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This report presents the results of marine and freshwater scientific work carried out under the Fishing Industry Science Alliance (FISA).

# *Determination of the distribution of the resident inshore and offshore migratory cod populations around Shetland (IVa) and westwards into VIa*

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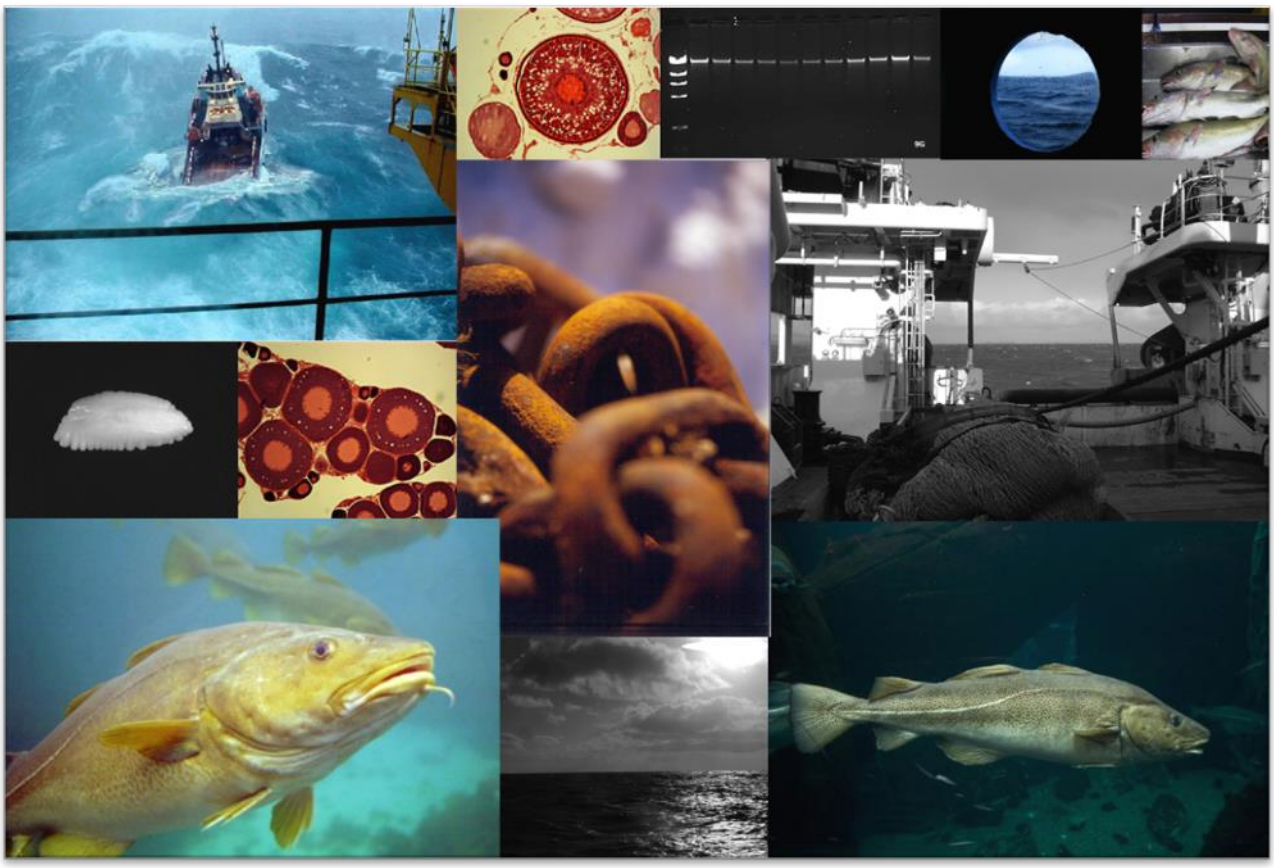

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## Summary

The current genetic analysis alludes to finer scale structuring of Atlantic cod stocks in the IVa and VIa stock units than had previously been reported by Heath *et al*. (2014). Consistent with previous studies of maturation, cod from Viking sampled in 2014 matured at a later age and larger size than other areas, providing a phenotypic population marker.

During spawning time there was no indication that the Viking group extended beyond the > 100 m waters of the northern North Sea. Indeed, the new genetic and maturity evidence suggests that Shetland coastal cod (ShIE) appear to extend into waters > 100 m east of Shetland.

The possible separation of cod from Scottish inshore waters from those offshore is also reminiscent of the inshore-offshore division seen in the northern North Sea.

There is some indication of mixing of populations outside the breeding season in the genetic analysis as well as the observation of large immature cod present in west coast samples.

The present study has considerably expanded our understanding of the Viking cod from northern IVa and when combined with the studies by Poulsen *et al*. (2011) and Heath *et al*. (2014), provides a good indication of population extent at spawning time and suggests a split around 0030 W.

## Introduction

A ‘stock’ is assumed to be a discrete group of fish that shows little mixing with adjacent groups having the same growth and mortality parameters across a particular geographical area (Gulland, 1983). Recent studies on Atlantic cod from the North Sea and Scottish west coast have identified considerable population structuring within these stocks. Through genetic analysis, otolith shape and microchemistry, maturation schedule analysis and tagging studies, two populations have been identified in the North Sea – one predominantly inhabiting regions < 100m and the other found in deeper offshore waters to the east of Shetland (Galley *et al*., 2006; Neat *et al*., 2006; Wright *et al*., 2006a, b; Wright *et al*., 2011; Heath *et al*., 2014; Neat *et al*., 2014). The offshore component has been referred to as ‘Viking’ as it is centred around the Viking Bank. Both populations are known to inter-mix as juveniles off Shetland’s east coast, but little is known of the stock dynamics west of the Shetlands, or the western extent of the offshore component, which may extend into the offshore waters west of Scotland (VIa). Much of the previous data comes from waters to the east of Shetland and less is known of the inter-mixing to the west of Shetland or indeed the western extent of the offshore ‘Viking’ component.

The aims of this project were to determine the western extent of the offshore cod sub-population that inhabits the waters around Shetland as juveniles, and to determine the separation at maturity between coastal and offshore populations of cod during spring and autumn.

## Methods

### Sample Analysis

Biological material (gonad samples, otolith and gill clipping for genetics), along with other biological measures (length, sex & macroscopic maturation stage) were collected from six areas; Shetland east coast - inshore and offshore (ShIE, Viking), Shetland west coast – inshore and offshore (ShIW, ShOW), and Scottish west coast – inshore and offshore (ScIW, ScOW) (Figures 1 & 2). ShIW corresponds to the coastal cod group known to show high site fidelity to the western waters of Shetland (Neat *et al*., 2006). Samples were taken during the autumn when mixing among populations may occur, and again in February and March during the spawning season. The requested sampling protocol for samplers is given in Appendix 1. The NAFC Marine Centre collected samples from the east and west of Shetland whilst SFF collected samples from VIa. Due to poor weather conditions NAFC were unable to collect many samples from offshore areas but fortunately, additional material was

provided by MSS from commercial and research vessels so that all six areas had at least the minimum sample requirements for analyses.

In total 1524 cod were obtained (721 from November - December, 803 from February – March; see Figure 1 & 2 for breakdown). For samples from research vessel catches, weight measurements were also obtained. The samples obtained complement past sampling programmes (e.g. see Heath *et al*., 2014) and considerably expand the westward extent of past sampling. All biological samples have been relocated to the MSS Marine Laboratory, Aberdeen. To ensure optimal quality of samples, all gill clippings received were transplanted (and occasionally sub-sampled) into fresh vials of ethanol to minimise degradation. Similarly, ovary samples were topped up with fresh NBF to ensure maximal fixation.

## Determination of Maturation Stage

For all cod from the February/March data set and all male cod from the October/November dataset, maturation stage was determined macroscopically during initial processing using the staging system defined by the ICES Workshop on Maturity Staging of Cod, Whiting, Haddock and Saithe (Bucholtz *et al.,* 2007). Female cod (n=418) from the November/December dataset were staged histologically to ensure that early maturation commitment, visible at the cellular level, was identified (Figure 3). Ovary tissue was fixed and stored in 10 % neutral buffered formalin (NBF) solution before being embedded in paraffin wax, sectioned (2µm) using a rotary microtome RM2155 (Leica Instruments GmbH), and stained with Haematoxylin and Eosin. Slides were then observed under a light microscope. Early ovarian development was classified based on the developmental stage of the most advanced oocytes, using Wallace & Selman's (1991) classification system; perinuclear (PN), circumnuclear ring stage (CNR), cortical alveolus (CA) and vitellogenic (VIT) (Figure 3). Only sections containing oocytes (min >10%) at the cortical alveolus stage or later were considered to be maturing individuals.

## Age Estimation

Prior to age estimation, images of all intact sagittal otoliths were taken for future shape analysis to compliment the genetic analyses. To date, otoliths (n = 843) from the spawning survey have been prepared and examined. Otoliths were embedded in black resin and sectioned, following standard protocols developed by CEFAS. Age was then estimated for each individual at x 10 magnification using transmitted light.

## DNA Extraction and RAD Library Construction

DNA was extracted using a SSTNE/salt extraction method and treated with RNase to remove residual RNA from the sample. Each sample was quantified by spectrophotometry (Nanodrop) and fluorescence (Quibit) quantitation methods and quality assessed by agarose gel electrophoresis. DNA samples from 20 individuals from all six locations from the Feb/March sampling window were taken forward into ddRAD analysis. A double digest RAD library was constructed (Peterson *et al.,* 2012) using the restriction enzymes Sbf1 and Sph1 while individual-specific combinations of P1 and P2 adapters allowed subsequent post-hoc segregation of all samples. The library was run twice on an Illumina MiSeq platform (v2 chemistry, 150 base paired-end reads). Data was compiled and processed using STACKS (Catchen *et al*., 2013). Due to variability in the sequencing outputs two data processing scenarios were run as described below. Following data processing loci with a minor allele frequency ≥0.15 in at least one of the test populations and an Fst ≥ 0.03 were further considered:

### All Locations:

In this scenario we considered all locations and all individuals and selected loci that were present in at least 17 individuals in each location.

### Higher Stringency Panel:

In this dataset we applied a higher stringency in data pre-processing where we filtered out individuals with relatively low numbers of individual reads in order to improve our confidence in the detection of heterozygote loci. This dataset ultimately included a total of 60 individuals from 5 locations (ScIW 17, ScOW 15, ShIW 6, ShIE, 10 & Viking 12) in subsequent analysis. The Shetland inshore samples were initially

pooled for loci identification but in subsequent analysis both pooled and segregated scenarios were considered.

SNP Analysis

Of the 90 total loci analysed from the ddRAD dataset, 13 loci - 3 from the “all locations” panel and 10 from the “high stringency” panel, were taken forward for a more in-depth genotyping analysis. Selection of the loci was prioritised based on the potential resolving power in relation to the study areas (Fst value), as well as technical constraints associated with the assay design criteria (KASP on demand, LGC Genomics, UK). A total of 689 samples were genotyped by LGC genomics including samples from the spawning period (including a reanalysis of the ddRAD sample set), as well as samples from the autumn period (n = 44 – 75 per location per time period.

Non-genetic Analysis

Differences in length at age among areas in the Feb/Mar samples were examined using a generalised linear model (GLM) with a Gaussian distribution. Length was the response variable with age and sex treated as factors. As there were few cod aged >5 the analysis was restricted to ages 2 – 5.

Maturation was modelled using a binomial generalised linear model according to:

$$logit(m) = length \times factor(area) \times factor(age)$$

where *m* is the proportion mature and *area* and *age* (2 to 5) are treated as factors.

Due to over dispersion in the survey data arising from the similarity in maturity within hauls, a quasi-binomial link function was used where variance is given by the dispersion parameter multiplied by the mean. Model selection for GLMs was through backwards step wise selection of variables based on model deviance compared using ANOVA in R using mgcv and MASS libraries.

## Genetic Analysis

Descriptive statistical analysis of markers as well as population genetics analysis was performed using a variety of packages. Hardy Weinberg equilibrium, fixation index (Fst) and pair wise population differentiation was performed using GENEPOP v4.2 (Raymond & Rousset, 1995; Rousset, 2008). ARLEQUIN (V 3.5.1.2) was used

to examine for signatures of directional selection by implementing a hierarchical island model (20,000 simulations, 100 demes per group and 10 groups). A Bayesian clustering analysis (STRUCTURE v2.3.4) was performed using an admixture model and correlated allele frequencies among populations, as well as providing sampling information as a prior in order to improve accuracy in detecting population structure. Results were compiled using CLUMPAK (Kopelman *et al*., 2015). The ddRAD datasets was also processed using a discriminant analysis of principal components using the ADEGENT program.

## Results

### Population Variation in Length and Maturity

Information on the proportion mature and mean length of cod in samples by area and time period is given in Table 1. The mean length of samples and proportion mature ranged from 49 – 70.5 cm and 0.37 – 1, respectively. Figure 4 shows the length at age for all 6 areas from the Feb/Mar (spawning) samples. Age, area and the interaction had a significant effect on length (Table 2). However, sex had no effect on the relationship. ScIW and Viking cod were smaller at age 2 but length at age increased more rapidly than other areas leading to similar lengths at age 5 across samples.

During the Feb/Mar collection period all ScOW cod and most males from other areas were mature. For the spawning period all males were mature at age 2, except for Shetland inshore samples and Viking. During the same period all age 2 females were mature from ScOW and a high proportion from ShOW. In contrast, a proportion of females up to age 3 in Shetland inshore samples and up to age 4 in Viking samples were immature. The high proportion of mature males and females in some areas did not allow for any formal analysis of maturity - length relationships for some areas. For the 4 samples (Viking, ScOW, ShIE and ShIW) exhibiting some variation in maturity, there was no significant difference in maturity at length between ShIE and ScOW but Viking cod were considerably larger and later to mature than cod from all other sample locations (Figure 5).

There were significant differences in slope of maturity - length relationships between samples collected in autumn and winter for the ShIE samples ($p<0.001$), with a wider range of sizes at maturity and larger immature cod being found in the autumn (Figure 6). A wide range of large and small immature female cod was also found in samples from ScIW, ScOW and ShOW, and consequently no significant maturity - length relationship could be fitted to those samples.

## Genetic Analysis

The initial analysis focused on description of the ddRAD sequencing output and the prioritised identification of candidate markers which are potentially informative for population locations.

### All Locations

In this processing scenario a total of 40 loci were considered informative (Fst ≥ 0.03) and taken forward into analysis. Single locus Fst values ranged from 0.03 – 0.08 (Table 3). No loci deviated from Hardy-Weinberg equilibrium across all populations and only one locus deviated in the majority of populations (4 pop. of 6). No loci were deemed to be outliers based on the ARLEQUIN analysis, thus we can consider this to be a "neutral" panel of markers (Excoffier *et al.,* 2010). Using these 40 loci, population segregation was possible using Fisher's probability test (Table 4). This suggested three discernible groups consisting of a common "central cluster" including ScOW, ShOW, ShIW and ShIE which is then flanked on either side by ScIW and Viking. Which is supported by the structure analysis (Figure 7) as well as a discriminant analysis of principal components (data not shown). As a whole the proportion of the total dataset variance explained by the PCA model was 75.5%, with 8.2% for PC1, 6% for PC2 and 5.6% for PC3.

### Higher Stringency Panel

In this processing scenario a total of 50 loci were considered informative (Fst >0.03) and taken forward into analysis. Single locus Fst vales were low with only 4 SNPs showing an Fst greater than 0.1 (Table 3). One locus showed departure from Hardy Weinberg disequilibrium in all populations while no loci were identified to be outliers. Using these 50 loci population segregation was possible using Fisher's probability test (Table 5). With the Shetland Inshore samples pooled, all 4 locations were distinct; when the Shetland inshore samples were segregated ShIW was comparable with ScIW and ShIE while all other comparisons were distinct. Bayesian clustering analysis showed, as with the "all locations" analysis, that ScIW and Viking are distinct however, there is evidence of finer scale structuring within the previously declared main cluster (Figure 7). When the dataset was processed using discriminant analysis of principal components ScIW and ScOW are isolated when comparing PC1 to PC2 while ShOE is isolated in PC3 (data not shown). As a whole the PCA model explained 86.4% of the total dataset variance with PC1, 2 and 3 accounting for 7.34%, 6.06% and 5.4% respectively.

SNP Analysis

Of the 90 total loci analysed from the ddRAD dataset, 13 loci - 3 from the “all locations” panel and 10 from the “high stringency” panel, were taken forward for a more in-depth genotyping analysis. These were selected based on their ability to segregate sampling locations, based on the ddRAD output, as well as technical constraints of the flanking sequence information which can restrict assay design. Among the 13 loci applied to the spawning period dataset ($n$ = 383), single locus Fst values were low, ranging from 0 – 0.033 (Table 6), with no loci being found to deviate from the Hardy-Weinberg equilibrium. Population segregation by Fischer’s probability tests distinguished Viking from all areas except ShIE, though this comparison was near significance ($p$ = 0.086) (Table 7). ScIW was also considered isolated from the Shetland offshore areas as well as ShIE, but could not be distinguished from the ScOW ($p$ = 0.109) and ShIW ($p$ = 0.064) areas. For the clustering analysis, initial K value optimisation suggested a most likely K value of 2 by both the log probability and the Evanno method. Results show a weak structuring from west to east with ScIW and Viking showing greatest divergence from the “central grouping” (Figure 7), which was supported by the Fischer’s pair-wise results.

The same markers were profiled in samples from all locations in the “Autumn” sampling window ($n$ = 306). Analysis of this dataset is complicated by the weak structure evident through the spawning season. However, single locus Fst values were low, ranging from 0 – 0.042 with all loci being considered neutral based on the Hardy-Weinberg equilibrium analysis. The Fischer’s probability tests identified significant differences in allele frequencies between the autumn and the spawning sample periods in both Viking ($p$ = 0.01) and ScIW ($p$ = 0.026) locations only. All other locations showed no significant differences in allele frequency between the two sampling periods.

## Discussion

The current genetic analysis is suggestive of finer scale structuring of Atlantic cod stocks in the IVa and VIa stock units than had previously been reported by Heath *et al*. (2014). The analysis had an iterative approach by firstly identifying novel markers within a condensed sample set that would be potentially informative for geographic origin (within the study area) and then secondly apply these markers in a wider sample series to explore evidence of structuring. A total of 90 informative SNPs were identified by this study. Population structure analysis within the limited dataset used for marker identification alluded to fine scale structuring that may differentiate ScIW and Viking from a common admixed cluster ranging from ShIE to ScOW. When a wider sample set was investigated using 13 SNPs the evidence of structuring during the spawning season was weaker than the ddRAD suggested. However, there was still a differentiation of the ScIW and the Viking sample locations from the common admixed area with the Viking location appearing most distinct from all other sample sites. This weak structuring during the spawning season complicated the interrogation of the autumn sample set where the intention had been to look for evidence of stock mixing. As such only ScIW and the Viking locations showed a significant difference in allele frequencies between the two sample points. This is suggestive of mixing in these two locations between the autumn and spawning sample periods. The lack of evidence of detectable structuring in the other locations during spawning, negates the possibility to assess for mixing in the autumn samples from ScOW, ShOW, ShIW and ShIE.

Consistent with previous studies of maturation, cod from Viking sampled in 2014 matured at a later age and larger size than other areas (Yoneda & Wright, 2004; Wright *et al*., 2011). Maturity in cod is positively influenced by autumn temperature (Yoneda & Wright, 2005a, b) which possibly explains why cod on the west coast mature so early. The present day maturity schedule of west coast cod is similar to that reported for the 1970s and early 2000s (Yoneda & Wright, 2004). In contrast, an analysis of historic trends in North Sea cod populations that took account of regional temperature differences found that the current early and small size at maturity seen in shallow water cod such as ShIE and ShIW reflects a downward shift in the maturation reaction norm since the 1970s (Wright *et al*., 2011). Hence, present day Viking cod are the only North Sea population to reflect the historic maturation schedule and the maturity ogive used in the ICES North Sea cod assessment up until 2015. Due to the population level differences in maturity at age the ICES NSSK (2015) workshop proposed a change to the maturity age key used to estimate SSB that weights proportion mature by population abundance.

Genetic and maturity evidence from this study seems consistent with a reproductively isolated 'Viking' cod population. Importantly, the new data greatly help to define the distributional extent of this population. During spawning time there was no indication that the Viking group extended beyond the > 100 m waters of the northern North Sea. Indeed, the new genetic and maturity evidence suggests that Shetland coastal cod (ShIE) appear to extend into waters > 100 m east of Shetland. This means that the population model of Heath *et al*. (2014) is likely to have included some coastal cod. Indeed, that study assumed that all cod from ICES roundfish area 1 would have a maturity-size relationship reflective of Viking. As ICES roundfish area 1 includes not only the present Viking stations but also ShOW, ShIW and ShIE it is not surprising that the reported length at 50% maturity had fallen to 48 cm by 2006 in the Heath *et al*. (2014) study. In contrast, the 73 cm length at 50% mature for Viking cod in the present study was comparable to that reported by Wright *et al*. (2011) for just the Viking population area.

The indication that ScIW cod may be genetically distinct from other groups could explain the limited dispersal of tagged cod from that region (Wright *et al*., 2006a) and that >90% of 0-group cod from nursery areas recruited locally (Wright *et al*., 2006b). The possible separation of a ScIW from ScOW is also reminiscent of the inshore-offshore division seen in the northern North Sea.

The limited genetic evidence of mixing in autumn is in agreement with the maturation evidence of mixing of populations outside the breeding season as large immature cod were present in ScIW, ScOW and ShOW. This suggests that the distributional range of Viking cod may extend westwards particularly along the edge of the continental shelf. The maturity - length relationship of ShIE cod in autumn was also different to that during spawning being characteristic of Viking cod. However, this probably may not reflect a shift in Viking distribution as the samples were taken further north than the spawning sample. In contrast to these changes to the composition of small and large immature cod there was no significant change in the maturity - length relationship of ShIW cod. The autumn and spawning samples came from the same site (Scalloway Deep) and electronic tagging of this cod group has indicated that they show high site fidelity (Neat *et al*., 2006). Based on the maturity information it is unlikely that there is a complete seasonal shift in population distribution as there was no change for Viking and ShIW and other autumn samples were comprised of both small and large immature cod consistent with mixing rather than a south - westward population migration.

The present study has considerably expanded our understanding of the Viking cod from northern IVa and when combined with the studies by Poulsen *et al*. (2011) and Heath *et al*. (2014) it provides a good indication of population extent at spawning time and suggests a split around 0030 W (Figure 8). Importantly, at this time there appears to be no overlap with VIa. However, whilst preliminary, the maturity data do suggest a westward extension of Viking cod distribution outside the spawning period.

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Tables & Figures

**Table 1.** Summary of maturity and length data collected for both males and females at all sites during the autumn and spring sampling periods.

| TIME PERIOD | AREA | SEX | n | PROPORTION MATURE | MEAN LENGTH | S.E. |
|---|---|---|---|---|---|---|
| **Nov/Dec** | **ScIW** | **m** | 25 | 0.72 | 49.2 | 2.5 |
| | | **f** | 25 | 0.84 | 55.1 | 3.5 |
| | **ScOW** | **m** | 100 | 0.94 | 64.4 | 1.4 |
| | | **f** | 186 | 0.73 | 61.0 | 0.8 |
| | **ShIE** | **m** | 31 | 0.58 | 61.3 | 2.2 |
| | | **f** | 57 | 0.79 | 70.5 | 1.5 |
| | **ShIW** | **m** | 50 | 0.80 | 62.4 | 1.2 |
| | | **f** | 149 | 0.90 | 66.0 | 0.9 |
| | **Viking** | **m** | 19 | 0.68 | 69.4 | 3.0 |
| | | **f** | 29 | 0.62 | 65.8 | 3.1 |
| | **ShOW** | **m** | 21 | 0.86 | 59.7 | 2.2 |
| | | **f** | 27 | 0.52 | 58.8 | 1.3 |
| **Feb/Mar** | **ScIW** | **m** | 92 | 0.76 | 58.7 | 1.6 |
| | | **f** | 136 | 0.68 | 64.0 | 1.8 |
| | **ScOW** | **m** | 12 | 1.00 | 54.2 | 2.1 |
| | | **f** | 60 | 1.00 | 64.6 | 1.4 |
| | **ShIE** | **m** | 50 | 0.74 | 65.7 | 1.5 |
| | | **f** | 150 | 0.88 | 66.7 | 1.0 |
| | **ShIW** | **m** | 50 | 0.72 | 54.9 | 1.5 |
| | | **f** | 150 | 0.55 | 59.7 | 1.0 |
| | **Viking** | **m** | 23 | 1.00 | 49.2 | 3.5 |
| | | **f** | 27 | 0.37 | 57.9 | 4.4 |
| | **ShOW** | **m** | 25 | 1.00 | 56.7 | 2.0 |
| | | **f** | 25 | 0.96 | 56.6 | 2.3 |

**Table 2.** GLM model coefficients, standard errors, z values and significance of effects of age, area and age:area interaction on cod length.

| Term | Estimate | Std. Error | t value | Pr(>\|t\|) |
|---|---|---|---|---|
| **(Intercept)** | 13.8357 | 1.5262 | 9.065 | < 2e-16 |
| **Age** | 13.6888 | 0.4227 | 32.384 | < 2e-16 |
| **factor(ScOW)** | 15.6327 | 3.3435 | 4.676 | 3.45e-06 |
| **factor(ShIE)** | 13.4746 | 2.4323 | 5.540 | 4.12e-08 |
| **factor(ShIW)** | 15.9101 | 2.0258 | 7.854 | 1.31e-14 |
| **factor(Viking)** | -2.4832 | 2.9133 | -0.852 | 0.39427 |
| **factor(ShOW)** | 16.9304 | 3.3440 | 5.063 | 5.14e-07 |
| **age:factor(ScOW)** | -3.7117 | 0.9602 | -3.865 | 0.00012 |
| **age:factor(ShIE)** | -3.5326 | 0.6511 | -5.425 | 7.68e-08 |
| **age:factor(ShIW)** | -4.9172 | 0.5688 | -8.645 | < 2e-16 |
| **age:factor(Viking)** | 0.3206 | 0.8583 | 0.374 | 0.70882 |
| **age:factor(ShOW)** | -5.5616 | 0.9994 | -5.565 | 3.58e-08 |

**Table 3.** Summary of locus Fst frequency distribution for the ddRAD analysis.

| Fst | No. |
|---|---|
| ***All locations panel*** | |
| **>0.07** | 3 |
| **0.06 – 0.07** | 2 |
| **0.05 – 0.06** | 4 |
| **0.04 – 0.05** | 6 |
| **0.03 – 0.04** | 25 |
| ***"High stringency" panel*** | |
| **>0.07** | 3 |
| **0.06 – 0.07** | 2 |
| **0.05 – 0.06** | 4 |
| **0.04 – 0.05** | 6 |
| **0.03 – 0.04** | 25 |

**Table 4.** Summary of Fisher's exact probability test of pairwise comparisons of populations using the “all locations” ddRAD panel according to Raymond & Rousset, (1995). Significant pairwise differences are indicated by *.

| Population pair | $Chi^2$ | df | P-Value |
|---|---|---|---|
| **ScIW & ScOW*** | 160.7238 | 78 | 0 |
| **ScIW & ShIE*** | 146.3031 | 80 | 0.000009 |
| **ScOW & ShIE** | 81.23598 | 76 | 0.319492 |
| **ScIW & ShIW*** | 136.4423 | 80 | 0.000086 |
| **ScOW & ShIW** | 92.32437 | 80 | 0.163432 |
| **ShIE & ShIW** | 95.0154 | 78 | 0.0923 |
| **ScIW & Viking*** | 155.6585 | 78 | 0 |
| **ScOW & Viking*** | 132.1057 | 80 | 0.000223 |
| **ShIE & Viking*** | 175.2926 | 80 | 0 |
| **ShIW & Viking*** | 137.6236 | 80 | 0.000066 |
| **ScIW & ShOW*** | 153.0484 | 80 | 0.000002 |
| **ScOW & ShOW** | 92.40974 | 80 | 0.161924 |
| **ShIE & ShOW** | 79.59016 | 76 | 0.366689 |
| **ShIW & ShOW** | 97.11887 | 76 | 0.051671 |
| **Viking & ShOW*** | 127.1095 | 80 | 0.000633 |

**Table 5.** Summary of Fisher's exact probability test of pairwise comparisons of populations according to Raymond & Rousset, (1995) using the "Higher stringency panel" from the ddRAD analysis with Shetland inshore samples pooled (top) and separated (bottom). Significant pairwise differences are indicated by *.

| Population pair | $Chi^2$ | df | P-Value |
|---|---|---|---|
| **ScIW & ScOW*** | 152.2672 | 100 | 0.000593 |
| **ScIW & ShIW/E*** | 146.6885 | 100 | 0.001644 |
| **ScOW & ShIW/E*** | 187.9149 | 196 | 0 |
| **ScIW & Viking*** | 142.5286 | 100 | 0.003378 |
| **ScOW & Viking*** | 181.7205 | 98 | 0.000001 |
| **ShIW/E & Viking*** | 189.8842 | 100 | 0 |

| Population pair | $Chi^2$ | df | P-Value |
|---|---|---|---|
| **ScIW & ScOW*** | 152.0939 | 100 | 0.000612 |
| **ScIW & ShIE*** | 141.322 | 100 | 0.004134 |
| **ScOW & ShIE*** | 180.9773 | 96 | 0 |
| **ScIW & ShIW** | 87.29053 | 100 | 0.813853 |
| **ScOW & ShIW*** | 127.7331 | 94 | 0.011825 |
| **ShIE & ShIW** | 84.64239 | 88 | 0.581582 |
| **ScIW & Viking*** | 143.4781 | 100 | 0.002875 |
| **ScOW & Viking*** | 181.8705 | 98 | 0.000001 |
| **ShIE & Viking*** | 167.3609 | 100 | 0.000028 |
| **ShIW & Viking*** | 123.4184 | 98 | 0.04222 |

**Table 6.** Summary of individual locus as well as overall Fst values for 13 SNP markers as assessed in the spawning period (*n* = 371).

| **Locus** | **$F_{st}$** |
|---|---|
| GM_ALL120_1219 | 0.0329 |
| GM_All120_1400 | -0.0017 |
| GM_ALL120_2904 | 0.0165 |
| GM_HS60_276 | 0.0040 |
| GM_HS60_2938 | 0.0052 |
| GM_HS60_3664 | -0.0039 |
| GM_HS60_4684 | 0.0132 |
| GM_HS60_4814 | -0.0027 |
| GM_HS60_5331 | 0.0283 |
| GM_HS60_5602 | -0.0041 |
| GM_HS60_6695 | -0.0017 |
| GM_HS60_7086 | 0.0022 |
| GM_HS60_7185 | 0.0043 |
| **All Loci** | 0.0075 |

**Table 7.** Summary of the Fischer's exact probability test of pairwise comparisons of populations from the spawning period using the 13 SNP loci (*n* = *371*). Significant pairwise differences are indicated by *.

| POPULATION PAIR | | | $CHI^2$ | DF | P-VALUE |
|---|---|---|---|---|---|
| **ScIW** | **&** | **ScOW** | 35.14217 | 26 | 0.109 |
| **ScIW** | **&** | **ShIE*** | 49.074322 | 26 | 0.004 |
| **ScOW** | **&** | **ShIE** | 37.494085 | 26 | 0.067 |
| **ScIW** | **&** | **ShIW** | 37.777335 | 26 | 0.064 |
| **ScOW** | **&** | **ShIW** | 33.392896 | 26 | 0.151 |
| **ShIE** | **&** | **ShIW** | 19.987422 | 26 | 0.792 |
| **ScIW** | **&** | **ShOW *** | 39.167656 | 26 | 0.047 |
| **ScOW** | **&** | **ShOW** | 18.711906 | 26 | 0.848 |
| **ShIE** | **&** | **ShOW** | 15.508253 | 26 | 0.947 |
| **ShIW** | **&** | **ShOW** | 25.452188 | 26 | 0.494 |
| **ScIW** | **&** | **Viking *** | Infinity | 26 | 0.000 |
| **ScOW** | **&** | **Viking *** | 50.089517 | 26 | 0.003 |
| **ShIE** | **&** | **Viking** | 36.342655 | 26 | 0.086 |
| **ShIW** | **&** | **Viking *** | 45.99028 | 26 | 0.009 |
| **ShOW** | **&** | **Viking *** | 40.932504 | 26 | 0.032 |

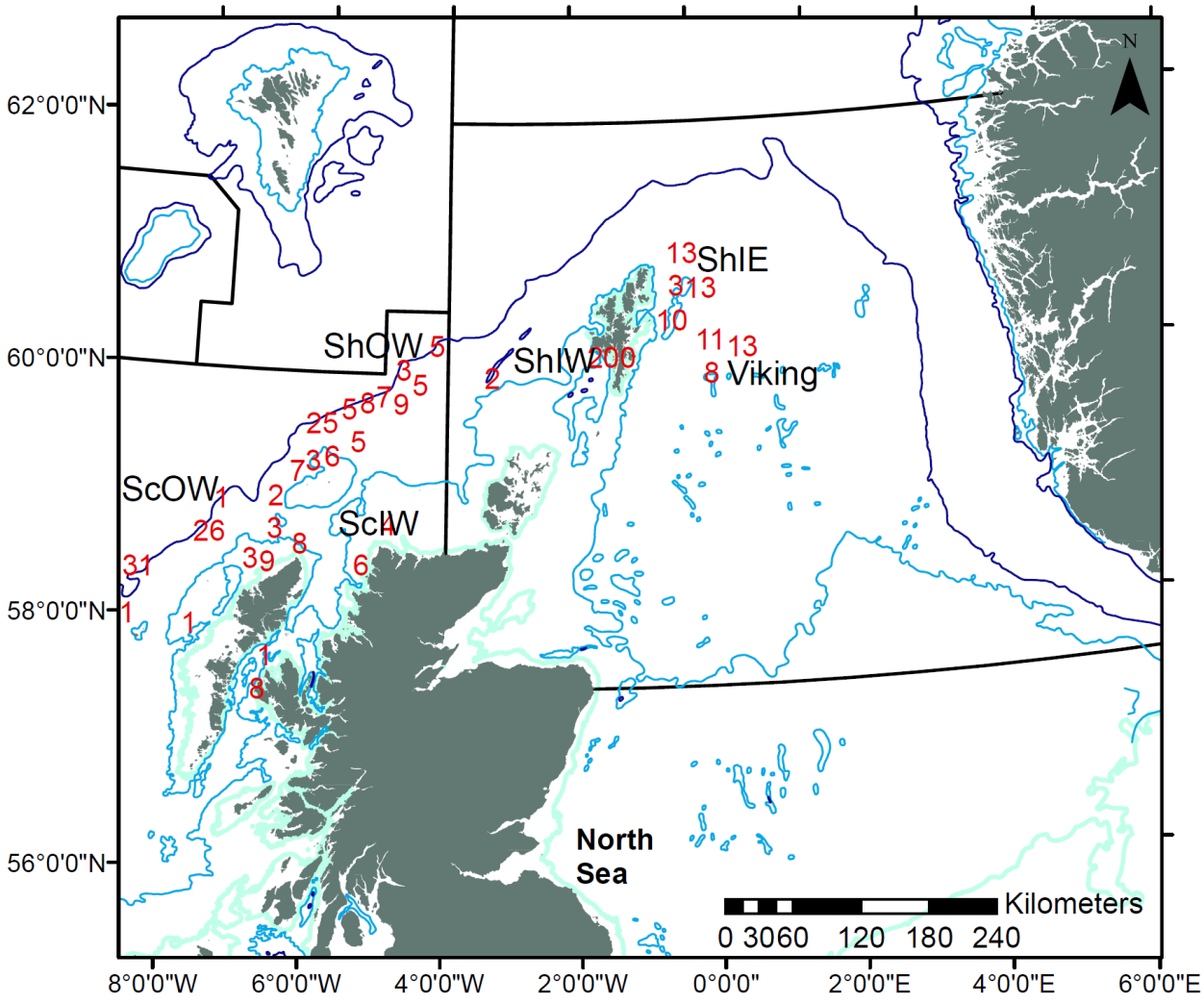



**Figure 1:** Sites sampled in November and December 2013. Numbers refer to total adult cod per site. Shetland Inshore West (ShIW), Shetland Offshore West (ShOW), Shetland Inshore East (ShIE), Shetland Offshore East (Viking), Scotland Offshore West (ScOW), and Scotland Inshore West (ScIW).

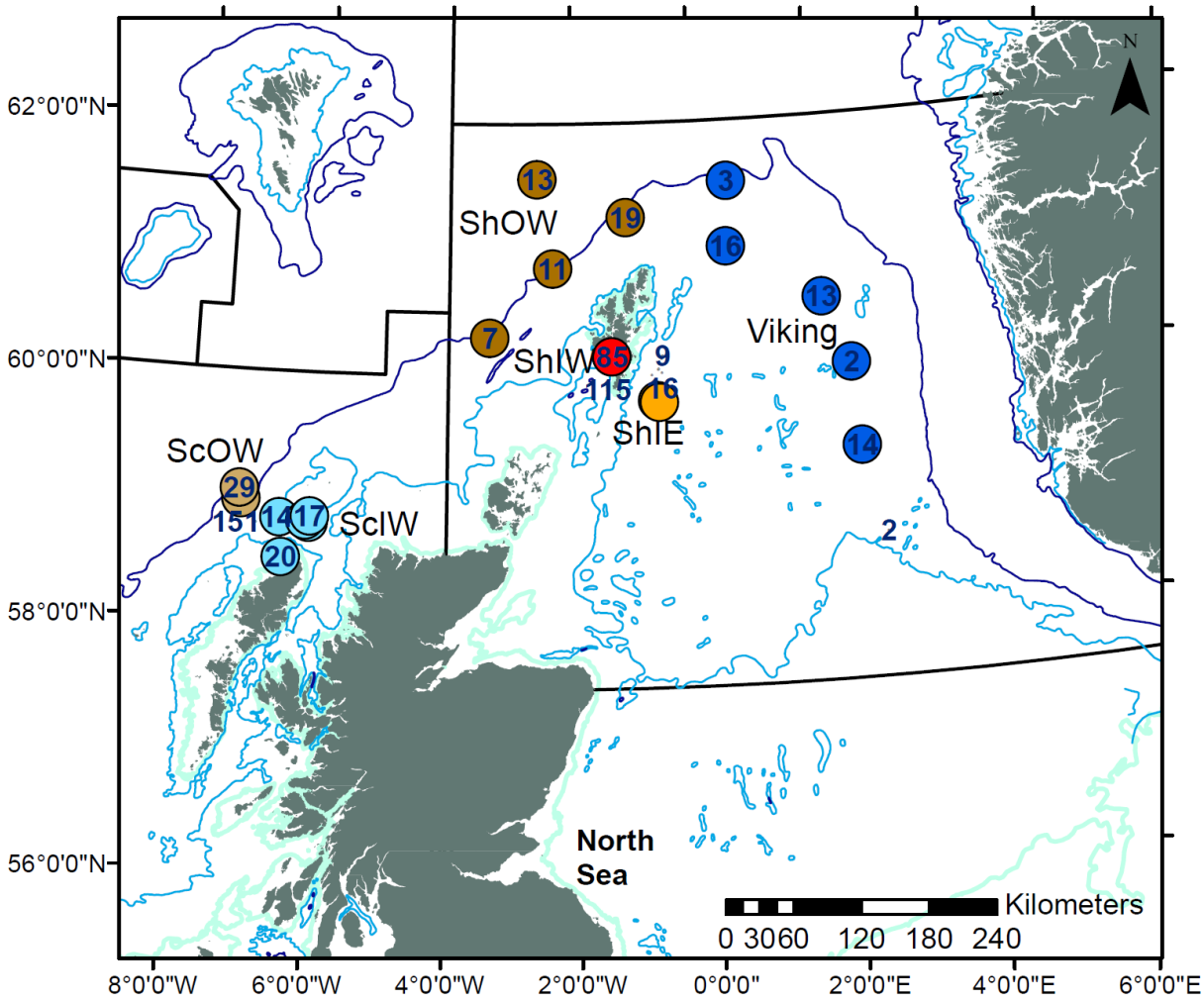



**Figure 2:** Sites sampled January to March 2014. Numbers refer to total adult cod per site. Coloured spots indicate location of genetic samples analysed.

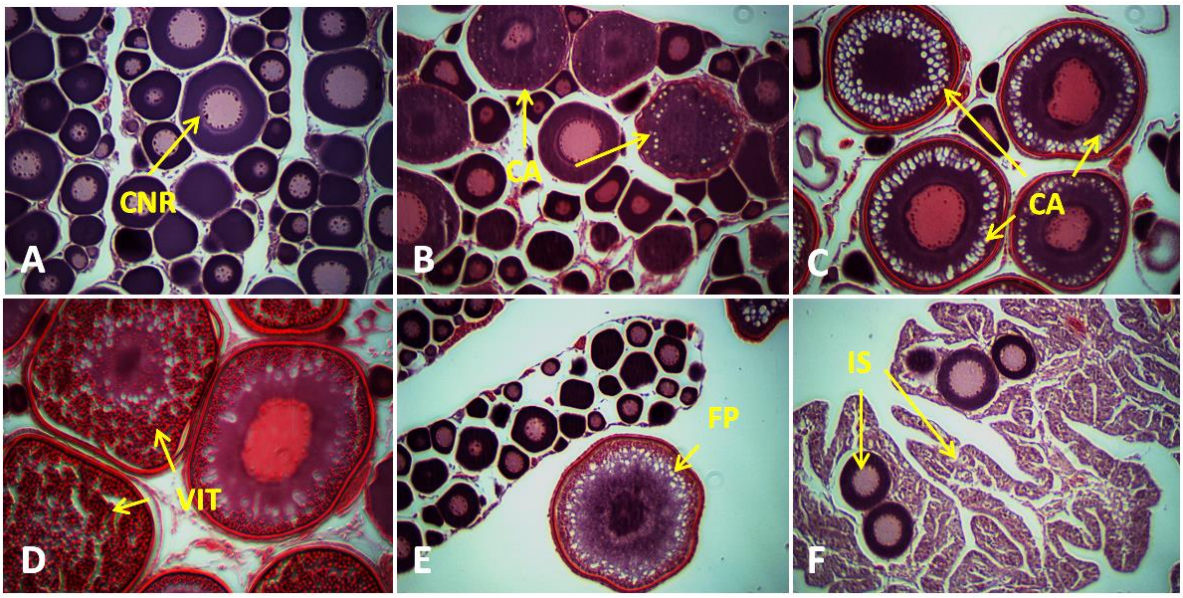



**Figure 3:** Histology stages present within autumn samples (x100 magnification). A) early CNR stage oocytes, B) late CNR and early CA oocytes, C) late stage CA, and D) shows the vitellogenic (VIT) stage of oocyte development. Image E) highlights a false positive (FP) where a small number of oocytes appear to be developing (< 10 %). This may be caused by carry over during sectioning. Image F) shows an unusual individual with both male and female cells. Macroscopically these individuals are usually classified as female (see Bucholtz *et al*., 2007). However, intersex individuals accounted for < 0.05 % of the dataset and were omitted from any analyses.

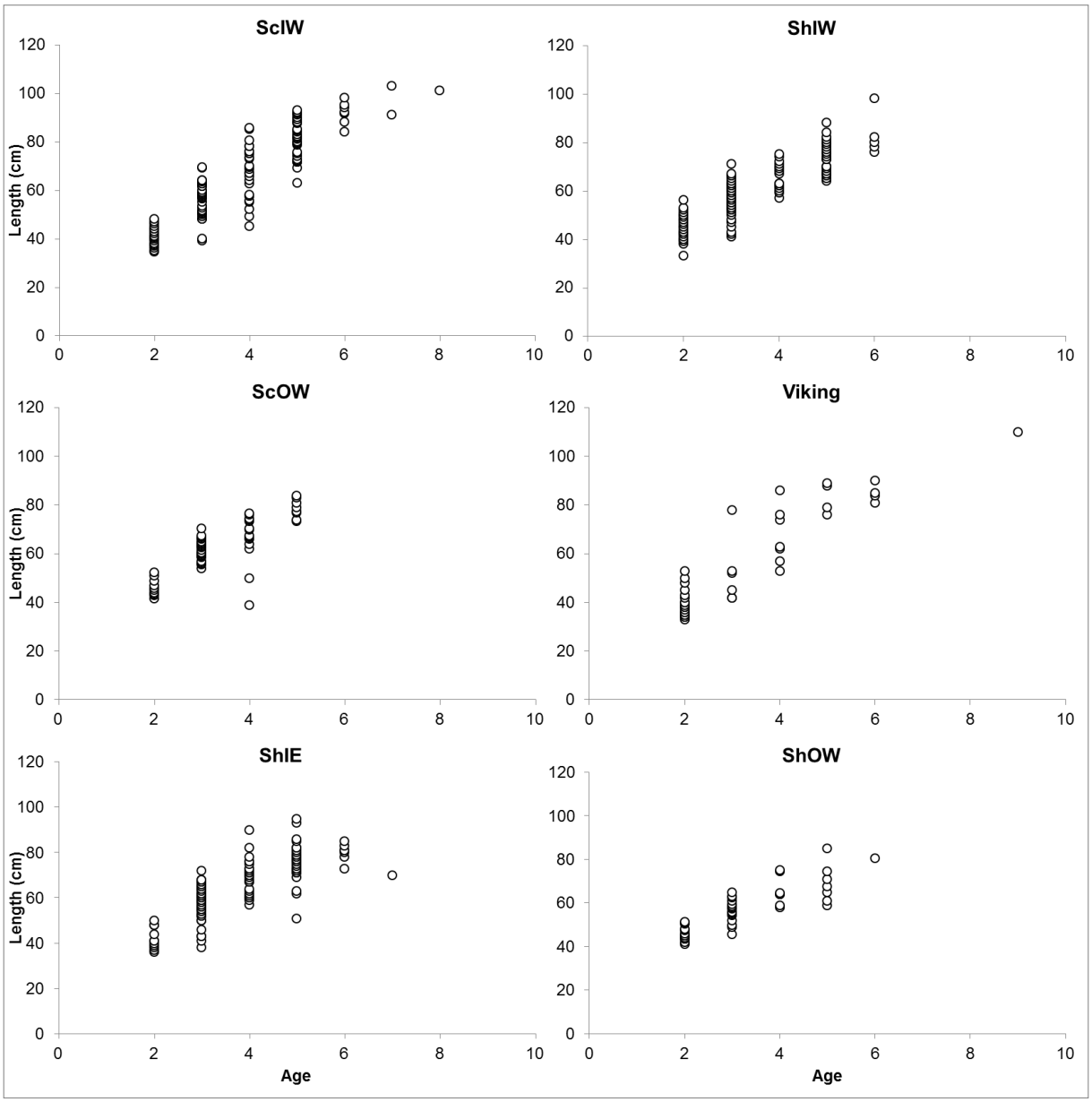



**Figure 4:** Length ranges for each age represented at each area during the Feb/Mar time period.

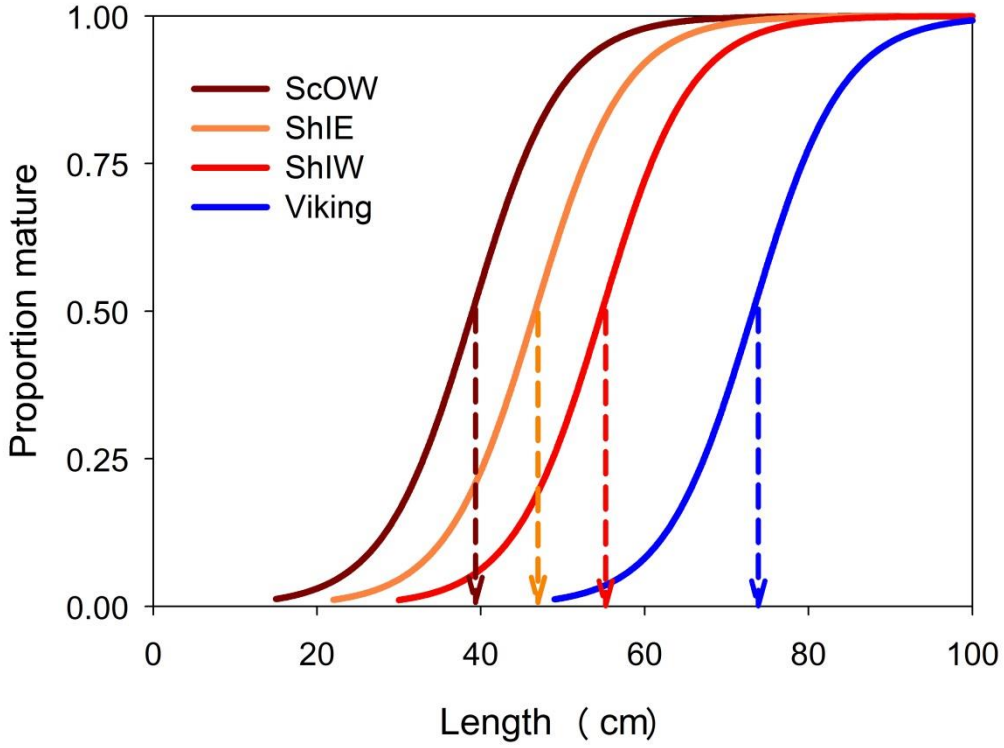



**Figure 5:** Predicted proportion mature at length for age 3 females from 4 sample areas in the Feb/Mar samples, based on model coefficients in **Table 4**.

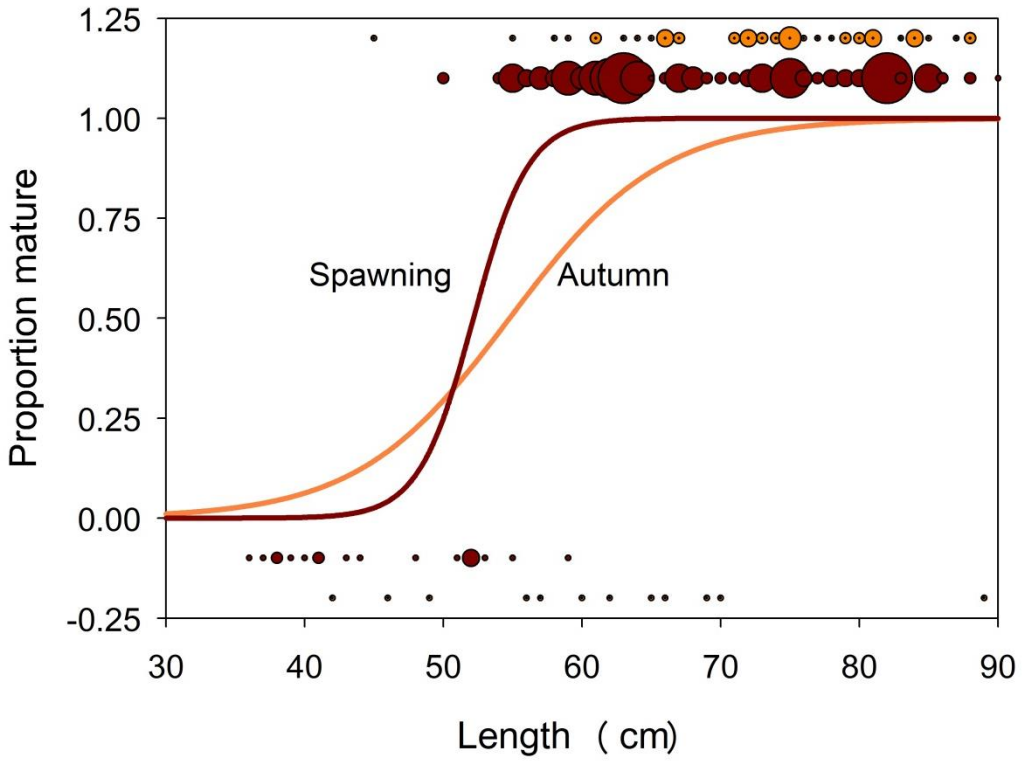
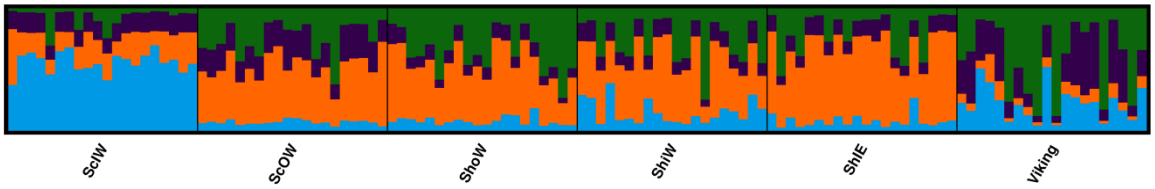
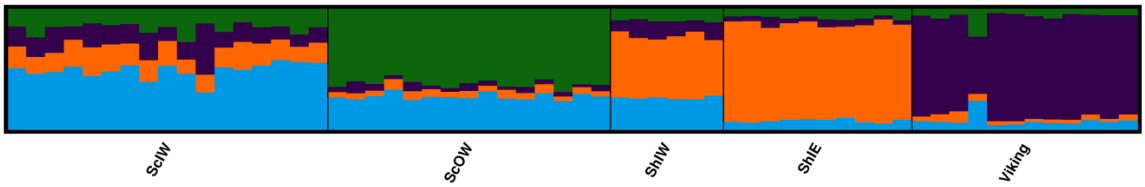



**Figure 6:** Predicted proportion mature at length for female cod from ShIE for the spawning and autumn sample periods. Circles represent the numbers at length of immature (below 0.00) and mature (above 1.00) females upon which the fit is based.

**a)**




**b)**




**c)**

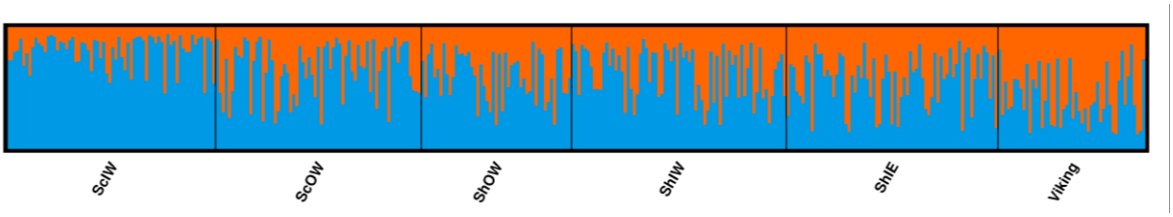



**Figure 7:** Results from clustering analysis for the spawning season sampling for a) the ddRAD "all locations" analysis (K=4), b) the ddRAD "high stringency" analysis (K = 4) and c) the 13 loci SNP analysis (K=2). Clustering was generated using STRUCTURE.

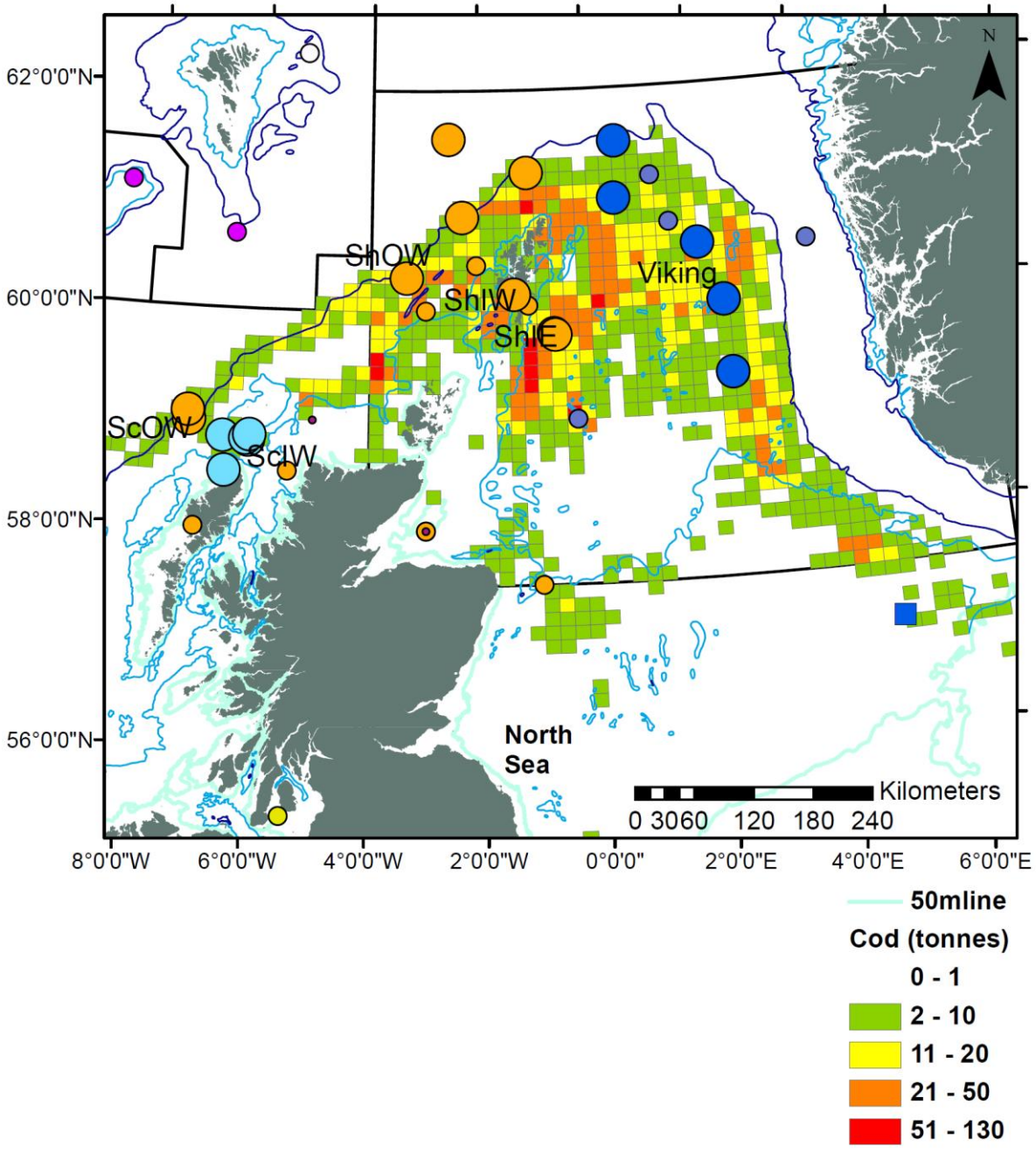



**Figure 8:** Location of population groups from SNP evidence. Large circles refer to results from the current study, small circles refer to Heath *et al*. (2014) samples and square refers to Poulsen *et al*. (2011). Dark blue = Viking, orange = shallow water deme, light blue = new structuring indicated in ScIW by this study. Population samples are overlaid on estimated landings per 1/16$^{th}$ ICES rectangle in 2011 to show approximate distribution of major fishery.

Appendix 1

FISA Cod Genetics Project – Sampling Protocol

**Purpose:**

The purpose is to collect biological data and samples from adult cod (>20cm) so that analysis can determine genetically whether sub-populations separate out to spawn or remain mixed as occurs during feeding periods.

**Equipment required:**

At sea

- Measuring board ( measuring to 0.5cm)
- Sampling sheets
- Sample vials (labelled & pre-filled with ethanol for gill samples)
- Sample vials (labelled & pre-filled with 10% NBF for gonads)
- Sealable bags (for ovaries)
- Scissors
- Tweezers/Forceps
- Tissue & Ethanol/Ethanol wipes

**Instructions:**

We require the following samples/measurements from **100 adult female cod** and **50 adult male cod** (>20cm) from each location (inshore VIa & offshore VIa)

For each fish:

- **Measure** the total length of the fish to the nearest 0.5cm
- **Sex & Stage gonads**
- **Females only – take a 1cm$^3$ sub-sample of ovary and** place in pre-labelled vial containing 10% NBF. For minimum 50 individuals place the ovary remains into a sealable plastic bag (provided, pre-labelled) and store cold/frozen. If ovary is exceptionally large only keep one lobe. These will be weighed back at marine lab to allow for fecundity analysis
- **Remove 1 otolith** and place in pre-labelled envelope

- **Take ~1cm$^2$ gill sample** to avoid cross contamination, gill sample must be taken using aseptic techniques. Please rinse tweezers and scissors in ethanol and fully clean using an ethanol soaked tissue between each fish

**Notes:**

All samples/measurements must be fully traceable back to the fish from which they came. Provided are sampling sets for each fish (1 envelope, 1 sealable bag for gonad, 1 vial with 10% NBF of ovary samples & 1 vial with 100% ethanol for gill) which have been pre-labelled with a 6-digit code. Also provided are sampling sheets. For each fish, please choose a sample set. The code from this sample set can then be written in to the "sample id" column on the sample sheets along with the other measurements taken for that fish.

Ovary samples are to be taken where possible to allow for fecundity analysis. A minimum of 50 samples are required from each site (inshore and offshore) for a complete analysis. These samples should be bagged and sealed in the bags provided, and frozen or chilled immediately after sampling, so they can be returned to the lab to be weighed on a sensitive balance. Prior to bagging, please take a sub-sample of the female gonad tissue (~1cm$^2$) and place it, along with its label (provided inside kit bag) into an unmarked vial filled with 10% NBF (provided). Please do not forget the label – if it is missing, please mark the vial with the sample id using permanent marker.

Vials containing the gill and ovary samples can be stored upright in a cold room (not frozen). Otolith envelopes can be placed in a bag and stored at room temperature.