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Temporal isolation between two strongly differentiated stocks of the Greenland halibut (*Reinhardtius hippoglossoides* Walbaum, 1792) from the Western Barents Sea

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Abstract: Identification of discrete stocks of Greenland halibut is an important aspect of proper fisheries exploitation. Available literature data indicated a lack of significant inter-area differences between Greenland halibut populations from the Northeast Atlantic. To define the population diversity, two aspects were taken into account: enzyme-genetic diversity and the concentration of heavy metals in tissues. Seven allozyme loci variations were used to characterize the genetic structure of four populations of Greenland halibut from the Western Barents Sea region. The samples were collected from the spawning area in the period when this species took migration to spawn. The sample RH4 was significantly different from the other samples collected from the same location (RH2 and RH3) and depth for over two days. Another sample (RH8), collected from the nearby area 6 days later was similar to the samples RH2, RH3. We noticed a significant divergence between the sample RH4 and the three remaining samples, where the value of the index F_{ST} fluctuated about 0.40 and approximately 0.01 between three similar populations. This genetic fluctuation negates the thesis of a panmictic character of the Western Atlantic population.

Feeding groups of Greenland halibut are moving along the Barents Sea shelf and they are exposed to different heavy metals concentrations according to the food preferences or the exact place of feeding. We identified similar concentrations of heavy metals, i.e., Zn, Cu, Cd, and Pb in all samples. Trace metal analysis of aquatic organisms from the Barents Sea can provide important information on the degree of environmental contamination, and the potential impact of seafood consumption.

Keywords: Arctic, allozymes, discrete stocks, genetic diversity, heavy metals.

Introduction

Greenland halibut (*Reinhardtius hippoglossoides*) occurs in the North Atlantic (Bowering and Nedreaas 2001), at a depth of about 200 to 1500 meters (Jorgensen 1997). In the Barents Sea, along the shelf, Greenland halibut occurs in three different water masses: cold surface water (to the 200 m depth), warm Atlantic water between 200–900 m depth, and cold near-bottom water (from 900 m depth to the bottom) (Løyning 2001; O'Dwyer *et al.* 2001).

Migrations of Greenland halibut depend on gender and degree of maturity (Albert 2003). In the North Atlantic Ocean, a seasonal migration between feeding and spawning areas of the Greenland halibut (Jørgensen 1997; Albert 2003), can be involved in the phenomenon of panmixia (Roy *et al.* 2014). In the Barents Sea, the spawning ground of the Greenland halibut is stretched along the continental slope between the 71º and 75º north latitudes (Nizovtsev 1991). The peak spawning occurs during the winter and summer periods (Albert *et al.* 2001).

Consequently, species with planktonic larvae in their life cycle (which is transported by sea or ocean currents at long distances) should be relatively genetically homogeneous. Non-neutral molecular markers such as allozymes allow insight into the adaptive aspect of genetically homogeneous populations, depending on their habitat. Allozymes are an important part of several mechanisms that regulate and protect the homeostasis of cells or tissues.

Many aquatic species characterize low levels of polymorphisms enzyme (Frank-Lawal 2005). Additionally, the effect of lack of geographical barriers in marine ecosystems is gene flow between populations and weak structure (Ward 2000). This is congruent with the observation of Vis *et al.* (1997) and Igland and Nevdal (2001) who reported that the entire Atlantic population of Greenland halibut is genetically homogenous and panmictic. Nevertheless, there can be some environmental factors of isolation like salinity and temperature gradient (Nielsen *et al.* 2003) which could affect the structure of subpopulations. This observation was supported by Fairbarn (1981) who reported that genetic variation at allozyme loci suggests the existence of two separate subspecies of Greenland halibut in the Pacific and Atlantic Oceans (The Bering Sea *vs* The Gulf of St. Lawrence and The Northwest Atlantic). Environmental factors often limit the gene flow between populations and in a sufficiently long time can lead to the emergence of physiological races, populations, and subspecies. Most recently, Westgaard *et al*. (2017) performed an analysis of genetic population structure in Greenland halibut from the Atlantic area using Single Nucleotide Polymorphisms (SNP) markers derived from Restriction-site Associated DNA (RAD-seq) sequencing. The Authors indicated a weak but significant population structure of Greenland halibut and hypothesized that it could be connected with the egg and larval drift as well as migratory behavior. These results are also crucial to proper fishery management. Feeding groups of Greenland halibut are moving along the Barents Sea shelf and they are exposed to different heavy metals

concentrations according to the food preferences or the exact place of feeding. Trace metal analysis of aquatic organisms from the Barents Sea can provide important information on the degree of environmental contamination, and a potential impact of seafood consumption. Predominant inputs to the Arctic occur by long-range transport, via oceanic water mass exchanges and atmospheric processes, or run-off from land and industrial emissions (Macdonalds and Bewers 1996). These contaminations, deposited by numerous processes undoubtedly influence marine fauna.

Cadmium (Cd) has only been produced commercially in the twentieth century. It is a by-product of the zinc industry; its production is thus determined essentially by that of zinc. According to Food and Agriculture Organization of the United Nations (FAO) datasheet the concentration of cadmium in fish is in the range of 1-50 μg/kg. Marine Teleosts incorporating cadmium by drinking seawater and eating the invertebrates (Petri and Zauke 1993) accumulate this metal mainly in the liver (Miliou *et al.* 1998) but also in muscles. According to a special way of intake concentrations of this metal were used as main/ environmental reference in a statistical analysis of correlations between concentrations of copper (Cu) and zinc (Zn). Cadmium occurs mainly in association with the sulphide ores of zinc, lead, and copper. Trace metal contamination of the Greenland halibut may have a significant impact on this organism, potentially contaminating the marine food chain. The lead (Pb) isotopic compositions of aquatic organisms may further assist the identification of possible sources of contamination and biological pathways.

Decomposition of solid samples is an important step in combined analytical methods. In most cases, when using highly sensitive measuring methods, such as Flame Atomic Absorption Spectrophotometry (FAAS), graphite furnace AAS (electrothermal atomic absorption spectrometry – ETAAS), Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), Inductively Coupled Plasma Mass Spectrometry (ICP-MS), or inverse voltammetry, the sample is measured in an aqueous solution (Knapp 1991). Combined analytical methods are favoured for multi-element analysis of environmental and biological samples at very high speed. Sequential and simultaneous determinations of the elements can be made using the above analytical techniques. Heavy metal (Zn, Cd, Cu, Mn, Pb, Fe, Ni) concentrations have been determined in fish and biological samples using FAAS and ICP-OES (Zhuang *et al.* 1995; Karadede and Ünlü 2000; Cid *et al.* 2001; Chen *et al.* 2000).

Graphite furnace atomic absorption spectrometry (ETAAS) is one of the suitable methods for the determination of trace metals in food and biological samples because of its speed, minimum need for sample preparation, the possibility of automation, good sensitivity, and low detection limit (Blust *et al.* 1988; Lynch and Littlejohn 1989; Bin and Zhe-Ming 1996; Acar *et al.* 2000; Doner and Akman 2000; Huang *et al.* 2000; Acar 2001; Cid *et al.* 2001; Tüzen 2003; Mendil *et al.* 2005; Detcheva and Grobecker 2006; Vitak and Volynsky 2006). However, the

determination of heavy metals in fish samples by ETAAS is difficult because the influence of a complicated matrix greatly affects the analytical results. Therefore, different chemical modifiers are used for the stabilization of the analyte.

The research aimed to determine the possibilities of identification discrete stocks of the Greenland halibut in the same spawning area in a short period (in connection with the appearance of moving shoals) on the level of non-neutral (adaptive) molecular markers and heavy metals.

Materials and Methods

The samples were collected during the commercial cruise at the beginning of the Greenland halibut spawning migration. Two samples from the same location and two other samples nearby were collected in a short period. Sampling details are outlined in Table I and Fig. 1 (see also Karpiej *et al.* 2013 – presented probes were acquired together with our samples during the same commercial cruise of M/Tr *Polaris*). All of the individuals were identified morphologically

Table 1.

Collection information for *Reinhardtius hippoglossoides* sampled in 2006 from Western Barents Sea region.

Population	No. of individuals	Date	Beginning of trawl		End of trawl		Depth
			φ	λ	φ	λ	d (m)
RH2	66	9 October	74°52'N	15°30'E	75°05'N	15°16'E	670–723
RH3	70	10 October	74°50'N	15°36'E	74°39'N	15°56'E	545–590
RH4	19	11 October	74°51'N	15°35'E	74°38'N	15°59'E	596–575
RH8	52	17 October	75°58'N	14°01'E	76°18'N	14°22'E	652–656

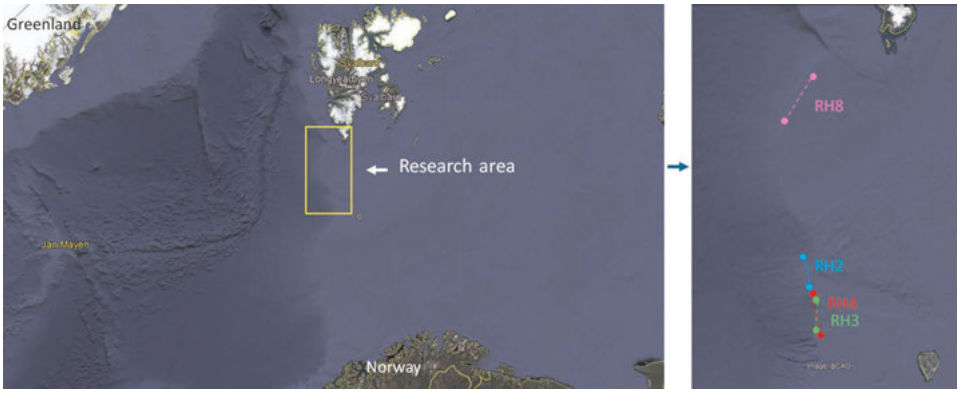


Fig. 1. Study area: Western Barents Sea region, spring 2006. Abbreviations of trawls: sample RH2 – blue line, sample RH3 – green line, sample RH4 – red line, sample RH8 – violet line. Dots indicate beginning and end of trawl.

to specimens of Greenland halibut. The collected individuals from each sample were similar in terms: age (10–16 years), a spectrum of weight (0.55–2.75 kg), a spectrum of a Condition Factor (0.504–1.160). Part of individuals from each haul was checked for sex (predominance of males 65:35) and maturity of gonads (mixed 2–4 stage). The samples about 2 cm^3 cuts of white muscle and liver were immediately fixed and transported in dewar with liquid nitrogen. In a laboratory, the samples were stored at -80º C until analyses.

To identify allozyme loci variation electrophoresis on cellulose acetate plates was carried out according to standard protocols (Hebert and Beaton 1989). Interpretations of zymogrames and characteristics of allozymes were followed by genetic nomenclature for protein-coding loci (Richardson *et al.* 1986; Buth 1990). Some of the isoenzymes were reported in the literature as the most differentiating for the Greenland halibut and thus the most effective for the present analysis, i.e., Igland and Nævdal (2001) studied 20 putative loci and only three of them were polymorphic at the 0.95 criterion: GPI-1, IDHP-2, and PGM. In our study, in total seven enzyme systems were stained: alcohol dehydrogenase (ADH, E.C.1.1.1.1), adenylate kinase (AK, E.C.2.7.4.3), glucose-6-phosphate isomerase (GPI, E.C.5.3.1.9), isocitrate dehydrogenase (IDH, E.C.1.1.1.42), lactate dehydrogenase (LDH, E.C.1.1.1.27), 6--phosphogluconate dehydrogenase (6PGDH, E.C.1.1.1.44) and phosphoglucomutase (PGM, E.C.2.7.5.1).

The observed heterozygosity (H_O) and expected heterozygosity (H_E), the number of private alleles were estimated using GenAlEx software (Peakall and Smouse 2006). This program was also used to perform Principal Component Analysis (PCA). *F*-statistics and tests of conformity to Hardy-Weinberg equilibrium were calculated in Arlequin v.3.5 software (Excoffier and Lischer 2011). Alpha-levels were adjusted using the Bonferroni correction for multiple comparisons (Rice 1989).

Similarity relationships between populations were reconstructed using the Biosys-1 software package (Swofford and Selander 1981). Populations v.1.2.30 (Langella 1999) was used to examine genetic relationships between individuals. Bayesian clustering analysis in Structure v. 2.3.2 (Pritchard *et al*. 2000) was used to infer the number of populations within the dataset. The appropriate number of potential genetic clusters (*K*-value) was determined by plotting the log probability $L(K)$ and ΔK across multiple runs as implemented in Structure Harvester (Earl and von Holdt 2012). To confirm the genetic structuring of samples obtained in previous analyses, Structure v. 2.3.2 was used for checking the strength of differentiation. Two datasets were used. The original dataset contained the original number of individuals. The second dataset consisted of sample RH4 supplemented by randomly sampled individuals from other samples, to check the potential impact of unequal group sizes. Equally acceptable samples were analyzed again to check if the number of individuals has some influence on the analyses.

Samples. – All samples had been collected during the cruise of trawler M/Tr *Polaris* in October of 2006 at the Barents Sea area (Table 1). Individuals from three trawls, differentiated by the depth and geographical locations have been used for the determination of heavy metals (Zn, Cu, Cd, and Pb) concentration.

Samples preparation and determination of elements by FAAS and ETAAS. – Samples were lyophilized and homogenized. Approximately 0.30 g of lyophilized sample was weighed into a Teflon beaker. Afterward, 4 cm^{-3} of HNO_3(s.p) were added and sample subjected to closed vessel microwave digestion (UniClever BM-1z, Plazmatronika, Poland) at a maximal power of 100%, pressure 70 atm, hold 10 min, cooling 20 min.

The clear solutions obtained were quantitatively transferred to 10 cm^{-3} polyethylene graduated tubes and filled to mark with Milli-Q ultrapure water. Zn and Cu were determined by flame atomic absorption spectrometry (FAAS) in the air – acetylene flame at 213.8 nm, and 324.8 nm, respectively. Background correction was performed using a deuterium lamp. A TJA Solutions Model SOLAAR M6 atomic absorption spectrometer equipped with a deuterium lamp and Zeeman effect background correction system was used. Cd, Cu, and Pb were determinated by electrothermal atomic absorption spectrometry (ETAAS) using Zeeman background correction. The ETAAS instrumental and operating conditions are listed in Table 2. Twenty µl of the sample was injected in a pyrolytic graphite furnace tube by an autosampler and run with the parameters included in Table 3.

Table 2.

Optimal instrumental parameters for ETAAS determination by Pb, Cu, Cd.

Parameter	Pb	Cu	Cd
Wavelength	217.0 (nm)	324.8 (nm)	228.8 (nm)
Spectral width slit	0.5 (nm)	0.5 (nm)	0.5 (nm)
Lamp current	90 (%)	80 (%)	50 (%)
Matrix modifier	$Mg(NO_3)_2$ (5 µl)	$Mg(NO_3)_2$ (5 µl)	$Pd(NO_3)_2$ (5 µl)
Dry			
Temperature	100 °C	100 °C	100 °C
Time	30 s	30 s	30 s
Pyrolysis			
Temperature	800 °C	850 °C	800 °C
Time	20 s	20 s	20 s
Atomizing[a]			
Temperature	1200 °C	2100 °C	1000 °C
Time	3 s	3 s	3 s
Cleaning			
Temperature	2500 °C	2500 °C	2500 °C
Time	3 s	3 s	3 s

[a] The purge gas flow rate (200 ml min^{-1} argon) was stopped.

Standards used in the calibration procedure were prepared in the same acids (Suprapur® nitric acid, Merck, Germany) as samples. A standard stock solution of Zn, Cu, Cd, and Pb (1000 ± 2 mg/dm^3) was purchased also from Merck.

The accuracy of the method was verified (10 – replicate) using certified reference material (Dogfish) CRM DOLT – 3. Recoveries were above 90 % for all trace metals measured (Table 3).

Table 3.

Results of heavy metal concentration in certified reference material after microwave digestion of simple.

Metal	Certified values (µg/g)	FAAS (µg/g)	Recovery %	ETAAS (µg/g)	Recovery %
Zn	86.6 ± 2.4	87.1 ± 3.2	100.6	-	-
Cu	31.2 ± 1.0	30.6 ± 0.4	98.1	-	-
Cd	19.4 ± 0.6	18.0 ± 0.9	92.8	20.0 ± 0.6	103.1
Pb	0.32 ± 0.05	-	-	0.33 ± 0.07	103.1

One of the factors taken into account as comparable aspect describing individuals and subpopulations was Condition Factor (CF) estimated using the weight and the total length of the fish according to the formula proposed by Fulton (1902) [Fafioye and Oluajo 1999]: $CF = C\frac{100\ W}{L^3}$ where W = weight in grams and L = total length (cm) of the fish.

Other factors were biometric data like weight and length, collected during sampling. Their use for comparison revealed which factor was most linked with levels of heavy metals concentrations.

Results

The analyzed allozymes showed a specificity of tissues: IDH showed expression from liver and ADH, GPI, LDH, PGM from white muscles as it was reported for part of them (GPI, IDH, and PGM) by Igland and Naevdal (2001). There was no expression for AK and 6PGDH enzyme systems and thereby these allozymes were removed from further analysis. Three loci were polymorphic at the 0.95 criterion: GPI-1, IDH-1, and PGM. Loci ADH, GPI-2, and LDH were monomorphic in samples RH2, RH3, and RH8 but polymorphic with other alleles in the sample RH4. In the LDH locus, one genotype in the sample RH4 was shared with one individual from the sample RH8. Private alleles were found only in the RH4 population (Table 4). In all populations, the mean expected heterozygosity (H_E) was higher than the mean observed heterozygosity (H_O) (Table 4). The number of different and effective alleles and the results of the tests for Hardy-Weinberg (HW) equilibrium are listed in Table 4. After adjusting levels of significance by the

Table 4.

Genetic variability at six allozymes.

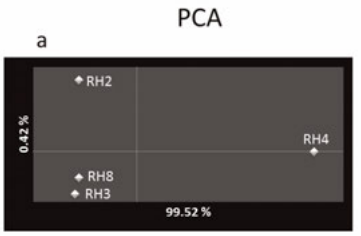
Pop/N	Locus	Allele	Allelic frequencies	Na	Ne	H_O	H_E	No. of private alleles	HWE-p/ signification
RH2/ 60	ADH	b	1.000	1.000	1.000	0.000	0.000	-	m
	GPI-1	a b c d	0.016 0.575 0.175 0.234	4.000	2.420	0.617	0.587	-	0.350/ns
	GPI-2	b	1.000	1.000	1.000	0.000	0.000	-	m
	IDH-1	a b c	0.592 0.358 0.050	3.000	2.079	0.183	0.519	-	0.000/***
	LDH	b	1.000	1.000	1.000	0.000	0.000	-	m
	PGM	a b c	0.033 0.283 0.684	3.000	1.824	0.483	0.452	-	0.936/ns
Mean				2.167	1.554	0.214	0.260		
SE				0.543	0.259	0.112	0.117		
RH3/ 63	ADH	b	1.000	1.000	1.000	0.000	0.000	-	m
	GPI-1	a b c d	0.040 0.571 0.143 0.246	4.000	2.445	0.619	0.591	-	0.036/ns*
	GPI-2	b	1.000	1.000	1.000	0.000	0.000	-	m
	IDH-1	a b c	0.794 0.190 0.016	3.000	1.501	0.127	0.334	-	0.000/***
	LDH	b	1.000	1.000	1.000	0.000	0.000	-	m
	PGM	a b c	0.048 0.317 0.635	3.000	1.959	0.603	0.489	-	0.015/ns*
Mean				2.167	1.484	0.225	0.236		
SE				0.543	0.248	0.124	0.111		
RH4/ 18	ADH	a c d	0.556 0.389 0.055	3.000	2.160	0.000	0.537	3	0.000/***
	GPI-1	b c d e f	0.556 0.139 0.111 0.111 0.083	5.000	2.781	0.667	0.640	1	0.001/***
	GPI-2	a c	0.333 0.667	2.000	1.800	0.000	0.444	2	0.000/***
	IDH-1	c d	0.622 0.378	2.000	1.906	0.222	0.475	1	0.024/ns*
	LDH	a c	0.556 0.444	2.000	1.976	0.000	0.494	1	0.000/***

Pop/N	Locus	Allele	Allelic frequencies	Na	Ne	H_O	H_E	No. of private alleles	HWE-p/ signification
	PGM	a b c d	0.028 0.444 0.472 0.056	4.000	2.356	0.222	0.576	1	0.021/ns*
Mean				3.000	2.163	0.185	0.528	1.5	
SE				0.516	0.147	0.106	0.029	-	
RH8/ 45	ADH	b	1.000	1.000	1.000	0.000	0.000	-	m
	GPI-1	a b c d	0.022 0.622 0.133 0.223	4.000	2.199	0.622	0.545	-	0.372/ns
	GPI-2	b	1.000	1.000	1.000	0.000	0.000	-	m
	IDH-1	a b	0.756 0.244	2.000	1.658	0.200	0.397	-	0.003/**
	LDH	b c	0.978 0.022	2.000	1.045	0.000	0.043	-	0.000/***
	PGM	a b c	0.011 0.389 0.600	3.000	1.987	0.356	0.497	-	0.122/ns
Mean				2.333	1.482	0.196	0.247		
SE				0.494	0.220	0.104	0.106		

Na = the number of different alleles, Ne = the number of effective alleles, H_O = observed heterozigosity, H_E = expected heterozigosity, SE = Standard Error values, HWE = probability of Hardy-Weinberg equilibrium with signification after Bonferroni correction, ns = not significant, *P<0.05, **P<0.01, ***P<0.001, m – monomorphic locus. Pop/N = population/number of individuals.

Bonferroni correction, significant deviations of genotypic frequencies from those expected under Hardy-Weinberg equilibrium were found at the IDH-1 locus for all samples tested and at the GPI-1 and PGM loci for the RH4 sample.

The Principal Component Analysis (PCA) also confirmed the difference between sample RH4 and the other three samples (Fig. 2). There are visible two groups, one consisting of the samples RH2, RH3, RH8, and another one representing the sample RH4 (Fig. 3). Likewise, the analysis of similarity relationships among the individuals studied revealed a deep subdivision between the populations represented by samples RH2, RH3, RH8, and the very divergent RH4 population (Fig. 4). Similarity relationships at the individual level are congruent with the results obtained at the population level (Fig. 5). Moreover, the pairwise F-statistics (F_{ST}) revealed a clear separation of the RH4 sample from the remaining studied samples. The F_{ST} values for the comparisons between samples RH2, RH3, and RH8 were around 0.01, which could indicate the phenomenon of panmixia. The F_{ST} values between these three samples and the RH4 sample were around 0.60, which, in turn, suggests that these two groups represent genetically-isolated populations (Fig. 2).



FST

	RH2	RH3	RH4	RH8
RH2	0.00000			
RH3	0.02159	0.00000		
RH4	0.58448	0.61151	0.00000	
RH8	0.01158	-0.00403	0.58357	0.00000

	RH2	RH3	RH4	RH8
RH2	0.00000			
RH3	0.08199	0.00000		
RH4	0.71880	0.77659	0.00000	
RH8	0.03363	0.00139	0.72766	0.00000

	RH2	RH3	RH4	RH8
RH2	0.00000			
RH3	-0.00466	0.00000		
RH4	0.03136	0.01878	0.00000	
RH8	0.00100	-0.00592	0.00776	0.00000

Fig. 2. Patterns of the genetic structure of *Reinhardtius hippoglossoides* revealed Principal Component Analysis (PCA) and pairwise population F_{ST} values; a – analysis for all allozymes loci ADH, IDH-1, LDH, GPI-1, GPI-2, PGM; b – allozymes catalyze crucial metabolic reactions using the substrate synthesized intracellularly: ADH, IDH-1, LDH; c – less specifical allozymes which catalyze reactions using the substrates derived from the extracellular environment: GPI-1, PGM without less differentiated locus GPI-2.

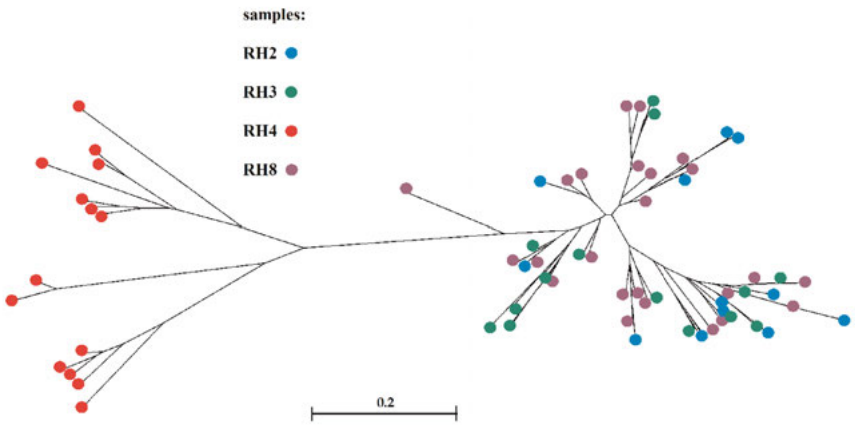


Fig. 3. Genetic relationships between individuals of Greenland halibut. Abbreviations: blue dots – individuals from sample RH2, green dots – individuals from sample RH3, red dots – individuals from sample RH4, and violet dots - individuals from sample RH8.

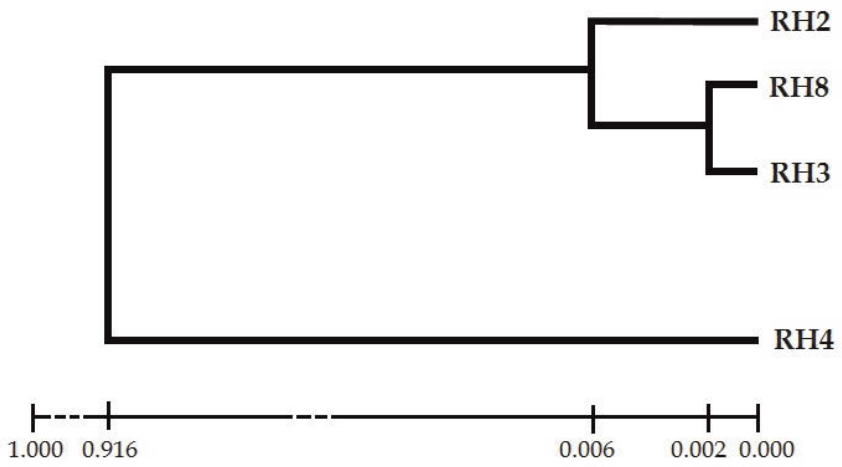


Fig. 4. Dendrogram based on genetic distance matrix Nei'a (1972).

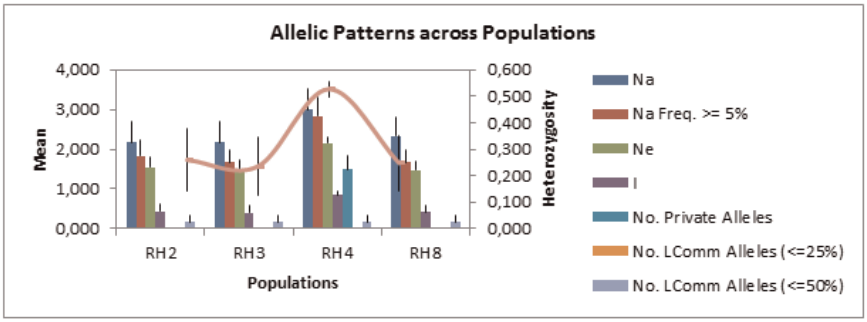


Fig. 5. Allelic patterns across populations. Abbreviations: Na – the number of different alleles, Ne – the number of effective alleles, I – Shannon's Information Index, pink line defines the expected heterozygosity.

The structure analysis also resulted in the highest likelihood Ln P(D) value for *K*=2 and indicated that the sampling locations were represented by two genetically discrete populations. Two analyses, one for unequal samples and another with RH4 sample supplemented with randomly chosen individuals, were consistent – the RH4 sample remained separate from RH2, RH3, and RH8 samples independently from the abundance of the sample (Fig. 6).

In systems of enzymes PGM and IDH, we observed polyploids individuals which were removed from the statistical calculations (Table 5). Information about polyploids was based on unusual for diplonts numbers of bands on zymograms (Richardson *et al.* 1986). Analysis of polyploid shown the same differences: population RH4 was composed of individuals of different genotypes than the rest of the populations (Fig. 7).

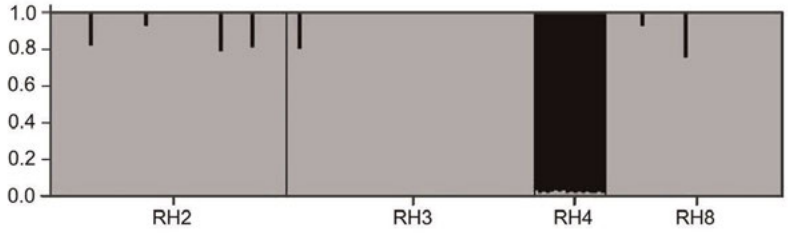


Fig. 6. Summary plot of the structure analysis of 6 allozymes loci with $K = 2$ hypothetical clusters.

Table 5.

Genotypes of polyploid individuals in enzyme systems IDH-1 and PGM.

Locus	Genotypes	Numbers of genotypes			
		RH2	RH3	RH4	RH8
IDH-1	a^nc	4	1	0	6
PGM	a^nb^kc	1	2	1	2
	bc^md^s	1	4	0	0

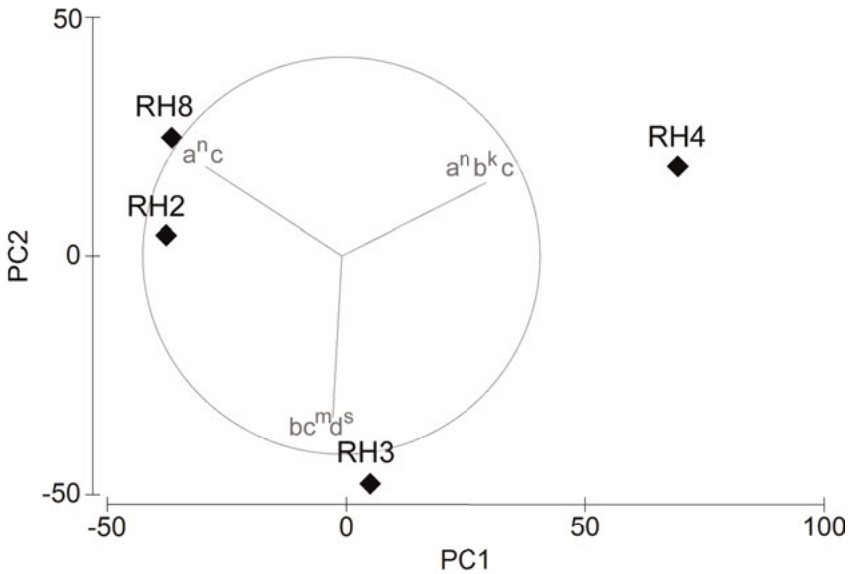


Fig. 7. PCA cluster of *Reinhardtius hippoglossoides* polyploid similarity.

Heavy metals. — Analysis of populations RH2 and RH4 (Table 1), revealed similar concentrations of zinc, copper, cadmium, and lead (Tables 6 and 7).

Small fluctuations between concentration values of heavy metals in a whole group containing two populations could be a result of different diet or age.

Table 6.

Heavy metal content in muscle tissue (fresh weight), population RH2.

No. of individuals	Zn [mg/kg]	Cu [mg/kg]	Cd [µg/kg]	Pb [µg/kg]
2.1	3.14	0.25	31.7	2.2
2.2	3.08	0.20	40.9	-
2.3	2.64	0.17	15.3	3.9
2.4	3.25	0.23	11.3	-
2.5	3.49	0.36	48.1	4.1
2.6	4.10	0.32	30.5	4.3
2.7	2.15	0.14	17.5	5.1
2.8	2.38	0.09	14.1	-
2.9	3.35	0.14	38.4	3.2
2.10	2.89	0.15	10.3	-
2.11	4.21	0.30	46.1	-
2.12	3.28	0.14	38.4	2.0
2.13	3.05	0.21	19.7	1.7
2.14	2.41	0.17	15.6	4.3
2.15	3.64	0.19	20.6	5.8
2.16	2.51	0.21	25.7	2.1
2.17	2.84	0.18	9.1	-
2.18	2.94	0.20	39.8	4.3
2.19	3.56	0.40	48.3	2.3
2.20	2.32	0.21	30.8	-
2.21	3.19	0.23	19.1	-
2.22	1.98	0.15	10.5	5.9
2.23	4.72	0.26	71.2	-
2.24	3.64	0.11	18.4	-
2.25	1.14	0.12	9.2	6.0
2.26	3.56	0.31	46.2	3.9
2.27	4.92	0.35	60.2	-
2.28	2.59	0.22	29.8	3.9
2.29	3.31	0.14	20.3	-
Average value	**3.11**	**0.21**	**28.9**	**3.82**
n	29	29	29	17
Min value	1.14	0.09	9.10	1.70
Max value	4.72	0.40	71.20	6.00
Standard deviation SD	0.80	0.08	16.3	1.40

Table 7.

Heavy metal content in muscle tissue (fresh weight), population RH4.

No. of individuals	Zn [mg/kg]	Cu [mg/kg]	Cd [µg/kg]	Pb [µg/kg]
4.1	1.59	0.14	20.6	2.6
4.2	2.73	0.18	19.7	5.3
4.3	3.41	0.12	15.6	7.8
4.4	4.06	0.30	35.7	-
4.5	1.44	0.15	10.8	2.4
4.6	2.77	0.14	11.1	-
4.7	2.85	0.21	13.2	-
4.8	1.44	0.16	16.0	4.9
4.9	1.68	0.12	21.0	-
4.10	3.36	0.19	101.9	-
4.11	3.10	0.07	51.6	-
4.12	2.96	0.11	37.6	-
4.13	3.07	0.30	19.1	5.9
4.14	3.27	0.19	81.9	-
Average value	**2.70**	**0.17**	**32.6**	**4.8**
n	14	14	14	6
Min value	1.44	0.07	10.8	2.4
Max value	4.06	0.30	101.9	7.8
Standard deviation SD	0.83	0.07	27.9	2.1

Nonetheless, sex as a factor affecting the concentration of heavy metals in muscle tissue is excluded. The next factor was Condition Factor (CF). Results showed a strong, a statistically supported correlation between CF and heavy metals concentrations (Table 8).

The 3D analysis revealed that an average level of concentration of copper and zinc according to cadmium, are characteristic for each analyzed subpopulation RH2 and RH4 of Greenland halibut (Fig. 8)

Table 8.

Correlations between concentrations of heavy metal and condition factor (CF). Unpaired student test, P<0.05.

	Average	SE	N	Difference	SD Diff.	t	df	p
CF	1.02205	0.68331						
Cd	27.20217	15.22251	46	-26.1801	15.29496	-11.6092	45	0.000000
Cu	0.200435	0.080082	46	0.821611	0.690135	8.074418	45	0.000000
Zn	3.041957	0.719693	46	-2.01991	0.989500	-13.8451	45	0.000000
Pb	3.925926	1.695150	27	-2.73122	2.413544	-5.88009	26	0.000003

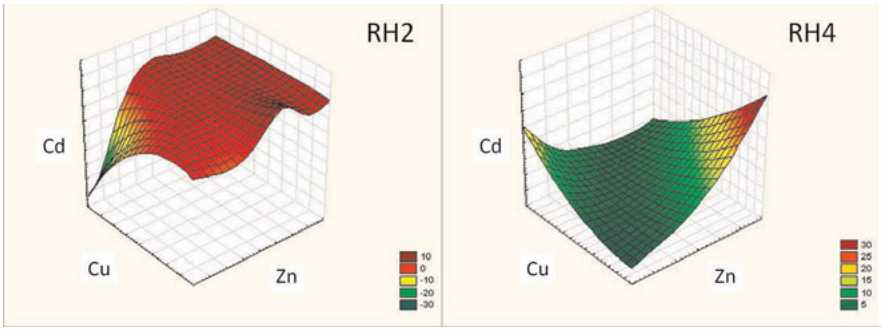


Fig. 8. 3D interpolation analysis: Zn, Cd, Cu concentration data of populations RH2 and RH4.

Discussion

Our results showed the high value of F_{ST} between sample RH4 and other samples (RH2, RH3, and RH8), which is a very surprising fact in the light of previous literature data. Private alleles found in the sample RH4 were an additionally strong signal of the presence of the Greenland halibut separate stock. However, deviations from Hardy-Weinberg equilibrium suggested that the sample RH4 could be homogenous. There were two possibilities: 1. specimens could originated from a small number of ancestors, 2. allozymes were modified by some environmental factors (e.g. temperature, pressure or heavy metals). However, heavy metals concentrations were very similar in both populations and probably did not affect the results. In turn, Riget *et al.* (1992) showed that there is also a possibility of identification of subpopulations of Greenland halibut. The most obvious reasons for "high differentiation" obtained in our study were eliminated: each sample consisted of randomly selected fish among individuals from one haul. All of them were characteristic of the Greenland halibut morphology. All samples were collected (however, the RH4 sample consisted of only 19 individuals and three other samples were more numerous, i.e., from 52 to 70 individuals per sample), carried and stored in the same way

and place. The results of analyzed allozymes were consistent among three homogenous samples and different only for one sample. These facts have verified that the potential risk of procedural mistakes is eliminated.

The Western part of the Barents Sea was a region in which Greenland halibut spawns and feeds (Bogstad *et al.* 2007). The material for the study was collected in October at the beginning of halibut's spawning (Morgan *et al.* 2003). A decrease of the continental shelf could be a zone where ecologically isolated populations migrate during spawning time. There is a probability that the sample RH4 came from another ecological environment (Nielsen *et al.* 2003).

The systems of enzymes PGM and IDH observed polyploid individuals which were removed from the statistical calculations. Polyploid organisms were found in many fish (Hebert and Beaton 1989; Mateos and Vrijenhoek 2005). However, the induction of polyploids in animals is very seldom, it happens among asexually reproducing organisms. We have a lot of causes of polyploidization in fish, for example meiotic and mitotic gynogenesis, androgenesis (Felip *et al.* 2001), and hybrydogenesis (Mateos and Vrijenhoek 2005). Polyploids may also arise due to changes in the environment – physical factors such as thermal shock caused by heat or cold, and shock pressure (Holmefjord and Refstie 1997 — Atlantic halibut (*Hippoglossus hippoglossus*) revealed the presence of triploid individuals by heat and cold shocks in experimental conditions), which negatively effects on ripening egg. Due to the different environmental conditions (biotic and abiotic) on different depths, should be observed adaptation mechanisms (mainly on the metabolism, as an answer gives the protein), resulting in different enzymes, enzyme-substrate interactions. In the analyzed case, the diversity of polyploids in populations could explain their different origins, with different thermal conditions.

Given the fact that the previous data indicated low genetic diversity of the Greenland halibut populations, which are present on the shelves of the North Atlantic, our obtained results allow us to hypothesize that the level of genetic diversity of investigated populations is related to the depth of its occurrence. Therefore, we hypothesize that the population RH4 arrived from deep regions of the Norwegian or the Greenland Sea as well as we could also identify the Pacific and the Atlantic lineages of Greenland halibut in the spawning area; however, still deeper analysis is needed to resolve this issue. Due to the different environmental conditions (biotic and abiotic) in different depths, adaptation mechanisms mainly on the metabolism level (as an answer gives the protein) should be observed. It could result in non-neutral (adaptive) molecular markers – different enzymes, enzyme-substrate interactions. Moreover, due to sampling in close locations in a short period (one day), the result shows that the migration of this species is very dynamic. The repetition of sampling in the same area of study at an interval of one day allowed the observation of violent migration of Greenland halibut's shoals. In turn, Lindholm (2016) indicated the possibility of the vertical distribution of the population. Nevertheless, this phenomenon could not explain our results without further research. However, due to moving juvenile stages of halibut in the surface

layers of water and sea currents, genetic diversity may relate to the period of the adult life of the individual. Presented results, a new look at the genetic diversity of Greenland halibut, should be taken into account in proper fisheries management of commercial fish exploitation in spawning regions.

Heavy metals – Results of the analysis suggest the presence of weak differentiation between local shoals of Greenland halibut. Similar concentrations of heavy metals along the south-north transect suggest that the diet is the dominant factor affecting the differentiation. The sampling was performed in October when fish were ready for spawning (gonads mature index – 3,4,5, mainly 4^{th} stage). At this time mixing of Greenland halibut can be a result of spawning migrations (Albert *et al*. 2001) and, in effect, could decrease the level of differentiation of populations.

The high Cd levels in some Arctic fish gives support to the thesis of a general 'cadmium-anomaly' in polar waters, which is derived mainly from analyses of pelagic and benthic marine invertebrates, such as crustaceans and molluscs (Demoreno *et al.* 1997; Ritterhoff and Zauke 1998; Bustamante *et al.* 1998). The upwelling phenomena on local scales may increase the bioavailability of Cd as discussed for calanoid copepods from the northern North Sea by Zauke *et al.* (1996). Secondly, Petri and Zauke (1993) hypothesized that some invertebrates might suffer from a general trace element deficiency and might thus have developed very efficient uptake mechanisms during evolution. Since these mechanisms are most probably not element-specific, non-essential elements like Cd might be taken up along with essential elements like Cu and Zn. This process is eventually very pronounced in organisms with developed mechanisms of detoxification, as discussed by Ritterhoff and Zauke (1998) for metallothioneins in hyperiid amphipods from the Greenland Sea. In this way, the presence of cadmium in seawater affects the level of Cd incorporating by Greenland halibuts straightly by drinking seawater and in a trophic way. Hovde *et al.* (2002) found statistically significant differences in diet composition according to the longitude and depth but also the sampling's period and predator's size. In present studies linear character of cadmium concentration suggests that there is a correlation between predator's size but there is equally possible that the cadmium accumulates independently just by drinking water. In turn, changes in levels of cadmium concentrations in seawater localities affect fauna inhabiting the relevant areas so indirectly affects the level of accumulation of cadmium in a subpopulation. Our results revealed the different levels of Cd-concentration according to the geographic origin of the sample. Anyway, a constant process of cadmium accumulation is explaining the strong correlation between Cd concentration and the length or weight of Greenland halibut.

Nonetheless, the 3D analysis revealed that an average level of concentration of copper and zinc according to cadmium, are characteristic for each analyzed subpopulation of Greenland halibut. These levels of concentrations were observed only in an aspect of geographical differentiation or species differences but very rarely in the case of geographical subpopulations of one species from the same

area. The genetic differentiation of Greenland halibut populations is affected, according to Knutsen *et al.* (2007) by a passive drift of pelagic eggs and larvae with ocean currents. This would imply an important role of oceanic currents in shaping the genetic structure of this fish. Nonetheless, this structure would implicate that we are observing structure affected by drift and uncontrolled dispersion but not relations between feeding or nursery groups. Characteristics of these territories can depict specific concentrations of heavy metals. Statistic differences between groups included in present studies support the theory that each group of fish should be treated as a potentially different unit according to their characteristics due to environmental pollutions. The present method offers also the possibility for detailed tracking of food chain influences and even characterization of a diet.

An additional argument supporting this hypothesis is the fact that the studied populations are very similar in terms of the concentration of heavy metals, although the 3D analysis (Fig. 8) indicates a different interpolation of data. Since the concentration of heavy metals in the examined tissues shows slight fluctuations for the studied populations, and the genetic structure is different, it may indicate the pressure of another environmental factor (not the diet), possibly temperature, or additional pressure (vertical stratigraphy) influencing the genetic diversity of the halibut population (Holmefjord and Refstie 1997; Nielsen *et al.* 2003). Previous studies (Vis *et al.* 1997; Igland and Nevdal 2001) conducted for shoals at a similar depth (shelf), but in other regions of the North Atlantic, indicated the homogeneous nature of the halibut population. The obtained results are therefore a premise for the concept of further studies on the diversity of the halibut population related to the vertical distribution of individual populations.

Conclusion

The obtained results indicate the existence of two isolated populations. The results allow for several hypotheses: 1. genetic diversity at the level of allozymes is formed in shoals during ontogenesis, 2. genetic diversity is probably related to vertical stratification: depth of occurrence (pressure) and/or composition of bottom sediments (e.g. concretions) and may have a modifying effect on allozymes, 3. shoals of Greenland halibut move rapidly with the change of thermal conditions in the area of their occurrence, 4. the genetic diversity of Greenland halibut, should be taken into account in proper fisheries management of commercial exploitation.

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