by the nucleus and by the width of the first winter ring (D1) show as red lines (see text for explanation).

3.3 Microsatellite testing and multiplex development

3.3.1 Testing of published microsatellite markers

An extensive search for published microsatellite markers for Atlantic and Pacific herring gave a list of 47 markers. Some of these markers were not used in this study because of various reasons, like poor PCR amplifications, stutter problems and artefacts peaks. Information about repeat motif, primer sequences, publication and GenBank accession numbers of 34 markers used in this study can be found in Table 3. In addition, Table 3 shows the final multiplex systems that were designed for these loci. Not all of the markers produced clear, readable PCR products, , and therefore the primers were redesigned by using the software CloneMgr Suite 7, Primer3 (http://frodo.wi.mit.edu/primer3/) and GenBank sequence information. The new redesigned primers sequences for eleven microsatellite markers are listed in Table 4, however none of these markers were used in the final multiplex systems.

Table 3. Information on 34 published microsatellite markers for Atlantic and Pacific herring that were tested in this project.

Marker*	Repeat	Forward Primer	Reverse primer	References	GenBank**	Multiplex
Cha1005	GACA	TGCAAGATAGAGTCACAG	GGGGACAGAACCAACTTCAC	1	AF304359	
Cha1014	GATA	TCCTAAACCAACCCCTGTGA	ATTATTGTGTTAAATTTGACAGACC	1	AF304360	
Cha1017	GATA	GGTCTCATTATCTTCTCACTCTTTTG	TCTCCCTATGTGTATTGTTTTACTGTG	1	AF289096	4
Cha1020	GACA	CCTGGAGAGACAGATAGAAAA	GAGTTTAGCAGACGCTTTA	1	AF289095	2
Cha1027	GACA	ATTCAACCCCCTACAAGC	TGAGGCAGCAGACGATACAC	1	AF290885	6
Cha1045*	GATA	CATTAGGGATGGCTCTGC	CCAGAAAAGAAGTCCCAGATG	1	AF304361	
Cha1059	GACA	CATCTACCACCTCCGACTCC	AATCTAAAGGAAGCCCACTC	1	AF289094	2
Cha1202	GACA	TTTCCGTTACACTTTCACATCG	GTGCCTCAGTTTTCACATACA	1	AF304363	7
Cha104	TG	ACGTAGGCGCAGACAT	GTTTGCTCAAGTCAATGTGATTTTTA	2	AF309791**	
Cha107	TC	GCATTACACAGAGAGGAAT	GTTTAGATACGCCTCTCTCTTT	2	AF309792**	
Cha108	CA	CTTGACATACAGTATGTTCAAAT	GTTTCTGTGAGCTGTACACCA	2	AF318286**	
Cha113_Miller	CT	CAGTCAGAAAGAAGGAGA	GTTTCCTCCTCGTGCTCTTT	2	AF309793**	
Cha120*	CA	ACACGCTTGCCTTGAGAT	GTTTGATCTGATTATCTTGAAAATTTG	2	AF309795**	
Cha27*	GACA	CACATTTATCAATTTCTTTG	GTTTCAGAAAGAGAATCTAACCTCT	2	AF309799**	
Cha4	GACA	CTTATCTGTCTGACTGCCTATTTG	GTTTCTTCTCTGCTCCACCCAGAA	2	AF309800**	9
Cha6	GATA	GTGTGAGTTTGCTCCAAA	GTTTGTACCAATGAATGATTACAA	2	AF309801**	
Cha8*	GACA	GATCCTTCTTTTAAGGAAAA	GTTTGACAGAACTTACTATCTCAGA	2	AF309804**	
Cha113	GT	CTCTTCCATTTCGCTACGG	GCAGTCTGACTTTACAATGA	3	AF019994	2
Cha123*	GT	GGGACGACCAGGAGTG	AAATATAGTTTTATGATTGGCT	3	AF019992	
Cha17	GT	GAGACTTACTCTCATCGTCC	GCACAGTAGATTGGTTCCAC	3	AF019993	2
Cha20	GT	GTGCTAATAGCGGCTGCTG	TTGTGGCTTTGCTAAGTGAG	3	AF019989	7
Cha34*	GT	CCTATGCTATCCTAACGATGG	GCTATGACCATGATTACGG	3	AF019991	
Cha63	GT	TGCCTGCTGAAGACTTCC	CCCCTAAATGTGTTCTTTTAGC	3	AF019987	6
Cpa101	ATCT	CATTGCCACCTACTGACCTG	CACCCTGAAGATGATGAGGA	4	AF406937	6
Cpa102	ATCT	TTGCACCCAGTCAGCTAAAC	GCGGCAAAGTCATAACCTG	4	AF406938	9
Cpa103	TAGA	GACTCACAGGTTCTCCTCAACA	TGGAGGGATTGGAACATTT	4	AF406939	4
Cpa104	ATCT	TGATTGGGTCCTTTTGAACAT	GCAATGACTGACACAGCAAA	4	AF406940	7
Cpa107	ATCT	ATGATTTTTCGCCTTTTGCT	CCCAGAAACAAGAGCTAGGC	4	AF406943	
Cpa108	TAGA	TTGTGTATGTATGTCGGTGAGG	CAGTATGTAGGGAGGGTGGTC	4	AF406944	4
Cpa110	TAGA	CTGACAACCCTCGACATACAT	ACAATTTGCACTGGTTTGTAGTAG	4	AF406946	
Cpa111	TAGA	TGTCCAGTAAAACATGCCTGA	GCTCCGTTCTCTTTCTTGCT	4	AF406947	2
Cpa112	TAGA	GAGAGGGAGTTAAAATTGACAGC	GGCACAAGATGAGAGTGCAG	4	AF406948	4
Cpa113*	ATCT	TGTCCATCTGTCCATTCAGC	ACCACACAGCACATTTACAGG	4	AF406949	4
Cpa114	ATCT	GCGTTTGTCCATACCACATT	CAGCTCTGAAAACCCAGACA	4	AF406950	7

*Markers that were redesigned, see Table 4.

**Reverse primers from Miller et al. 2001 are all with GTTT tail at 5´end.

References; 1: McPherson et al. 2001., 2: Miller et al. 2001, 3: O´Connell et al. 1998., 4: Olsen et al. 2002.

Table 4. Redesigned primers for published markers.

Marker	Repeat	New forward Primer	New reverse primer	References*	GenBank
MCha1045	GATA	GCCAGTCAGGAGAAAGTC	AAAGAAGTCCCAGATGTG	1	AF304361
MCha120	CA	GGGCTTAGCTGAACTTAGGC	CGCTCTAGAACTAGTGGATCTG	2	AF309795
MCha27	GACA	CTGATGGGCCAATGCTTTCG	TGTTGTAAGGGTTGTTAGC	2	AF309799
MCha8	GACA	CTCATCTGCATCCTGATGAG	TGGCAGACAGCAAGACAGAAC	2	AF309804
MCha123	GT	CTACAGACACAGCCCACAAC	ATGATTGGCTATCATTCCATCC	3	AF019992
MCha34	GT	GATGGTGGTGGAGGATGTG	CCTCATTAGGGACGGAGAAC	3	AF019991
MCpa113	ATCT	TACCTCCGTCTGTCCATCTG	ACAGCACATTTACAGGTTGAC	4	AF406949
MCha134	CA	TTCTCTACAAAGGGCATATAGC	GGCCATTATATTACATACCATTG	2	AF309798
MCha7	GATA	CATACTTACAGCTGCAAGTC	CCTATGTGTATGTGATGTATG	2	AF309803
MCha38	GT	GTAACGGTCTGCACACTCAC	CGAAAGGACGAGCCAATAAC	3	AF019990
MCha70	CA	GCAGAGGACACTTTATCTTG	GTGTGGTCCATTTCTTAAAC	3	AF019988

*References; 1: McPherson et al. 2001., 2: Miller et al. 2001, 3: O´Connell et al. 1998., 4: Olsen et al. 2002.

3.3.2 Development of new microsatellite markers

A next generation sequencing (NGS) technique was used to develop new microsatellite markers for Atlantic herring. In total 32 markers were developed and tested at Matís and the methodology of the developmental work and characteristics of the 14 best markers have been published (Libungan et al. 2012) (see Appendix 1). Table 5 lists the information on all markers for Atlantic herring developed in this project using the NGS technique.

Table 5. Information on all markers for Atlantic herring developed in this project by using the NGS technique.

Marker	Forward	Reverse	Repeat	Size Range	Multiplex
MSild01*	CTGAGACTCAGTCAGTCATATC	TACTGCTGCTCGCATCTG	CA	91-119	
MSild02*	GCGTATCTTTGCGTAGTTGTG	ATCTCCCACGGTTCTTTGTC	CA	105-177	
MSild03*	AGTTGGACATACATGCATTC	TTTGGTCTGGTCGACATCTG	CA	107-205	
MSild04	AAGCAGCTTTGATCCACAAC	AACCCTAAACTTAAGCATAGTG	CA	140+	
MSild05	GAGAGCGGAGATCAGAAAGG	CAAATACAGAGCTGAAGACTATATG	CA	171-196	
MSild06	GAGGGCTTGCTTCTCCATAC	TTTCGTTCCGTCCGTACC	CA	206+	
MSild07	TCTGGCACAGAGTCTGAAG	AACGTAAGTAACGTAACCGTAACC	CA	220-226	
MSild08	TAGTCAAGCGTGCTCATGTG	TTACGTCGAACTTGCGTGTC	CA	407-409	
MSild09	AGCCCACCCAGGAGAAGAC	CCCGGTCCTCCTCGTCTC	GA	95+	
MSild10	TTCCTGTCATTCGCGTAGAG	CGTACGTCCGTACCGTACC	GA	185+	
MSild11	TCCTGTGTTGCCGTTTAATG	GCTTCCATTTGCACCTAAGC	AGT	130-163	
MSild12*	CCTGAGTTGACTGGGAGTTTAG	GTCATCTGATGGCCGTGGAG	CTT	73-139	6
MSild13*	TGCAGATCCTGCATGTTC	TTCGCTTTAGATCAAAGTGTCTG	GAT	176-251	9
MSild14	ATGATAGCCAACTTTCAGAG	ACTGGGCAGCAGTTCAGTTC	TAA	154+	
MSild15*	CCAGTCATGCCCATCAAATC	CCAGCAGCATGCAGATTATTC	TTC	217-286	
MSild16*	GAGAGGGTCAAAGCGTTCTG	CCATTTCCAATTTCACTCTTAC	ATGA	326-434	
MSild17*	GTTTCTCCTCGGGATTCTGG	AACTTGCCTACATGTCTATTTGC	CATA	336-420	13
MSild18*	AGTTCCATTGCCATGTTAGC	ATCCATACTCTGCCAGACAC	GAGT1	200-264	
MSild19	ACACCATGGCACTAACAAGG	GCGGAGAATATTGACTATTTCATTG	GCAC	294-314	
MSild20	CAGGCAGGACGTCAGATG	CACTGTCTGTCTGCCTGCTC	GTGA	403+	
MSild21	GGGTGCTTGTGAGTGAACAG	GGCGAGCTCAGCTTGTTG	CA	74-105	
MSild22	TCTTTGGCTACAGAGGACAGG	GGAAGGGCAGTGGAGGATAG	CA	120-171	
MSild23	GGATGCTGCAGTAATGAGAGG	ATGCATGCTGGTGGGTAGTC	CA	121+	
MSild24*	GGGTTGTGCTGACCTTTGAC	GAGTCTGTGAATGCCATGTG	CA	165-351	13
MSild25	AGCAAGATAGGAAGAAGATAGTATG	GTCTCTGAGCAACGCTGATG	CA	242-343	
MSild26	TGTCCTGCGACTCTGACATC	ACACTGGATGGGTGCAATTC	CA	322-379	
MSild27*	AGAGGCCACAGTGGATCAGAG	CACTTTGAGCTGCATGAAAGG	GAT	185-233	13
MSild28	TGACAGTAGCAGTGCCTGTAAG	GCTTCCCATTCCTGTGTTTG	ACA	216+	
MSild29*	TTTCTGCTCCGGCAAGTG	CAGTGCTGTGATGCTTATAATG	ATG	256-319	
MSild30*	GAATATGGCAAGCTGCAACC	CATTGTAAATGAGGGTCTTATTCC	ATTG	97-137	
MSild31	ACAGGAAATGCGTAATACATACTC	CCACATGGCCTCCTTGTTAG	TACA	75-135	
MSild32*	GGTCCACCTGGTTCACAATAG	ACAGGCTTGCTCCAAATCTC	TAGA	172-272	13

*Markers that were published in (Libungan et al. 2012).

+Estimated PCR product size from the NGS sequences.

3.3.3 Multiplex development of published and Matís developed microsatellite markers

The markers that were polymorphic and gave clear, readable peaks with high signal were used for further testing and multiplex development. Both published, redesigned and Matís developed markers were included in multiplexes. More than 60 multiplex setups were tested; Table 6 lists the final six multiplex systems that were used in the project.

Table 6. Multiplex systems for all markers used in the project.

Multiplex	Markers	µl*	Tm	Dye	Allele range	Genotyping quality **
SildPrint2	Cha113	0,1	58	PET	104-156	97
	Cha17	0,18	58	6FAM	85-189	99
	Cha1059	0.03	58	NED	63-127	98
	Cha1020	0,14	58	VIC	153-245	90
	Cpa111	0,16	58	VIC	256-295	91
SildPrint4	Cpa113	0.06	57	PET	118-230	93
	Cha1017	0,15	57	VIC	161-213	98
	Cpa103	0,13	57	6FAM	163-263	93
	Cpa112	0,14	57	VIC	232-416	92
	Cpa108	0,1	57	NED	233-275	96
SildPrint6	MSild12	0.03	58	VIC	73-139	97
	Cha1027	0,1	58	PET	113-213	100
	Cha63	0,1	58	NED	137-181	100
	Cpa101	0.06	58	VIC	169-321	98
SildPrint7	Cha20	0,1	60	VIC	92-212	70
	Cpa104	0.08	60	NED	180-506	97
	Cpa114	0.08	60	VIC	178-282	98
	Cha1202	0,1	60	6FAM	97-173	100
SildPrint9	Cha4	0.07	58	VIC	106-194	99
	Cpa102	0.06	58	NED	128-420	99
	MSild13	0,16	58	6FAM	176-251	99
SildPrint13	MSild17	0,1	58	VIC	336-420	95
	MSild24	0,15	58	PET	165-351	96
	MSild27	0.06	58	6FAM	185-233	99
	MSild32	0,1	58	VIC	172-272	99

*Primer amount (Forward and Reverse primer mix of an equal concentration in 10 µl PCR mix).
** The percentage of the 1130 samples genotyped with the particular marker.

3.4 Genotyping with neutral and non-neutral microsatellite markers

The objective of this work package was to improve genetic information on the herring stocks within Icelandic waters by using the multiplex systems developed in the project (Table 6). A total of 1477 baseline samples from nine populations were analysed for 25 loci (Table 6) with an ABI PRISM 3730 sequencer using the GeneScan-500 LIZ size standard. Genotypes were scored using GeneMapper 4.0 from Applied Biosystems. In total 1130 baseline samples that were scored with 16-25 markers, which were then used for the statistical analysis. The average genotyping success of analysed baseline samples of all markers was 96%, ranging from 70% (marker Cha20) to 100% (Cha1027, Cha63 and Cha1202) (Table 6). In addition, 369 physicochemical samples were analysed with 13 markers (SildPrint4, 6 and 7; Table 6) and 232 of these samples had good scoring capacity and were used for genetic analyses (8-13 markers).

3.5 Statistical analysis

3.5.1 Statistical analyses of microsatellite markers

The genetic diversity assessed as the number of alleles per locus was high, ranging from 9 (Cpa111) to 65 (Msild24) (Table 7). The expected heterozygosity (H_e) per sample ranged from 0.821 to 0.859 (data not shown). Genotypic proportions were out of HWE in 31 out of 312 exact tests. A total of four tests remained significant after Bonferroni correction for multiple tests (Rice 1989) (α = 0.05/312 =

0.00016) and were not attributable to any specific loci or samples. An overview of the general marker characteristics for all 24 markers used in the study is given in Table 7 (Cha20 was not used for statistical analysis as it was uninformative).

Table 7. General primer characteristics: Marker name; k is number of alleles; N is number of individuals genotyped; H_o is observed heterozygozity; H_e is expected heterozygosity; FIS is the inbreeding coefficient (Hardy-Weinberg equilibrium); Pval is the P-value of HWE.

Marker	k	N	*Ho*	*He*	FIS	Pval
Cha4	22	1118	0.878	0.879	0.041	0.634
Cha17	43	1108	0.927	0.948	0.013	0.060
Cha63	22	1117	0.861	0.859	-0.006	0.377
Cha113	26	1091	0.936	0.886	0.016	0.166
Cha1017	14	1106	0.819	0.812	0.055	0.005
Cha1020	25	1009	0.927	0.926	-0.010	0.073
Cha1027	27	1111	0.934	0.936	0.029	0.001
Cha1059	17	1102	0.649	0.692	0.071	∞
Cha1202	15	1117	0.746	0.739	0.006	0.0276
Cpa101	32	1070	0.921	0.921	0.021	0.719
Cpa102	55	1108	0.935	0.934	0.005	0.114
Cpa103	24	1050	0.885	0.886	0.071	0.110
Cpa104	55	1095	0.834	0.831	0.063	0.001
Cpa108	11	1078	0.492	0.491	0.021	0.409
Cpa111	9	1021	0.442	0.443	0.011	0.783
Cpa112	32	1031	0.891	0.891	-0.015	0.926
Cpa113	26	1049	0.936	0.936	0.014	0.277
Cpa114	24	1101	0.917	0.917	0.036	0.359
MSild12	22	1070	0.885	0.884	0.017	0.069
MSild13	23	1108	0.902	0.898	0.002	0.593
MSild17	20	1044	0.891	0.889	0.0287	0.393
MSild24	65	1069	0.960	0.960	0.010	∞
MSild27	16	1092	0.799	0.805	0.017	0.545
MSild32	20	1105	0.909	0.909	0.014	∞

Detection of loci under selection using LOSITAN (Antao et al. 2008) resulted in the discovery of three potential outlier loci, namely Cpa111 (positive selection), Msild13 (positive selection) and Cap101 (balancing selection). No other loci showed signs of being influenced by selection (see Figure 8).

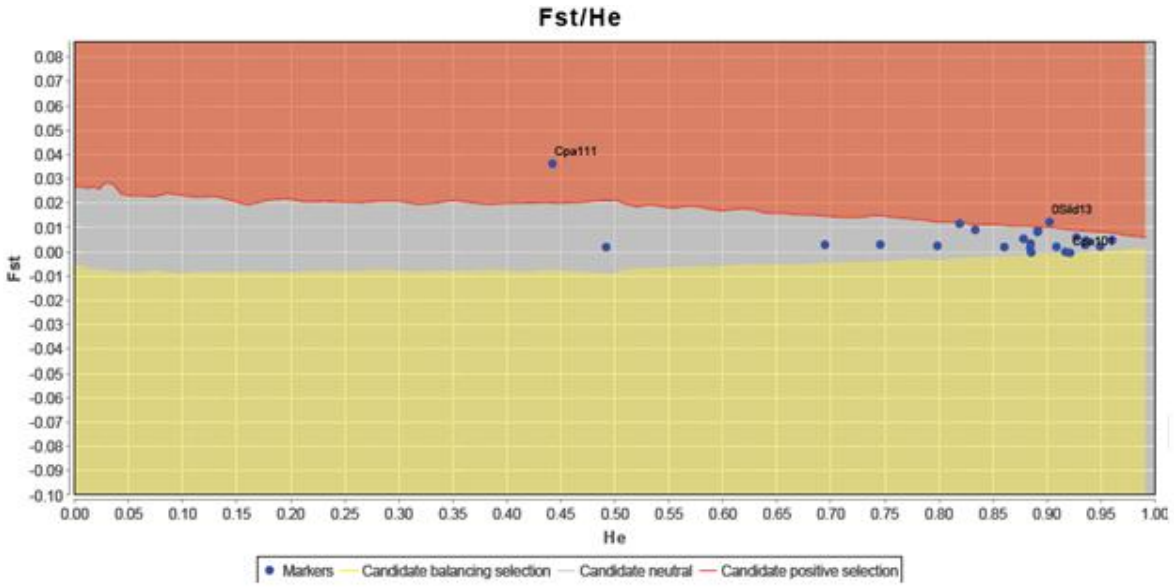


Figure 8. A graphical representation of the F_{ST} outliers methods results from the selection detection workbench Lositan for all samples.

The implication for management and conservation of genetic markers under selection, especially microsatellite loci, which were expected to be neutral, has been discussed for years (Nielsen et al. 2006; Pampoulie et al. 2006; Pampoulie et al. 2011; Skarstein et al. 2007; Westgaard and Fevolden 2007). Selection at a specific locus will typically lead to variation in allele frequencies in a shorter and faster time than will drift alone, and result in a higher level of differentiation at markers under selection than at neutral ones over a similar time-scale evolution. The combination of neutral and non-neutral genetic markers has been shown to convincingly reveal adaptive divergence in many marine species (Hauser and Carvalho 2008; Hemmer-Hansen et al. 2007; Pampoulie et al. 2006; Zane 2007), hence the utility of the three outliers detected in LOSITAN.

3.5.2 Population structure of herring in the North Atlantic Ocean (all samples)

The overall estimates revealed significant level of differentiation among the collected samples (F_{ST} = 0.006, $p < 0.001$, 95% CI: 0.0013 - 0.0291) and FIS values (FIS = 0.022, $p < 0.05$, 95% CI: -0.0061 – 0.0708). This genetic pattern was reflected in the pairwise F_{ST} comparisons of samples as 33 of the 78 comparisons were significant after Bonferroni corrections (Table 8) (Rice 1989). However, the significant values mainly involved samples from NLSSH, e.g. the local spawning herring from the fjords located in Norway. No other comparisons were significant.

Table 8. Pairwise F_{ST} and significance (bold values) among 13 samples of Atlantic herring based on allelic frequencies after Bonferroni corrections.

	FASH	FSSH	ISSH-2009	ISSH-2010	NASH-Lofoten	NLSSH-Landviksvannet	NLSSH-Lindas	NLSSH-Trondheim	NSSH-Gulroy	NSSH-Ringbas	NSSH-Store	NSSH-2012	NSASH
FASH	0												
FSSH	-0.001	0											
ISSH-2009	0.000	0.000	0										
ISSH-2010	0.000	0.000	0.000	0									
NASH-Lofoten	0.000	0.000	0.000	0.000	0								
NLSSH-Landviksvannet	**0.013**	**0.015**	**0.014**	**0.013**	**0.011**	0							
NLSSH-Lindas	**0.010**	**0.011**	**0.011**	**0.012**	**0.009**	**0.016**	0						
NLSSH-Trondheim	**0.008**	**0.008**	**0.008**	**0.008**	**0.007**	**0.018**	**0.008**	0					
NSSH-Gulroy	0.000	0.000	0.000	0.000	-0.001	**0.012**	**0.008**	**0.007**	0				
NSSH-Ringbas	0.000	-0.002	-0.002	-0.001	0.000	**0.006**	**0.012**	**0.009**	0.001	0			
NSSH-Store	0.001	0.001	0.001	0.000	0.001	**0.015**	**0.016**	**0.008**	0.001	-0.002	0		
NSSH-2012	0.002	0.003	0.003	0.003	0.001	**0.015**	**0.010**	**0.008**	0.000	0.003	0.002	0	
NSASH	0.001	0.000	0.001	0.001	0.000	**0.012**	**0.011**	**0.008**	0.000	-0.002	0.001	0.003	0

Emboldened values differ significantly from zero (Fisher's exact test, $p < 0.05$) after Bonferroni correction.

The Bayesian cluster analysis (STRUCTURE) (Pritchard et al. 2000) performed without *a priori* designation of the sampling location or any assumption about population structure, confirmed the observed pattern with the pairwise F_{ST} comparisons by clustering separately the populations from the North Atlantic and populations from the three NLSSH samples. It therefore detected that the most likely number of populations contained in our samples was $K = 2$ (Figure 9), e.g. 1) North Atlantic populations (ISSH, NSSH, NSASH, NASH, FASH and FSSH) and 2) NLSSH Landviksvannet, NLSSU Lindas and NLSSH Trondheim.

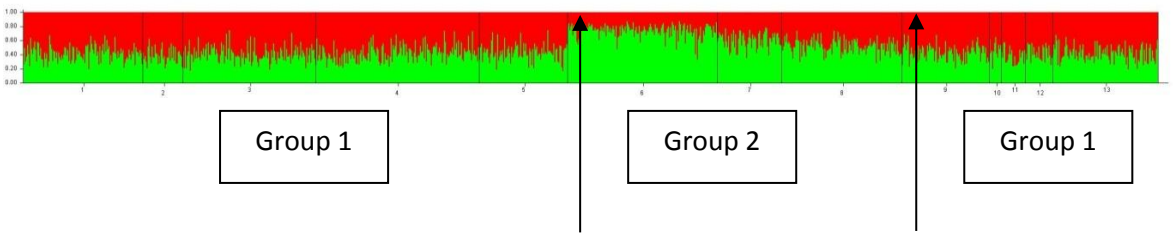
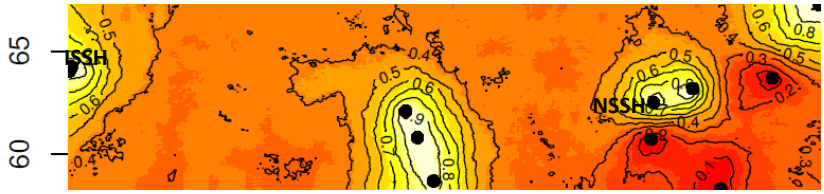


Figure 9. Structure clustering and assignment analysis (Pritchard et al. 2000). Bar plots represents individual assignment score to the two populations detected. Each bar represents one individual which is either assigned to North Atlantic (group 1, RED) of NLSSH (group 2, GREEN). Numbers refer to samples analysed (1. FASH, 2. FSSH, 3. ISSH2009, 4. ISSH 2010, 5. NASH, 6. NLSSH Landviskvannet, 7. NLSSH Lindas, 8. NLSSH Trondheim, 9. NSSH Gullroy, 10. NSSH Ringbas, 11. NSSH Storegg, 12. NSSH 2012, 13. NSASH).

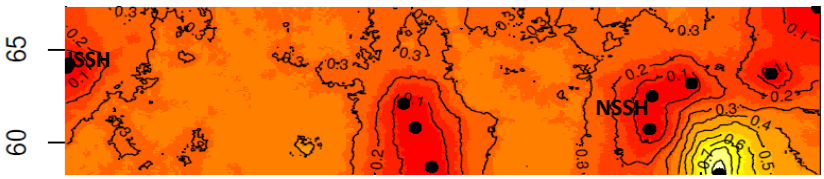
All runs to infer the number of subpopulations (K) performed in GENELAND (Guillot et al. 2005) and using spatial data (geographical coordinates) indicates that the most likely number of subpopulations K was 3 (Figure 10). The 5 runs performed to assign individuals with a K fixed to 3 were consistent across each of the runs. These geographically distinct subpopulations were composed of samples from the 1) North Atlantic, from 2) NLSSH-Landviksvannet, and 3) NLSSH-Lindas and NLSSH-Trondheim.

All the presented results suggested a strong differentiation among the North Atlantic samples and the samples collected from the Norwegian local fjords (NLSSH). The genetic differentiation mainly originated from the loci under selection (F_{ST} around 0.02), but not only as few neutral microsatellite loci also exhibited a significant but lower differentiation (F_{ST} around 0.002). Although adaptive divergence depends on the strength of selection vs. gene flow, the current results suggested that adaptive divergence has occurred in the local fjord of Norway.

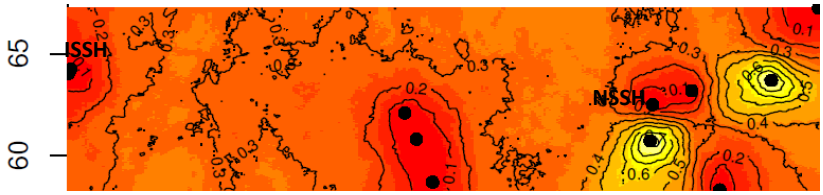
(1) North Atlantic samples



(2) NLSSH-Landviksvannet



(3) NLSSH-Lindas and NLSSH-Trondheim



(4) Compilation of population membership

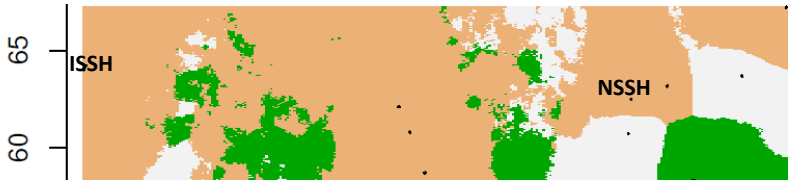


Figure 10. Map of posterior probability of belonging to subpopulations detected in the GENELAND analysis (Guillot et al. 2005). Subpopulation 1 (1) consists of samples from the North Atlantic, subpopulations 2 (2) of samples collected at NLSSH Landviksvannet and subpopulation 3 (3) from sample collected at NLSSH Trondheim and Lindas. (4) shows a compilation of population membership. ISSH and NSSH stocks are located on the maps.

3.5.3 Population structure of herring in the North Atlantic Ocean (excluding NLSSH)

Despite the exceptionally high level of differentiation among NLSSH populations and samples collected from the North Atlantic population, pairwise F_{ST}'s comparison also gave other important information. For example, samples collected in 2010 in NSSH few miles away from each other (Gullroy, Ringbas and Storegg) are not genetically different, and samples collected in 2009 and 2010 at Icelandic ground (ISSH) are also not genetically different (see Table 8).

However, high levels of differentiation among some of the samples collected might override the genetic differentiation among other samples, and therefore additional analyses were performed among samples belonging to the North Atlantic group, e.g. by removing the NLSSH fjord samples. In addition, samples from Gullroy, Storegg and Ringbas were clustered together to form the NSSH 2010 sample.

By removing the NLSSH samples, the overall estimates did not reveal a significant level of differentiation among the collected samples (F_{ST} = 0.0001, p > 0.05), but the overall F_{IS} value remained significant (F_{IS} = 0.020, p < 0.05). This genetic pattern was reflected in the pairwise F_{ST} comparisons of samples as none of the 28 comparisons were significant after Bonferroni corrections (Rice 1989) (Table 9).

Table 9. Pairwise F_{ST} among eight samples of Atlantic herring based on allelic frequencies. None of the values were significant.

	FASH	FSSH	ISSH 2009	ISSH 2010	NASH	NSSH 2010	NSSH 2012	NSASH
FASH	0							
FSSH	-0.001	0						
ISSH-2009	0.000	0.000	0					
ISSH-2010	0.000	0.000	0.000	0				
NASH	0.000	0.000	0.000	0.000	0			
NSSH-2010	0.000	0.000	0.000	0.000	-0.001	0		
NSSH-2012	0.001	0.001	0.001	0.001	0.000	0.000	0	
NSASH	0.001	0.000	0.001	0.001	0.000	0.000	0.000	0

The Bayesian cluster analysis (STRUCTURE) performed without *a priori* designation of the sampling location or any assumption about population structure, confirmed the observed pattern with the pairwise F_{ST} comparisons by detecting only one population within the samples (K=1) as did all runs performed in GENELAND using spatial data (geographical coordinates).

To be sure of the observed pattern visual inspection was performed of the individual genotype by performing PCA in Adegenet (Jombart 2008), (see Figure 11). The coinciding centroids of the stars-in the PCA clearly suggests that populations of herring in the North Atlantic are not genetically differentiated. The lack of clear differentiation between the populations (each shown in a different colour in Figure 11) demonstrates that it is not possible to perform Individual Assignments (AI) using this marker set, as individuals might not be assigned correctly to their baseline population of origin.

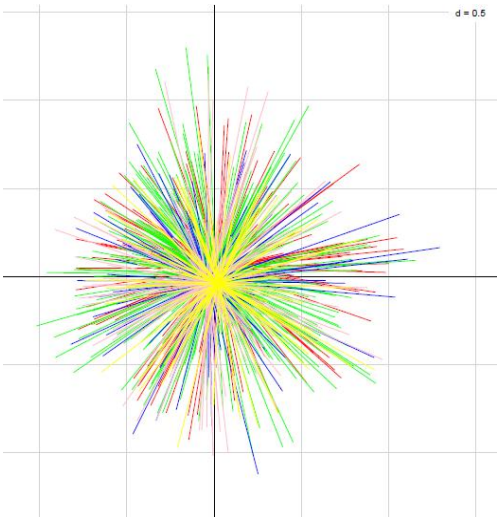


Figure 11. Result of the PCA analyses performed in Adegenet (Jombart 2008). Each sample is represented with a different colour while each line represents an individual.

The observed lack of genetic structure among populations/stocks of the North Atlantic can be explained by several hypotheses. One common concern is that the lack of detection power is due to the genetic marker used and the sampling scheme design. More than 900 individuals were genotyped at all 24 microsatellite loci within this study, was and further analysis with the POWSIM software (Ryman and Palm 2006) as undertaken to determine whether the investigation scheme was sufficient to detect any level of true genetic structure among the North Atlantic populations (excluding the fjords samples).

The resolution power is assessed by simulating different expected level of F_{ST} according to the effective population size (N_e) and generations (t) and to Nei´s formula (Nei 1987): $F_{ST} = 1 - (1 - 1/2N_e)^t$. The significance, evaluated using Fisher's exact tests and χ^2 tests, reflects the power to detect a given level of differentiation (Average F_{ST}) with the sampling design developed during the study. N_e values used during the test are based on estimates calculated from fisheries data for the smallest stock. "Runs" denotes the number of simulations performed. The setting $F_{ST} = 0$ and t = 0 estimates α (type I error; in the absence of genetic drift).

The estimate of the statistical α (type I) error, e.g. the probability of rejecting the null hypothesis (H_o: genetic homogeneity) when it is true, varied from 0.075 with Fisher's exact tests to 0.077 with χ^2 tests (Table 10), which is slightly higher than the 5% limit for significance, but still at a reasonable level. The simulation analyses on the power of the microsatellite loci revealed that the combination of the microsatellite loci and sample sizes used, conferred a statistical power sufficient to detect a very low ($F_{ST} = 0.001$) level of differentiation if it was present in the data (Table 10). Therefore the hypothesis, that the lack of genetic differentiation observed among the North Atlantic samples/areas is due to the lack of power of the genetic technique used, can be rejected.

Table 10. Estimate of the resolution power of the microsatellite loci using POWSIM (Ryman and Palm, 2006).

Expected F_{ST}	Average F_{ST}	χ2-test	Fisher's test	Ne	Generation (t)	Runs
0,0000	0,0000	0.077	0.075	1,000	0	1,000
0,0000	0,0000	0.089	0.076	5,000	0	1,000
0,0010	0,0010	1.000	1.000	500	1	1,000
0,0010	0,0010	1.000	1.000	1,000	2	1,000
0,0010	0,0010	1.000	1.000	5,000	10	1,000
0,0025	0,0025	1.000	1.000	1,000	5	1,000
0,0050	0,0050	1.000	1.000	1,000	10	1,000

3.6 Physicochemical properties

The condition of the raw material and physicochemical properties of ISSH and NSSH herring from six commercial catches (Table 11) were measured to obtain information on where and when the best quality of herring is obtained, in relation to colour, WHC, gaping and etc. The measurements were done in two trials (Table 11). The results of the colour, pH, fat- and water content as well as WHC, gaping, CF and GSI were measured and calculated in two trials. Trail 1 included water content, fat, WHC, CF and GSI. Trial 2 included the same properties as in Trial 1 in addition to colour, pH and gaping.

Table 11. Sampling of the Norwegian spring spawning herring (NSSH) and the Icelandic summer spawning herring (ISSH). Spawning time for NSSH is in April, and in July for ISSH.

	Catching season	Catching area	Stock	Number of samples
Trial 1	September 2010	Heradsdjup	NSSH	38
Trial 1	October 2010	Norwegian sea	NSSH	106
Trial 1	January 2011	Breidafjordur	ISSH	81
Trial 2	November 2011	Breidafjordur	ISSH	50
Trial 2	August 2011	Rauda torgid	NSSH	49
Trial 2	September 2011	Heradsdjup	NSSH	45

3.6.1 Trial 1

Length, weight and condition factor

The average length of the ISSH was 33.6 cm (min 30 cm; max 37 cm), and the average weight was 301 g (min 220.7 g; max 387.4 g). The average length of the NSSH was 31.7 cm (min 21 cm; max 36 cm), and the average weight was 299 g (min 112.8 g; max 388.7 g). There was a significant differences ($p<0.001$) in CF between the herring from different catch areas (Figure 12). CF was also significantly higher ($p<0.001$) in the NSSH compared with ISSH (Figure 13).

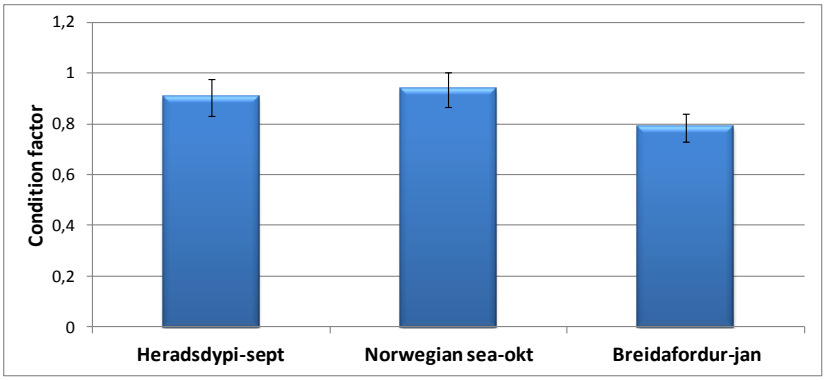


Figure 12. Condition factor of herring from the ISSH and NSSH fishing stocks at different fishing area and season (Trail 1).

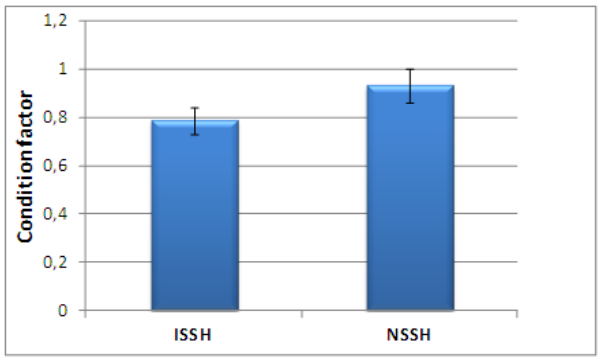


Figure 13. Condition factor of herring from the ISSH and NSSH fishing stocks (Trail 1).

Physicochemical measurements

The fat content in ISSH was significantly lower ($p<0.05$) than in NSSH caught in Heradsdypi and Norwegian Sea (Figure 14). The fat content was significantly higher in NSSH ($p<0.05$) than in the ISSH fish. The average fat content for ISSH was 10.9% and 13.9% for the two NSSH samples.

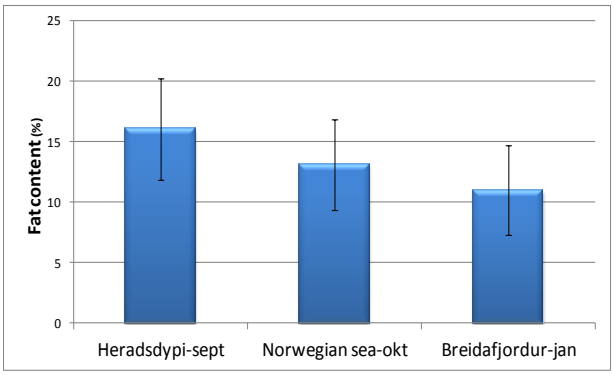


Figure 14. Fat content in herring from different catching area and fishing time (Trail 1).

The water content in herring caught in Breidafjordur was significantly higher than in herring caught in Heradsdypi and in Norwegian Sea ($p<0.001$) (Figure 15). At lower water content, the fat content

increases. There were no significant differences in the sum of the water and fat content, which suggests no difference in protein content. No significant differences were found in WHC between the herring from different catching area (Figure 16).

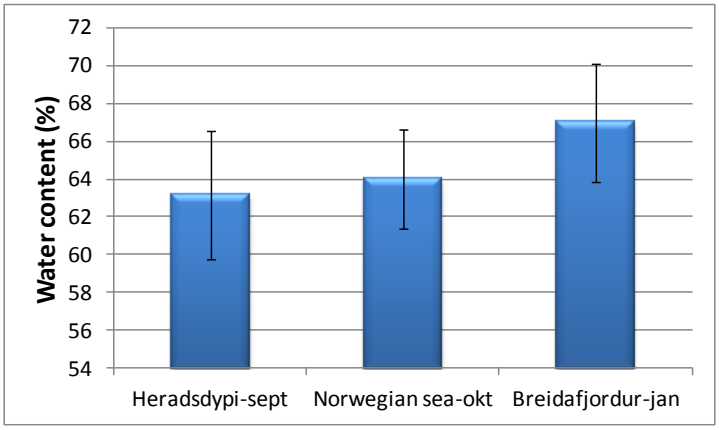


Figure 15. Water content in herring form different catching area (Trial 1).

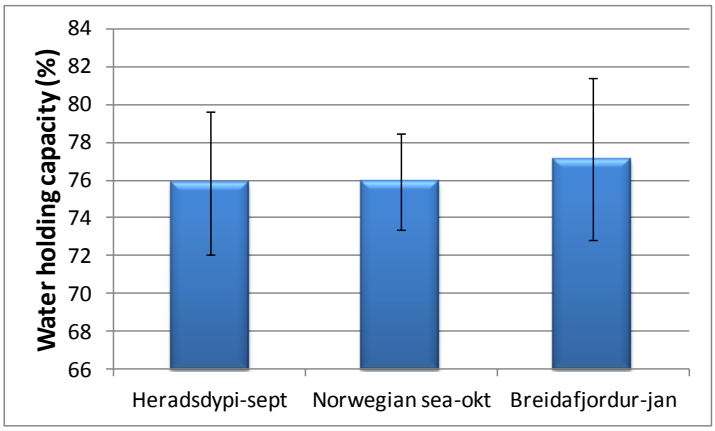


Figure 16. Water holding capacity in herring from different catching area (Trial 1).

From the Pearson correlation coefficient Table (Table 12), the length and weight are correlated positively high, also the age and length. The CF is correlated inversely with maturity and age, but correlated positively high with the weight of the gonads and the GSI. The gonads are correlated positively high with GSI. Water and fat content are inversely correlated.

Table 12. Pearson correlation coefficient for different parameters in Trial 1 (red figures denote to significant differences between parameters p<0.05).

	Length	Weight	CF	Maturity	Age	Gonads	GSI	Water	Fat	WHC
Length	1									
Weight	0,816	1								
CF	-0,519	0.0516	1							
Maturity	0,383	0.0212	-0,625	1						
Age	0,818	0,634	-0,518	0,377	1					
Gonads	-0,263	0,162	0,652	-0,624	-0,277	1				
GSI	-0,272	0.0658	0,646	-0,596	-0,314	0,981	1			
Water	0,164	-0,140	-0,497	0,397	0,312	-0,356	-0,335	1		
Fat	0.0738	0,298	0,320	-0,209	-0.0554	0,153	0,146	-0,818	1	
WHC	0,188	0.0643	-0,208	0,172	0,251	-0,135	-0.0979	0,328	-0,155	1

Multivariate analysis on weighted principal components (PCA) was performed on all data to identify similarities and differences between catch area, catch season and stock on different parameters. The PC1 represent 42% of the total variation, mainly described by the effects of WHC and water content, as well as the maturity of the gonads and CF. The second principle component represented 24% of the total variation, described by the effects due to fat content, weight, length and age. For PC1, the weight of the gonads was closely and positively related to CF and GSI, and WHC, maturity and water content were closely related. For PC2, the length and age were closely related (Figure 17). WHC was the only variable which did not affect the model. The NSSH caught in the Norwegian Sea in October 2010 was characterized by high CF, GSI and gonads, while the ISSH was more characterized by high WHC and high maturity stage as well as length and age (Figures 17-20). The ISSH stock is well separated in the score plot, while the NSSH is more clustered together. The separation is probably due to maturity stage. All the ISSH were in maturity stage 8 (resting time after spawning), while the NSSH was in different maturity stage.

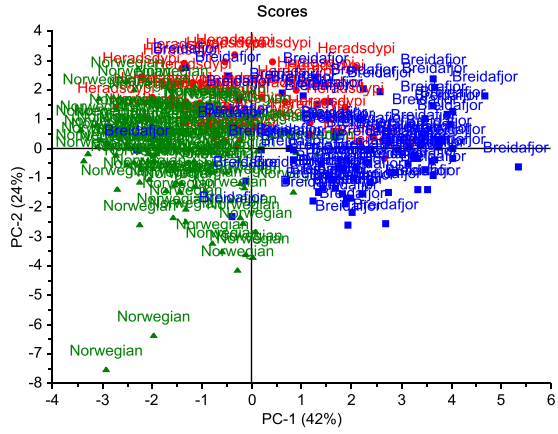


Figure 17. PCA score plot for the different catch area (Trial 1).

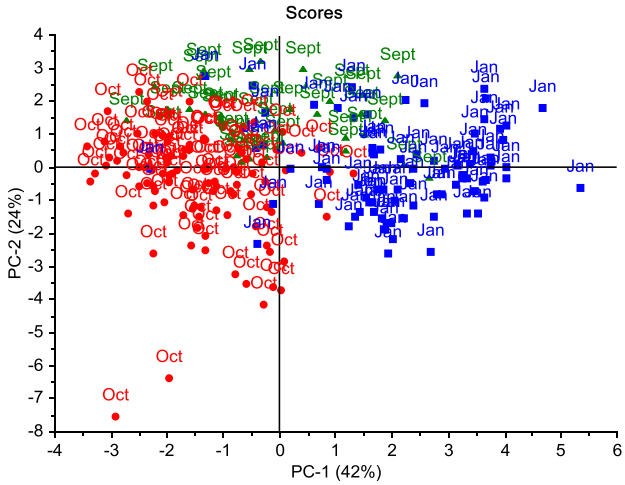


Figure 18. PCA score plot for the different catch seasons (Trial 1).

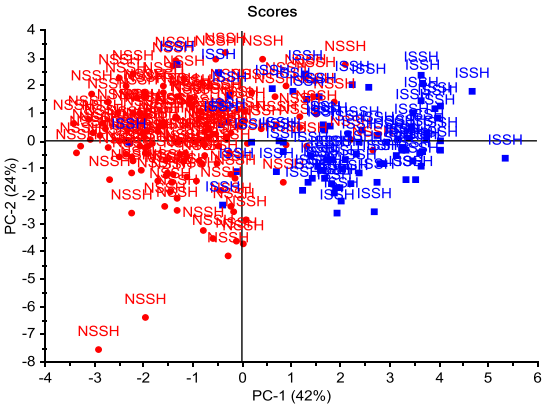


Figure 19. PCA score plot for different stocks (Trial 1).

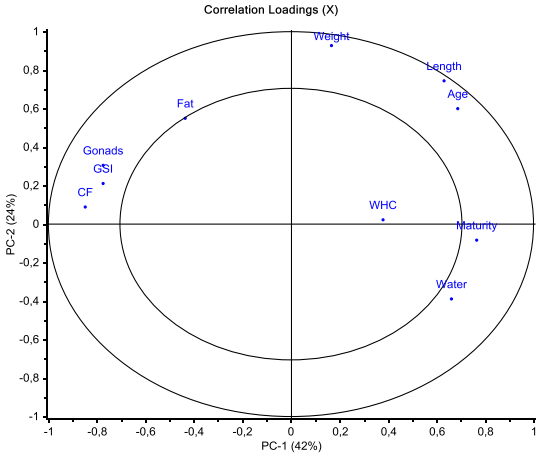


Figure 20. PCA correlation loadings plot (Trial 1).

3.6.2 Trial 2

Length, weight and condition factor

Herring sampled in three fishing grounds in 2011 were under study. The average length of the ISSH individuals was 32 cm (min 28 cm; max 35 cm), and the average weight was 286 g (min 163.1 g; max 425.6 g). The average length of the NSSH was 33.7 cm (min 31 cm; max 37 cm), and the average weight was 370 g (min 302 g; max 460.4 g).

CF was significantly lower ($p<0.001$) for ISSH fish caught in Breidafjordur compared to the two NSSH groups (Figure 21).

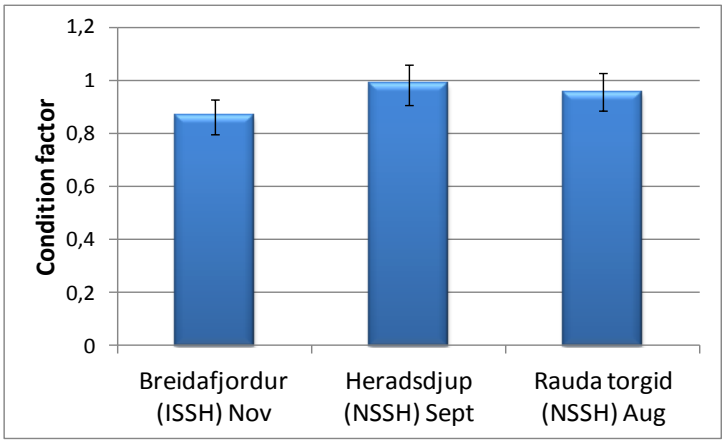


Figure 21. Condition factor of herring from the ISSH and NSSH fishing stocks at different fishing area and season (Trial 2).

Physicochemical measurements

No significant differences ($p>0.05$) were observed in fat content between the three herring groups regarding different fishing areas and seasons (Figure 22).

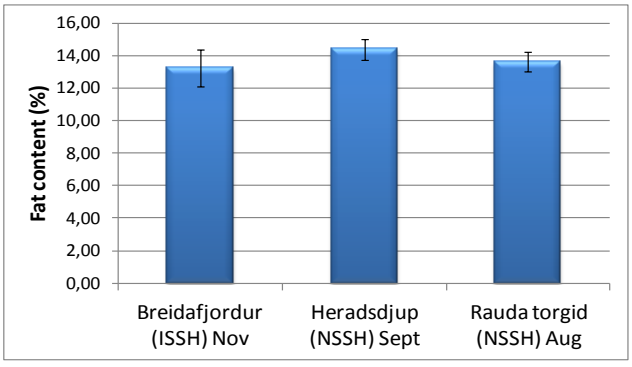


Figure 22. Prediction of fat content with NIR analysis in herring from different seasons and catch areas (Trial 2).

ISSH caught in Breidafjordur in November had significantly higher water content ($p<0.001$) than the two NSSH groups (Figure 23).

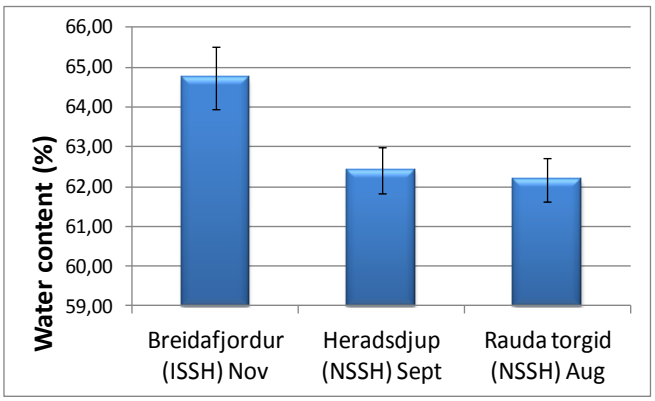


Figure 23. Prediction of water content with NIR analysis in herring from different season and catching area (Trial 2).

NSSH herring caught in Rauda torgid was significantly lower ($p=0.007$) in WHC than the other NSSH group caught in Heradsdjup and the ISSH caught in Breidafjordur (Figure 24).

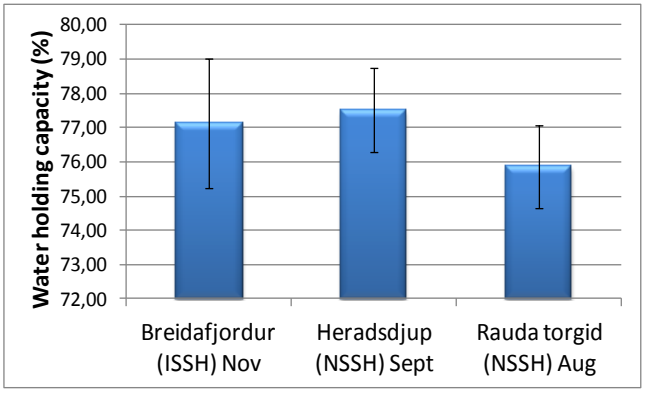


Figure 24. Prediction of water holding capacity with NIR analysis in herring from different season and catching area (Trial 2).

The colour (*L* value) of the ISSH herring caught in Breidafjordur in November had significantly darker muscle ($p<0.001$) than the two NSSH groups. No significant differences were found in the herring due to *a* value and *b* value (Figure 25). The *L* value is the intensity of white colour (dark=0, white=100), *a* value is the intensity of red colour (red = +, green = -) and *b* value is the intensity of yellow colour (yellow = +, blue = -).

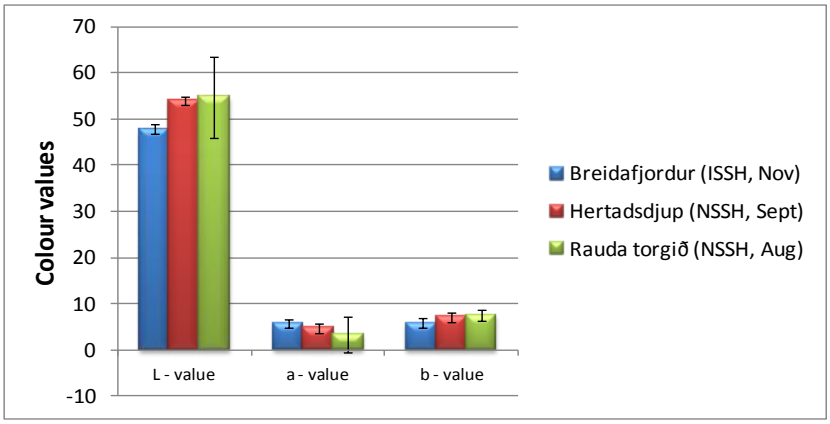


Figure 25. Colour measurements of herring from different seasons and catch areas (Trial 2).

The pH was significantly ($p<0.001$) higher in ISSH herring caught in Breidafjordur in November compared to the two NSSH groups (Figure 26).

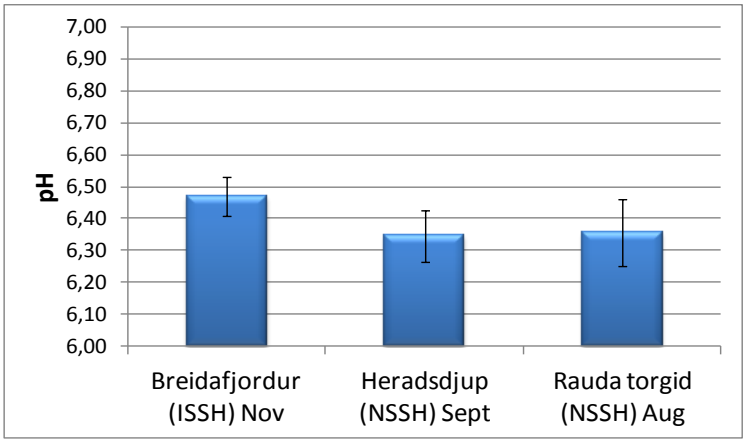


Figure 26. pH in herring muscle from different seasons and catch areas (Trial 2).

There was significantly ($p<0.05$) more gaping in NSSH herring caught in Rauda torgid compared to the other NSSH group caught in Heradsdjup and the ISSH caught in Breidafjordur (Figure 27).

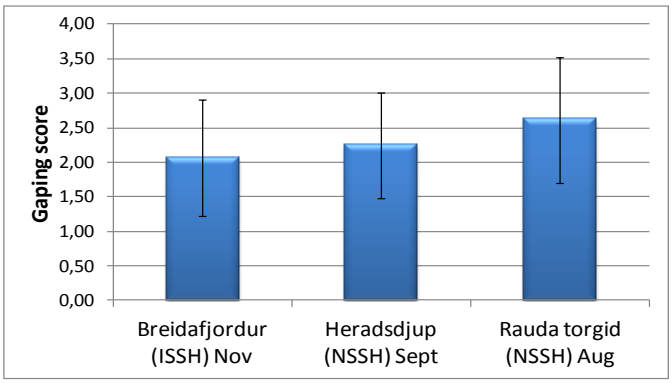


Figure 27. Gaping in herring muscle from different seasons and catch areas (Trial 2).

There is a relationship between pH and gaping, as at lower pH the gaping increases. The same has been observed in thawed fish (Love and Haq 1970). These results indicate a relationship between pH and WHC, as pH increases with increasing WHC. This is in line with previous reports (MacCallum et al. 1967; Rustad 1992).

From the Pearson correlation coefficient table (Table 13), the length and weight are positively correlated, as are the age and length. The CF is inversely correlated with maturity, but positively correlated with the weight of the gonads and the GSI. Additionally the CF is negatively correlated with water content and positively correlated with fat content. The gonads are positively correlated with GSI. Water and fat content are inversely correlated. At higher pH the gaping decreases and WHC increases.

Table 13. Pearson correlation coefficient for Trial 2 (red figures denote a significant correlation p<0.05).

	Length	Weight	CF	Maturity	Age	Gonads	GSI	Water	Fat	WHC	L value	a value	b value	pH	gaping
Length	1														
Weight	0,850	1													
CF	0.0537	0,562	1												
Maturity	-0,327	-0,515	-0,477	1											
Age	0,804	0,782	0,213	-0,345	1										
Gonads	0,302	0,572	0,616	-0,551	0,396	1									
GSI	0,257	0,522	0,602	-0,560	0,356	0,994	1								
Water	-0.081	-0,371	-0,592	0,402	-0,106	-0,376	-0,371	1							
Fat	0.0518	0,206	0,311	-0.0975	0.065	0,22	0,206	-0,765	1						
WHC	-0,125	-0,15	-0,126	0,216	-0.043	-0.073	-0.078	0,384	-0,221	1					
L value	0,207	0,477	0,599	-0,571	0,227	0,419	0,425	-0,549	0,233	-0,268	1				
a value	-0,204	-0,271	-0,197	0,249	-0,241	-0,242	-0,249	0,198	-0.0997	0,165	-0,588	1			
b value	0.091	0,356	0,54	-0,579	0,180	0,446	0,463	-0,425	0,175	-0,141	0,785	-0,416	1		
pH	-0,178	-0,38	-0,457	0,431	-0,198	-0,354	-0,354	0,382	-0,217	0,151	-0,613	0,323	-0,573	1	
gaping	0.0113	0,116	0,221	-0,154	-0.002	0.0727	0.082	-0,191	0.055	-0,218	0,293	-0.067	0,324	-0,339	1

The score plots (Figures 28-30) show that the ISSH herring, caught in Breidarfordur in November 2011 is differentiated from the NSSH caught in Heradsdjup (in September 2011) and Rauda torgid (in August 2011).

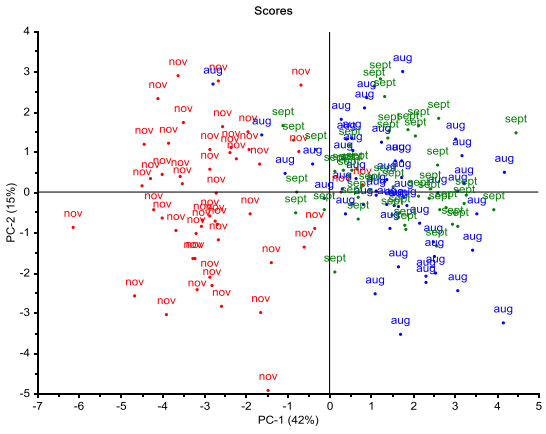


Figure 28. PCA score plot of different catch seasons (Trial 2).

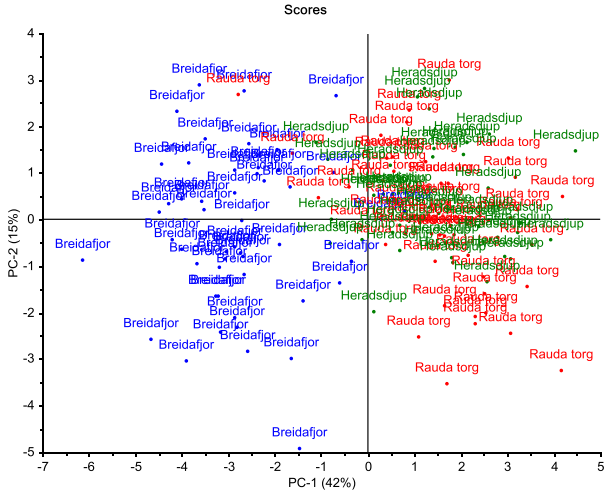


Figure 29. PCA score plot of different catch areas (Trial 2).

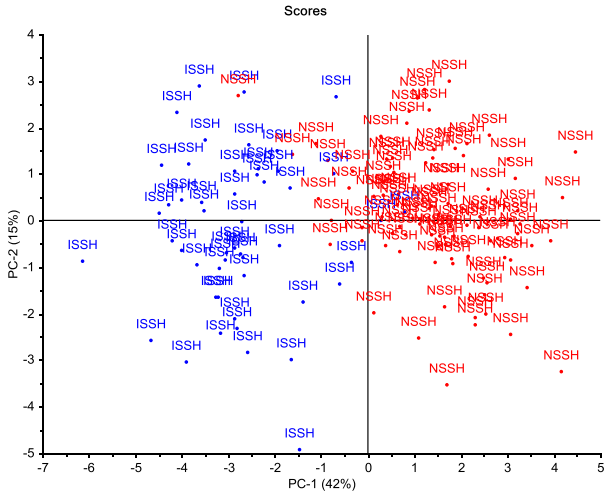


Figure 30. PCA score plot of different stocks (Trial 2).

Seven principal components described 91% of the variation between the samples. The first principal component, PC1, which represents 42% of the total variation, is mainly described by the effect of colour, CF, GSI, maturity and pH on the samples. The second principal component, PC2, which represents 15% of the total variation, described the variation in the samples due to length, weight, fat content and age (Figure 31). For the PC1, the weight of the gonads is closely related to GSI, and the CF is closely related to colour. For the PC2, length of the herring is closely related to age. ISSH caught in Breidafjordur in November 2011 was characterized by high water content and WHC, as well as high pH and high maturity stage and high red colour (Figures 28-31). It can be seen from these results that maturity is a dominating factor when it comes to the separation of the two stocks. All the ISSH is in maturity stage 8 when the NSSH is in different maturity stage.

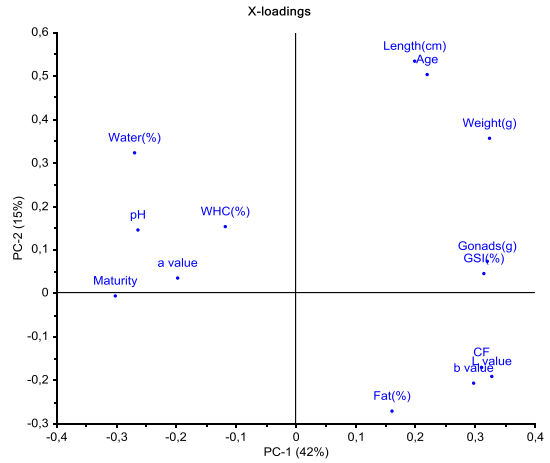


Figure 31. Loadings from PC1 and PC2 of all samples (Trial 2).

From the correlation loadings plot WHC, fat content, pH and *a* value had little effect on the model (Figure 32). The maturity stage had the dominant effect on the results, as all the ISSH herring have maturity stage 8, while the NSSH were in a mixed maturity stage.

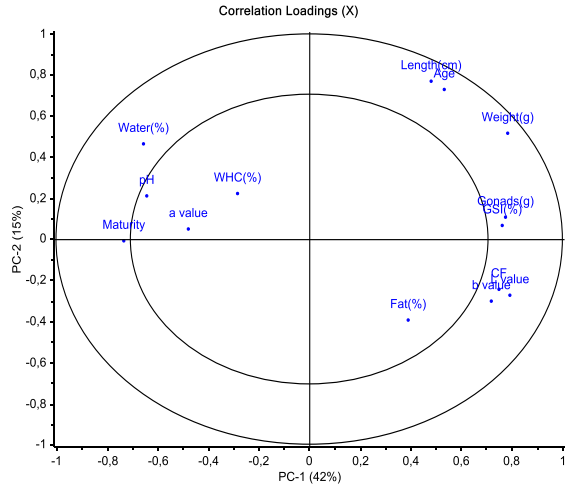


Figure 32. Correlation loadings from PC1 and PC2 from principal component analysis (PCA) of all samples and variables. Seven PCs described 91% of the samples variation (Trial 2).

3.7 Multidisciplinary analyses

While it was initially intended to conduct a combined analysis of the genetic, biological and functional characters of the herring samples, it has been shown in sections 3.5.2 and 3.5.3, that there is insufficient differentiation between the herring stocks with these markers to distinguish between the different stocks in the North East Atlantic. For this reason it was not possible to combine all the datasets produced in this project for the allocation of fish in mixed fisheries to their spawning components. However, as shown in section 3.6.2 the physicochemical properties by catch location and season were successfully combined with the biological properties and processing characteristics.

4. DISCUSSION & CONCLUSION

The novelty of the project was in the multidisciplinary approach of identifying the herring stock structure in the Northeast Atlantic Ocean. An extensive sample scheme was performed for the years 2008-2012 from the presumed nine stocks in the Northeast Atlantic Ocean, with collaboration between the Nordic and Scottish marine institutes MRI, IMR, FAMRI and MSS. New microsatellites for herring and novel multiplex systems containing 25 microsatellite markers were developed in the project and to the best of our knowledge no other such systems are available. This allowed a larger number of samples and smaller DNA quantities to be genotyped with reduced costs, time and error rate.

Within this project, the power of the microsatellite analysis method when large numbers of both samples and markers are used to genotype Atlantic herring in the Northeast Atlantic Ocean, was estimated. The knowledge that has been acquired during the last 3-4 years will be essential for applications for larger future projects of this consortium. An important part of the current work was to ensure high quality, representative samples of discrete herring populations in the Northeast Atlantic Ocean, which can be used in future projects. The genetic analyses on the current project were carried out on total of 1130 individuals collected from 21 sampling sites. Spawning herring was characterized, and alongside the genetic analyses, there was a combined analysis of the biological and physicochemical characteristics. Studies on the otolith microstructure between the different stocks are still on-going (PhD of Lisa A. Libungan).

The results from the successful combination of the physicochemical and biological properties of the catch can be used to optimize processing methods and increase the value of the products. Herring from the two stocks (ISSH and NSSH) differed mainly because of the colour and water/fat content. However, it is not possible to determine the root of these differences between the stocks, i.e. it is not known if these differences are due to genetic or environmental differences. The two stocks have been shown to be very different in relation to maturity and weight, especially in Trial 2. The maturity stage had the most dominant effect on the results, because all the ISSH herring was in high maturity stage while the NSSH herring were in mixed maturity stage.

While the multidisciplinary approach of this project was not able to be as extensive as had originally been hoped, the combination of the physicochemical and biological properties of the fish still highlighted some interesting differences. Further work is needed to determine the causes of these differences and to determine if the results can be applied within a practical framework to allow stock identification and management of mixed-stock fisheries, and to provide stakeholders with tools for more practical and sustainable fisheries. In doing so, this would enhance conservation and long-turn sustainable management of herring, which is one of the most important commercial fish stocks exploited in the Nordic region. Genetic markers have been intensively used to assess genetic structure of the Atlantic herring in its southern distribution (Bekkevold et al. 2005; Gaggiotti et al. 2009; Mariani et al. 2005), but we are among the first one to investigate herring population structure in detail in the Norwegian Sea and surrounding waters. The results of this study showed that, even with 24 microsatellite loci, the herring in the Northeast Atlantic did not exhibit any significant genetic differentiation among stocks of the investigated areas, although the NLSSH herring was indeed genetically differentiated from all other samples. Although the observed lack of genetic structure

among populations/stocks of the North Atlantic can be explained by several hypotheses, a primary concern, which was important to discount, was to determine whether the sampling scheme, sampling protocols and genetic design were all satisfactory to detect significant level of differentiation. Power analyses performed during this study revealed that the protocols and sampling schemes implemented were sufficient to detect a level of genetic differentiation around 0.001, which is very low.

Herring from ISSH and NSSH were not significantly different with the applied genetic method and therefore it was not to possible to distinguish between those stocks in the mixed-fishery using the techniques tested here. As a result, it was not possible to apply techniques to genetically distinguish between stocks in the mixed-fishery, or to combine the genetic results with the physicochemical and biological properties. However, the results from this project are promising for future research programs. First, it has often been claimed that genetic results could be improved by increasing both number of samples and of loci studied. Our study shows that in the case of the Atlantic herring, population size are likely to be large and that increasing both the number of microsatellite loci and samples will not lead to an increase power of detection as suggested by previous studies (Ryman et al. 2006). Second, as discussed in more detail below, this study recommends that other genetic markers such as Single Nucleotide Polymorphisms (SNPs) might be more appropriate to study these high gene-flow species as selective markers have the potential to reveal genetic differences on a more appropriate, ecological-time scale than neutral ones, i.e. looking at processes that have occurred over 10's generations rather than 1000's generations. Therefore, the lack of genetic structure observed during the present study is likely to be due to either a large effective population size of the stocks investigated or to a recent event of reproductive isolation. At neutral loci, given enough time, drift would have led to genetic differentiation of subpopulations, but marine populations in the North Atlantic tend to be young and to originate from ice-free refugia during the Last Glacial Maximum, some 20,000 years-ago, while these markers can answer questions on an evolutionary time scale, it is likely that other non-neutral genetic markers, will be better suited to address questions that have an ecological timescale. These non-neutral makers, which are under selective pressure, will capture a genetic signal that is not detected with the neutral set of microsatellite loci used here, due to the lack of time since divergence.

Although markers such as microsatellites can be under selective pressure (as shown in section 3.5.1), SNPs are another class of genetic markers, which are gaining in popularity for population scale studies in both terrestrial and marine species. SNPs are the most common mutation found within a genome, and rather than being a region where a short motif is repeated (as in microsatellites), it is a single nucleotide that has mutated, resulting in a polymorphic site. SNPs are attractive markers for many reasons (for reviews see Brumfield et al. 2003; Helyar et al. 2011; Morin et al. 2004), including the availability of high numbers of annotated markers, low-scoring error rates, relative ease of calibration among laboratories compared to length-based markers and the associated ability to assemble combined temporal and spatial data sets from multiple laboratories. Additionally, the potential for high-throughput genotyping, improved genotyping results for poor quality samples such as historical, noninvasive or degraded samples (Morin and McCarthy 2007; Smith et al. 2011), a simple mutation model, and the ability to examine both neutral variation and regions under selection offers unparalleled scope for expansive screening of genomes and large sample sizes from natural

populations. Although several early studies questioned the advantage of SNPs over neutral markers such as microsatellites (e.g. Rosenberg et al. 2003), more recent studies have shown that SNPs are also showing promise as highly informative markers, as many studies with access to very large numbers of SNPs (mainly human) have shown that a small fraction of the SNPs have a very high information content for population structure analysis (e.g. Lao et al. 2006; Paschou et al. 2007), outperforming microsatellites (Liu et al. 2005). Despite microsatellites typically displaying far greater allelic diversity per locus, individual SNPs can segregate strongly among populations (Freamo et al. 2011; Karlsson et al. 2011; Limborg et al. 2012).

Development of powerful genetic markers for the identification of herring stocks, would give increased opportunities for stock identification, which is fundamental for effective fisheries management. As a result of this project, many future studies have been identified, including;

(1) Stability in genetic variation in a single stock over number of generations: This can be used to test the stability in genetic variation over long period and to test for the existence of bottleneck effects, and the effects of fisheries induced evolution. This can be tested on ISSH where DNA can be extracted from fish-scales (Yue and Orban 2001) from the present back to the 1940s.

2) The kinship between ISPH and NSSH: This would test the present and historical kinship between the two spring spawning herring stocks by using fish scales. It is important to know if ISPH will ever recover or not. According to the data in the database at MRI, there is an indication that around 1% of the herring in Icelandic waters are spring spawners, but the proportion declined in the 1980s and 1990s (Óskarsson, personal communication). In catch samples of ISSH in the recent years, particularly in those taken off the southeast and south coast of Iceland, there have been varying proportions of spring spawners identified with ISSH shoals, however, it is not known if they belong to ISPH or NSSH and the spawning of spring spawners has not been observed in Icelandic waters in recent years. Thus, results of the project have the potential to: (a) protect the marine biodiversity, (b) ensure a sustainable exploitation of the living resources in the sea, and (c) distinguish between stocks in a mixed fishery, which is fundamental for their assessment and thereby, management of the stocks.

A comprehensive method for proper identification of the genetic diversity could be used in the future to detect warning signals about changes in genetic diversity and life history of Atlantic herring stocks as a result of overexploitation. For the rational management of herring in the Northeast Atlantic, a necessary requirement is a proper knowledge on the stock structure and migration patterns in order to protect each target stock. While this project has only partly succeeded in its aims, the project has been successful in advancing our knowledge of the interactions between the herring stocks in the Northeast Atlantic, and in highlighting future directions that the consortium can take. In addition, the advances made by the consortium, in producing a large, high quality archive of herring samples covering all the major populations of herring, which are available for future studies is an essential step to enable future work.

5. ACKNOWLEDGEMENTS

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