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Shift of grey seal subspecies boundaries in response to climate, culling and conservation

KATHARINA FIETZ,^{*,†} ANDERS GALATIUS,[‡] JONAS TEILMANN,[‡] RUNE DIETZ,[‡] ANNE KRISTINE FRIE,[§] ANASTASIA KLIMOVA,[¶] PER J. PALSBØLL,[†] LASSE F. JENSEN,^{**} JEFF A. GRAVES,^{††} JOSEPH I. HOFFMAN[¶] and MORTEN TANGE OLSEN^{*}

^{*}Evolutionary Genomics Section, Centre for Geogenetics, Natural History Museum of Denmark, University of Copenhagen, Øster Voldgade 5–7, DK-1350 Copenhagen K, Denmark, [†]Marine Evolution and Conservation, Groningen Institute for Evolutionary Life Sciences, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands, [‡]Department of Bioscience, Aarhus University, Frederiksborgvej 399, DK-4000 Roskilde, Denmark, [§]Institute of Marine Research, PO Box 1870 Nordnes, 5817 Bergen, Norway, [¶]Department of Animal Behaviour, University of Bielefeld, PO Box 10 01 31, 33501 Bielefeld, Germany, ^{**}Fisheries and Maritime Museum, Tarphagevej 2, DK-6710 Esbjerg V, Denmark, ^{††}Scottish Oceans Institute, School of Biology, University of St Andrews, Fife KY16 9TH, UK

Abstract

Identifying the processes that drive changes in the abundance and distribution of natural populations is a central theme in ecology and evolution. Many species of marine mammals have experienced dramatic changes in abundance and distribution due to climatic fluctuations and anthropogenic impacts. However, thanks to conservation efforts, some of these species have shown remarkable population recovery and are now recolonizing their former ranges. Here, we use zooarchaeological, demographic and genetic data to examine processes of colonization, local extinction and recolonization of the two northern European grey seal subspecies inhabiting the Baltic Sea and North Sea. The zooarchaeological and genetic data suggest that the two subspecies diverged shortly after the formation of the Baltic Sea approximately 4200 years BP, probably through a gradual shift to different breeding habitats and phenologies. By comparing genetic data from 19th century pre-extinction material with that from seals currently recolonizing their past range, we observed a marked spatiotemporal shift in subspecies boundaries, with increasing encroachment of North Sea seals on areas previously occupied by the Baltic Sea subspecies. Further, both demographic and genetic data indicate that the two subspecies have begun to overlap geographically and are hybridizing in a narrow contact zone. Our findings provide new insights into the processes of colonization, extinction and recolonization and have important implications for the management of grey seals across northern Europe.

Keywords: admixture, Baltic Sea, *Halichoerus grypus*, local extinction, recolonization

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Introduction

A central theme in ecology and evolution is to identify the processes that drive changes in the abundance and distribution of natural populations, and to assess how such changes may lead to colonization and adaptation to novel environments, diversification or extinction.

Advances in molecular techniques have greatly enhanced our ability to address such topics at both wide and narrow temporal and spatial scales (e.g. Bonnell & Selander 1974; Baker *et al.* 1990; Foote *et al.* 2013). Nevertheless, central questions remain, in particular relating to the combined roles of climate and humans in driving population divergence and extinction, the timescale over which these processes occur, how they may affect past, present and future population connectivity and diversity and how to manage

Correspondence: Morten Tange Olsen, Fax: +45 353210102; E-mail: morten.olsen@snm.ku.dk

natural populations experiencing temporal or spatial shifts in population boundaries.

Marginal ecosystems are particularly well suited to address such questions, as many species native to these systems will be genetically distinctive and may thus be particularly prone to environmental and anthropogenic effects. Negative impacts could, for example, result in the loss of entire genetic lineages. The Baltic Sea is a semi-enclosed brackish water basin in northern Europe that was formed following the last glacial retreat approximately 15 000 years BP and attained its current form approximately 8000 years BP (Ukkonen *et al.* 2014). Its relatively short history is characterized by several ecological shifts resulting from alternating climatic and geological events and associated periods of freshwater, brackish and marine environments, as well as by a long list of human impacts, ranging from Pleistocene exploitation to 21st century eutrophication, pollution and overharvesting (Osterblom *et al.* 2007; Varjopuro 2011; Korpinen *et al.* 2012). Given the Baltic Sea's history and its geographical and ecological marginality, many of its plant and animal species show genetic adaptations to local environmental conditions, are relatively isolated and have lower genetic diversity than conspecific populations in the neighbouring North Sea (Bekkevold *et al.* 2005; Johannesson & Andre 2006; Nielsen *et al.* 2009; Johannesson *et al.* 2011). It is generally recognized that such genetically atypical populations may require particular management and conservation efforts (Johannesson & Andre 2006). However, although much has been learnt about marine species' colonization and adaption to the Baltic Sea, the relative roles of environmental variation and human intervention in shaping these species' evolutionary and demographic histories remain unclear.

Many marine mammals are top predators in marine food chains and are thus considered good indicators of environmental change and status (Hooker & Gerber 2004; Sergio *et al.* 2008). Over time, five marine mammal species have been present regularly in both the North Sea and Baltic Sea, namely the grey seal (*Halichoerus grypus*), harbour seal (*Phoca vitulina*), ringed seal (*Pusa hispida*), the now locally extinct harp seal (*Pagophilus groenlandicus*) and the harbour porpoise (*Phocoena phocoena*). These species are all believed to have colonized the region shortly after the last glacial retreat and gradually moved into the central Baltic Sea as this became available (Møhl 1971; Ukkonen 2002; Sommer & Bencke 2003; Schmolcke 2008; Aaris-Sørensen 2009; Ukkonen *et al.* 2014). Subsequently, they experienced substantial shifts in abundance and distribution as a consequence of climatic and anthropogenic pressures (Harding & Härkönen 1999; Härkönen *et al.* 2005; Storå & Lougas 2005). Given their ability to relocate easily,

marine mammals are typically able to change their distribution more readily than many other species, such as marine invertebrates and plants, and are potentially less physiologically affected by local environmental conditions. Nevertheless, marine mammals in the Baltic Sea all show patterns of genetic and morphological divergence from neighbouring marine mammal populations, the underlying cause(s) of which remain poorly understood (Goodman 1998; Palo *et al.* 2001; Amano *et al.* 2002; Graves *et al.* 2009; Wiemann *et al.* 2010; Galatius *et al.* 2012; Klimova *et al.* 2014; Olsen *et al.* 2014).

The grey seal is a relatively large phocid seal with a cold temperate to sub-Arctic distribution along the coasts of the North Atlantic Ocean (Haug *et al.* 2007). Despite its high dispersal potential (McConnell *et al.* 1999; Brasseur *et al.* 2015b), the North Sea and Baltic Sea populations are morphologically (Chapksii 1975) and genetically differentiated (Boskovic *et al.* 1996; Graves *et al.* 2009; Fietz *et al.* 2013; Klimova *et al.* 2014) and are regarded as two separate subspecies: *H. grypus grypus* in the Baltic Sea and *H. g. atlantica* in the North Sea (Abadi *et al.* 2010; Berta & Churchill 2012; Olsen *et al.* 2016). In addition, and unlike other marine mammal species of this region, neighbouring populations of grey seals in the North Sea and Baltic Sea have contrasting breeding habitats and seasons. The North Sea grey seals breed on ice-free islands, sand banks and skerries in November–December, while the Baltic Sea ones breed on sea ice and skerries in February–April (Härkönen *et al.* 2007).

The zooarchaeological record indicates that grey seals have been exploited by humans since the postglacial colonization of the region, and it is well documented that hunting and human-induced habitat alterations resulted in the disappearance of the grey seal as a breeding species across most of mainland Europe in the 16th and 17th centuries (Summers 1978; Lambert 2002; Härkönen *et al.* 2007). In Skagerrak, Kattegat and the Baltic Sea (Fig. 1), grey seal numbers were reduced by up to 90% as a result of an internationally coordinated culling campaign, which by the 1930s had resulted in the extermination of grey seals in Danish waters and along the Baltic coasts of Poland and Germany (Søndergaard *et al.* 1976; Heide-Jørgensen & Härkönen 1988; Harding & Härkönen 1999). In the 1970s, reduced fertility due to environmental contaminants such as PCBs and DDTs caused further population declines (Helle *et al.* 1976). However, as a result of dedicated management and conservation efforts – including regulation of chemicals, restriction of hunting and designation of seal reserves – grey seal abundance has increased substantially and this species is now recolonizing large parts of its historic range. Throughout the region, this has led to renewed conflicts with fisheries (Königson *et al.* 2007, 2009; Gardmark *et al.* 2012) and debates over possible

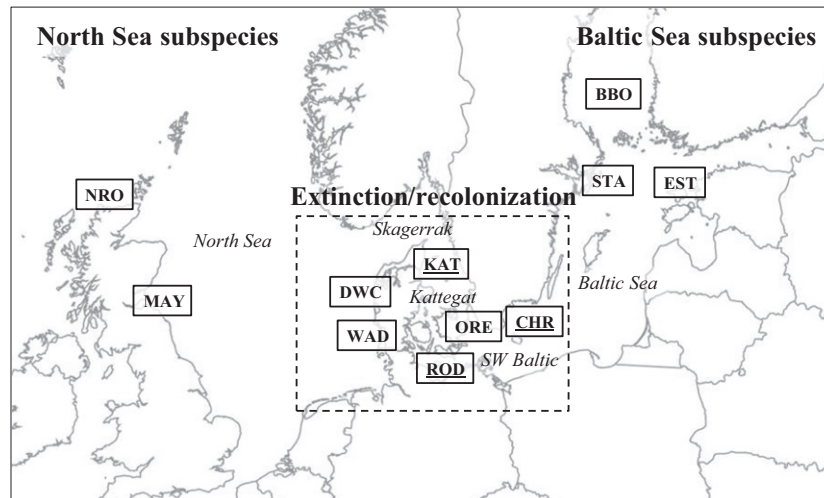


Fig. 1 Grey seal sampling sites in northern Europe, including localities where the North Sea and Baltic Sea subspecies are found, and the ‘transition’ zone of the geographically intermediate locations where grey seals went extinct but are currently recolonizing the area. Mitochondrial haplotypes were obtained from contemporary samples at all 11 sampling localities, representing both long-term breeding populations in the North Sea (NRO and MAY) and Baltic Sea (STA, EST and BBO), as well as the sites that are currently being recolonized in the North Sea (WAD and DWC), Kattegat (KAT) and southwestern Baltic (ORE, ROD and CHR). In addition, mitochondrial DNA was obtained from historic (late 19th century) skull samples from localities KAT, ROD and CHR (underlined), and microsatellite genotypes were obtained from five of the sampling sites for which high-quality samples were available (MAY, WAD, ORE, ROD and CHR). See Table S1 (Supporting information) for additional information.

counter-measures, such as hunting and culling (Naturvårdsverket 2006; DME 2013). Further implications may include increased interspecific competition and predation (Svensson 2012; Leopold *et al.* 2015; van Neer *et al.* 2015) and possible risks of intra- and inter-specific transmission of pathogens.

In this study, we integrate zooarchaeological, demographic, life history and genetic data from contemporary and extinct grey seal populations in northern Europe to shed light on the processes driving colonization, extinction and recolonization. Specifically, we ask the following questions: When and why did the two grey seal subspecies diverge? How has divergence been maintained? What were the genetic and phenotypic affinities of extinct breeding populations? Where do the current recolonizers come from? Will recolonization have effects on subspecies connectivity, life history and phenotype? We conclude with a discussion on what our new insights entail for grey seal conservation and management in northern Europe.

Materials and methods

Demographic data

Prehistoric abundance. Estimates of relative abundance of prehistoric grey seal populations in the North Sea and the Baltic Sea were based on published zooarchaeological records (Stora 2002; Fairnell 2003; Sommer &

Benecke 2003; Prummel & Heinrich 2005; Fairnell & Barrett 2007; Schmolcke 2008), whereas estimates for the inner Danish waters – here defined as Kattegat and the Southwest Baltic Sea – were based on the database held by the Quaternary Collection at the Natural History Museum of Denmark. As information on the number of identified specimens was not available for all sites and areas, we used the number of archaeological sites in which grey seal remains were present as a measure of relative abundances in each of the regions.

Historic and current abundance. Information on the development of grey seal populations in the North Sea and the Baltic Sea during the past century was obtained from the literature covering the periods 1910s, 1950s, 1970s and 2010s. Estimates for the North Sea were based on *ad hoc* counts and expert opinions from the British Isles (Davies 1957; Summers 1978; Lambert 2002) and the Wadden Sea (Reijnders *et al.* 1995), whereas the estimate for the Baltic Sea was based on hunting statistics (Harding & Härkönen 1999; Harding *et al.* 2007). Grey seals in inner Danish waters were close to absent for most of this period and were thus not included. All 2010 estimates originate from published survey data (SCOS 2011; Härkönen *et al.* 2013; Brasseur *et al.* 2015a; Sveegaard *et al.* 2015).

Recent population trends in the inner Danish waters. Recent grey seal population trends in inner Danish waters

were assessed using standard aerial surveys covering the period 2002–2014 (Heide-Jørgensen & Härkönen 1988; Teilmann *et al.* 2010; Galatius *et al.* 2014). Briefly, abundance in Danish Kattegat and Southwest Baltic was based on survey counts obtained from a single-engine high-winged Cessna 172 aircraft, flying at altitudes of 500–600 feet over seal haul-outs at speeds of 70–80 knots. Surveys were carried out between 0900 and 1500 hours and only when wind speed was <10 m/s and precipitation was absent. Two observers on the same side of the aircraft took photographs of haul-out sites using hand-held cameras equipped with 135- to 300-mm lenses. Afterwards, the number of grey seals was counted on high-quality digital photographs.

Genetic data

Sampling and DNA extraction. The genetic data were acquired from diverse types of material to obtain the best possible spatial and temporal distribution of samples (Fig. 1; Table S1, Supporting information). The historic pre-extinction material consisted of grey seal skulls dating from the years 1889–1890 from Kattegat and the southwestern Baltic Sea stored at the Natural History Museum of Denmark ($n = 42$). From each specimen, we collected 50–100 mg of bone powder using an Osada SuccesSTM 40 drill (Osada Electric Company Ltd., Japan) with a round-headed steel bur size 23, running at approximately 2.5 g (1000 rpm). Total genomic DNA was extracted using the Qiagen MinElute PCR Purification KitTM following manufacturer's instructions with modifications in step 6 of the manufacturer's manual where 600 μ L Buffer PE was added to the column, and in step 9 where 50 μ L Buffer EB was added. All laboratory work on unamplified DNA was carried out in a laboratory designated to ancient DNA analysis.

Contemporary samples were collected between 1941 and 2013, with more than 95% of these stemming from the period 1998 to 2013, representing the period of recolonization of Kattegat and the Southwest Baltic. Samples collected at recently recolonized haul-out sites consisted of shed hair ($n = 33$) and scat samples ($n = 49$), as well as skin biopsies collected remotely by biopsy darts ($n = 8$) or during handling of seals caught for GPS tagging and Argos satellite tagging ($n = 25$). In addition, skin or muscle samples from stranded and by-caught animals were collected opportunistically ($n = 59$). Finally, a small number of contemporary samples consisted of bone powder from skulls, which was collected in the same way as the pre-extinction material described above ($n = 8$) (Table S1, Supporting information).

Muscle and skin tissue samples were conserved in DMSO (Amos and Hoelzel 1991) or in sodium chloride,

whereas hair and scat samples were stored dry in plastic tubes or bags. All samples were stored at -20°C after collection. Total genomic DNA was extracted from ca. 25 mg of tissue or 1–5 hair shafts stemming from the same individual using the Qiagen Blood and Tissue KitTM according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). For each scat sample, total genomic DNA was extracted by cutting out a ca. 1 mm² and using the QIAamp DNA Stool Mini KitTM according to the manufacturer's instructions (Qiagen). Exceptions from the protocol included the use of 50 mg of scat and only half an InhibitEX tablet (Bohmann *et al.* 2011).

Mitochondrial DNA amplification and sequencing. For historic and contemporary samples, a 435-bp section of the mitochondrial control region was Polymerase chain reaction (PCR)-amplified using the newly developed primers HG001F (5'-CACCACCAGCACCCAAAG-3') and HG001R (5'-TCATAGCTGAGTGATACCG-3'). Polymerase chain reactions on DNA were performed in a total volume of 25 μ L including 1–4 μ L DNA template (1–100 ng/ μ L, depending on sample type), 2.5 μ L 10xPCR GoldTM buffer, 2.0 μ L MgCl₂ solution (25 mM), 1.0 μ L purified bovine serum albumin (100 \times , 10 mg/mL), 1.0 μ L of each forward and reverse primer (each at 10 μ M), 0.25 μ L dNTPs (each at 100 mM) and 0.2 μ L AmpliTaq GoldTM (5 U/ μ L). PCRs were performed in a VeritiTM 96-Well Thermal Cycler (Applied BiosystemSTM) using the following conditions: 5-min denaturation at 95 $^{\circ}\text{C}$, 35–40 cycles of 30-s denaturation at 95 $^{\circ}\text{C}$, 30-s annealing at 55 $^{\circ}\text{C}$ and 30-s extension at 72 $^{\circ}\text{C}$, and finally 7-min extension at 72 $^{\circ}\text{C}$. All amplifications included negative controls and amplification products were subsequently checked by electrophoresis through a 2% agarose gel in TBE with GelRedTM (Bio-nuclear, Scandinavia). All PCR products were Sanger-sequenced in both directions by Macrogen (Macrogen, Europe, the Netherlands). The resulting mitochondrial DNA (mtDNA) sequences were subsequently edited manually using GENEIOUS 6.0.4 (Drummond *et al.* 2011). In addition to the mtDNA data generated here, we included mtDNA sequences from 105 animals that had been sequenced and quality-checked as detailed by Fietz *et al.* (2013) bringing the total sample size for mtDNA analyses to 329 animals.

Microsatellite amplification and genotyping. The contemporary samples were amplified and genotyped at 14 previously developed microsatellite loci (Hg4.2, Hg6.3, Hg8.9, SGPV11, Hg8.10, Hg3.6, Lw26, SGPV9, Hg6.1, HI16, TBPV2, M11, LC5, LW7) (Allen *et al.* 1995; Gemmell *et al.* 1997; Goodman 1998; Burg *et al.* 1999; Hoelzel *et al.* 1999; Davis *et al.* 2002). PCR amplifications were performed in a total reaction volume of 2.5 or 5.0 μ L,

depending on sample type. For a reaction volume of 2.5 μL , we used 1.25 μL 1 \times Qiagen multiplex PCR master mix, 0.13 μL primer mix with primer concentrations ranging from 0.6 to 2.0 μM and 0.25–0.50 μL DNA. The same proportions between reagents were used for reaction volumes of 5 μL . Final primer concentrations in the total reaction volume ranged from 30 to 105 nM. PCRs were performed on 384-well plates on a Geneamp 9700 or a Veriti 384-Well Thermal CyclertTM (Applied BiosystemTM) in four multiplex reactions (multiplex 1: Hg4.2, Hg6.3, Hg8.9, SGPV11, Hg8.10; multiplex 2: Hg3.6, Lw26, SGPV9; multiplex 3: Hg6.1, HII16, TBPV2, M11; and multiplex 4: Hg6.1, LC5, LW7, Hg8.10). Conditions were as follows: initial activation at 95 °C for 15 min followed by 25–40 three-step cycles of denaturation at 94 °C for 30 s, annealing at 54–60 °C for 90 s, extension at 72 °C for 90 s followed by a final extension step at 72 °C for 10 min. The resulting products were resolved in POP-7 polymer on a 36-cm capillary array in an Applied Biosystems 3130xl Genetic Analyzer (Applied BiosystemTM) using GS500 LIZ internal size-standard and GENEMAPPER software (ver. 4.0; Applied Biosystems). The microsatellite genotypes were scored independently by two persons using the GENEIOUS microsatellite plug-in (Drummond *et al.* 2011). All scat and hair samples were genotyped 2–5 times for each locus to minimize genotyping errors. Any animals for which the replicates did not fully match were omitted from further analyses.

Genetic diversity, Hardy–Weinberg proportions and linkage disequilibrium. For the mitochondrial control region sequences, the number of unique haplotypes, haplotype diversity (h), nucleotide diversity (π) and number of polymorphic sites were calculated using DNASP v. 5.10.1 (Librado & Rozas 2009). For microsatellite data, the package ADEGENET (Jombart 2008) implemented in R Software (R Development Core Team 2015) was used to determine the overall number of alleles per locus and per locality, percentage of missing data per locality, and expected and observed heterozygosity per locality. To test for deviations from Hardy–Weinberg equilibrium, a Monte Carlo Markov chain approach with 10 000 permutations was used. Pearson's chi-square tests with simulated P -values (based on 10 000 replicates) were used to estimate P -values for deviation from Hardy–Weinberg equilibrium (also implemented in R Software (R Development Core Team 2015)). Linkage disequilibrium was tested with GENEPOP (Rousset 2008). The dememorization number was set to 10 000, the number of batches to 1000 and the number of iterations per batch to 10 000 for each test.

Genetic structure of historic and contemporary populations. For mitochondrial data, we used hierarchical

analyses of molecular variance (AMOVA) in ARLEQUIN 3.5 (Excoffier & Lischer 2010) to examine overall and pairwise spatial heterogeneity between major sampling areas using F_{st} (Weir & Cockerham 1984) with 10 000 permutations. Also, to illustrate potential shifts in haplotype distribution, we constructed mtDNA haplotype networks for pre-extinction and contemporary data using the software TEMPNET (Prost & Anderson 2011).

For the microsatellite data, overall and pairwise population differences were assessed in ARLEQUIN v. 3.5 (Excoffier & Lischer 2010) using F_{st} (Weir & Cockerham 1984). Statistical significance was assessed using 20 000 permutations of the data. To examine population structure, we employed the program STRUCTURE v. 2.3.4 (Pritchard *et al.* 2000; Hubisz *et al.* 2009). Estimations in STRUCTURE were performed under the admixture model, using the model of correlated allele frequencies between clusters and locations as priors. For each value of K from 1 to 5, five simulations were performed, each with 100 000 initial steps of burn-in followed by 1 000 000 iterations. Output data were processed using STRUCTURE HARVESTER (Earl 2009) and CLUMPP (Jakobsson & Rosenberg 2007) and were graphically displayed using DISSTRUCT (Rosenberg 2004). We applied Evanno's ΔK as an additional predictor of K (Evanno *et al.* 2005).

Demographic population history. The population history of grey seals in northern Europe was explored using an approximate Bayesian computation approach as implemented in DIYABC v. 2.1.0 (Cornuet *et al.* 2008, 2010, 2014). The analyses were based on 64 animals from five localities for which we had both mtDNA and microsatellite data. Being interested in broadscale demographic patterns, we used the STRUCTURE results to reduce the number of populations to three: the North Sea (MAY and WAD), Rødsand (ROD) and the Baltic Sea (ORE and CHR). Three alternative demographic histories were modelled, with Rødsand being genetically closer to either North Sea seals, Baltic Sea seals or a mixture of the two. We performed 12 simulations (I–XII) in which we used a range of upper prior bounds for divergence and admixture time spanning from realistic time frames relating to the postglacial opening of the Baltic Sea, to (higher) priors based on initial exploratory DIYABC analyses (Table S5, Supporting information). Rather than using a single prior, we used a prior range to evaluate how robust the demographic model choice and the corresponding posterior estimates for timing parameters were. For each simulation, we assumed uniform prior distributions for all estimated parameters. The microsatellite mutation rate was uniformly distributed between 1×10^{-4} and 1×10^{-3} substitutions/generation (Weber & Wong 1993). The control region mutation rate was uniformly distributed between 8.12×10^{-7} and

3.85×10^{-6} substitutions/site/generation (Phillips *et al.* 2009; Dickerson *et al.* 2010). For each demographic model, we simulated 1×10^6 data sets and calculated 18 summary statistics (Cornuet *et al.* 2008, 2010, 2014). For microsatellite data, these included the mean number of alleles and mean genetic diversity for one and two samples, the F_{st} , the classification index, the shared allele distance, the $(\delta\mu)^2$ distance for two samples and the maximum-likelihood coefficient of admixture (Choisy *et al.* 2004). For the mitochondrial control region, the summary statistics included the number of haplotypes and private segregating sites for one sample, the number of segregating sites, the mean of pairwise difference for one and two samples (within and between), the F_{st} for two samples and the maximum-likelihood coefficient of admixture. Choice of summary statistics was based on parameters they meant to capture (e.g. mean number of alleles for estimation of population size, $(\delta\mu)^2$ distance for population divergence and admixture summary statistics for estimation of admixture). Following this, we used a polychotomous weighted logistic regression on the 3×10^4 simulated data sets closest to the observed data set to determine the posterior probability for each demographic model. Based on the most probable demographic model, local linear regression on the 1% closest simulated data sets with a logit transformation was used to estimate the posterior parameter distributions (Beaumont *et al.* 2002). The next step was to evaluate the level of confidence for choosing the best-supported demographic model. Thus, we simulated 1×10^5 pseudo-observed data sets, estimated their posterior probability and measured the proportion of times the chosen demographic model had the highest posterior probability. The confidence in model choice was estimated by drawing scenario-parameter combinations into posterior distributions (Cornuet *et al.* 2014). Following this, we evaluated the performance of the method for parameter estimation by computing bias and precision as described in Cornuet *et al.* (2008, 2014). The number of simulated data closest to the observed data was set to 5000 for precise estimations of the accuracy measures. Finally, we performed an evaluation of how well each demographic model fitted the data by running a model-checking analysis. In order to avoid overestimating the quality of fit using the same summary statistics twice, we used different set of the summary statistics from those used for inferential steps above (Cornuet *et al.* 2010).

Results

Demographic data

Prehistoric abundance. The zooarchaeological data suggest that grey seals have been present in Kattegat and

the southwestern Baltic Sea for at least 9500 years and began to colonize the central Baltic Sea approximately 6000–4500 years BP (Fig. 2). In contrast, grey seal remains appear relatively rarely in the entire zooarchaeological record of the British Isles and the Wadden Sea region. Across all regions, the zooarchaeological record exhibits a marked drop in the number of grey seal remains from 4500 to 2500 years BP.

Historic and current abundance. The historic abundance of grey seals was much lower in the British Isles and Wadden Sea relative to the Baltic Sea, although this distribution shifted in the 1950s and 1970s when the British population increased and the Baltic population crashed. Since then, both populations have increased manifold, from a few thousand to more than 40 000 and 120 000 in the Baltic Sea and North Sea, respectively (Fig. 3).

Recent population trends in inner Danish waters. Grey seal surveys conducted from 2002 to 2014 show an overall increasing number of recolonizers at historic haul-out sites in Kattegat (Fig. 4A) and the Southwest Baltic (Fig. 4B). In both regions, grey seals are present during the North Sea moulting and Baltic Sea breeding season in January–March, as well as the Baltic Sea moulting season in May–June. Moreover, pups have been observed in both regions during the Baltic Sea breeding season. Surveys in Kattegat and the southwestern Baltic were not conducted during the North Sea breeding season in October–December.

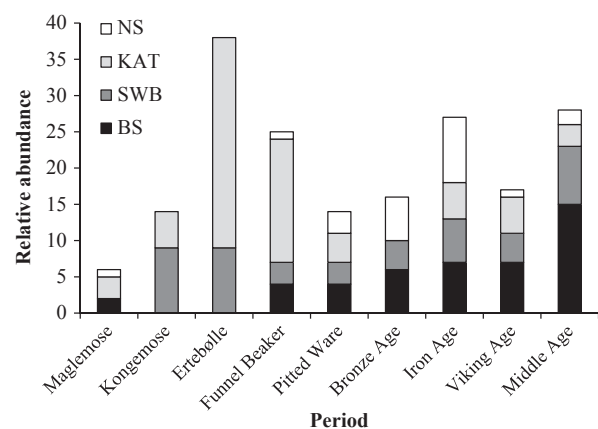


Fig. 2 Prehistoric relative abundance of grey seals in northern Europe based on the number of zooarchaeological sites with grey seal remains in the North Sea (NS; white) and Baltic Sea (BS; black), as well as the geographically intermediate Kattegat (KAT; light grey) and Southwest Baltic (SWB; dark grey) populations. The data indicates a drop in grey seal abundance in the Pitted Ware and Bronze Age periods (4500–2500 years BP), corresponding to a period during which northern Europe experienced a change towards colder and wetter climate.

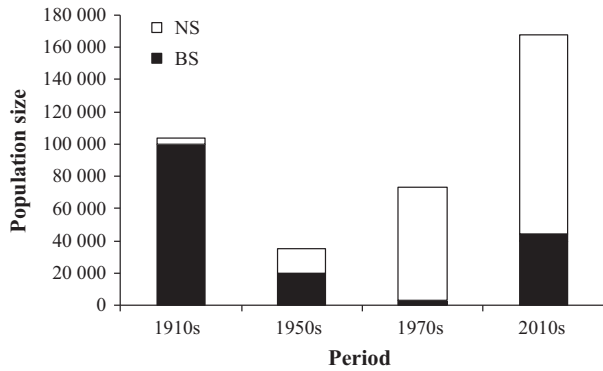


Fig. 3 Historic and contemporary abundance of grey seals in northern Europe, showing a shift in the relative population sizes of grey seals in the North Sea (NS; white) and the Baltic Sea (BS; black) between the 1910s and 2010s. The sizes of the recolonizing populations in Kattegat (KAT) and the Southwest Baltic (SWB) are in the lower hundreds and are therefore not included in the figure.

Genetic data

Genetic diversity, Hardy–Weinberg proportions and linkage disequilibrium. Our mitochondrial data set consisted of 329 grey seals from 11 localities across the North Sea and Baltic Sea, as well as the geographically intermediate ‘transition zone’ in the inner Danish waters. A total of 74 mtDNA haplotypes were observed, of which only one haplotype was shared between the North Sea and Baltic Sea animals (Table S2, Supporting information). The overall genetic diversity was high ($h = 0.960$ [95% CI = 0.9596–0.9604]; $\pi = 0.014$ [95% CI = 0.014–0.014]) and slightly higher in historic and contemporary Baltic samples than in North Sea seals (Table S3, Supporting information).

Microsatellite genotypes at 14 loci were obtained from 64 contemporary samples from five of the 11 sampling locations. Initially, we screened 133 samples for microsatellite variation, but approximately half of these

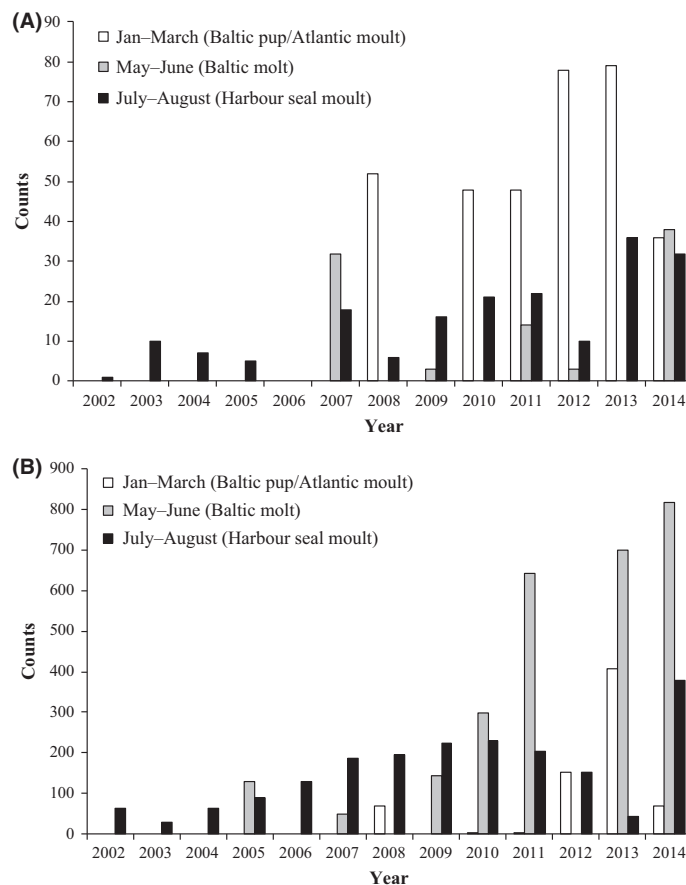


Fig. 4 Abundance of grey seals at haul-out sites in Kattegat (A) and Southwest Baltic (B), showing an overall increasing number of recolonizers and the presence of animals during both the North Sea subspecies’ moulting season and Baltic Sea subspecies’ pupping season (January/February–March; white), the Baltic Sea moulting season (May–June; grey) and the harbour seal moulting season (July–August; black). Note that there is a 10-fold difference in census size and that the Baltic pupping season overlaps with the North Sea moulting season. Surveys have not been conducted during the North Sea pupping period (October–December).

were excluded from further analyses because genotypes could not be reproduced with 100% consistency across replicates. This was particularly true for scat and shed hair samples collected at KAT and CHR. Variability at the microsatellite loci ranged from 4 to 12 alleles per locus and between 68 and 92 alleles per locality (Table S4, Supporting information). Six loci deviated significantly from the expected Hardy–Weinberg genotype equilibrium under random mating at some localities. Most deviations were detected at localities with relatively few samples. Pooling all data, indications for linkage disequilibrium were initially found in 12 of 92 pairs of loci. However, no pairs of loci were significantly in linkage disequilibrium after Bonferroni correction for multiple statistical tests.

Population structure. Overall mitochondrial genetic differentiation across historic and contemporary samples was estimated at $F_{ST} = 0.05$ (95% CI = 0.034–0.065) ($P < 0.001$). Comparing major sampling areas, the largest difference was evident between contemporary North Sea seals and historic individuals from the Southwest Baltic Sea, whereas contemporary North and Baltic populations differed slightly less from each other (Table 1). Based on the contemporary data, Kattegat was genetically more similar to the North Sea than to the Baltic Sea. No significant differences in population structure were detected between historic and contemporary seals from the Baltic Sea. We generated haplotype networks to visualize spatial and temporal mtDNA relationships (Fig. 5). These support the estimates of genetic differentiation, showing that the historic grey seal samples from Kattegat and southwestern Baltic share haplotypes with the contemporary Baltic Sea population, while only one haplotype is shared with contemporary North Sea seals (Fig. 5A). In contrast, most, but not all, of the contemporary samples from Kattegat share haplotypes with the contemporary North Sea population (Fig. 5B).

Using microsatellite data, there was no significant overall population genetic structure ($F_{ST} = 0.019$,

$P = NS$, Table 2). However, the STRUCTURE results were consistent with those based on the mitochondrial data, indicating a genetic split between animals sampled at the North Sea localities, Isle of May and the Wadden Sea, and animals at the Baltic Sea localities, Øresund and Christiansø (Fig. 6). In contrast to the mitochondrial results, microsatellites indicate that animals sampled at the Rødsand breeding site in the Southwest Baltic are of mixed ancestry.

Demographic population history. We analysed the combined mtDNA and microsatellite data within an ABC framework to estimate the divergence time of the North Sea and Baltic Sea populations and to explore the most likely origin of the current grey seal breeding population at Rødsand (Fig. 7; Table 3; Table S5, Supporting information). In all simulations, the most probable demographic model, with posterior probabilities ranging from 61% to 77% and posterior errors from 27.1% to 39.9%, was the one involving a split and subsequent population admixture at Rødsand between the North Sea and Baltic Sea lineages. The posterior parameter estimates for divergence time (t_2) and time of admixture (t_1) varied across simulations, with t_2 and t_1 posteriors increasing with broadening priors. For increasing t_2 priors in simulations VII–XII, the t_2 posterior levelled off at approximately 700 generations with upper and lower 95% CI at approximately 300 and 3850 generations, respectively, when averaging across simulations (Table S5, Supporting information). This corresponds to approximately 9800 years BP (95% CI = 4200–54 000 years BP) when assuming a generation time of 14 years (Härkönen *et al.* 2007). For t_2 using biologically plausible priors (simulation I–II), the posterior estimate was ca. 300 generations ago (95% CI = 131–943 generations ago), corresponding to 4200 years BP (95% CI = 1834–13 202 years BP; Table 3). The corresponding t_1 posterior was estimated at 17.6 generations ago (95% CI = 2.95–420 generations ago) with an admixture rate (R_a) of 27.5% (95% CI = 2–94.6%).

Table 1 Estimates of mtDNA genetic differentiation

Locality	Contemporary North Sea	Contemporary Baltic Sea	Historic Kattegat and SW Baltic	Contemporary Kattegat
Contemporary North Sea		<0.001	<0.001	0.011
Contemporary Baltic Sea	0.099 (0.066–0.129)		NS	<0.001
Historic Kattegat and SW Baltic	0.143 (0.086–0.196)	0.000 (0–0.0003)		<0.001
Contemporary Kattegat	0.021 (0.004–0.038)	0.059 (0.038–0.080)	0.071 (0.043–0.098)	

NS, not significant. Bold values are significant ($P < 0.05$).

P -values are above the diagonal, and F_{ST} values are below the diagonal.

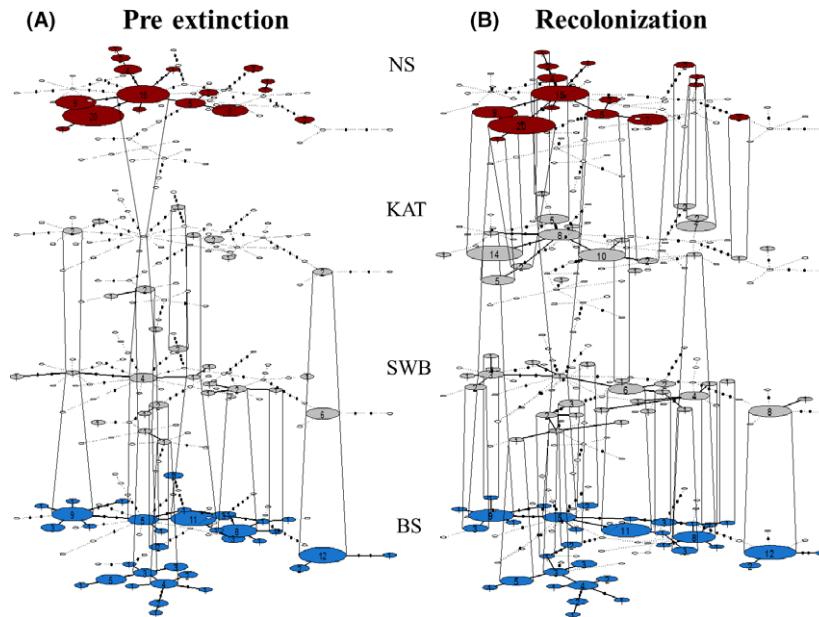


Fig. 5 Mitochondrial haplotype networks from pre-extinction (A) and contemporary recolonization (B) grey seal populations showing a temporal shift in subspecies boundaries and increased admixture in contemporary populations. Both networks are based on contemporary samples from the North Sea subspecies [NS; dark red (top); localities NRO, MAY, DWC and WAD] and Baltic Sea subspecies [BS; blue (bottom); localities EST, STA and BBO], as well as either pre-extinction (A) or contemporary recolonization (B) samples from Kattegat (KAT; grey; locality KAT) and Southwest Baltic Sea (SWB; grey; localities ROD, ORE and CHR). Vertical lines connect identical haplotypes between layers. Numbers refer to number of samples with a particular haplotype. Open white circles are haplotypes missing in that particular layer, but found in one of the other layers. Black dots represent mutations/haplotypes missing entirely.

Table 2 Estimates of pairwise genetic differentiation at microsatellite markers

Locality	MAY	WAD	ROD	ORE	CHR
MAY		NS	NS	0.012	0.017
WAD	0.000 (0.000–0.015)		NS	0.027	NS
ROD	0.008 (0.000–0.038)	0.007 (0.000–0.029)		NS	NS
ORE	0.046 (0.008–0.089)	0.027 (0.000–0.057)	0.008 (0.000–0.033)		NS
CHR	0.057 (0.004–0.120)	0.033 (0.000–0.071)	0.007 (0.000–0.046)	0.001 (0.000–0.027)	

NS, not significant. Bold values are significant ($P < 0.05$).

P -values are above the diagonal, and F_{ST} values are below the diagonal.

Discussion

The role of climate and humans in subspecies divergence

A long-standing question in ecology and evolution pertains to the processes that drive adaptation and divergence among populations. Here, we used multiple data types to assess when, where and how grey seal populations of northern Europe diverged to different breeding seasons and habitats. Although no method provided clear results on its own, our combined data with biologically plausible priors used for ABC analyses point to a

split 4200 years BP with a range of approximately 13 000–1800 years BP between the North Sea and Baltic Sea subspecies. This estimate overlaps with the 10 000 years BP estimated in a recent study (Klimova *et al.* 2014), supporting the hypothesis that the formation and initial colonization of the Baltic Sea during its formation phase approximately 15 000–8000 years BP was a major factor driving subspecies divergence.

Assuming that grey seals were continuously distributed, the question arises how the two subspecies became reproductively isolated. Following its formation, the Baltic Sea experienced dramatic ecological shifts resulting from climatic fluctuations and associated

alternating periods of freshwater, brackish and marine environments. One of the most dramatic events was a very rapid climate-induced environmental shift from brackish-marine to freshwater conditions at approximately 3500–4500 years BP (Berglund & Sandgren 1996; Andren *et al.* 2000; Emeis *et al.* 2003; Zillen *et al.* 2008).

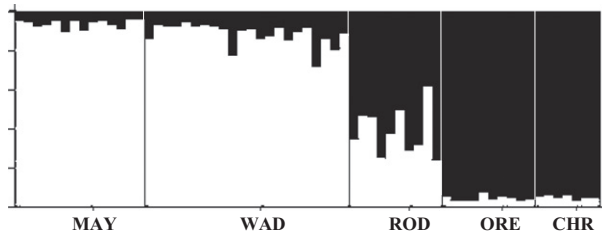


Fig. 6 Genetic differentiation and admixture among grey seal subspecies in northern Europe inferred from nuclear microsatellite markers using the program STRUCTURE (Pritchard *et al.* 2000; Hubisz *et al.* 2009). Each vertical bar represents a grey seal individual and the colouring its proportion of membership to the North Sea subspecies (localities MAY and WAD; white) and the Baltic Sea subspecies (localities ORE and CHR; black) at $K = 2$. The grey seals from the Rødsand locality (ROD) in the Southwest Baltic Sea exhibit mixed ancestry.

This shift was caused by gradual cooling and increased precipitation across northern Europe, which decreased the salinity and increased the winter ice cover of the Baltic Sea, resulting in marked changes in Baltic Sea diatom and bacterial communities and an overall decrease in primary productivity (Tuovinen *et al.* 2008; Seppä *et al.* 2009; Witkowski *et al.* 2009; Lyra *et al.* 2013; Willumsen *et al.* 2013). We hypothesize that the cooling was a major factor driving the reproductive isolation between North Sea and Baltic Sea grey seals: the former adjusting their breeding season and habitat to ice-free conditions in the North Sea, and the latter to a colder environment with annual periods of sea ice. Across their range, grey seals primarily breed in ice-free habitats. However, grey seal pups are born with a white lanugo fur, indicating that the species originally was an ice breeder and thus could utilize the increasing ice cover in the Baltic Sea for breeding. Weaning weights of Baltic grey seals born on ice are higher than for Baltic pups born on land (Jussi *et al.* 2008), indicating greater breeding success and a possible selective advantage of breeding on ice. Moreover, females breeding on ice give birth more synchronously than in land breeding

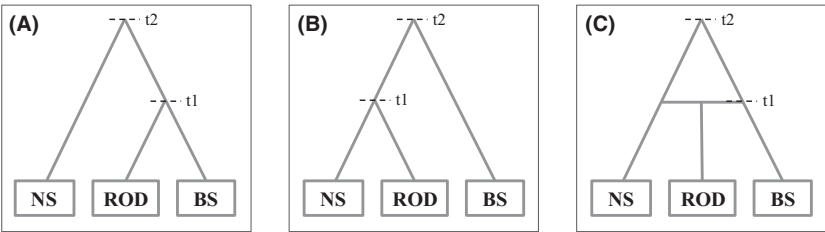


Fig. 7 Divergence and admixture of North Sea and Baltic Sea grey seal subspecies based on an estimation using an ABC approach (Cornuet *et al.* 2014). Three different demographic models were compared in which animals from the Rødsand locality in the Southwest Baltic originated from the Baltic Sea (A), from the North Sea (B) or were of admixed origin (C). Among these, the most probable demographic model was the one including admixture (C) (posterior estimates can be found in Table 3).

Table 3 Priors, posterior estimates and mean relative biases of the demographic parameters from the demographic model using biologically plausible priors under simulation I in DIYABC v. 2.1.0 (Cornuet *et al.* 2008, 2010, 2014)

Parameter	Prior	Posterior					MRB
		Mean	Median	Mode	2.5%	97.5%	
N1	10–100 000	17 000	10 900	5290	2570	71 700	0.41
N2	10–100 000	60 100	62 500	98 900	11 900	98 500	0.30
N3	10–100 000	30 500	22 100	7690	3710	90 900	1.362
Na	10–50 000	11 900	10 900	7290	3460	25 300	–0.10
t1	0.01–500	115	77	17.6	2.95	420	∞
t2	1–1000	472	437	308	131	943	0.27
ra	0.001–0.999	0.421	0.391	0.275	0.020	0.946	2.11

N1 (North Sea), N2 (Rødsand) and N3 (Baltic Sea) = current effective population sizes; Na = the ancestral population size; t1 = time of admixture; t2 = time of divergence; ra = rate of admixture; MRB = mean relative bias.

colonies (Haller *et al.* 1996), narrowing the time window of male mating opportunities and likely driving additional reproductive isolation between North Sea and Baltic Sea grey seals.

Given the drop we observed in grey seal numbers in the zooarchaeological record during the Pitted Ware and Bronze Age periods 4500–2500 years BP, we further hypothesize that climate-induced reductions in primary productivity also drove the physical isolation of the North Sea and Baltic Sea grey seals through a decrease in grey seal abundance and hence connectivity throughout the region. The drop corresponds to a 1000-year gap in the harp seal record from 4000 to 3000 years BP in the same region (Bennike *et al.* 2008), as well as a gradual population decline and reduction in body size of both harp seals and ringed seals (Stora 2002; Stora & Ericson 2004; Stora & Lougas 2005; Schmölcke 2008; Ukkonen *et al.* 2014). These observations could indicate that the period was characterized by limited prey resources and possibly substantial interspecific competition. The ecological shift may also have been a contributing factor to the divergence reported for many other Baltic Sea species, including fish and marine invertebrates (Johannesson & Andre 2006).

Finally, while rapid climate change and increasing sea ice cover appear to have initiated the reproductive and physical isolation of North Sea and Baltic Sea grey seals, we hypothesize that this isolation was maintained by periods of low abundance caused by repeated human disturbance. Grey seals are relatively rare in the zooarchaeological record of both the British Isles and the Wadden Sea area. In historic times, the Wadden Sea population went extinct as early as the late Middle Ages and permanent colonies were not re-established before the 1950s (Reijnders *et al.* 1995; Härkönen *et al.* 2007). Likewise, although the 16th–19th century abundance of grey seals in the British Isles is unknown, it is likely to have been low and was estimated at around 500–4000 animals in the early 20th century (Summers 1978; Lambert 2002). In contrast, the Baltic Sea subspecies is relatively abundant in the zooarchaeological record, and although it did experience a dramatic decline due to overexploitation and high contaminant loads in the 20th century (Harding & Härkönen 1999), the duration of this decline was <100 years. These observations fit with the lower genetic diversity of North Sea than Baltic Sea grey seals reported here and elsewhere (Graves *et al.* 2009; Klimova *et al.* 2014). That is, both genetic and demographic data suggest that the prehistoric and historic abundance of North Sea grey seals was low for long periods of time. We hypothesize that this may have been a major factor in maintaining the isolation of the North Sea and Baltic Sea subspecies.

Shifting subspecies boundaries and recent admixture in Denmark

As a result of dedicated management and conservation efforts, the grey seal is again becoming common across northern Europe and both subspecies are now recolonizing their historic ranges (Abt & Engler 2009; Brasseur *et al.* 2015b). Our analysis of mtDNA indicates that the historic breeding population in Kattegat and Southwest Baltic was genetically connected to the Baltic Sea population. In contrast, the analysis of contemporary mtDNA from the same region indicates that the recolonizers in Kattegat primarily originate from the North Sea, while animals recolonizing the Southwest Baltic Sea primarily originate from the central Baltic Sea population. This is also supported by recent GPS tagging studies which revealed that grey seals conduct long migrations of more than 800 km, but remain within the Baltic Sea (Dietz *et al.* 2015). Thus, it seems that historic hunting and recent conservation efforts have shifted the boundary between the North Sea and Baltic Sea grey seal subspecies (Fig. 8). Moreover, our microsatellite analyses strongly suggest that admixture is taking place at Rødsand in the Southwest Baltic. Although the DIY-ABC analyses were variable with regard to posterior estimates of timing, all simulations provided highest support for a demographic admixture model, which, when taken together with the results of the STRUCTURE analysis, are in line with a demographic history involving recent admixture between North Sea and Baltic Sea grey seals. Indeed, our population censuses document an increasing presence of recolonizers throughout the season, and there have been several observations of grey seal pups in the region during both the North Sea and the Baltic Sea breeding seasons (Härkönen *et al.* 2007; R. Dietz, A. Galatius, J. Teilmann, unpublished). This indicates a spatiotemporal overlap between the two subspecies.

What allows animals from different breeding populations with different breeding habitats and timings of reproduction to interbreed? On the British Isles, pupping follows a clockwise cline in the mean birth date starting in southwestern UK in August and ending in southeastern UK in December (SCOS 2011), while peak pupping time is December–January in the Wadden Sea (Brasseur *et al.* 2015b) and February–March in the Baltic Sea. Reports from the early 1800s document that grey seal pups were hunted on Anholt in Kattegat from the 2nd of February until mid-March and that the first females usually appeared in mid-December (Tauber 1880), which is consistent with a pupping time in mid-January. Thus, it seems that European grey seals exhibit a clinal pattern in breeding season from August to December in the British Isles, through December and January in the Wadden Sea and Danish waters to

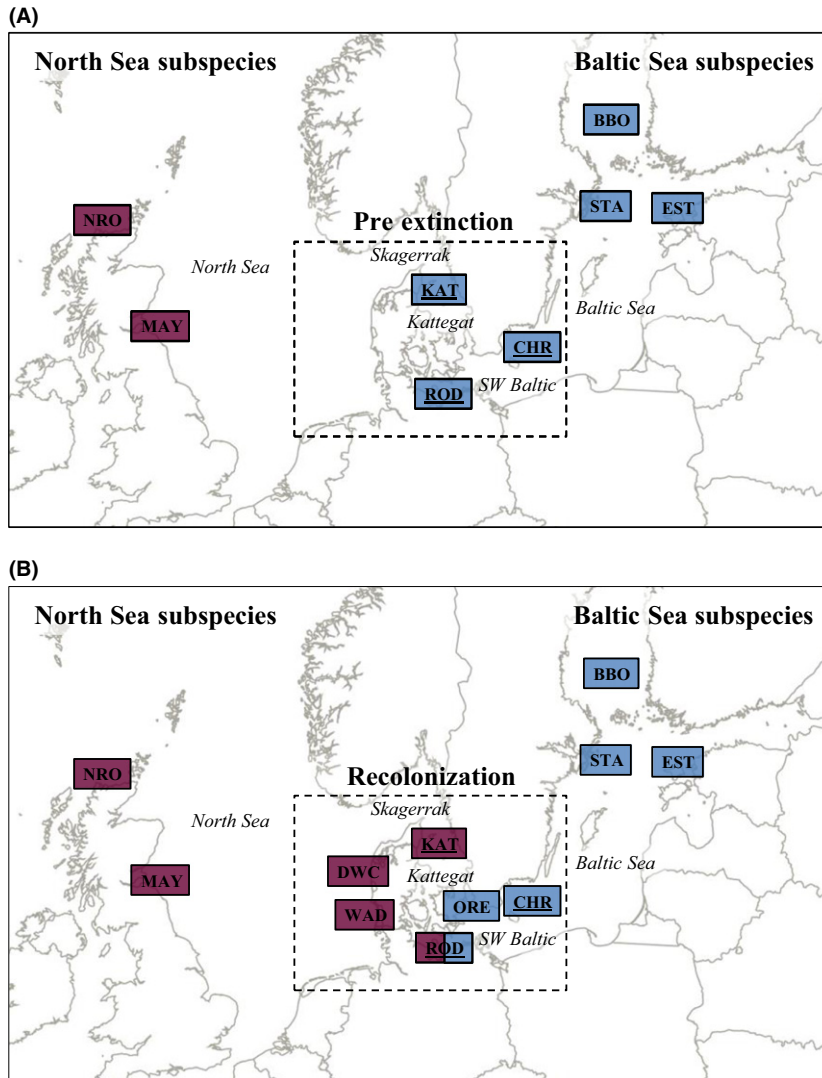


Fig. 8 Spatiotemporal shift in grey seal subspecies boundaries and admixture in Denmark. The figure summarizes results from the haplotype networks (Fig. 5), STRUCTURE analyses (Fig. 6) and estimates of divergence and admixture in DIY-ABC (Fig. 7, Table 3) to show the genetic structure of grey seals before their local extinction (A) and during the current recolonization (B). Dark red (dark grey) = North Sea subspecies; Blue (light grey) = Baltic Sea subspecies.

February–March in the central Baltic Sea. Such plasticity at the population level supports the existence of some degree of mating season plasticity also at the individual level, potentially allowing for admixture in cases when animals of the two subspecies meet. Given the high degree of site fidelity reported for grey seal females (Pomeroy *et al.* 2000; Karlsson *et al.* 2005), the likely more asynchronous mating season of land breeding grey seals (Haller *et al.* 1996) such as those in the North Sea, and the observed differences in genetic differentiation estimated at mtDNA and microsatellite markers, we find it plausible that such admixture primarily is driven by male grey seals from the North Sea.

Implications for the future management of grey seals in Europe

We have combined zooarchaeological, demographic and genetic data to elucidate several key aspects of the

northern European grey seals' evolutionary and demographic history. Still, our findings are not without limitations in that we do not have nuclear data from the historic population, precluding inference of historic male-mediated gene flow. Also, our inference of prehistoric grey seal abundance – and hence links between historic abundance and current genetic diversity – could be biased by the differential availability of zooarchaeological samples, for example due to variation in excavation effort and regional differences in sea level changes and associated flooding of prehistoric sites. Still, northern European sea levels appear to have been stable for at least 7000 years (Lambeck *et al.* 1990; Tornqvist & Hijma 2012), suggesting that the latter source of bias is probably negligible.

Bearing these caveats in mind, our study provides some valuable insights for the management of grey seals in northern Europe. First, it is clear that grey seals in Skagerrak, Kattegat and the southwestern Baltic Sea

cannot be managed as a single genetic or demographic unit, as they consist of animals of both the North Sea and the Baltic Sea subspecies. Second, future movements and hybridization between North Sea and Baltic Sea grey seals may gradually lead to a breakdown of subspecies and management boundaries across parts of the northern European range. Third, given these movements and hybridization, population surveys in Skagerrak, Kattegat and the southwestern Baltic should be extended to cover the breeding and moulting season of both North Sea and Baltic Sea grey seals. Fourth, without looking into the genetic information, it could be argued that the former grey seal population in the Kattegat area is now being re-established. This is true with respect to observed number of animals, but not with respect to genetic origin. Our study suggests that counts alone provide limited inference and that it is necessary to combine demographic with genetic data to understand changes to populations that can occur with recolonization.

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K.F. and M.T.O. designed the research; K.F., A.G., J.T., R.D., L.F.J., J.G. and M.T.O. collected samples and demographic data; M.T.O. compiled and analysed the zooarchaeological data; A.G. and M.T.O. performed the demographic analyses; K.F., A.K.F. and M.T.O. performed molecular laboratory work; K.F., A.G., A.K., J.H. and M.T.O. analysed the molecular data; K.F., A.K., J.H. and M.T.O. wrote the manuscript; A.G., J.T., R.D., P.P., L.F.J. and M.T.O. provided funding; all authors commented on previous versions of the manuscript and approved of the final version.

Data accessibility

All genetic and demographic data are uploaded as supplementary material, except for mtDNA haplotypes, which are uploaded in GenBank under Accession nos. KF483184–KF483221.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Sampling localities and sample types.

Table S2 Grey seal haplotype distributions across the sampling locations.

Table S3 Sample sizes and estimates of mtDNA diversity.

Table S4 Sample sizes and estimates of microsatellite diversity.

Table S5 Estimations of support for the best demographic model, posterior error and mode estimates for timing and admixture rate.

Appendix S1 Grey seal survey data from Kattegat and the southwest Baltic Sea.

Appendix S2 Microsatellite data from contemporary grey seal samples used in Fietz *et al.* (2016) Molecular Ecology.