

Genetic and environmental correlates of morphological variation in a marine fish: the case of Baltic Sea herring (*Clupea harengus*)

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Abstract: Baltic Sea herring (*Clupea harengus*) have been shown to exhibit morphological differences across the marked salinity and temperature gradients in the region. Here we analyse genetic (nine microsatellite loci), morphometric (skull shape), and meristic (pectoral fin rays and number of vertebrae) variations across seven samples of spawning herring collected from four spawning locations in the Baltic Sea to examine whether morphological variation correlates with genetic and (or) environmental factors. Results suggest that herring is adapting to its environment through a combination of selection and plastic responses. Skull shape, including and excluding size variation, differed significantly among samples, both temporally and spatially. Genetic and morphometric distances were correlated, especially when size variation was excluded from the analysis. When size variation was included, skull shape variation was more closely correlated with environmental distances among spawning locations. Vertebrate number differed among samples and was correlated with environmental distances, whereas the number of fin rays was not. Genetic and geographic distances among samples were not correlated.

Résumé : On a montré que les harengs (*Clupea harengus*) de la Baltique présentent des différences morphologiques le long des forts gradients de salinité et de température de la région. Nous analysons la variation génétique (neuf locus microsatellites), morphologique (forme du crâne) et méristique (rayons de la nageoire pectorale et nombre de vertèbres) dans sept échantillons de harengs en fraye récoltés sur quatre sites de reproduction dans la Baltique, afin de déterminer si la variation morphologique est en corrélation avec les facteurs génétiques et (ou) environnementaux. Nos résultats laissent voir que le hareng s'adapte à son environnement par une combinaison de réponses reliées à la sélection et à la structure. La forme du crâne (avec et sans la variation de taille) diffère significativement d'un échantillon à l'autre, aussi bien dans le temps que dans l'espace. Les distances génétiques et morphométriques sont en corrélation l'une avec l'autre, particulièrement lorsque la variation de taille est exclue de l'analyse. Lorsque la variation de taille est incluse, la variation de la forme du crâne devient plus fortement corrélée aux distances environnementales entre les sites de fraye. Le nombre de vertèbres varie d'un échantillon à l'autre et est en corrélation avec les distances environnementales, alors que le nombre de rayons de la nageoire ne l'est pas. Il n'y a pas de corrélation entre les distances génétiques et géographiques au sein des échantillons.

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Introduction

When a species' range covers a heterogeneous environment, a single phenotype is unlikely to be associated with high fitness throughout the range (Via et al. 1995). To succeed, a species must be able to match phenotypes with local environments. Historically, local marine fish populations (stocks) have been identified largely using phenotypic traits.

One difficulty when applying such methods is that phenotypic traits are influenced by both genetic and environmental variation and the relative importance of these two sources of variation is generally unknown (Swain and Foote 1999).

In the marine environment, the traditional view of nonexisting or biologically nonsignificant genetic differentiation at regional scales has been challenged by a number of recent studies. Biologically significant (albeit low) population ge-

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netic differentiation has been found in highly vagile marine fish (e.g., Atlantic cod (*Gadus morhua*; Ruzzante et al. 1998; Knutsen et al. 2003) and Atlantic herring (*Clupea harengus*; McPherson et al. 2001a; Bekkevold et al. 2005; Jørgensen et al. 2005)). The majority of these studies was based on neutral molecular loci (mostly microsatellites) and did not examine evidence of structure at the phenotypic level, though Jørgensen et al. (2005) and Bekkevold et al. (2005) have presented evidence of correlation between genetic structure and variation in environmental factors.

Morphological and ecological variability has been shown to reflect adaptive divergence by natural selection in sympatric populations and species of freshwater and anadromous fish, but marine examples are less common (reviewed by Guinand et al. (2004) and Conover et al. (2006)). Morphological population divergence has been found in studies of pelagic marine fishes such as herring (Ryman et al. 1984) and redfish (*Sebastes* spp.; Valentin et al. 2002). Populations inhabiting different environments have been shown to exhibit cogradient morphological divergence (e.g., gene expression in the killifish *Fundulus heteroclitus* (Schulte 2001), accumulation rate of somatic energy stores in Atlantic silverside (*Menidia menidia*; Schultz and Conover 1997)) and countergradient morphological divergence (e.g., body shape in juvenile cod (Marcil et al. 2006), growth rate in striped bass (*Morone saxatilis*; Conover et al. 1997)).

Morphological divergence between spawning groups may be due to differences in local environments only, or it could partly reflect genetic differentiation among the spawning groups in cases where there is pronounced homing to spawning locations and low mixing between spawning groups. To identify the most likely scenario in a natural environment, one may combine data on environmental, morphological, and neutral genetic divergence among spawning groups.

The Baltic Sea is one of the world's largest brackish water bodies and constitutes a complex ecosystem with several heterogeneous subunits, each based in a deep basin partially separated from other basins by sills. Surface water temperature decreases gradually from the southeast to the northwest, and inflows of saline water from the North Sea and freshwater from large rivers create a strong salinity gradient (Ojaveer and Elken 1997), which generates a generally unstable and heterogeneous environment.

Biologically significant genetic differences have been reported for Atlantic herring in the transition zone between the highly saline North Sea and the brackish Baltic Sea (Bekkevold et al. 2005; Ruzzante et al. 2006), an area that is generally described as a hybrid zone for a number of marine species (Nielsen et al. 2003, 2004). Within the Baltic Sea itself, herring spawning grounds are found throughout the region (Aro 1989), and geographically separated spawning groups are known to exhibit morphological differences (e.g., Popiel 1984; Parmanne 1990) that have been used for population identification. More recently, genetic studies based on microsatellite markers have identified low but statistically significant differences among herring from some of these areas (Jørgensen et al. 2005), yet it is not well known to what extent morphological and genetic differences correlate with each other.

In this study, we tested the hypothesis that morphological divergence among herring in the Baltic Sea is due to adapta-

tion to local environmental conditions by natural selection, at least in part. Salinity and temperature vary throughout the Baltic Sea, and embryos and early larval stages of herring from different spawning and nursery grounds are exposed to different salinity and temperature regimes (e.g., Klinkhardt 1996). Herring larvae from different areas of the Baltic Sea are thus likely to exhibit different tolerance levels to these two environmental parameters. Further, as the species composition of mesozooplankton communities is dependent on salinity and temperature (Möllmann et al. 2000), different prey items are available for young herring in the different areas of the Baltic Sea (Möllmann et al. 2004).

To test for correlation between genetic and morphological differentiation, as well as their correlations with environmental variability among spawning groups, we estimated (i) neutral molecular genetic differentiation using nine microsatellite loci, (ii) meristic differentiation among those same groups using the number of pectoral fin rays and the number of vertebrae, two meristic traits previously shown to vary geographically in herring, (iii) morphometric differentiation using a geometric morphometrics approach in which we tested for differences in morphological differentiation among samples and age groups using the relative position of homologous landmarks placed on herring skulls, and (iv) correlations of neutral genetic and morphological differentiation with geographic and environmental distances among spawning groups. Our study design did not allow for directly testing for genetically based local adaptation, but we tested for indications of local adaptation by natural selection using an indirect approach. The tests are expected to indicate which factors might influence morphological variation in herring in the Baltic Sea.

Materials and methods

Samples

Atlantic herring were collected from four spawning locations in the Baltic Sea: (i) near the island of Rügen (RU; two collections, March 2003 and May 2003), (ii) in the Gulf of Riga (RI; one collection, 2003), (iii) in the Åland Archipelago (AL; two collections, 2002 and 2003), and (iv) off the Swedish coast of the Bothnian Bay (BB; two collections, 2002 and 2003) (Fig. 1; Table 1). The sampling design was based on previously recognized spawning groups (Ojaveer and Elken 1997) located in different areas of the Baltic Sea that were defined based on zones of increased genetic and environmental differentiation (Jørgensen et al. 2005). Herring in six of the seven samples were in prespawning or spawning condition (maturity stage five or six; Anonymous 1962), the exception being the sample from AL in 2003 (AL03) in which the majority of the specimens were in postspawning condition. Regardless of maturity stage, they were all caught on their respective spawning grounds. The samples were frozen upon arrival at the Danish Institute for Fisheries Research and stored at -18°C . A piece of gill tissue was removed from each specimen and stored in 96% ethanol for subsequent DNA extraction.

Genetic analyses

DNA was extracted from gill tissue using the Chelex-proteinase K protocol by Estoup et al. (1996), the hot so-

Table 1. Catch data for the Atlantic herring (*Clupea harengus*) samples included in the present study.

Sample (code)	Sampling location	Sampling date	Sample size	
			<i>n</i> ^a	<i>n</i> ^b
Bothnian Bay (BB02)	63.38°N, 20.25°E	June 2002	94	116
Bothnian Bay (BB03)	63.38°N, 20.15°E	June 2003	59	115
Åland Archipelago (AL02)	60.20°N, 22.10°E	June 2002	55	115
Åland Archipelago (AL03)	60.20°N, 22.10°E	June 2003	88	109
Gulf of Riga (RI03)	ICES 44H2	May 2003	80	109
Rügen (RU0303)	54.14°N, 13.27°E	March 2003	54	100
Rügen (RU0503)	54.11°N, 13.26°E	May 2003	81	100

Note: All individuals were caught on the spawning grounds and in spawning condition. The position given for the Gulf of Riga sample refers to the ICES (International Council for the Exploration of the Sea) management area 44H2 in the northwestern part of the Gulf.

^aSample sizes in morphometric analyses.

^bSample sizes in genetic analyses.

Fig. 1. Sampling locations: RU, Greifswalder Bodden near the German island of Rügen; RI, the Gulf of Riga; AL, the Åland Archipelago; BB, the Southern Bothnian Bay at the Swedish coast. The exact position for each sample is shown in Table 1.



dium hydroxide and Tris (HotSHOT) protocol by Truett et al. (2000), or the DNeasy[®] Tissue Kit 250 (Qiagen Sample and Assay Technology, Hilden, Germany). Nine microsatellite markers were analyzed: *CHA1017*, *CHA1020*, *CHA1027*, *CHA1202* (McPherson et al. 2001b), *CPA101*, *CPA107*, *CPA111*, *CPA112*, and *CPA114* (Olsen et al. 2002). The microsatellite loci were polymerase chain reaction (PCR) amplified using standard reagents, and their annealing temperatures ranged from 54–60 °C for the different loci (exact protocols are available from the first author on request). The PCR products were analyzed on the BaseStation 51[™] DNA fragment analyzer (MJ Research, Inc., Waltham, Massachusetts), and gels were typed using the software Cartographer (version 1.2.6; MJ Geneworks, Inc., San Francisco, California). As a quality control measure to minimize the probability of genotyping errors (Bonin et al. 2004), we (i) included two standard individuals in each gel to improve typing consistency, (ii) re-analyzed between 10% and 20%

of the individuals from each sample, depending on the marker, to minimize the risk of misinterpretation of gels, and (iii) applied the software Micro-Checker (version 2.2.0; van Oosterhout et al. 2003) to test for the presence of null alleles, stuttering, and large allele dropouts.

Tests for conformity to Hardy–Weinberg expectations were conducted using GENEPOP (version 3.3; Raymond and Rousset 1995) according to the procedure by Guo and Thompson (1992). Weir and Cockerham's (1984) θ , an unbiased estimator of F_{ST} , and exact pairwise tests (Goudet et al. 1996) were used to estimate pairwise genetic differentiation between samples using FSTAT (version 2.9.3; Goudet 1995). The *P* values were compared with Dunn–Šidák significance levels (Sokal and Rohlf 1995) adjusted to $k = 21$ (the number of pairwise comparisons). These tests were supported by a hierarchical analysis of molecular variance (AMOVA) as implemented in Arlequin (version 3.11; Excoffier et al. 2005). With the AMOVA, we estimated the proportions of genetic differentiation that could be attributed to differentiation among spawning locations and between temporal samples within spawning locations, the significances of which were tested using 10 000 randomizations.

Meristic traits and age determination

In Atlantic herring, the number of pectoral fin rays and the number of vertebrae are well suited for the identification of separate spawning groups (Parsons 1972), and in the present study, we examined both traits. Age determination was based on the number of winter rings in otoliths following standard procedures (International Council for the Exploration of the Sea 2003). Kruskal–Wallis rank sum tests (Zar 1996) were used to test for differentiation in the medians of pectoral fin rays, vertebrae, and age among locations, and two-sample Wilcoxon rank sum tests were used to test for differentiation between early and late temporal samples using S-PLUS (version 6.1; Insightful Corp. 2002) and JMP (SAS Institute Inc. 1997).

Morphometric analyses

Digitizing homologous landmarks

Geometric morphometric methods have been used to compare body shape among species or populations of fish (e.g., Klingenberg et al. 2003; Neves and Monteiro 2003; Langer-

hans and DeWitt 2004). We chose the skull for these morphometric investigations because of its presumed ability to reflect any local adaptations in feeding biology. The large number of connected, inflexible bony structures in the head are not worn or broken by influences from the external environment. To detach soft tissue from the bony structures, heads were placed in boiling water. Skulls were carefully placed on modeling clay and photographed from above with a Nikon Coolpix 990 digital camera (Nikon Corp., Tokyo, Japan) fastened to a tripod and set at 1280 × 1024 pixel resolution. Each picture included a ruler for scaling. We applied the geometric morphometrics (GM) approach (e.g., Bookstein 1996). Advantages of this approach include (i) allowing for statistical analyses of overall shape variation and the generation of graphical illustrations of shape differences (e.g., Caldecutt and Adams 1998; Rüber and Adams 2001) and (ii) capturing all aspects of shape change among a set of landmarks without a priori knowing for which specific anatomical change to search (Adams and Rohlf 2000).

The GM analyses of skull shape were based on 26 digitized homologous landmarks. Any variation in shape due to position, rotation, or scale was removed by a generalized Procrustes analysis (described in Rohlf 1999; Adams et al. 2004). After this, shape was represented as partial warps, which describe the deviation in shape for each landmark between individual herring and the sample mean (Rohlf 1999). Landmark digitization was repeated five times on 10 randomly chosen specimens to estimate variability in digitization using separate analyses of variance (ANOVAs; PAST, version 1.21; Hammer et al. 2004). The variation among individuals was significantly higher than within individuals in 44 of 48 cases. The MS_{error} for all partial warps and the uniform component were in the order of 10^{-6} , and hence, measurement error is not a concern in this study.

Correcting for size effects

The skull size was estimated as the centroid size (CS), the square root of the sum of squared distances between the digitized landmarks and the centroid in all dimensions (Adams and Funk 1997). To test for overall size differences among the seven samples, we conducted a two-way ANOVA with spawning location and spawning time as independent variables and $\log(\text{CS}_{ij})$ as the dependent variable. Pairwise tests to compare centroid size in all pairs of samples (*t* tests and Wilcoxon two-sample tests) were conducted using PAST (version 1.21; Hammer et al. 2004).

Allometric growth effects could potentially create spurious differences among samples in shape analysis. Therefore, we used two approaches to minimize the impact of allometric growth effects. (i) All 46 partial warps and uniform component scores were adjusted for allometric effects using the following equation (Swain et al. 1991):

$$A_{ij} = W_{ij} + b_j[\log(\text{CS}) - \log(\text{CS}_{ij})]$$

where W_{ij} is the nonadjusted partial warp for individuals *i* in sample *j*, b_j is the regression slope for warp score vs. $\log(\text{CS})$ within group *j*, CS is the grand mean centroid size (here 60.180), and CS_{ij} is the centroid size for individual *i* in sample *j*. (ii) A canonical variates analysis using PAST (version 1.21; Hammer et al. 2004) was conducted on the ad-

justed data. Of the morphometric variance, 76.07% was contained along the first axis, 8.28% was contained along the second axis, and 15.65% was distributed along the remaining canonical variates axes (CVAs). The correlation between CVA 1 and $\log(\text{CS}_{ij})$ was still considerable (Pearson's $r = 0.616$), indicating that shape variation was not independent from size variation despite the adjustment for allometric effects. Because of this scaling effect on CVA 1, all following tests were conducted both including and excluding CVA 1 to identify any impact on the results from scaling effects.

Testing for variation in shape

To test for differentiation in shape among samples, we first performed a two-way multivariate analysis of variance (MANOVA) with spawning location and spawning time as independent variables and shape (represented by the seven first CVAs) as the dependent variable to test for differences in shape among samples in S-PLUS (version 6.1; Insightful Corp. 2002). To identify any confounding dissimilarities among age groups within spawning locations, we then conducted one-way MANOVAs (PAST, version 1.21; Hammer et al. 2004) for each age group separately with spawning location as the independent variable. Each age group consisted of a cohort (e.g., individuals of age 3 in 2002 and age 4 in 2003 at the same spawning location), forming a total of six age groups. The results of pairwise tests for shape differentiation supported MANOVA results and are not presented.

The loadings along the first two canonical axes were plotted for each specimen to illustrate variation within, versus among, locations (Fig. 2) (multigroup discriminant analysis; Hammer et al. 2004). Finally, the mean landmark configuration for each of the seven samples was compared with the overall consensus, and the resulting deviations were illustrated as deformations of so-called thin-plate splines using the software tpsRegr (version 1.28; Rohlf 2003) (Fig. 3b).

Spatial analyses

To test for correlations between genetic, geographic, and morphometric distances among spawning groups, we performed partial Mantel tests (Legendre and Legendre 1998) using the program IBD (version 1.5; Bohonak 2002). Genetic distances were represented by pairwise θ values. The input matrix for geographic distances contained the shortest waterway distances between sampling locations, estimated using the GIS program ArcMap (version 8.3; ESRI Corporation, Redlands, California). Morphometric distances were represented as the pairwise Mahalanobis' distances among samples. This distance measure describes the similarity between data sets while taking into account the correlation between data in the data sets, and it is not scale-dependent (Manly 1994). Genetic and geographic distances were log-transformed, and each test was based on 10 000 randomizations. We then tested for correlation between geographic and morphometric distances using the Mantel test option in Genetix (version 4.04; Belkhir 2000). The input file contained log-transformed geographic distances and pairwise Mahalanobis' distances estimated both including and excluding CVA 1. Similar procedures were used to test for correlations between morphological and environmental distances (differences in monthly mean values of minimum, maximum, and mean salinity and surface water temperature on the spawning and (or) nursery locations; further details on environmental data are given in

Fig. 2. Canonical variates (CVA) plots including scaling effects in the left column and excluding scaling effects in the right column: (a) age group 1 (3 + 4 years); (b) age group 2 (4 + 5 years); (c) age group 3 (5 + 6 years); (d) age group 4 (6 + 7 years); and (e) age group 5 (7 + 8 years). Southern Bothnian Bay at the Swedish coast (BB), ×; the Åland Archipelago (AL), ○; the Gulf of Riga (RI), △; Greifswalder Bodden near the German island of Rügen (RU), □. Ellipses represent 95% confidence areas. Note different scaling on the y axes.

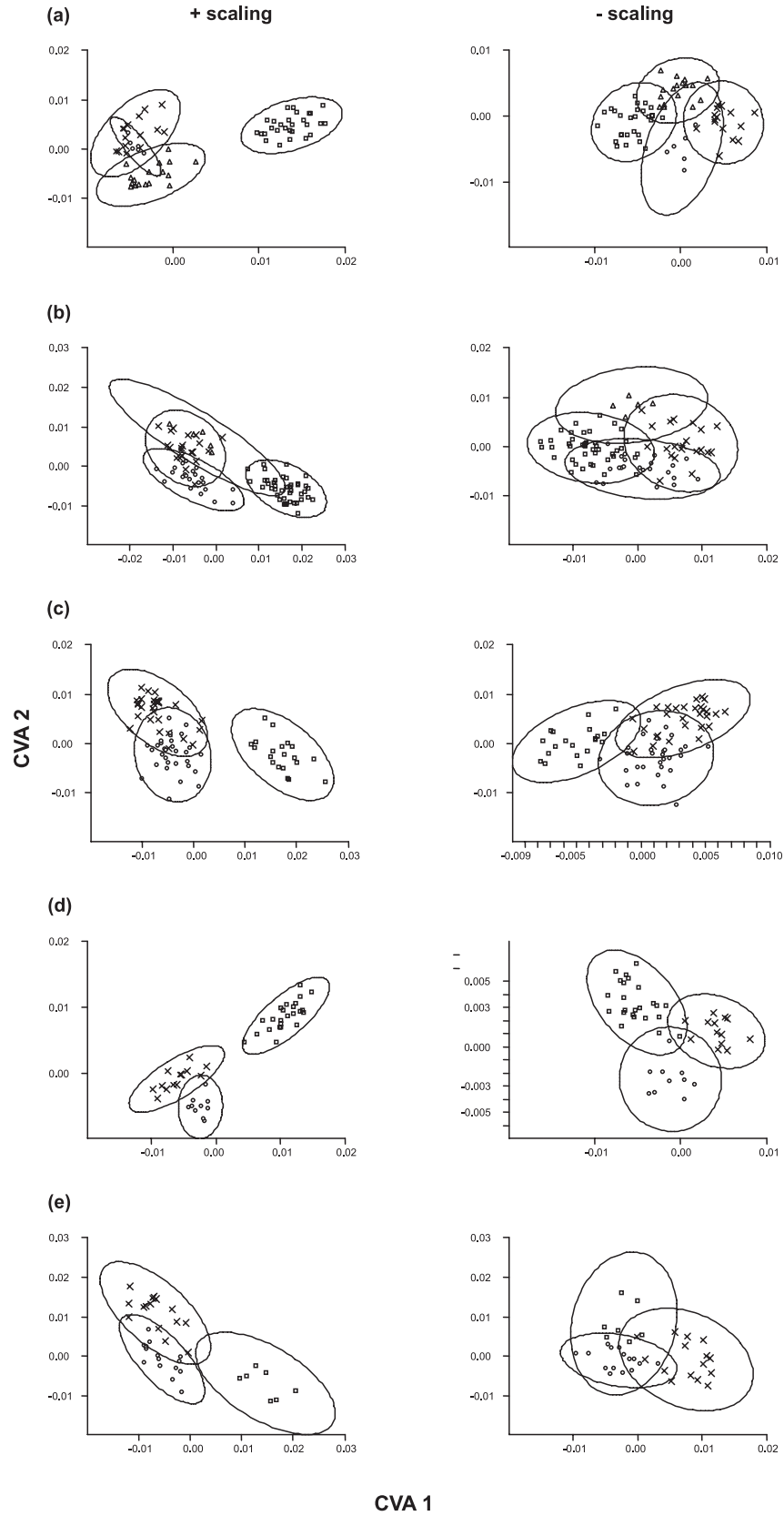
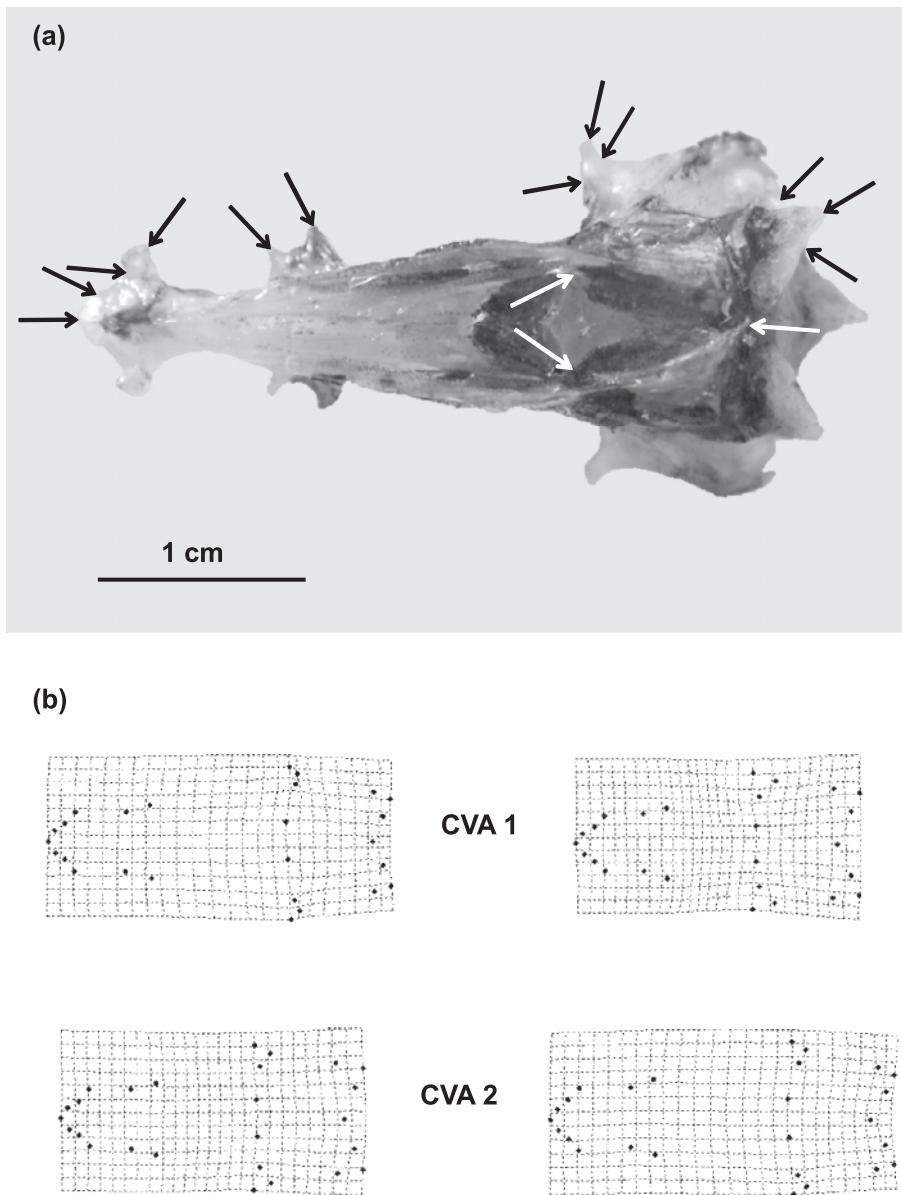


Fig. 3. (a) The homologous landmarks digitized on each specimen. There were 26 landmarks in total, but only one of each pair of bilateral landmarks is indicated by arrows in the photo. (b) Thin-plate spline representations of the variation in shape along CVA 1 and CVA 2 after adjusting partial warps and uniform components for size according to Swain et al. (1991). The deformations are shown in a 3× magnification.



Jørgensen et al. (2005)). Each Mantel test was based on 10 000 permutations.

Results

Genetic analyses

Of the 63 tests for departure from Hardy–Weinberg expectations, only two (3.2%, i.e., fewer than the 5% expected by chance alone) yielded a significant outcome (Appendix A). Before the multiple-test Dunn–Šidák adjustment of the α level, the RU samples differed from all other samples, and the temporal samples from the Åland Archipelago (AL02 and AL03) differed from each other. These differences disappeared after adjustment for multiple tests (Table 2). The hierarchical AMOVA supported the pairwise tests. The vari-

ance among spawning locations did not contribute significantly to the overall genetic variance ($F_{CT} = 0.005$, $p = 0.11$), whereas the variance between temporal samples within spawning locations did contribute significantly ($F_{SC} = 0.001$, $p = 0.03$).

Meristic and age analyses

The mean number of pectoral fin rays ranged from 17.33 (AL02) to 17.85 (RU0303). The mean number of vertebrae ranged from 54.86 (AL03) to 55.98 (RU0503). Overall, this led to a significant difference in the number of vertebrae among spawning locations ($P < 0.001$) and between temporal samples within location at Rügen (RU, $P = 0.005$, mean number of vertebrae 55.98 and 55.62) but not at the other locations for which temporal samples were available (BB,

Table 2. Pairwise θ values (Weir and Cockerham 1984) between samples of Atlantic herring (*Clupea harengus*).

	BB02	BB03	AL02	AL03	RI03	RU0303	RU0503
BB02	0						
BB03	-0.0008	0					
AL02	0.0009	-0.0001	0				
AL03	-0.0004	0.0018	0.0033	0			
RI03	0	-0.0005	0.0013	0.0001	0		
RU0303	0.0097	0.0113	0.0109	0.0106	0.0068	0	
RU0503	0.0129	0.0147	0.0158	0.0147	0.0111	0.0012	0

Note: None of the exact pairwise tests (Goudet et al. 1996) showed significant differentiation when compared with an α level Dunn-Sidak adjusted to $k = 21$. Values in bold type indicate significant differentiation before Dunn-Sidak adjustment of the α level. See Table 1 for explanation of sample codes.

Table 3. One-way multivariate analyses of variance (MANOVAs) testing for shape differences among Atlantic herring (*Clupea harengus*) spawning locations for each age group separately.

Age group	Wilk's λ	df	F	P	Pillai's trace	df	F	P	Total SS	Within-group SS	F	P
CVA 1 included												
1	0.001	144,45.9	3.559	<0.001	2.652	144,51	2.696	<0.001	0.002	0.001	6.129	<0.001
2	0.007	144,120.8	3.652	<0.001	2.256	144,126	2.654	<0.001	0.002	0.002	9.668	<0.001
3	0.019	96,58	3.797	<0.001	1.629	96,60	2.746	<0.001	0.002	0.001	9.070	<0.001
4	0.002	80,10	2.823	0.038	1.887	80,12	2.495	0.041	0.001	0.001	9.640	<0.001
5	0.021	42,28	3.935	<0.001	1.679	42,30	3.739	<0.001	0.001	0.001	7.476	<0.001
6	% correctly assigned: 96.55, $p = 0.580$								Mahalanobis' distance = 2.478, $p = 0.578$			
CVA 1 excluded												
1	0.003	141,48.9	2.173	0.001	2.507	141,54	1.948	0.003	0.001	0.001	2.029	0.001
2	0.028	141,123.8	2.035	<0.001	2.044	141,129	1.955	<0.001	0.002	0.002	3.163	<0.001
3	0.038	94,60	2.637	<0.001	1.562	94,62	2.349	<0.001	0.001	0.001	2.206	<0.001
4	0.007	78,12	1.747	0.142	1.837	78,14	2.030	0.069	0.001	0.001	2.433	<0.001
5	0.107	40,30	1.544	0.110	1.308	40,32	1.512	0.115	0.001	0.001	2.500	<0.001
6	% correctly assigned: 100, $p = 0.310$								Mahalanobis' distance = 2.298, $p = 0.297$			

Note: Results from the parametric MANOVA are shown as Wilk's λ (with associated df, F, and P values) and Pillai's trace (with associated df, F, and P values). Results from the nonparametric MANOVA are shown as sum of squares (SS) values and associated F and P. Age group 6 was only represented in AL and BB; therefore, pairwise tests were conducted for this age group. P values in bold italic type indicate significant differences in shape among spawning locations.

$P = 0.117$, mean number of vertebrae 55.11 and 55.31; AL, $P = 0.833$, mean number of vertebrae 55.45 and 54.86). The number of pectoral fin rays showed overall nonsignificant differentiation among spawning locations ($P = 0.231$), whereas differences were significant between temporal samples at AL ($P = 0.032$, mean number of rays 17.33 and 17.65). Age distributions differed significantly among locations ($P < 0.001$) and between temporal samples at AL and RU (BB, $P = 0.093$; AL, $P < 0.001$; RU, $P < 0.001$). Ages for the samples, expressed as sample (mean age in years, median age in years), were as follows: SB02 (5.83, 6); SB03 (5.18, 5); AL02 (8.04, 8); AL03 (6.33, 6); RI03 (3.38, 3); RU0303 (5.98, 6); and RU0503 (5.17, 5).

Morphometric analyses

Fish size as represented by $\log(CS_{ij})$ differed significantly among samples, both spatially and temporally ($P(\text{location}) < 0.001$; $P(\text{time}) < 0.001$; $P(\text{location} \times \text{time}) = 0.622$). Skull shape also differed among samples, both spatially and temporally ($P(\text{location})$, $P(\text{time})$, and $P(\text{location} \times \text{time})$ were all < 0.0001 including and excluding CVA 1). Parametric MANOVAs and nonparametric one-way NP-MANOVAs revealed that skull shape (including CVA 1) differed signifi-

cantly among the four spawning locations ($0.001 < P \leq 0.041$; Table 3) for age groups 1 to 5 (the results are shown in Fig. 2). Landmark configurations are presented as thin-plate splines and show that the shape variation is largest in the neurocranial region of the skull, whereas the snout is less variable (Fig. 3b). Along CVA 1, the shape change is mainly an expansion-contraction of the central skull, and the shape change along CVA 2 represents mainly width changes of the posterior part of the skull.

Spatial tests

Partial Mantel tests showed no significant correlation between genetic distances (θ ; Weir and Cockerham 1984) and geographic distances (Table 4a). Morphometric distances, however, were significantly correlated with genetic distances (Table 4a). If geographic distance was controlled for, morphometric and genetic distances were correlated when size differences were included (i.e., including CVA 1) but not when size differences were removed (i.e., CVA 1 excluded) (Table 4a). Skull shape differences were correlated with geographic distances (Pearson's $r = 0.517$, $P = 0.026$) when size differences were included (i.e., including CVA 1) but not otherwise (Pearson's $r = -0.367$, $P = 0.782$). Morphometric distances

Table 4. (a) Partial Mantel tests for correlation between geographic, genetic, and morphometric distances among samples of Atlantic herring (*Clupea harengus*). (b) Mantel tests for correlation between morphometric distances and environmental distances between the spawning and (or) nursery grounds in the month of peak spawning activity with salinity in the upper panel and temperature in the lower panel.

(a) Partial Mantel tests for correlation between geographic, genetic, and morphometric distances.						
	CVA 1 included		CVA 1 excluded			
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>		
Genetic + morphometric distances	0.7472	0.0401	0.5269	0.0266		
– fixed geographic distances	0.7421	0.0329	0.4885	0.0593		
Genetic + geographic distances	0.2268	0.1765	0.2268	0.1718		
– fixed morphometric distances	–0.1877	0.8094	0.0158	0.4645		

(b) Mantel tests for correlation between morphometric distances and environmental distances.						
	Minimum salinity		Mean salinity		Maximum salinity	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
CVA 1 included	0.862	0.029	0.901	0.018	0.887	0.019
CVA 1 excluded	–0.029	0.546	0.005	0.459	–0.001	0.488

	Minimum temperature		Mean temperature		Maximum temperature	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
CVA 1 included	0.555	0.043	0.522	0.034	0.509	0.088
CVA 1 excluded	–0.030	0.493	–0.015	0.474	–0.052	0.510

Note: Partial Mantel tests were conducted using IBD (version 1.5; Bohonak 2002); Mantel tests were conducted using GENETIX (version 4.04; Belkhir 2000); *r* values represent Pearson's *r* (Manly 1994). *P* values in bold italic type indicate significant correlations between morphometric distance and environmental distance.

were correlated with environmental distances when size was included but not when size was excluded (Table 4b).

Discussion

Spatial population structure

Using genetic, meristic, and morphometric approaches, we found evidence of spatial and temporal differentiation among samples of spawning Atlantic herring. Genetic and morphometric distances were correlated even when size was controlled for, but they were not correlated with environmental distances. Conversely, shape differentiation including size variation and mean vertebrate counts was correlated with environmental distances. Below we discuss these findings and their implications in detail.

Molecular genetic population structure

Genetic differentiation was generally low, as is typically the case in marine fishes (DeWoody and Avise 2000). The question is whether the differentiation is significant or not. Tests for pairwise differentiation usually involve a relatively high number of repeated tests, resulting in a conservative α level when table-wide significance levels are applied. Therefore, we found no significant pairwise genetic differentiation overall. An AMOVA also showed nonsignificant genetic variation among spawning location, but this is in contrast to a previous AMOVA that included more samples (Jørgensen et al. 2005). In addition, the θ values from tests involving Rügen (RU) samples showed significant genetic differentiation when tests were considered separately. The four spawning locations included in the present study were found to belong to three areas characterized by relatively high genetic differentiation among areas and very low differentiation

within areas (Jørgensen et al. 2005). Our RU samples are separated from the remaining Baltic Sea herring. Gulf of Riga (RI) herring belong to a Baltic Proper associated area, whereas the Åland Archipelago (AL) and Bothnian Bay (BB) samples belong to groups of herring feeding and spawning in basins to the northeast of the Baltic Proper.

No isolation by distance was found among spawning locations, which, in part, may be due to the genetic differentiation between temporal samples (which have a zero geographic distance). Though low, temporal genetic differentiation was significant according to the AMOVA.

Population structure of meristic traits

Although the number of vertebrae differed significantly among the four spawning locations in this study, the number of pectoral fin rays did not. There was a significant correlation between differences in salinity between localities and differences in vertebrate numbers, indicating that the variation among spawning groups may be due to different salinities on the spawning and (or) nursery grounds. No such correlation was found for the number of fin rays.

Morphometric population structure

Genetic and morphometric distances were correlated even after controlling for geographic distances. The skull bones were expected to reflect differences in feeding among populations. Morphometric analysis of the skulls shows that herring from different spawning locations differ in skull shape, and these results are consistent with the molecular data. Both types of data indicate that RU samples show relatively large differentiation (high θ values and large Mahalanobis' distances) from other samples, implying that herring from this location were distinct from herring from other locations.

This is consistent with the fact that RU herring feed in the Danish waters where salinity is much higher than in the basins of the Baltic Sea that constitute feeding areas of the remaining herring included here (Aro 1989).

Morphometric distances were significantly correlated with geographic distances when size variation (CVA 1) was included, but nonsignificant when size variation (CVA 1) was excluded. Size was probably correlated to geographic distance mainly because the Rügen (RU) samples have by far the largest distances to the remaining samples, both when size and geographic distance are considered.

The number of samples is relatively low, and consequently the results of Mantel tests should be treated with caution. Nevertheless, two results — (i) the lack of morphometric isolation by distance when excluding size variation (CVA 1) and (ii) significant correlation between genetic and morphometric distances — may indicate that skull shape without the influence of size variation is more closely associated with neutral genetic variation (assuming that microsatellites are neutral).

Temporal population structure

At BB and RU we found no basis for rejecting the null hypothesis of temporal genetic stability, but the Åland (AL) 2002 and 2003 samples differed genetically before Dunn–Šidák correction. Nevertheless, despite the absence of temporal genetic differences at localities BB and RU, we found significant differentiation in multivariate shape between temporal samples within locations, even when the scaling effect was removed. Furthermore, the number of vertebrae and the number of pectoral fin rays differed significantly between temporal samples at RU and AL, respectively.

We tested for confounding effects due to temporal population structure at three locations, BB, AL, and RU. The samples from BB and AL were taken 1 year apart, whereas the samples from RU were taken at the beginning and the end of the spring-spawning season of 2003. It is likely that the temporal morphological differentiation is caused by different environmental conditions at the time of hatching and early growth as temporal genetic differentiation was only found at AL.

Because mean age differed significantly among samples, shape was compared independently for different age groups. There were differences in shape among spawning locations within all age groups for which we had representative samples from all locations.

Factors shaping population structure

We compared several distance measures by correlation analyses and found that genetic and morphometric distances were correlated, especially when size variation was excluded from the analysis. When size variation was included, skull shape variation was more closely correlated with environmental distances among spawning locations. This suggests that shape may be more strictly genetically controlled, whereas size shows plastic responses to environmental factors. Similar conclusions were reached in studies on morphological structures as different as the skull shape of Iberian lynx (*Lynx pardinus*; Pertoldi et al. 2005), molar teeth in lab mice (Workman et al. 2002), and wing venation in fruit fly (*Drosophila melanogaster*; Birdsall et al. 2000). Genetic and geographic distances among herring samples were not correlated, making an isolation by distance population structure unlikely.

By means of the canonical variates analysis of the partial warps adjusted for allometric effects, we placed all shape variation due to scaling effects along the first canonical axis. Approximately 76% of the total shape variance was explained by this axis. It should be noted that body size variation is not just a “noise component”, but an important variable that needs to be considered in shape analyses (Swain and Foote (1999) and references therein). Therefore, all analyses in this study were conducted with and without CVA 1. From this, we can conclude that most of the shape variation among spawning locations is due to the significant size-induced differences, but the remaining 24% of the shape variation still explains a significant proportion of the differentiation. Conversely, a minor part of the shape variation may be removed when excluding CVA 1, and this may impact the correlation analyses by lowering the probability of significant correlations between skull shape and, e.g., environmental variables.

The low θ values found among the spawning locations could indicate high gene flow, which is normally expected to lower the likelihood of local adaptation by natural selection, but the actual degree of adaptation is determined by an interaction between selective pressure and gene flow (e.g., Hendry et al. 2001). It is therefore possible for local populations to exhibit adaptation despite high gene flow (e.g., Saint-Laurent et al. 2003), and such a scenario could explain the significant variation in skull shape among locations. However, because of the migration patterns of the four spawning groups included here (Aro 1989), it can be questioned if there is much gene flow among them. This raises the possibility that the θ values are biased towards 0 because of large effective population sizes and lack of equilibrium between migration among spawning groups and random genetic drift within spawning groups, a process that is expected to take longer with increasing effective population sizes in each group and with a low number of migrants among groups (Chakraborty and Leimar 1987). Corresponding inaccuracies in morphological distances may arise when loci coding for morphological traits such as skull shape are not in selection–migration equilibrium. The present day connection between the Baltic Sea and the North Sea was formed through the Danish Straits 9 500 – 10 000 years ago (Ignatius et al. 1981). This is perhaps insufficient for the herring in the Baltic Sea to reach equilibrium.

To test directly whether differentiation in morphological traits is due to local adaptation by natural selection and not simply phenotypic plasticity, one may scan the entire genome of the organism under study as suggested by, e.g., Storz (2005) and Beaumont and Balding (2004) and compare genetic differentiation among populations at different loci (regions). To identify the ecological, environmental, or other factors that have major impacts on fish phenotypes, it may be necessary to conduct a common garden experiment or a reciprocal transplant experiment (e.g., Kawecki and Ebert 2004). Direct estimates of phenotypic plasticity in fish are so far uncommon, except for tests of the dependence of a few meristic traits on temperature during development, but a meta-analysis of 23 species of northern freshwater fish in postglacial lakes (Robinson and Parsons 2002) concluded that plasticity was a common response to varying environmental factors.

The significant morphometric differentiation among herring spawning groups excluding size variation was corre-

lated to neutral molecular genetic differentiation but not to environmental distances in the present study. This suggests that shape is influenced by other, possibly biotic, factors not studied here. One such factor might be the species composition of mesozooplankton in the different areas of the Baltic, as this would lead to variation in feeding among spawning groups (Möllmann et al. 2004). In addition, we found that morphometric distances including size variation, as well as differences in mean vertebrae count, were correlated with environmental distances. These results suggest that herring in the Baltic Sea adapt to local environments in part by phenotypic plasticity, which has been argued to be the optimal strategy to coping in a heterogeneous environment, such as the Baltic Sea, on a short time scale (Robinson and Wilson 1994). It follows that although variation in herring morphology may indicate a potential for local adaptation to spawning and (or) nursery areas, morphological variation alone would be a dubious indicator of herring population structure on a regional scale.

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Appendix A

Table A1. Data summary for microsatellite markers.

	<i>CHA1017</i>	<i>CHA1020</i>	<i>CHA1202</i>	<i>CHA1027</i>	<i>CPA101</i>	<i>CPA107</i>	<i>CPA111</i>	<i>CPA112</i>	<i>CPA114</i>
BB02^a									
No. alleles	10	20	12	21	21	4	5	13	20
<i>H</i> _{EXP}	0.804	0.909	0.789	0.936	0.915	0.571	0.376	0.764	0.916
<i>H</i> _{OBS}	0.703	0.903	0.781	0.939	0.901	0.693	0.395	0.759	0.938
<i>P</i> (HW _{eq})	0.885	0.560	0.382	0.551	0.530	0.049	0.747	0.200	0.831
BB03									
No. alleles	12	18	10	21	20	4	5	13	17
<i>H</i> _{EXP}	0.811	0.908	0.757	0.929	0.910	0.626	0.411	0.741	0.913
<i>H</i> _{OBS}	0.779	0.929	0.771	0.860	0.938	0.576	0.421	0.821	0.911
<i>P</i> (HW _{eq})	0.739	0.222	0.980	0.101	0.963	0.099	0.641	0.102	0.686
AL02									
No. alleles	10	19	10	20	19	4	7	14	16
<i>H</i> _{EXP}	0.778	0.911	0.732	0.923	0.914	0.625	0.422	0.753	0.913
<i>H</i> _{OBS}	0.777	0.911	0.685	0.929	0.930	0.685	0.438	0.759	0.929
<i>P</i> (HW _{eq})	0.116	0.007	0.104	0.055	0.215	0.153	0.931	0.158	0.028
AL03									
No. alleles	9	21	12	22	18	5	6	14	18
<i>H</i> _{EXP}	0.807	0.916	0.810	0.928	0.906	0.547	0.391	0.755	0.919
<i>H</i> _{OBS}	0.771	0.889	0.780	0.908	0.861	0.537	0.385	0.697	0.887
<i>P</i> (HW _{eq})	0.749	0.070	0.059	0.208	0.297	0.854	0.337	0.402	0.053
RI03									
No. alleles	11	18	12	19	22	5	5	13	17
<i>H</i> _{EXP}	0.799	0.914	0.776	0.914	0.917	0.566	0.390	0.717	0.914
<i>H</i> _{OBS}	0.752	0.898	0.761	0.907	0.926	0.459	0.358	0.741	0.906
<i>P</i> (HW _{eq})	0.244	0.474	0.183	0.898	0.372	0.001	0.505	0.107	0.267
RU0303									
No. alleles	8	17	11	18	19	4	6	16	16
<i>H</i> _{EXP}	0.816	0.872	0.715	0.926	0.908	0.511	0.439	0.799	0.909
<i>H</i> _{OBS}	0.755	0.796	0.765	0.920	0.910	0.505	0.515	0.847	0.919
<i>P</i> (HW _{eq})	0.632	0.430	0.977	0.828	0.379	0.007	0.119	0.545	0.693
RU0503									
No. alleles	9	17	9	18	20	4	6	16	18
<i>H</i> _{EXP}	0.801	0.891	0.721	0.920	0.897	0.473	0.501	0.798	0.861
<i>H</i> _{OBS}	0.737	0.916	0.730	0.889	0.857	0.677	0.580	0.847	0.847
<i>P</i> (HW _{eq})	0.801	0.131	0.807	0.403	0.645	0.000	0.056	0.927	0.032

Note: *H*_{EXP}, expected heterozygosity; *H*_{OBS}, observed heterozygosity; *P* values in bold type indicate departure from Hardy–Weinberg equilibrium (HW_{eq}), as revealed using the test by Guo and Thompson (1992) and Dunn–Šidák adjusting the α to 0.0073 ($k = 9$, the number of loci used for each sample).

^aSee Table 1 for explanation of sample codes.