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Sperm, egg, and embryo proteins critical for genetic adaptation of herring to low salinity in the Baltic Sea

Cheng Ma[a,b,1], Fahime Mohamadnejad Sangdehi[a,b,1], Mari Kawaguchi[c,2], Kaori Sano[d,2], Svenja V. Dannenberg[e,2], Mats E. Pettersson[a,b], Andreas Wallberg[a,b], Joshua L. Wort[f,3], Yumeng Yan[e], Sergei Moshkovskii[e,g], Florian Berg[h], Arild Folkvord[h,i], Christof Lenz[e,g], Henning Urlaub[e,g], U. Benjamin Kaupp[f,j], Shigeki Yasumasu[c], and Leif Andersson[a,b,k,4]

Affiliations are included on p. 10.

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How species genetically adapt to new environments is a central question in evolutionary biology. Here whole-genome sequencing combined with functional analysis is used to dissect how Atlantic herring, a marine fish, has adapted to the brackish Baltic Sea. Genes involved in reproduction and early development emerge as primary targets of natural selection, with key changes in a sperm-specific anion channel (*LRRC8C2*), a zona pellucida protein (*ZPBA1*), a cluster of three genes for fish transglutaminase (*FTG1-3*), and a copy number expansion of a fish hatching enzyme gene (*HE1C*). The large diameter of LRRC8C2 homomers facilitates transport of ions and osmolytes, likely preventing swelling of sperm when spawning in low salinity. Altered ZPBA1 sequence together with modified FTG1-3 enzyme activity produces a harder egg envelope that prevents egg swelling in brackish waters, while the enhanced activity of the adapted HE1C enzyme enables larvae to digest this reinforced egg envelope during hatching. Baltic Sea herring populations reproducing in brackish water are fixed or nearly fixed for variant alleles at these four unlinked loci, each carrying multiple amino acid substitutions compared to the alleles prevalent in the Atlantic Ocean populations. The alleles at two of these loci (*FTG1-3,* and *HE1C*) have been introgressed from the sister species Pacific herring. These findings reveal concrete molecular mechanisms by which a marine species has adapted to a novel, low-salinity environment.

molecular evolution | ecological adaptation | genome biology | natural selection | reproduction

Significance

In species with external fertilization, sperm, eggs, and embryos are directly exposed to the environment and must therefore undergo genetic adaptation to the local conditions. Using genetic and functional analyses, we investigated how Atlantic herring, adapted to the high salinity of the Atlantic Ocean, have adapted to the low salinity of the Baltic Sea. Our results show that genetic changes in genes governing reproduction and early development are particularly critical. These include genes encoding i) a sperm-specific anion channel, ii) one of the most abundant egg envelope proteins, iii) an enzyme that makes the egg envelop harder and thereby protects the egg from swelling at low salinity, and iv) an enzyme that degrades the reinforced egg envelope at hatching.

A major goal in population genomics is to identify the genes and molecular mechanisms causing evolutionary change. Key questions include the life stages at which selection acts and the relative contributions of changes in protein structure versus regulatory modifications. Genome-wide studies have mapped many regions associated with phenotypic variation, but translating these associations into defined causal variants and mechanisms remains difficult, largely because most traits are highly polygenic (1). Human height is a classical example, with thousands of loci each explaining only a minute fraction of the variance (2). Nevertheless, a number of large-effect alleles that drive phenotypic variation have been identified in natural populations, allowing to link specific genes to distinct traits (3). Notable examples include genes influencing morphological variations in sticklebacks (4) and genes determining beak morphology in Darwin's finches (5, 6).

Atlantic herring (*Clupea harengus*) is abundant throughout the North Atlantic and Baltic Sea, whereas its sister species, Pacific herring, inhabits the North Pacific and Arctic Ocean (Fig. 1*A*). Atlantic herring is an excellent model for genetic studies of ecological adaptation because it combines i) enormous population size, which minimizes genetic drift (7), ii) subdivision into locally adapted ecotypes and subpopulations, and iii) a recent colonization (within the last 8,000 y) to a new environment, the brackish Baltic Sea, where salinity drops to 2 to 3‰ in the inner Bothnian Bay compared to 34 to 35‰ in the North Atlantic. Previous whole-genome sequencing revealed hundreds of loci exhibiting striking genetic differentiation between Atlantic and Baltic herring, with some loci reaching complete fixation (7). Variants changing protein sequence are highly enriched among those displaying the largest allele-frequency differences, underscoring the central role of coding changes in adaptation to the Baltic Sea (8).

Our previous work suggested that genetic changes affecting reproduction in brackish water are central to adaptation. We identified a strongly differentiated genomic region between Atlantic and Baltic herring that contains multiple copies of fish hatching enzyme genes (8), which are specifically expressed in hatching gland cells of prehatching embryos (9, 10). However, the contig-level assembly used then was too fragmented to fully characterize this polymorphism. We also found robust signatures of selection in Baltic herring

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[1] C.M. and F.M.S. contributed equally to this work.

[2] M.K., K.S., and S.V.D. contributed equally to this work.

[3] Present address: Division of Molecular and Cellular Function, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester M13 9PT, United Kingdom.

[4] To whom correspondence may be addressed. Email: leif.andersson@imbim.uu.se.

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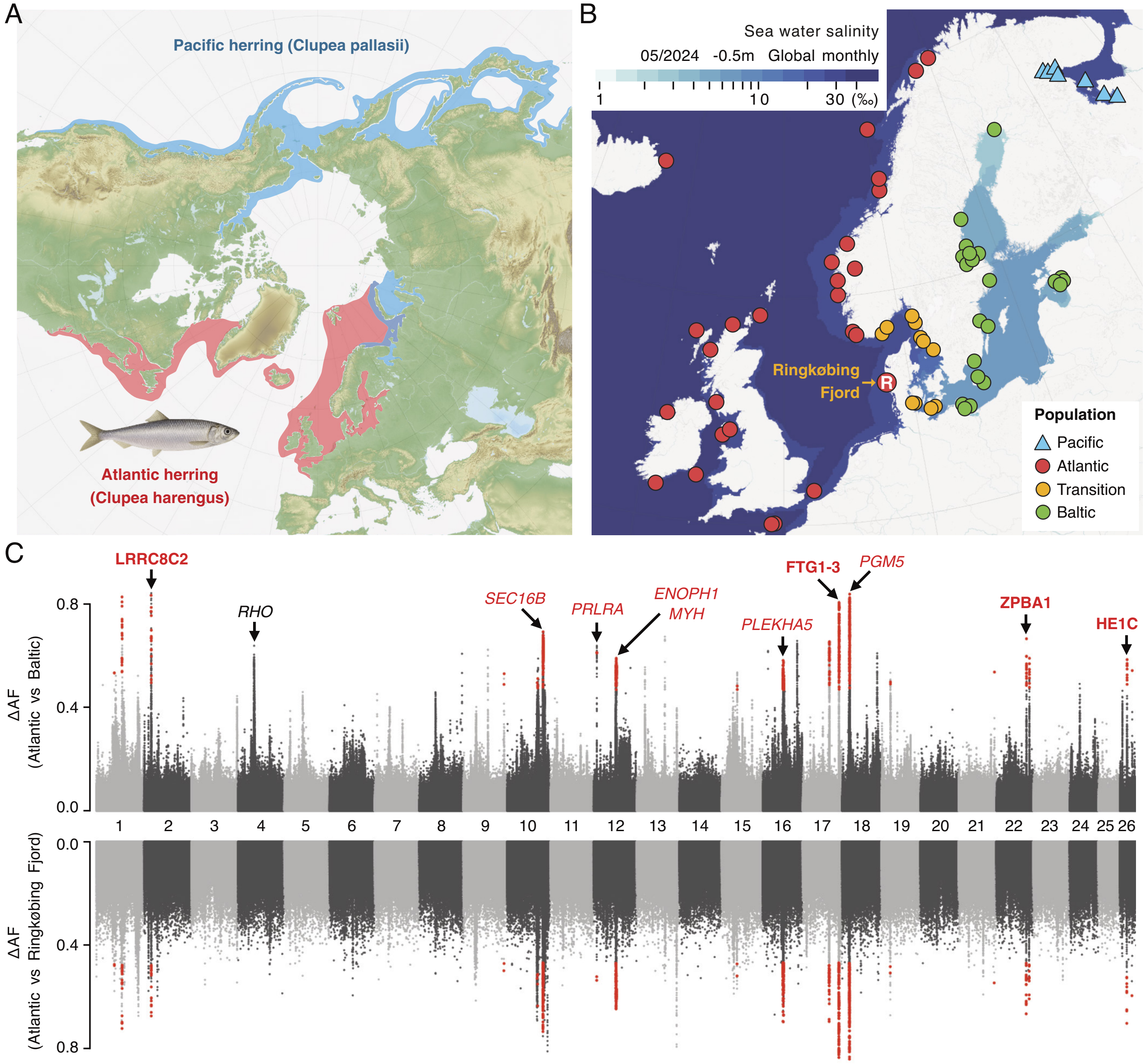

Fig. 1. Species distribution, sample locations, and genomic screen. (*A*) Species distribution of Atlantic and Pacific herring. (*B*) Sampling locations for whole-genome sequencing. The *Inset* shows color code for salinity. (*C*) Allele frequency contrasts comparing Atlantic spring-spawners against Baltic spring-spawners and Ringkøbing Fjord herring. The upward-facing track shows absolute delta allele frequencies in the Atlantic-Baltic contrast, the downward-facing one the Atlantic-Ringkøbing contrast. SNPs highlighted in red have values above 0.5 in both contrasts. Gene labels in red indicate notable genes in regions of overlap.

shared with some Atlantic populations that spawn in brackish water but spend their adult life in fully marine environments, notably the population spawning in Ringkøbing fjord on the Danish west coast, where salinity is 12 to 15‰ (11).

Here, we combine a genetic screen of Baltic herring, Atlantic herring, and an Atlantic population spawning in the brackish Ringkøbing fjord with high-resolution proteomic analyses and functional analysis of sperm and eggs to identify genes underlying adaptation of reproduction to brackish waters. We identify two key genes: a sperm-specific volume-regulated anion channel (VRAC) gene (*LRRC8C2*) and a zona pellucida protein (*ZPBA1*), a major egg-envelope constituent (12). Additionally, we identify two relevant gene clusters: three linked copies of a fish transglutaminase gene (*FTG1-3*), encoding an enzyme that crosslinks ZPBA1 proteins during egg hardening (13), and a cluster of fish hatching enzyme genes (*HE1C*) encoding the enzymes which digest the egg envelope during hatching (9, 10). Genetic, computational, and functional characterization show that these loci have tightly interconnected evolutionary histories that enabled herring to thrive and become a keystone species within the Baltic Sea ecosystem.

Results

Ringkøbing Fjord: A Rosetta Stone for Understanding the Evolution of Baltic Herring. We reanalyzed our previously published whole-genome sequencing data from 69 population samples (Fig. 1*B*), focusing on pairwise comparisons between Atlantic herring (spawning in marine environment) versus Baltic herring or Atlantic herring from Ringkøbing Fjord, which both spawn in brackish water (Fig. 1*C*). We identified 51 genomic regions with one or more SNPs exceeding a delta allele frequency (dAF) of 0.50 (*SI Appendix*, Table S1). All these regions had strong statistical support (*SI Appendix*, Fig. S1). These included the *rhodopsin* (*RHO*) locus where a missense mutation Phe261Tyr is underlying adaptation to the red-shifted light environment of the Baltic Sea (14). Another previously highlighted locus is *prolactin-receptor locus A* (*PRLRA*), a gene with a well-established role in osmoregulation (15). This locus shows strong signatures of selection in populations of herring and the closely related sprat adapted to the brackish Baltic Sea. The lack of missense mutations in herring and sprat suggest underlying regulatory mutations that have not yet been identified (16).

Approximately half of the loci (n = 26) that were identified comparing Atlantic and Baltic herring were also highly significant in the contrast between Atlantic herring and herring from Ringkøbing Fjord. Ringkøbing Fjord and Baltic Sea herring shared alleles at these loci (*SI Appendix*, Table S1) which did not include the *rhodopsin* locus. The results imply that genetic polymorphisms within the shared genomic regions are important for reproduction under brackish conditions.

Comparative analysis of loci under strong selection in herring populations spawning in brackish water (*SI Appendix*, Table S1) revealed four candidate genes of particular interest: i) a C isoform of the VRAC (*LRRC8C2*) on chromosome 2, ii) a zona pellucida gene (*ZPBA1*) on chromosome 22, iii) a cluster of three fish transglutaminase genes (*FTG1-3*) on chromosome 17, and iv) a cluster of fish hatching enzyme genes (*HE1C*) on chromosome 26. These genes were selected for further genetic and functional analysis.

LRRC8C2: A Sperm-Specific VRAC Unique to the Clupeiformes order. Cells tightly regulate their volume in response to changes in osmolarity. VRACs play a vital role in preventing cells from excessive swelling (17–19). These channels open upon membrane stretch during cell swelling and conduct efflux of anions—primarily Cl^- ions—or osmolytes. VRACs usually assemble as heteromeric complexes composed of subunits encoded by the leucin-rich repeat-containing protein 8 (LRRC8) gene family. Vertebrates express five distinct isoforms encoded by *LRRC8A-E*.

In herring, genomic analysis revealed two distinct loci on chromosome 2 and 10 that encode C isoforms of VRAC, designated *LRRC8C2* and *LRRC8C1*, respectively (*SI Appendix*, Table S2). These genes were characterized through phylogenetic analysis, which shows that *LRRC8C2* represents a gene duplication unique to members of the order Clupeiformes (Fig. 2*A* and *SI Appendix*, Table S3). PAML analysis showed that *LRRC8C2* exhibits an accelerated rate of protein evolution as indicated by a much higher ratio of nonsynonymous (d_N) to synonymous substitutions (d_S) compared with *LRRC8C* genes in mammals and in fish (Fig. 2*B*), likely reflecting the acquisition of a more specialized function. Rapid evolution is a common feature of proteins involved in sexual reproduction (20). Importantly, the accelerated evolution does not reflect a loss of functional importance: the open reading frame is conserved across all analyzed clupeiformes with an *LRRC8C2* gene and the *dN/dS* ratio well below 1.0 (Fig. 2 *A* and *B*), despite these species diverging roughly 70 Mya (21).

Four of the most strongly differentiated SNPs between Atlantic and Baltic herring in the chromosome 2 region are missense mutations in *LRRC8C2*. We identified four alleles: i) *Atlantic*, fixed in Atlantic herring spawning in marine habitat; ii) *Intermediate*, predominant in herring spawning in brackish waters with intermediate salinity such as Ringkøbing Fjord and the Atlantic–Baltic transition zone; iii) *BalticS*, predominant in the southern Baltic Sea; and iv) *BalticN*, prevalent in the northern Baltic Sea with the lowest salinity (Fig. 2 *C* and *D* and *SI Appendix*, Fig. S2). The *Atlantic* and *Intermediate* alleles differ by two amino acid substitutions, while two additional substitutions occur in *BalticS* and *BalticN* (Fig. 2*C*), suggesting a pattern of stepwise adaptive evolution to decreasing salinity. The strong correlation between allele frequencies and spawning site salinity is remarkable (Fig. 2*D*).

To examine the expression pattern of *LRRC8C1* and *LRRC8C2* gene products, we performed proteomic analyses on spawned sperm, eggs, and five somatic tissues (brain, heart, liver, skeletal muscle, spleen) from three to five individual Baltic herring using liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Dataset S1) with data-dependent and -independent acquisition (DDA and DIA). In addition, we used parallel reaction monitoring (PRM) to complement the DDA and DIA data. LRRC8C1 was readily detected in four of the five somatic tissues, but was absent from sperm and oocytes. By contrast, DIA and DDA LC–MS/MS analyses revealed LRRC8C2 in sperm, which was further confirmed by PRM-MS (*SI Appendix*, Fig. S3), but LRRC8C2 was not detected in any somatic tissue or in oocytes. We therefore applied a limit-of-detection (LOD) approach (23) to quantitatively assess whether LRRC8C2 is indeed absent from these tissues. For all somatic tissues, the LOD was 0.005 fmol per µg of protein (*SI Appendix*, Table S4 and Fig. S4). Accordingly, if LRRC8C2 is expressed in any of these tissues, its abundance must lie below this level. Using the same approach, we determined that LRRC8C1 is either absent from herring sperm or present at levels below 0.005 fmol per µg of protein (*SI Appendix*, Table S4 and Fig. S3). These findings indicate that LRRC8C2 is a sperm-specific protein in clupeiformes, whereas LRRC8C1 exhibits broad tissue expression, like the single *LRRC8C* orthologue in other vertebrates.

According to intensity-based absolute quantification (iBAQ), LRRC8C2 is highly abundant in sperm (Fig. 2*E* and *SI Appendix*, Table S5). Other abundant prominent proteins include protein kinase A (PKA), A-kinase anchoring proteins (AKAPs), Na^+/K^+-ATPase and Ca^{2+}-ATPase subunits, as well as signaling proteins such as several CatSper channel subunits, four HCN channel isoforms, soluble adenylate cyclase ADCY10, and the sperm-specific Na^+/H^+ exchanger SLC9C. Many proteins implicated in oocyte-sperm recognition at the "fertilization synapse" are also present (Fig. 2*E*).

Strikingly, LRRC8C2 is the only LRRC8 isoform present in sperm (*SI Appendix*, Fig. S5 and Dataset S1*A*); only a single peptide was detected for LRRC8Aa (Dataset S1*A*), likely reflecting contamination, and no peptides from subunits B, D, and E were detected, suggesting that VRAC in sperm is a homomeric channel. This is unexpected because VRACs are typically heteromers that require the A subunit for function, and only LRRC8CA had previously been shown to form functional homomeric hexamers in heterologous systems (24, 25). Recent structural work shows that mammalian LRRC8C subunits can assemble into homoheptamers (26, 27), potentially constituting a functional ion channel with a large pore diameter (*SI Appendix*, *Supplementary Text*, Figs S6–S8, and Table S6).

In conclusion, the sperm-specific expression, the perfect association between missense mutations and salinity combined with the established function of VRACs imply that the Baltic LRRC8C2 variants likely protect herring sperm from swelling in brackish water, but this conjecture needs to be further explored by experimental work.

Altered Protein Sequences of a Major Zona Pellucida Protein (ZPBA1) and Fish Transglutaminase (FTG) are Associated with Thicker and Harder Egg Envelope in Baltic Herring. ZPBA1 is a major structural component of the egg envelope (12) and the ZP domains of these proteins polymerize to form fibrous structures, with the N-terminal portion looping out and bundling fibers together (28). Three genes encode different isoforms of zona

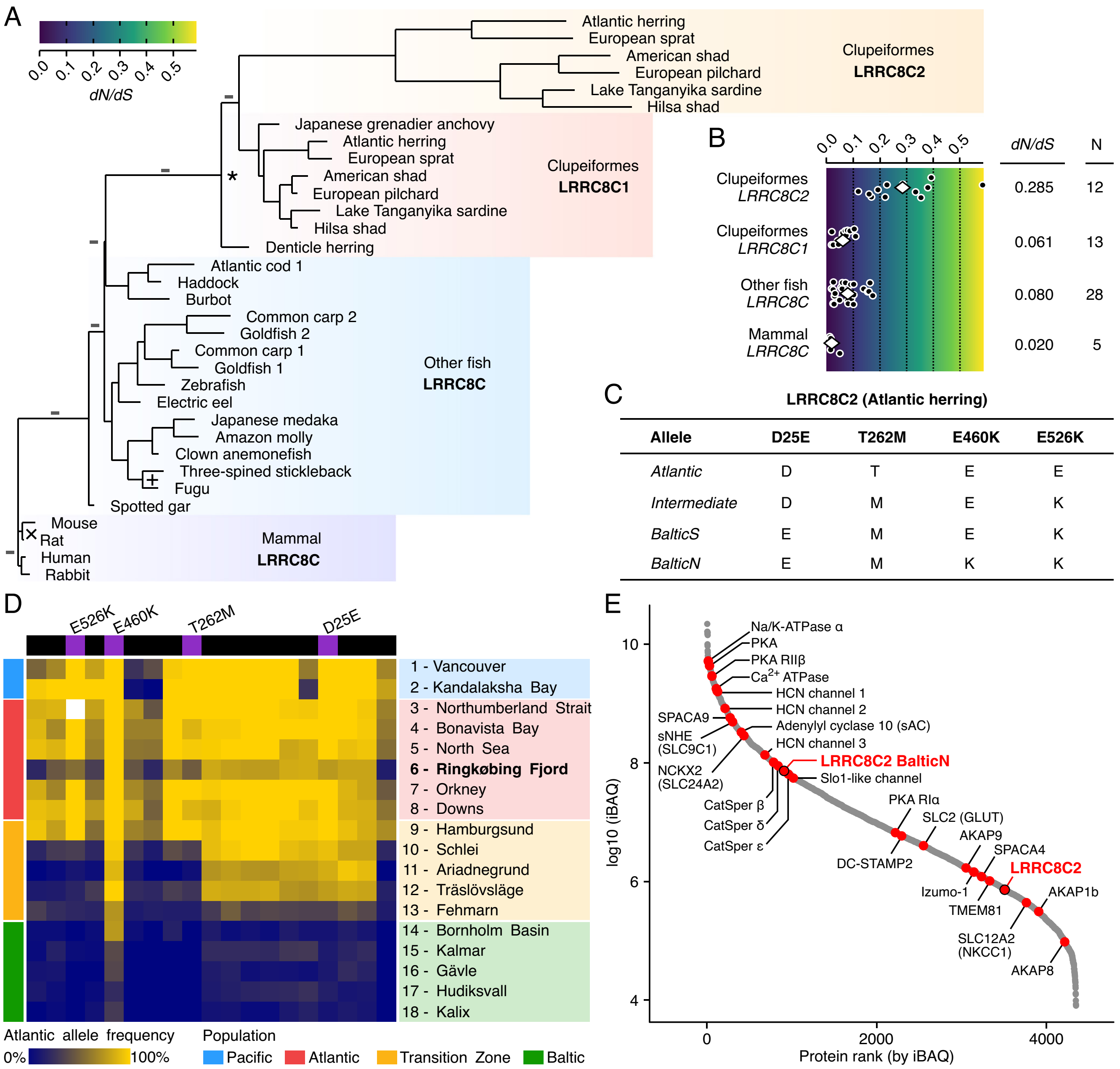

Fig. 2. Characterization of the sperm-specific *LRRC8C2* gene and its evolution. (*A*) A Bayesian gene tree based on a coding DNA alignment of *LRRC8C* sequences from fish and other vertebrates. Branches were rescaled by d_N/d_S using a free-ratio branch model under maximum likelihood. Both lengths and colors of the branches represent the d_N/d_S ratio. All nodes except three have posterior probabilities greater than 0.95 (× = 0.85; + = 0.61; * = 0.82). Ancestral branches not included in any group are indicated with a minus sign. (*B*) Distribution of d_N/d_S ratios of different groups of branches (black = d_N/d_S per branch; white = mean d_N/d_S of the group). The mean d_N/d_S and number of branches of each group are provided. (*C*) Amino acid combinations making up the four common *LRRC8C2* alleles in Atlantic herring. Amino acid positions are based on the following NCBI entry: XP_012675982.2. (*D*) Allele frequency heatmap showing highly differentiated SNPs at the *LRRC8C2* locus in selected population samples of Pacific, Atlantic, and Baltic herring. Purple boxes above the heatmap indicate missense positions. Note that *LRRC8C2* is encoded on the negative strand, reversing the SNP order. (*E*) Sperm proteins ranked by iBAQ intensity (22), visualizing the distribution of abundance across the proteome. Key sperm proteins are indicated, highlighting LRRC8C2 in red. Detailed data for highlighted proteins are in *SI Appendix*, Table S5.

pellucida protein B in the herring genome (*SI Appendix*, Table S2), strong signatures of selection are evident only for the *ZPBA1* gene on chromosome 22, at 20.87 Mb. *ZPBA1* is located within a closely linked gene cluster, but the peak of association is precisely at this gene, and the top SNP is a *ZPBA1* missense mutation (*SI Appendix*, Fig. S9). Postfertilization hardening of the envelope is mediated by a transglutaminase (FTG) unique to fish, which crosslinks ZPBA proteins, in the N-terminal region, transforming the egg envelope into a rigid structure (13). The *FTG* gene evolved by duplication from the *coagulation factor 13A* gene (*F13A*) early during the evolution of teleosts (13) (Fig. 3*A* and *SI Appendix*, Table S7). Similar to *LRRC8C2*, *FTG* shows accelerated protein evolution after duplication and neofunctionalization (Fig. 3*A* and *SI Appendix*, Table S8). Furthermore, a copy number expansion has occurred before the split between Atlantic and Pacific herring, both have five copies *FTG1-5* located on two chromosomes, while the closely related European sprat (*Sprattus sprattus*) has a single copy (Fig. 3*A*).

Population genetic analyses revealed near-complete fixation of different alleles at the *ZPBA1* locus on chromosome 22 and at

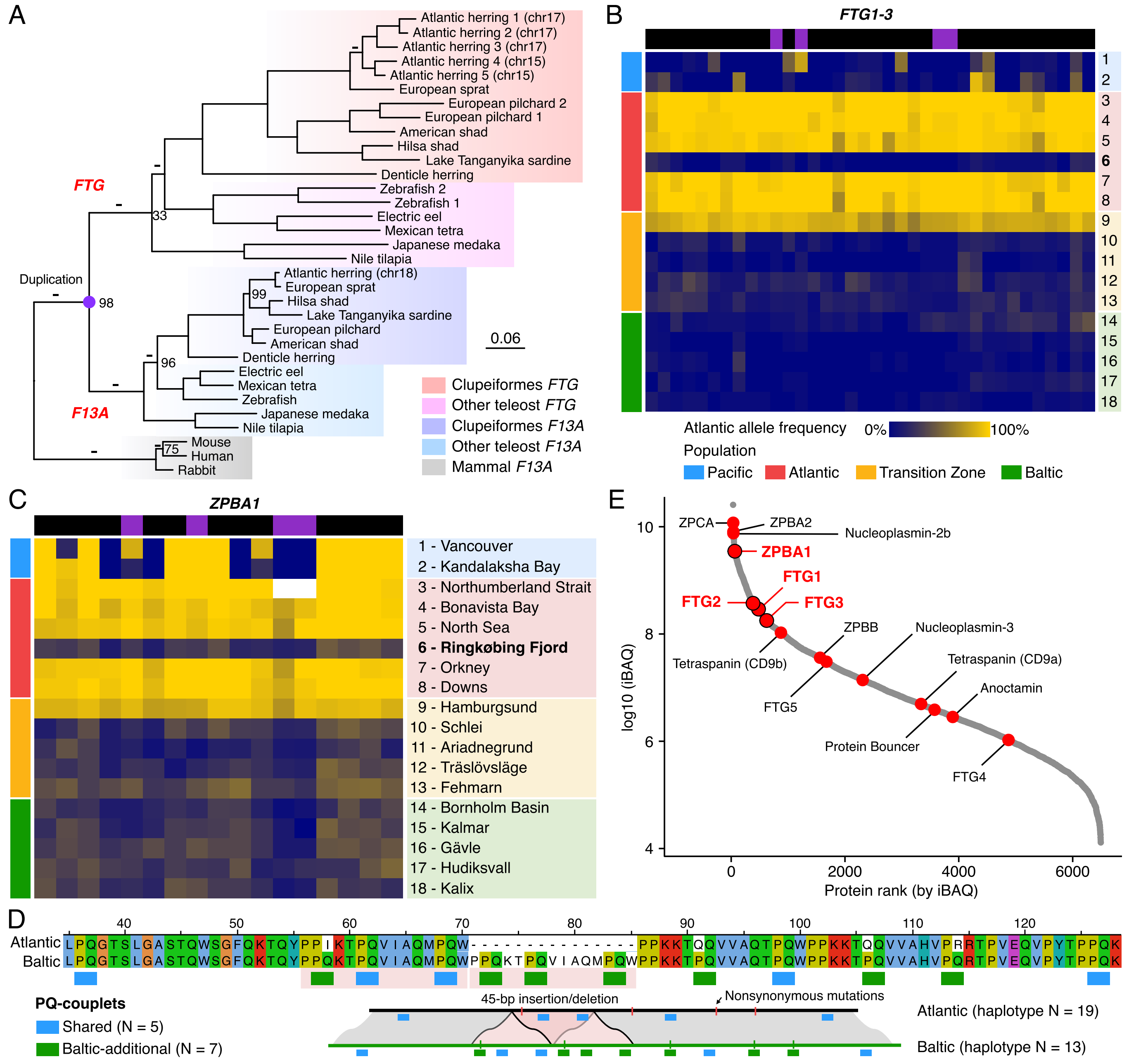

Fig. 3. Genetic analysis of fish transglutaminase (*FTG1-3*) and zona pellucida protein (*ZPBA1*) genes. (*A*) Maximum-likelihood phylogeny of *FTG* and *F13A* coding sequences from Atlantic herring and representative teleost species, rooted using mammalian *F13A* genes as an outgroup. The tree topology was inferred under a codon-based substitution model, and branch lengths were rescaled according to nonsynonymous substitution rates (d_N) estimated under a free-ratio branch model. The duplication separating *FTG* and *F13A* lineages is marked. Mean d_N, d_S, and d_N/d_S values for the defined branch groups are reported in *SI Appendix*, Table S8. Branches marked with a minus sign were not assigned to any branch group. Bootstrap support values are shown at nodes where support was below 100%. (*B*) Allele frequency heatmap showing highly differentiated SNPs at the *FTG1-3* locus in selected populations of Pacific, Atlantic, and Baltic herring. Purple boxes in the top row indicate missense positions. (*C*) Corresponding heatmap showing highly differentiated SNPs at the *ZPBA1* locus. (*D*) Alignment of the N-terminal region of representative ZPBA1 sequences from Atlantic and Baltic herring. Green boxes indicate PQ-couplets detected only in alleles found in Baltic herring, blue boxes indicate shared PQ-couplets. The insertion/deletion polymorphism represents a copy number variation of a 15 aa sequence motif. − = gaps in alignment. (*E*) Oocyte proteins ranked by iBAQ intensity (22), visualizing the distribution of abundance across the proteome. Key oocyte proteins are indicated, highlighting ZPBA1 and FTG1-3 in red. Detailed data for highlighted proteins are in *SI Appendix*, Table S9.

the *FTG1-3* gene triplet on chromosome 17 between Atlantic and Baltic herring (Fig. 3 *B* and *C* and *SI Appendix*, Figs. S10 and S11). Notably, the Ringkøbing Fjord population shares alleles with Baltic herring at both loci. The allele frequency heat map for the *FTG1-3* region strongly suggested that the Baltic haplotypes represent adaptive introgression from Pacific herring (Fig. 3*B*). This was confirmed by constructing genetic distance trees for individually sequenced Atlantic, Baltic, and Pacific herring (*SI Appendix*, Figs S12 and S13). A strong signature of a selective sweep in the Baltic herring was noted as their *FTG1-3* haplotypes formed a tight cluster within the cluster of Pacific haplotypes. Interestingly, some haplotypes present in Atlantic herring, most likely representing local Norwegian fjord herring, also carried Pacific haplotypes but clearly different from the ones present in Baltic herring (*SI Appendix*, Figs. S12 and S13), implying that Pacific *FTG1-3* haplotypes occur in some Atlantic herring populations and were selected when the Baltic Sea was colonized.

The allele frequency heat map for the *ZPBA1* locus did not give a clear indication of the origin of the selected haplotype (Fig. 3*C*), but the genetic distance tree clearly showed that the adaptive haplotypes present in Baltic herring have evolved from Atlantic herring haplotypes (*SI Appendix*, Fig. S12*B*).

The predicted protein sequences of the Atlantic and Baltic *ZPBA1* alleles show striking divergence in the N-terminal region of the protein: a 15 amino acid insertion/deletion at residues 70 to 84 and complete or near-complete fixation for different variants at residues 58, 91, 106, and 114 (Fig. 3*D*). This is precisely the region of ZPBA1 targeted by fish transglutaminase (12). The transglutaminase is forming ϵ-(γ-glutamyl) lysine cross-links in ZPBA proteins by targeting Pro-Gln-X repeats and the most common Baltic *ZPBA1* allele carries 12 such sites whereas the Atlantic allele has only 5 (Fig. 3*D*). No fixed differences between Baltic and Atlantic alleles are observed elsewhere in the *ZPBA1* sequence.

The signal of genetic differentiation on chromosome 17 peaks at a cluster of three fish transglutaminase genes which exhibit a distinctive arrangement with the central copy oriented in the opposite direction relative to the flanking copies (Fig. 4*A*). Atlantic herring host five *FTG* copies: two on chromosome 15 (*FTG4-5*) and three on chromosome 17 (*FTG1-3*), whereas the closely related European sprat carries a single copy (Fig. 4*B*). The nucleotide distance tree, derived from PacBio-based haplotypes from Atlantic and Baltic herring, shows no differentiation at the *FTG4-5* loci on chromosome 15 in sharp contrast to the perfect sorting of alleles at each of the three loci on chromosome 17 (Fig. 4*B*). There is a striking reduction in nucleotide diversity (π) at the *FTG1-3* region in Baltic herring, a classical sign of a selective sweep (Fig. 4 *B* and *C*). Further, PAML analysis demonstrates accelerated protein evolution of the *FTG* genes in herring following divergence from European sprat about 12 Mya (dN/dS = 2.4; *SI Appendix*, Table S8). Alignment of FTG1-3 protein sequences revealed a total of 35 amino acid substitutions showing fixed differences between Atlantic and Baltic alleles, with Baltic *FTG* haplotypes belonging to the Pacific herring lineage (Fig. 4*D*). We identified Pacific haplotypes that were identical to Baltic haplotypes at all these amino acid sites that passed quality filtering (n = 30) (*SI Appendix*, Fig. S13*B*). Furthermore, the Baltic haplotypes belong to the cluster of *FTG* haplotypes more common in Arctic Pacific herring than in Pacific herring sampled close to Vancouver (*SI Appendix*, Fig. S13*B*).

Proteomic analysis of spawned eggs from Baltic herring confirmed the presence of ZPBA1 and FTG1-5 proteins in unfertilized oocytes (Fig. 3*E* and *SI Appendix*, Table S9). The enzymes encoded by the *FTG1-3* locus under selection are clearly more abundant in the egg proteome than FTG4-5 according to our iBAQ analysis. Further, assays of transglutaminase enzyme activity in extracts from unfertilized eggs revealed that Baltic alleles exhibit higher activity at low-salinity conditions (6‰), while Atlantic alleles are more active at high salinity (35‰), matching the distinct salinity profiles of the Baltic Sea and the Atlantic Ocean (Fig. 4*E*).

We conducted in vitro fertilizations using sperm and eggs from Atlantic and Baltic herring at three different salinities (6, 16, and 34‰) with the aims to investigate enzyme activities in eggs and embryos and to measure egg envelope characteristics. This experiment was not designed to compare fertility rates because we cannot mimic the natural environmental conditions at spawning grounds, involving for instance mechanical forces generated by waves and currents. However, we noted that Atlantic females showed low fertility at low salinity but maintained high fertility at higher salinity (*SI Appendix*, Table S10). Conversely, three out of four Baltic females exhibited lower fertilization rates at high salinity (34‰) compared to low salinity (6‰). These results suggest salinity-associated differences in fertility rates, though the limited sample size prevents statistical significance.

The isolated Atlantic fertilized egg envelopes appeared less rigid compared to those of Baltic herring (*SI Appendix*, Fig. S14). The density of the egg envelope protein was estimated using egg diameter, egg envelope thickness, and protein content per egg envelope. The protein content per unfertilized and fertilized egg envelope was significantly higher in Baltic herring eggs than in Atlantic herring eggs, and egg envelopes of Baltic herring were more than twice as dense as Atlantic herring (*SI Appendix*, Fig. S15 and Table S11), suggesting a denser protective envelope in the Baltic population.

Measurements of the diameter of fertilized eggs revealed substantial swelling in Atlantic herring eggs when incubated at low salinity (a >30% increase in diameter at 6‰). In contrast, eggs from Baltic herring remained largely resistant to swelling under the same conditions (Fig. 4*F*), indicating that the Atlantic egg envelope expands and the perivitelline space enlarges in brackish environments. This leads to a weakening of the protective function of the egg envelopes for the embryos during their development. In conclusion, genetic changes in the ZPBA1 and FTG1-3 protein sequences are associated with a harder egg envelope that protects eggs from swelling during spawning in brackish environments such as the Ringkøbing Fjord and Baltic Sea.

A Substantial Copy Number Amplification of Fish Hatching Enzyme Genes (*HE1C*) Is Linked to Degradation of Hard Egg Envelopes in Brackish Environments. Hatching enzymes cleave N-terminal Pro-Gln repeat sequence in ZPBA1 that contains Gln-Lys cross-links formed by fish transglutaminase (29, 30). Genes encoding fish hatching enzyme have undergone dynamic evolutionary changes, including gene duplication and diversification among teleosts (31). In Euteleostei, the hatching enzymes are classified into two types: high and low choriolytic enzyme, each with distinct biochemical properties. Manual annotation of hatching enzyme genes using the most recent Atlantic herring reference genome (Ch_v3.1, GCA_040183275.2) identified 37 copies distributed across seven loci—each separated by at least 1 Mb—across five chromosomes (*SI Appendix*, Tables S2 and S12); *HE1A-E* and *HE2* encode high choriolytic enzyme while *HE3* encodes low choriolytic enzyme. Expression analysis in Pacific herring showed that *HE1* is more highly expressed than *HE2* and *3*, indicating *HE1* is the major hatching enzyme in herring (32). Notably, *HE1A*, *HE1B,* and *HE1C* at chr20:1.9 Mb, chr22:25.9 Mb, and chr26:6.3 Mb harbor tandem arrays of 6, 7, and 20 gene copies in the reference genome, respectively (*SI Appendix*, Table S2). A maximum likelihood phylogeny of hatching enzyme genes (*SI Appendix*, Fig. S16) reveals that all herring *HE1* subtype genes cluster in a species-specific clade, indicating concerted evolutionary processes within lineages.

Whole-genome sequencing of Atlantic and Baltic herring populations revealed strong genetic differentiation at the *HE1C* gene cluster on chromosome 26 replicated in Ringkøbing Fjord (Figs. 1*C* and 5*A*). The allele frequency heat map shows, as for the *FTG1-3* locus, that the Baltic haplotypes represent adaptive introgression from Pacific herring. A genetic distance tree based on individually sequenced herring supports this conclusion (*SI Appendix*, Fig. S12). Haplotype-phased PacBio long-read assemblies—representing eight Atlantic herring and seven Baltic herring revealed striking differences in copy number: all Baltic haplotypes, except one, carry about 20 *HE1C* copies, while Atlantic haplotypes possess at most two copies, most with none, mirroring the corresponding region in European sprat (Fig. 5*B*).

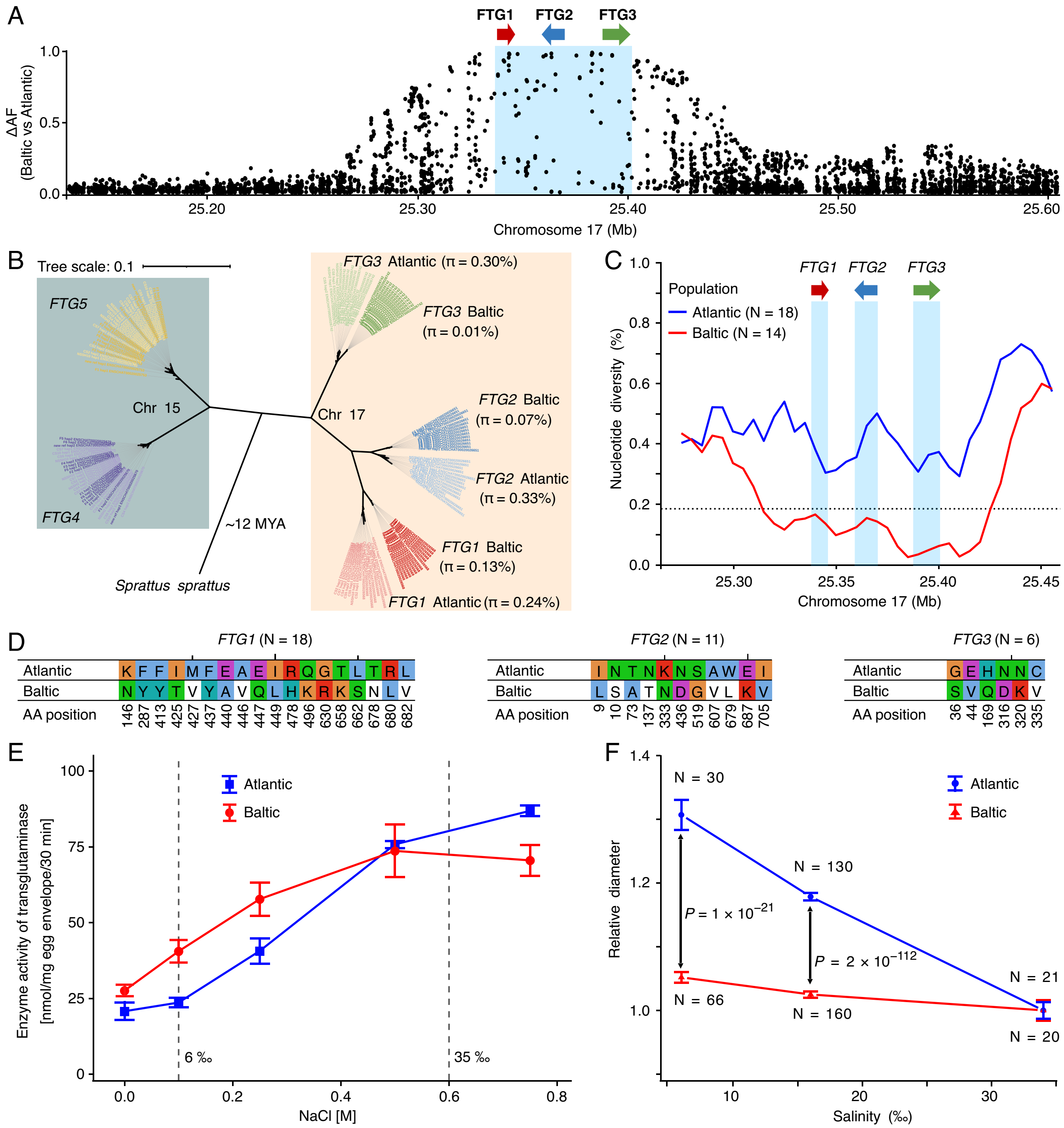

Fig 4. Natural selection at fish transglutaminase genes *FTG1-3* and functional consequences. (*A*) Delta allele frequency (ΔAF) between Atlantic and Baltic herring in part of chromosome 17. (*B*) Maximum-likelihood tree of *FTG* coding sequences from haplotype-phased PacBio assemblies of 8 Atlantic and 7 Baltic herring using European sprat (*S. sprattus*) as outgroup. Tip labels in lighter and darker colors denote Atlantic and Baltic sequences, respectively. Nucleotide diversity (π) for *FTG1–FTG3*, calculated separately for Atlantic and Baltic sequences, is shown in parentheses. (*C*) Nucleotide diversities (π) for Atlantic and Baltic herring across the *FTG1–FTG3* genomic region. (*D*) Amino acid alignment of FTG1 - FTG3 sequences of sites showing fixed differences between Atlantic and Baltic herring. N = number of fixed amino acid changes between Atlantic and Baltic alleles. (*E*) Enzyme activity of fish transglutaminase (±SEM), estimated from the amount of monodansylcadaverine (MDC) incorporated in the egg envelopes of unfertilized eggs from Atlantic and Baltic herring in relation to salinity. (*F*) Diameter of fertilized eggs from Atlantic and Baltic herring incubated at 6‰, 16‰, and 34‰ salinity. N = total number of eggs measured. These were collected from 4 Atlantic and 10 Baltic herring females.

Both Atlantic and Baltic populations carry multiple *HE1* copies on chromosomes 20 and 22, but only Baltic herring possess the additional 20 copies on chromosome 26, fundamentally altering their HE1 protein repertoire (*SI Appendix*, Fig. S17).

Population-level whole-genome sequencing data demonstrated that copy number of *HE1C* genes on chromosome 26—distinguished by unique nucleotide variants—correlates strongly with ambient salinity at sampling sites ($R^2 = 0.95$, $P < 2.7 \times 10^{-41}$) (Fig. 5*C* and *SI Appendix*, Table S13), indicating that copy number is under strong natural selection in response to salinity during embryonic development. Notably, Pacific herring spawning in the White Sea, where the salinity can be as low as 10 to 12‰ in bays during spring, carry more

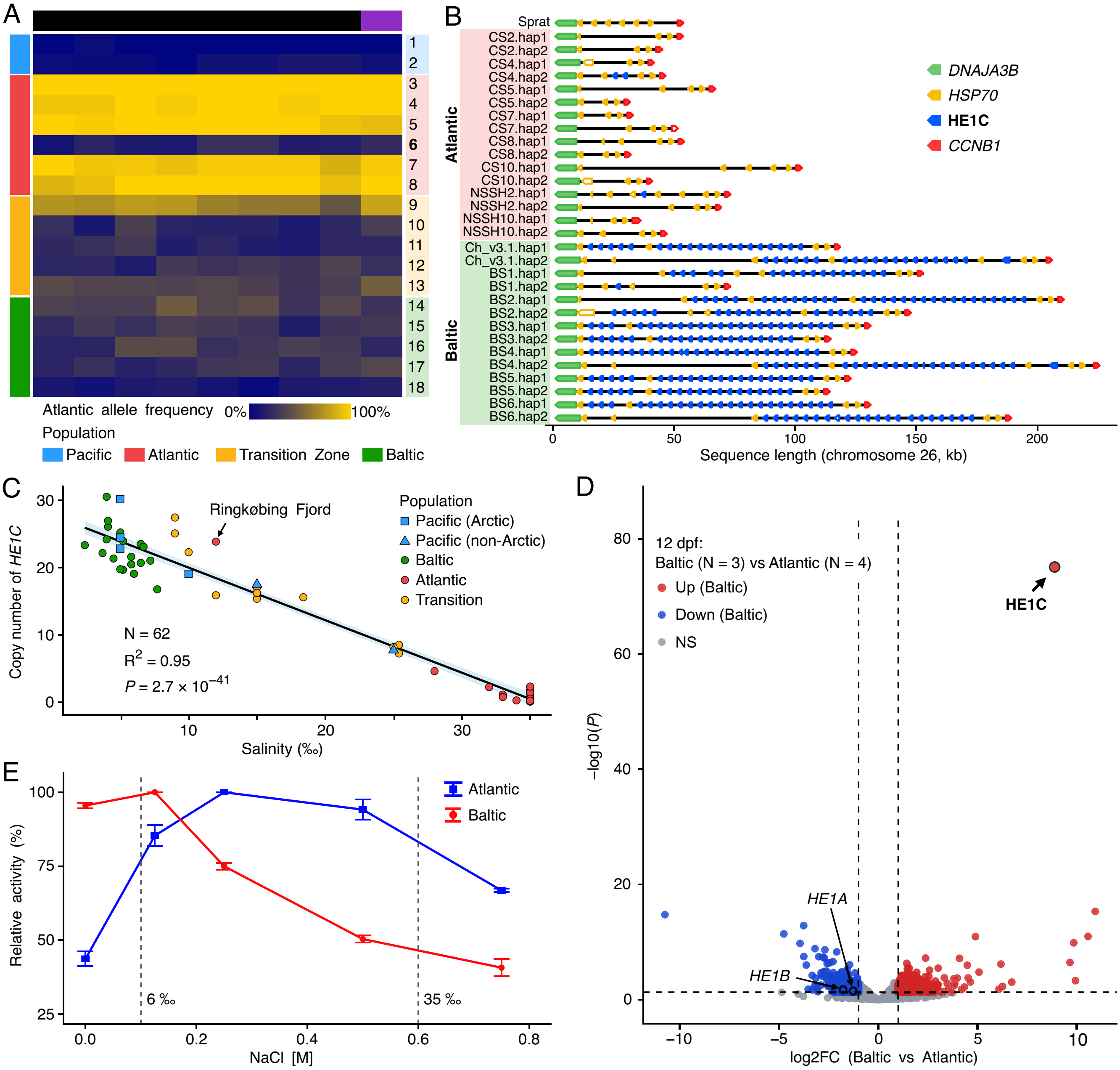

Fig. 5. Copy number expansion and altered enzyme activity of fish hatching enzyme (*HE1C*). (*A*) Allele frequency heatmap showing highly differentiated SNPs at the *HE1C* locus on chromosome 26. Purple boxes in the top row indicate missense positions. Population numbers and heatmap colors are as in Fig. 2*D*. (*B*) Organization of the *HE1C* locus on chromosome 26 (4.96 to 5.00 Mb) based on haplotype-phased PacBio assemblies of 8 Atlantic and 7 Baltic herring. Background shading denotes population origin: red for Atlantic and green for Baltic haplotypes. The syntenic region on European sprat chromosome 1 is included for comparison. Each arrow represents a gene copy, with disrupted copies shown as empty arrows. (*C*) Correlation between *HE1C* copy number and salinity inferred from previously reported whole-genome sequencing of Atlantic and Baltic herring population samples. (*D*) Volcano plot documenting highly significant upregulated expression of *HE1C* mRNA from Baltic herring compared with Atlantic herring based on mRNA-seq analysis of embryos collected 12 d after fertilization. (*E*) Relative enzyme activity (±SEM) of Baltic vs. Atlantic fish hatching enzyme, partially purified from Atlantic and Baltic hatching liquid, in dependence on salinity.

copies than those from the Pacific Ocean (Fig. 5*C*). RNA-seq analysis of whole embryos 6, 12, and 18 d after fertilization showed that *HE1C* genes are the most differentially expressed genes between Atlantic and Baltic herring, driven by the massive copy number expansion in Baltic herring, leading to high RNA levels (Fig. 5*D* and *SI Appendix*, Fig. S18).

Enzyme assays further showed that Baltic hatching liquid exhibits much higher specific activity than Atlantic (15.0 ± 1.25 and 2.05 ± 0.69 mg $\text{protein}^{-1}\ \text{h}^{-1}\ \text{mL}^{-1}$, respectively), suggesting that degradation of the dense Baltic egg envelope requires large amounts of enzymes. The hatching enzyme was partially purified from hatching liquid using cation-exchange chromatography (*SI Appendix*, Fig. S19). Enzyme activity was reduced to 5.5 ± 0.9% in Baltic and 8.1 ± 1.3% in Atlantic in the presence of EDTA, indicating that the enzyme is a metalloprotease, consistent with its identity as a hatching enzyme (*SI Appendix*, Fig. S20*A*). Using the partially purified HE, we further examined optimal salinity and found that Baltic activity peaks at low salinity, whereas Atlantic peaks at high salinity (Fig. 5*E*). Substrate specificity was assessed using 18 kinds of peptides. Both the Baltic and Atlantic enzymes showed high activity to cleave the C-terminal of Trp and Tyr, but the latter had relatively broader specificities (*SI Appendix*, Fig. S20 *B* and *C*) These findings indicate that not only the enzyme activity differs but also that Baltic herring express gene variants that are adapted to brackish environments.

Discussion

This study demonstrates that genetic changes in genes regulating reproduction and early development are particularly critical for species with external fertilization adapting to new environments. At least half of the loci showing strong differentiation between Atlantic and Baltic herring are tied to natural selection acting during reproduction or early embryonic development (Fig. 1*C*). Because externally fertilizing fishes release gametes directly into water masses, environmental gradients in ambient temperature, salinity, and pH impose immediate selection on sperm and eggs, potentially resulting in locally adapted gamete phenotypes and altered fertilization patterns with effects that may also carry over to larval performance and population recruitment (33–35). In contrast, birds and mammals, with internal fertilization and early development within controlled environments, are less likely to exhibit such pronounced ecological adaptation. Our findings illustrate how effectively natural selection can operate in a species with large population sizes, multiple subpopulations, and high fecundity. The evolutionary patterns observed in Atlantic herring align with Sewall Wright's shifting balance theory (36), which predicts rapid evolution in large, subdivided populations with restricted gene flow. According to this model, genetic drift combined with natural selection within subpopulations leads to alternative adaptive peaks, and beneficial gene variants can later spread through gene flow. Atlantic herring subpopulations, which exhibit strong homing behavior (37, 38), can thus locally adapt and spread advantageous gene variants to facilitate colonization of new environments, as seen in our findings.

Our recent work indicates that about 10% of strongly differentiated loci between Atlantic and Baltic herring result from adaptive introgression from Pacific herring (39). Two of the four loci investigated here have been introgressed from Pacific herring, which diverged from Atlantic herring roughly 3 Mya (40). Notably, the spawning behavior of Atlantic and Pacific herring differs: Atlantic herring spawns in deeper marine waters, whereas Pacific herring prefer shallower, often brackish environments (41). Thus, the brackish spawning conditions of Baltic herring resemble those of Pacific herring particularly in the White Sea, providing a clear rationale for adaptive introgression of gene variants affecting the hardening and degradation of the egg envelope. Our previous studies indicated that adaptive introgression likely occurred from Arctic Pacific herring to Baltic herring (39) which appears most plausible given the close proximity and the evidence for hybridization in the subarctic contact zone between Atlantic and Pacific herring populations (42). This interpretation is best supported in the present study by the similarity in *HE1C* copy number between Arctic Pacific and Baltic herring (Fig. 5*C*) and the clustering of Baltic *FTG1-3* haplotypes with the most common Pacific haplotypes from the White Sea area (*SI Appendix*, Fig. S13*B*).

Three of the four genes identified in this study exhibit notable evolutionary trajectories, *LRRC8C2* and *FTG* arose through gene duplication, acquired specialized functions and tissue-specific expression in sperm and egg, respectively, and their protein sequences evolved rapidly following duplication (Figs. 2 *A* and *B* and 3*A*). Fish hatching enzyme genes display highly dynamic evolution, characterized by copy number expansion and contraction, as previously shown (10) and reinforced by our findings. Notably, orthologous *HE* gene copies could not be identified between Atlantic herring and the closely related European sprat (*SI Appendix*, Fig. S16), highlighting rapid divergence. The *HE1A*, *HE1B,* and *HE1C* genes have experienced intense diversifying selection, leading to extensive amino acid variation among predicted proteins (*SI Appendix*, Fig. S17).

A notable feature of the four loci is that alleles at each locus differ by numerous mutations changing their protein sequences, with possible additional contributions from regulatory mutations to adaptive evolution. This phenomenon is especially apparent at the *HE1C* locus on chromosome 26, where a massive copy number expansion results in upregulated gene expression and increased enzyme activity at low salinity. The accumulation of many sequence differences in these genes is partially explained by adaptive introgression: two gene clusters—*FTG1-3* and *HE1C*—originated from introgressive events between Atlantic and Pacific herring, allowing adaptive mutations to accrue over roughly 3 My since their divergence from a common ancestor (40).

The evolution of adaptive alleles and haplotypes via the accumulation of multiple causal mutations appears to be a defining characteristic of adaptive processes in natural populations. This dynamic helps explain the pronounced genetic differentiation observed at loci contributing to ecological adaptation in Atlantic herring (Fig. 1), a species in which each nucleotide site mutates millions of times in each generation given its huge population size. If selection acted solely on single-nucleotide variants irrespective of sequence context, distinct haplotypes showing strong genetic differentiation would be less prevalent than reported here (Figs. 2*C*, 3 *B* and *C*, and 5*A*). Notably, the formation of "adaptive" haplotypes through multiple causal mutations is also well documented in domesticated animals, despite their brief evolutionary histories (43).

Human sperm (23) and probably mammalian internal fertilizers in general do not contain VRAC channels. By contrast, many fish species are external fertilizers that migrate between freshwater, brackish water, and marine habitats, but most are specialized to spawn in either marine (e.g., eels) or freshwater environments (e.g., many salmonids). Some species, however, have evolved ecological adaptations that enable spawning in both high and low salinity. Previous studies have suggested that modifications to sperm function may be crucial for successful reproduction in low-salinity environments (44). Our data indicate that genetic changes in the sperm-specific VRAC, encoded by *LRRC8C2,* were fundamental to Atlantic herring's adaptation to brackish environments. Vertebrates possess five VRAC isoforms, which play vital roles in protecting cells from excessive swelling throughout the body (17, 18). Future studies are required to determine what sets apart somatic LRRC8C homomeric channels from heteromeric channels that contain the principal A subunit, and LRRC8C1 from LRRC8C2 channels in particular. The evolution of a sperm-specific VRAC in clupeiformes may have contributed significantly to their ability to spawn under diverse environmental conditions. Interestingly, Clupeiformes includes an exceptionally high proportion of diadromous species (fish that migrates between marine and freshwater), as well as both marine and freshwater forms (45).

Protecting the integrity of herring egg in brackish water is a challenge comparable to maintaining sperm function under such conditions. Notably, Baltic herring eggs are shielded from the swelling observed in Atlantic herring eggs when exposed to low salinity—a phenomenon caused by the permeability of the egg to water and ions (Fig. 4*F*). Genetic changes at the *ZPBA1* and *FTG1-3* loci appear most critical for the evolution of this denser egg envelope in Baltic herring, although other genes identified in our genomic screening (*SI Appendix*, Table S1) may also contribute, pending further functional characterization.

A major challenge in fish genome biology stems from the broad and deep phylogeny of teleosts and the limited knowledge of gene functions. This is illustrated by the misannotation of the fish transglutaminase (*FTG*) gene as coagulation factor 13A (*F13A*) in all current assemblies of teleost genomes; *FTG* actually originated from a duplication of *F13A* early in teleost evolution (13) (Fig. 3*A*). The FTG enzyme plays a crucial role in hardening the egg envelope by cross-linking ZPBA1 proteins at specific Pro-Gln-X sites (Fig. 3*D*). While most teleosts, including European sprat carry one or two *FTG* copies, Atlantic and Pacific herring possess five functional genes distributed across chromosomes 15 and 17 (Figs. 3*A* and 4*B*). This copy number expansion likely represents an adaptive response to the transition from pelagic spawning, as seen in European sprat, to demersal spawning in herring, where sticky eggs are deposited on the bottom or among sea weeds (41). Eggs from demersal spawners have much thicker egg envelopes than pelagic spawners, providing enhanced resistance against mechanical forces caused by waves (46). The existence of five FTG isoforms in herring may enable more complex cross-linking of envelope proteins, resulting in a stronger and more resilient egg envelope.

The harder egg envelope of Baltic herring, though protective, presents a challenge at hatching because it must be degraded for larvae to emerge. Baltic herring have evolved a solution of this problem through a massive expansion in copy number of *HE1C* genes on chromosome 26, addressing both the need to digest the denser envelope and the low activity of Atlantic herring HE1 enzymes at low salinity (Fig. 5*E*). HE enzymes break down the egg envelope by cleaving the N-terminal region of ZPBA1 that contains ε(γ-glutamyl)-lysine cross-links introduced by the FTG enzyme (13). Our findings, together with a previous study (47) in killifish showing that a single-amino acid substitution can alter the optimal salinity preference of hatching enzyme, highlight the central role of this enzyme in enabling ecological adaptation to diverse salinity conditions at spawning sites, consistent with the dynamic evolution of this gene family (*SI Appendix*, Fig. S16).

Positive genetic epistasis likely promoted the evolution of altered egg envelope and its degradation and facilitated adaptive introgression from Pacific to Baltic herring at two unlinked loci on chromosomes 17 and 26. Modifications in the ZPBA1 protein make it a better substrate for FTG, which itself has evolved enhanced enzymatic activity at low salinity (Fig. 4 *D* and *E*). As a result, Baltic herring produce a denser egg envelope, while the *HE1C* alleles on chromosome 26 confer higher enzyme activity to degrade this denser envelope at low salinity, further boosting fitness (Fig. 5*E*). This triplet of unlinked genes provides an example of a "coadapted gene complex" (48).

Historically, Linnaeus classified Baltic herring as a subspecies (*C. harengus membras*) of Atlantic herring (*C. harengus harengus*), primarily based on the smaller and leaner ecotypes common in the Central and Northern Baltic regions compared to the herring in the southern Baltic Sea and in the Atlantic Ocean. However, we now reveal that populations of herring spawning anywhere in the Baltic Sea, regardless of body size, are fixed (or nearly so) for Baltic-specific alleles at these four key loci, as well as at some other loci (Fig. 1*C*). Here we propose a unified taxonomic treatment for all Baltic Sea ecotypes and propose the Baltic herring could even warrant taxonomic species status (*Clupea membras*) due to its marked genetic divergence at loci essential for ecological adaptation. The lack of genetic differentiation at neutral loci implies ongoing gene flow between Atlantic and Baltic herring, but herring is a broadcast spawner with no mate choice which means that some gene flow is inevitable in the absence of complete geographic separation. The criteria for species recognition as regards the presence of gene flow should be different for a marine species like herring compared with species with strong mate choice in which prezygotic selection may evolve rapidly (49).

Materials and Methods

A genetic screen comparing Atlantic and Baltic herring was performed using whole genome sequencing from multiple populations. The expression of herring proteins in sperm, eggs, and other tissues was studied using high-resolution mass spectrometry. Four genes showing strong genetic differentiation between herring spawning in marine or brackish water and expressed in sperm, egg, or in the embryo were selected for functional studies. Their evolutionary history was analyzed by phylogenetic analysis. Fertilized and unfertilized eggs were collected to characterize the egg envelope in Atlantic and Baltic herring, and enzyme activities of fish transglutaminase and fish hatching enzyme were characterized using eggs and embryos, respectively. Materials and methods are detailed in *SI Appendix*.

Data, Materials, and Software Availability. The WGS data presented here consist of previous published datasets (7, 42). The read sets are deposited with SRA, PRJNA1137584 (7), and PRJNA642736 (42), while per-population frequencies are available at https://doi.org/10.17044/scilifelab.22361761 (50). Alignments of new or previously published gene and protein sequences are deposited with Zenodo at https://doi.org/10.5281/zenodo.17880297 (51). The MS data for the herring sperm and oocyte samples are available at the ProteomeXchange consortium (https://doi.org/10.1093/nar/gkac1040) under MSV000100270 (MassIVE repository) (52) and PXD072133 (ProteomeXchange) (53). MS data for herring tissues, brain, heart, liver, skeletal muscle, and spleen are available under MSV000101199 (MassIVE) (54) and PXD076015 (ProteomeXchange) (55). PRM-MS data are available in Panorama Public (56) and under PXD076334 in ProteomeXChange (57). RNAseq data have been uploaded to the NCBI-SRA with Accession No.: PRJNA1390293 (58). All code used to analyze genotype data are available at https://github.com/LeifAnderssonLab/Ringkobing_Rosetta (59).

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Author affiliations: [a]Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala 75123, Sweden; [b]SciLifeLab, Uppsala University, Uppsala 75123, Sweden; [c]Department of Materials and Life Sciences, Faculty of Science and Technology, Sophia University, Tokyo 102-8554, Japan; [d]Department of Chemistry and Biological Science, Faculty of Science, Josai University, Saitama 350-0295, Japan; [e]Bioanalytical Mass Spectrometry Research Group, Max Planck Institute for Multidisciplinary Sciences, Göttingen 37077, Germany; [f]Life & Medical Sciences Institute, Unit 4: Chemical Biology and Medicinal Chemistry, University of Bonn, Bonn 53115, Germany; [g]Bioanalytics, Department of Clinical Chemistry, University Medical Center, Göttingen 37075, Germany; [h]Institute of Marine Research, Bergen N-5817, Norway; [i]Department of Biological Sciences, University of Bergen, Bergen N-5020, Norway; [j]Biophysics of Cellular Signal Transduction, Max-Planck-Institute for Multidisciplinary Sciences, Göttingen 37077, Germany; and [k]Department of Veterinary Integrative Biosciences, Texas A&M University, College Station, TX 77843

Author contributions: H.U., U.B.K., S.Y., and L.A. designed research; C.M., F.M.S., M.K., K.S., S.V.D., M.E.P., A.W., J.L.W., Y.Y., S.M., F.B., A.F., C.L., H.U., U.B.K., S.Y., and L.A. performed research; C.M., F.M.S., M.K., K.S., S.V.D., M.E.P., A.W., J.L.W., Y.Y., S.M., F.B., A.F., C.L., H.U., U.B.K., and S.Y. analyzed data; and U.B.K. and L.A. wrote the paper.

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