Identification of herring populations

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2015

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Dissertation submitted in partial fulfillment of a
Philosophiae Doctor degree in Biology

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Bibliographic information:
Lísa Anne Libungan, 2015, *Identification of herring populations*, PhD dissertation, Faculty of Life and Environmental Sciences, University of Iceland, 135 pp.

ISBN 978-9935-9243-2-2

Printing: Háskólaprent, Fálkagata 2, 101 Reykjavík, Iceland
Reykjavik, Iceland, June 2015

Abstract

Information on population structure is important for the successful management of harvested species and for the understanding of the distributional range, migration behaviours and for protection of biodiversity. Atlantic herring (*Clupea harengus* Linnaeus 1758) is one of the most abundant fish species in the world and due to its commercial value, an understanding of its biology, including its population dynamics, is needed for the sustainable management of the resource. The biogeography of herring is highly complex and populations are often defined on the basis of where and when they spawn. In this study, I have developed two tools to discriminate between herring populations. Microsatellite markers for genetic analysis and a statistical package (shapeR) to study otolith shape. Extensive sampling of two herring species, Atlantic and Pacific herring (*Clupea pallasii* Valenciennes 1847), was conducted throughout the North Atlantic, along the coast of Norway, Russia, and the Pacific. Analysis of variation in the microsatellite markers did not detect any differentiation among the herring stocks in the North Atlantic, however, otolith shape variation was detected. These differences could be traced to three morphological structures on the otolith outlines which showed a correlation with the stocks spawning time. A classifier based on the shape differences was able to discriminate with 94% accuracy between the Icelandic summer-spawners and the Norwegian spring-spawners, which are known to mix at feeding grounds east of Iceland. In separate studies on local populations in Norway, variation in otolith shape was detected, and among local populations along the coast, a latitudinal gradient emerged where neighbouring populations were more similar to each other than to those sampled at larger distances. These morphological differences are likely to reflect environmental differences but also indicate low dispersal among the local herring populations. At the species level, a comparison in otolith shape was conducted between Atlantic and Pacific herring and among subspecies of Pacific herring which revealed similarity of herring occupying the Bering Sea in the NW-Pacific, Balsfjord in N-Norway and the SE-Barents Sea in Russia, results which are in accordance with former genetic studies. The results of these studies show that otolith shape can serve as a marker to identify herring populations, subspecies and species at small and large geographic scales.

Útdráttur

Þekking á stofnlíffræði fiskistofna er mikilvæg fyrir árangursríka fiskveiðistjórnun og fyrir skilning á útbreiðslu, farmynstri og til verndunar á líffræðilegum fjölbreytileika. Atlantshafssíldin (*Clupea harengus* Linnaeus 1758) er ein af þeim tegundum í heiminum sem státar af mestum lífmassa allra sjávarfiska og hefur verðmæti hennar orðið til þess að áhersla hefur verið lögð á rannsóknir er snúa að stofnstærðarmati, lifnaðarháttum, líffræði og stofnlíffræði hennar. Líflandafræði síldarinnar er afar flókin og eru stofnar gjarnan skilgreindir út frá hrygningarsvæðum og hrygningartíma. Í þessari rannsókn hef ég þróað tvær aðferðir sem hægt er að nota til þess að aðgreina síldarstofna: erfðamörk (örtungl) sem hægt er að nota í erfðarannsóknum og hugbúnað (shapeR) til að rannsaka útlitseinkenni kvarna. Umfangsmikil sýnataka var framkvæmd á tveimur tegundum af síld, Atlantshafssíld og Kyrrahafssíld (*Clupea pallasii* Valenciennes 1847), víðsvegar í Norður Atlantshafi, meðfram strandlengju Noregs, Rússlandi og í Kyrrahafi. Niðurstöður samanburðar sem byggði á örtunglum gat ekki greint erfðafræðilegan mun á milli stofna í Norður Atlantshafi, hins vegar fannst munur á kvarnaútliti. Þennan breytileika var hægt að rekja til þriggja svæða á kvörnunum og var fylgni á milli útlitsbreytileika þeirra og hrygningartíma. Flokkari sem byggði á þessum útlitseinkennum gat greint í sundur stofna sem blandast á fæðuslóð, íslensku sumargotssíldina og norsk-íslensku vorgotssíldina, með 94% nákvæmni. Í tveimur rannsóknum á fjarðarstofnum í Noregi var hægt að nota kvarnaútlit til að aðgreina stofnana og í samanburði á stofnum meðfram strandlengju Noregs kom í ljós að stofnar sem voru nær hver öðrum í fjarlægð voru líkari í kvarnalögun en stofnar sem voru fjær. Þetta sýndi fram á að breytileiki í kvarnaútliti er tengdur breiddargráðu í fjarðarstofnunum í Noregi og líklegt er að þessir stofnar hafi takmarkað far og séu einangraðir. Kvarnaútlit var borið saman meðal tveggja síldartegunda, Atlantshafssíldar og Kyrrahafssíldar, og meðal undirtegunda Kyrrahafssíldarinnar. Niðurstöður sýndu að síld í Beringshafi í NV-Kyrrahafi var líkari síld í N-Noregi og Barentshafi en síld í NA-Kyrrahafi, en þær niðurstöður eru í samræmi við erfðarannsóknir á þessum sömu stofnum. Niðurstöður þessara rannsókna sýna að hægt er að nota útlitseinkenni kvarna til að aðgreina síldarstofna, undirtegundir og tegundir á stórum og smáum landfræðilegum kvarða.

To Kjartan, for always being there for me

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List of Original Papers

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Paper II: Libungan LA and Pálsson S. 2015. ShapeR: an R package to study otolith shape variation among fish populations. PLoS ONE 10 (3): e0121102

Paper III: Libungan LA, Óskarsson GJ, Slotte A, Jacobsen JA, Pálsson S. 2015. Otolith shape: A population marker for Atlantic herring *Clupea harengus*. Journal of Fish Biology 86: 1377-1395

Paper IV: Eggers F, Slotte A, Libungan LA, Johannessen A, Kvamme C, Moland E, Olsen EM, Nash RDM. 2014. Seasonal Dynamics of Atlantic Herring (*Clupea harengus* L.) Populations Spawning in the Vicinity of Marginal Habitats. PLoS ONE 9 (11): e111985

Paper V: Libungan LA, Slotte A, Husebø Å, Godiksen JA, Pálsson S. Latitudinal gradient in otolith shape among local populations of Atlantic herring (*Clupea harengus*) in Norway (*in review*)

Paper VI: Libungan LA, Slotte A, Otis EO, Pálsson S. Classifying Pacific herring (*Clupea pallasii*) subspecies based on otolith shape (*in review*)

A peer-reviewed paper not included in the thesis:

Pampoulie C, Slotte A, Óskarsson GJ, Helyar S, Jónsson Á, Ólafsdóttir G, Skírnisdóttir S, Libungan LA, Jacobsen JA, Joensen H, Nielsen HH, Sigurðsson SK, Daníelsdóttir AK. 2015. Stock structure of Atlantic herring *Clupea harengus* in the Norwegian Sea and adjacent waters. Marine Ecology Progress Series 522: 219-230

Acknowledgments

First, I would like to express my sincere gratitude to my advisor Prof. Snæbjörn Pálsson. His excellent supervision, problem solving skills, lightning fast responses, positive attitude and encouragement throughout the years made me more passionate about science than I thought I could ever be. It has been a priviledge working with you and I hope I will be fortunate enough to work with you again in the nearest future.

I thank Dr. Guðmundur J. Óskarsson at the Marine Research Institute for his guidance and for sharing his passion and extensive knowledge on herring. It has been very inspiring working with you and has made me appreciate how amazing this species is. Thanks to Prof. Gunnar Stefánsson, for comments on my work and Dr. Christophe Pampoulie at the Marine Research Institute, for useful discussions and help during the genetics part of my studies.

A large part of this thesis was in collaboration with the Institute of Marine Research in Bergen, Norway. Dr. Aril Slotte, I would like to greatly thank you for providing me with the opportunity to work on Norwegian and Barents Sea herring. Åse Husebø, also at IMR, thank you for our collaboration, positive comments and motivation. I thank Dr. Edward O. Otis (Ted) at Alaska Fish and Game in USA for several discussions on herring and our collaboration with the herring populations in the Pacific.

My opponents, Prof. Albert K. Imsland, University of Bergen & Akvaplan Niva and Prof. Henrik Mosegaard, Technical University of Denmark (DTU Aqua), I thank for allocating their time to review my work.

During my studies, I was fortunate to work in a positive and inspiring environment at the University of Iceland surrounded by friends and great scientists. Thank you all for fun times, great discussions and support. I thank my mother Margrét and Gústav, my sister Berglind and Bjarni, and my friends for their support. My grandmother Gerda (1923-2013) and father Dominador (1942-2011) I thank for motivating me to pursue higher education. I am greatful to my parents-in-law Katrín and Benedikt for their support and thank my inspiring family-in-laws: Gullý and Jan, Jón Atli and Stefanía, Kristín and Jón Pétur, Anna Þóra, Helgi and María.

I thank my husband Kjartan Benediktsson, for his love, help, patience, and for always believing in me, even at times when I did not. Thank you honey, I could never have done this without you. Our two cheerful sons, Seifur Ísak and Stígur Sær, have been my inspiration but at the same time reminders of what matters most in life.

This work was funded by the Icelandic Research Fund for Graduate students (nr. 100710001), the Assistant teachers grant at the University of Iceland and received an award from the Entrepreneur Fund of Íslandsbanki. Further funding agents and acknowledgments are listed in each paper in the thesis.

1 Introduction

Identification of populations and estimation of their relative contribution to mixed fisheries is important for effective management of harvested species, for the understanding of their distributional range and migration behaviour. Disregard of population structure in fisheries management can lead to overexploitation of local non-targeted populations and may result in loss of genetic variation (Nelson and Soulé, 1987; Smith et al., 1991), which may be vital for adaptation in an ocean that is affected by natural and anthropogenically induced variability, such as climate change.

Determining the geographic scale over which fish populations are connected is important for the understanding of dynamics of marine population (Conover et al., 2006; Jones et al., 2007; Leis et al., 2011). The connectivity has been classified as being either evolutionary or ecological, emphasizing processes occurring at different time scales (Leis et al., 2011). Evolutionary connectivity refers to the movements of genetic variants and their segregation over time, which often happens over large geographic areas in the marine environment. Ecological connectivity refers to dispersal of individuals during their life time, such as migration towards feeding and spawning grounds, a common occurence for many pelagic species. Many marine species have good dispersal capacities and as geographic barriers are often lacking in the marine environment, little genetic differentiation among populations is commonly observed (Hauser and Ward, 1998). Furthermore, many marine species are characterized by large population sizes, which reduces the rate of divergence of separated populations. Although geographic barriers may be lacking, oceanic patterns, such as prevailing currents and location of nursery areas, may reduce mixing of larvae, which can cause isolation among fish populations.

Markers used to obtain information on stock structure are of three main types: natural markers, artificial markers, and biological markers. Natural markers include using the genetic composition of fish, morphometric and meristic markers such as vertebrae number and otoliths, or using parasites as biological tags. Populations with low ecological connectivity, i.e. limited dispersal capabilities, have shown both genetic subdivision (Baus et al. 2005, Doherty et al. 1995, Planes, 1998) and divergence in phenotypic variation (Campana and Casselman, 1993; Hulme, 1995; Elsdon and Gillanders, 2004). Phenotypic variation among populations can be measured with meristic characters such as the number of vertebrae, which is influenced by both temperature (negatively) and salinity (positively) experienced during the incubation period (Hulme, 1995). Otolith chemistry, can be used where the concentrations of elements and isotopes in otoliths are compared to those in the water in which the fish inhabits can be used to identify its spawning origin (Elsdon and Gillanders, 2004; Campana, 1999). Otolith shape, where the morphology of the internal growth rings (Burke et al., 2008b) or the morphology of the perimeter (outline) is studied (Campana and Casselman, 1993) can be used for the

same purpose. Using parasites to track fish populations can be conducted by identifying an endemic area of parasites (MacKenzie and Abaunza, 1998). Subsequently, if fish are caught outside this area and are infected by the parasite, one can infer that these fish have been within the parasitic area at some time in their past history. Variation in various types of molecular genetic markers have been applied to study the genetic connectivity or variation among populations, e.g. in allozymes, microsatellites, DNA sequence of targeted genome regions, and more recently in Single Nucleotide Polymorphism (SNP) throughout the genome. Other markers include artificial markers using externally and internally attached tags to track movements of individual fish with mark and recapture methods and biological markers in terms of life-history traits, such as maturity stage, age at maturation, and spawning time.

The subject of this thesis is an analysis of the population structure of Atlantic herring (*Clupea harengus*), a pelagic marine fish species in the North Atlantic, by analysing natural markers to assess genetic variation and variation in otolith shape among populations. The final study in the thesis focuses on otolith shape at the species level between the genetically distinct (Grant 1986) Atlantic and Pacific herring (*Clupea pallasii*) and among Pacific herring subspecies.

1.1 Herring biology

The Atlantic herring is an iteroparous clupeid pelagic fish which aggregates into large schools and inhabits both sides of the North Atlantic Ocean, between latitudes 35°N and 70°N (Blaxter, 1985)(Fig 1.1). The adaptability and plasticity observed throughout the different life-history stages of herring makes it one of the most successful species of marine fish. It is known for variable adaptation among populations and it has thus been proposed that the adaptability is a basic trait specific to this species (Geffen, 2009). Accordingly, herring show an impressive range of reproductive strategies, favouring the survival of eggs and larvae in different environments (Haegele and Schweigert, 1985), such as spawning in shelf areas with stony and rocky bottom substrates (Runnstrøm, 1941a; Dragesund, 1970), inside brackish lakes (Eggers et al., 2014) and within fjords (Aasen, 1952, 1953), all of which can comprise a wide range of temperature and salinity gradients (Blaxter, 1985). In general, herring mature at the age of 3-4 years (Reid et al., 1999) and may spawn at different times of the year (Sinclair and Tremblay, 1984) where spawning can span over a four week period (Devold, 1967; Johannessen et al., 1995; Óskarsson and Taggart, 2009). Eggs hatch in 10-15 days, dependent on temperature (Bigelow and Schroeder, 1953). Based on this variation and geographical separation of spawning grounds, several populations of herring have been identified in the North Atlantic. Herring often have specific nursery grounds, for example the larvae of the Icelandic summer-spawners are carried clockwise with the coastal and Irminger Currents from their spawning grounds south and west of Iceland towards the north, where the main nursery areas are found in fjords (Einarsson, 1956). Similarly, larvae of the Norwegian spring-spawners drift with the coastal current northwards along the western Norwegian coast to nursery areas in the Barents Sea (Dragesund, 1970). Although spawning among oceanic populations, such as the Icelandic summer-spawners

Figure 1.1. Atlantic herring (Clupea harengus). *Drawing by Jón Baldur Hlíðberg.*

and Norwegian spring-spawners occurs upstream relative to the nursery grounds in the open marine environment, the larvae of other populations may be retained near their site of spawning. As an example, the larvae of the Scotia–Fundy herring in Canada (Iles and Sinclair, 1982) and several local populations in Norway are thought to be confined to specific areas with limited dispersal drift (Aasen, 1953; Lie et al., 1978; Johannessen et al., 2009).

1.1.1 Population structure

Atlantic herring has a complex population structure (Iles and Sinclair, 1982; Geffen, 2009) which is often defined based on where and when they spawn. Nearly thirty separate herring populations have been defined in the North Atlantic (Hay et al., 2001) based on the location and timing of spawning. These populations have a wide range of life history strategies (Geffen, 2009), and some even show sub-population structuring (Broch, 1908; Runnstrøm, 1941b; Rasmussen, 1942; Aasen, 1952; Lie et al., 1978; Jørstad and Pedersen, 1986; Hognestad, 1994; Husebø et al., 2005; Johannessen et al., 2009). In the Norwegian Sea and adjacent waters there are at least six herring stocks identified on the basis of spawning time and area. They are: the Icelandic summer-spawners, the Icelandic spring-spawners, the Norwegian spring-spawners, the Norwegian autumn-spawners, the Faroese autumn-spawners, and the North Sea autumn-spawners. In addition to these stocks there are a number of local herring populations which occupy fjords (Aasen, 1953; Lie et al., 1978; Jørstad et al., 1994), semi-enclosed coastal ecosystems (Johannessen et al., 2009; Langård et al., 2014) and the brackish Lake Landvik in southern Norway (Eggers et al., 2014).

1.2 Herring in Iceland

Three herring stocks are found around Iceland: the Icelandic spring- and summer-spawners which spawn along the southern coast of Iceland from northwest to east (Óskarsson and Taggart, 2009; Jakobsson et al., 1969), and the Norwegian spring-spawners which spawn mainly around Møre on the west coast of Norway (Runnstrøm,

1941a) but migrate east of Iceland to feed (see further in section 1.3). The two herring stocks which are local to Iceland (Icelandic spring- and summer-spawners) used to be found in relatively equal portions before they both collapsed in the late 1960s (Jakobsson, 1980). Until then, the herring fishery in Icelandic waters was directed at these three herring stocks. Except around their spawning season (Icelandic summer-spawners in July and Icelandic spring-spawners and Norwegian spring-spawners in March), the stocks were mixed to a varying degree over the year (Jakobsson et al., 1969). Accordingly, it was often a mixed fishery in Icelandic waters. Following the collapse, the Norwegian spring-spawners disappeared from Icelandic waters (Dragesund et al., 1997) but started to reappear around mid 2000s (Utne et al., 2012). The Icelandic summer-spawners recovered rather quickly and the current stock size indicates a successful fisheries management since the collapse (MRI, 2014). The Icelandic spring-spawners have, however, not recovered yet. As the Norwegian spring-spawners and Icelandic spring-spawners could not be distinguished by spawning time and fecundity, Jakobsson (1980) suggested they should be considered as one component.

1.3 Admixture at feeding grounds

The Norwegian spring-spawning herring is one of the largest herring stocks in the world. They undertake extensive clockwise feeding migrations in the Norwegian Sea, where they move from the west coast of Norway, towards the Faroe Islands and into Icelandic waters, before returning back to Norway where they spawn along the coast (Fig 1.2). The Norwegian spring-spawners mix with local populations in Iceland and the Faroe Islands and therefore a mixed fishery can take place in these areas. Individuals from these four stocks (Faroese autumn-spawners, Faroese spring-spawners, Icelandic summer-spawners and Norwegian spring-spawners) not only have the same external characteristics but also grow to similar sizes, making it problematic to separate them in mixed fisheries based on body features alone. In the early days of herring population discrimination in Icelandic waters, populations were separated on the basis of vertebrae number and by examining the transparency of the otolith nucleus (Einarsson, 1951). The vertebrae number for the Icelandic spring-spawners was reported to be slightly higher (mean number 57.19-57.23) than for the Icelandic summer-spawners (56.93-56.98), which was used as a population marker. Also, because the larvae and post-larvae of the Norwegian spring- and Icelandic summer-spawners develop during different seasons and growth conditions, it could be seen in the structure of their otolith nucleus. The Norwegian spring-spawners have an opaque nuclei which is less transparent, whereas the Icelandic summer-spawners have a hyaline nuclei. Nowadays, the method to discriminate between populations which are caught together in the mixed fishery (the Norwegian spring-spawners and the Icelandic summer-spawners) is to separate them based on maturity stage, since they spawn in different times of the year (Jakobsson et al., 1969). However, determining stocks solely based on maturity stage lacks precision because the method requires visual examination and relies upon subjective judgment by the sampler. Stock separation based on maturity is also subject to error due to potential overlap in the timing of gonad development between the two stocks. For example, if mat-

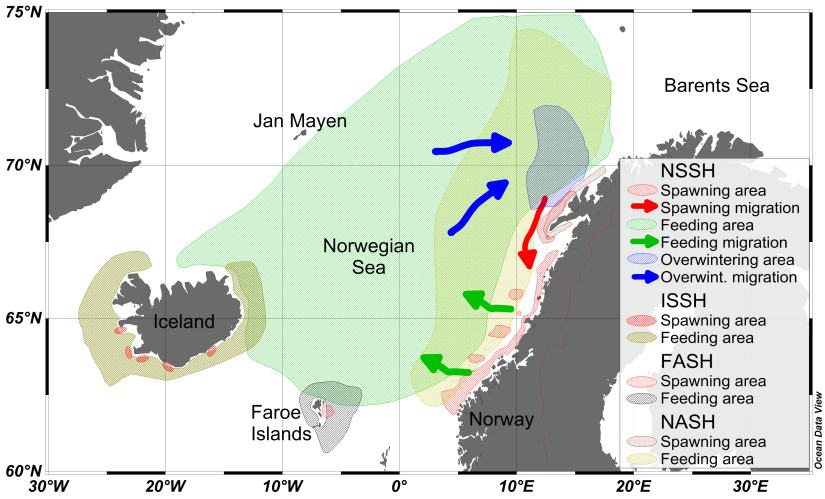

Figure 1.2. Mixing of herring populations in the Northeast Atlantic. Current migration pattern of adult Norwegian spring-spawning herring (NSSH) and interactions with other surrounding stocks, i.e. Icelandic summer-spawning herring (ISSH), Faroese autumn-spawning herring (FASH), and Norwegian autumn-spawning herring (NASH). From Pampoulie et al (2015).

uration and gonad growth of the Norwegian spring-spawning individuals starts late and the Icelandic summer-spawning individuals have just spawned, both stocks can be in the resting stage simultaneously. Another example is in the feeding areas north of the Faroes, where during late summer the Norwegian spring-spawners have begun to develop gonads after the feeding season and are found mixed with the Faroese autumn-spawners, which are in a similar or marginally more advanced maturity stage.

1.4 Herring in Norway

Several local herring populations in Norway have been identified based on biological characteristics and geographical distribution, such as the Balsfjord, Lysefjord and Østerbø herring (Aasen, 1953), Borge poll herring (Rasmussen, 1942), Lindåspollene herring (Dahl et al., 1973), Lusterfjord herring (Aasen, 1952), Lake Landvik herring (Eggers et al., 2014), Lake Rossfjord herring (Hognestad, 1994), and Trondheimsfjord herring (Sars, 1891; Runnstrøm, 1941b). The local herring populations are thought to complete their entire life-cycle within fjords (Aasen, 1952), lakes (Eggers et al., 2014) and semi-enclosed coastal systems (Langård et al., 2014). They differ from their oceanic counterparts by having small population sizes, a shorter life cycle, low vertebrae number, slower growth rate (Aasen, 1952), and smaller size-at-age (Lie et al., 1978; Johannessen et al., 2009). In conjunction with these differences, they also have

higher relative fecundity since local populations do not migrate over long distances and therefore invest less energy into growth and more into egg production than oceanic population (Hognestad, 1994; Jørstad and Nævdal, 1981; Sørensen, 2012; Silva et al., 2013). In addition to the local herring populations in Norway, there are two oceanic herring populations: the Norwegian spring-spawners, which are highly migratory and disperse all over the Norwegian Sea, and the Norwegian autumn-spawners, which are thought to be mainly around Lofoten (Husebø et al., 2005) and are managed as part of the Norwegian spring-spawners.

1.5 Herring species

Three allopatric species are found within the genus Clupea: the Atlantic herring (*C. harengus* Linnaeus 1758), Pacific herring (*C. pallasii* Valenciennes 1847) from the North Pacific Ocean, and the Chilean herring (*C. bentincki* Norman 1936). Remote populations of Pacific herring have been found in the SE-Barents Sea and White Sea in Russia, and Balsfjord in N-Norway (Laakkonen et al., 2013). The SE-Barents Sea herring has been classified as a separate subspecies (*C. pallasii suworowi*) as well as the herring in the White Sea (*C. pallasii marisalbi*). The European *C. pallasii* herring are thought to be early post-glacial colonists from the NW-Pacific (Laakkonen et al., 2013). The Balsfjord herring has been shown to be closely related to the White Sea and SE-Barents Sea herring, based on variation in mitochondrial DNA, and also to the *C. pallasii* populations from the NW-Pacific (Laakkonen et al., 2013). A mixture of herring populations in Balsfjord has been observed based on genetic studies using allozymes and mitochondrial markers (Jørstad and Nævdal, 1981) and an introgressive hybridization has been reported from the Atlantic herring into the Pacific herring in Balsfjord (Laakkonen et al., 2015).

1.6 Identifying herring populations

1.6.1 Genetic markers

Genetic markers based on allozyme variation, restriction length polymorphism, and microsatellites have shown uniformity among herring occupying the offshore waters of the Northeast Atlantic and over large geographical distances (Ryman et al., 1984; King et al., 1987; Kornfield and Bogdanowicz, 1987; Dahle and Eriksen, 1990; Pampoulie et al., 2015). Relatively low levels of genetic differentiation has been found among herring populations that may overlap geographically during feeding migrations (Bekkevold et al., 2005; Jørgensen et al., 2005; Mariani et al., 2005; Ruzzante et al., 2006; Gaggiotti et al., 2009; André et al., 2011; Lamichhaney et al., 2012; Corander et al., 2013; Teacher et al., 2013). Recent studies on population genomics have, however, revealed clear differentiation in the Baltic Sea (Corander et al., 2013) and genetic differences have been found between geographically isolated populations in Norway (Jørstad et al., 1994; Turan et al., 1998; Pampoulie et al., 2015).

1.6.2 Phenotypic markers

Phenotypic markers used for herring population identification include the number of vertebrae and variation in otoliths in terms of their microstructure, chemistry or shape (see further details in section 1.7). Vertebrae count of adult herring is an indicator of spawning grounds and time of spawning, as the number of vertebra are influenced by both temperature (negatively) and salinity (positively) during the incubation period (Hempel, 1953; Blaxter, 1957; Hempel and Blaxter, 1961). Otolith microstructure can be used to measure differing growth rates (fast growers and slow growers) where the increment width patterns in the otoliths of juvenile herring can be measured and used as an indicator of population variation (Brophy and Danilowicz, 2002). Differences in the elemental concentrations, the otolith chemistry, has been used to assign juvenile herring to nursery grounds in the Irish Sea, Scottish sea lochs, and the Minch (Geffen et al., 2011). Otolith shape analysis has been used to discriminate between stocks of Irish Sea and Celtic Sea herring in the Irish Sea by analysing the shape of inner growth rings of juveniles (Burke et al., 2008a). Outline analysis of otoliths has been applied in a comparison of the two herring species from the Atlantic and Pacific (Bird et al., 1986). In this thesis I apply more extensive analyses to study variation among several Atlantic herring populations in the North Atlantic (**Paper III**), along the coast of Norway (**Paper V**), among three populations in a small region in southern Norway (**Paper IV**) and between Atlantic and Pacific herring (*C. pallasii*), including Pacific herring subspecies (**Paper VI**).

1.7 Otoliths

Otoliths are calcified structures found in the inner ear of teleost fish. Otolith composition is relatively pure compared to most biological and mineralogical structures, the major elements are calcium, oxygen and carbon, which make up the calcium carbonate ($CaCO_3$) matrix (Campana, 1999). There are three pairs of otoliths (sagitta, lapillus and asteriscus), with the sagitta being the largest in most species, thus being most used in research (Hecht, 1978). Otoliths are located in chambers in the inner ear where they play a role in hearing and sense of equilibrium (Popper et al., 2005). Otoliths acquire yearly growth rings, or annuli, and have concentric rings around year 1 at the center. They can be thought of as metabolically inert environmental recorders, since all elements and compounds which accrete onto the growing surface are retained, and the continued growth of the otolith, from before the time of hatch to the time of death, spans the entire life of the fish (Campana and Neilson, 1985). Differences in the shape of the otolith tend to reflect phylogeny and development, which has led to their widespread use in taxonomy (Hecht, 1978; Nolf, 1985, 1995) and food web studies (Frost and Lowry, 1981). Otolith morphometrics have thus been used in species identification and to study geographical variations in populations and stocks of fish (Castonguay et al., 1991; Campana and Casselman, 1993; Friedland and Reddin, 1994). Otolith shape is influenced by genetic factors (Cardinale et al., 2004) and ontogenetic processes where otolith size changes in relation to body growth, temperature, and food quantity (Einarsson, 1951; Geffen, 1982; Folkvord et al., 2000; Feet et al., 2002; Fox et al., 2003;

Vignon, 2012). Higher food rations during early life can lead to a higher number of larger lobes and a more rectangular otolith (Hüssy, 2008). Fish may thus be marked in their otoliths for life by the environment they were spawned in and exposed to as they grow, layers are added to the otoliths and shaped by the initial shape that was formed in their early life stages. Variable spawning time among fish stocks can thus contribute to variation in shape, as it not only reflects early life temperature, but it can also be a proxy for ecological differences or variation in seasonal resource availability during the first year of the individual's life (Hempel and Blaxter, 1967; Burke et al., 2008b).

1.8 Aims of the thesis

The main objectives of this thesis are twofold. Firstly, to develop tools to identify herring populations and allocate herring that are caught together in mixed fisheries to their origin of spawning. Secondly, to analyse the divergence among herring populations. Two methods were developed for the identification of the herring populations, one was designing primers to assess variation in microsatellite markers (**Paper I**) and the other was a statistical method to analyse otolith shape (**Paper II**). The microsatellite markers were used in a study on populations from the North Atlantic (Pampoulie et al 2015, not included in this thesis) and the shape analysis method was applied to analyse the variation for the same populations (**Paper III**), among local Norwegian populations (**Papers IV, V**) and the divergence between the Atlantic and Pacific herring, including subspecies of Pacific herring (**Paper VI**).

2 Methods and Results

2.1 Sampling

Samples were obtained from two herring species: Atlantic herring (*C. harengus*), in Canada, the Faroe Islands, Iceland, Ireland, Norway, and Scotland and Pacific herring (*C. pallasii*), in Alaska USA, N-Norway (Balsfjord), and Russia. In total, samples were collected from 30 locations and are representative of eight countries and three different systems, ie. the open ocean, fjords, and a lake (Fig 2.1). Spawning herring or those ripe or close to spawning, were sampled on the different spawning grounds with pelagic trawls and purse seines on commercial fishing and research vessels. Sampling areas and timings were selected based on knowledge of spawning behaviour for each population, ensuring that individuals sampled at each locality belonged to the spawning stock of that site. Total length (cm) was recorded for each fish and maturity stage according to an 8-point scale: immature = 1 and 2, maturing = 3 to 5, running/spawning = 6, spent = 7, recovering/resting = 8 (Mjanger et al., 2011). Tissue samples were collected for the populations from Iceland and Lake Landvik in S-Norway for the development of the microsatellite markers. The sagittal otoliths were removed, washed in clean water, and stored in plastic trays. All fish were aged from their scales or otoliths using standard ageing techniques (DeVries and Frie, 1996).

Figure 2.1. All sampling areas in the study (black triangles). Samples were obtained from two herring species: Atlantic herring (C. harengus)*, in Canada, the Faroe Islands, Iceland, Ireland, Norway and Scotland and Pacific herring* (C. pallasii)*, in Alaska USA, N-Norway (Balsfjord) and Russia. In total, samples were taken from 30 locations and are representative of eight countries and three different systems, ie. the open ocean, fjords, and a lake.*

2.2 Testing two identification methods

2.2.1 Genetic method

In order to increase the statistical power of the microsatellite analysis new microsatellite markers were developed which could be used in addition to existing markers. Based on shotgun sequencing of the genomic DNA library, 32 primer pairs were designed and tested (**Paper I**). Fourteen of those were further analysed for two samples, one from Iceland ($n = 39$) and one from Norway ($n = 49$). Deviations from Hardy-Weinberg were analysed and the occurrence of null alleles estimated for the different markers. The resulting markers contain di, tri and tetranucleotide repeats, are polymorphic (7–30 alleles), their observed heterozygosity ranges between 0.69 and 1.00, and expected heterozygosity is between 0.55 and 0.97.

Six out of 14 of these microsatellites (msild12, msild13, msild17, msild24, msild27, msild32) were selected and combined with 18 other microsatellites to study the stock structure of Atlantic herring in the Norwegian Sea and adjacent waters (Pampoulie et al., 2015). The main results showed that the observed level of genetic differentiation was significant among the populations but low ($F_{ST} = 0.007$) and mostly attributable to the differentiation of the local Norwegian fjord populations. One of the locuses, Cpa111, was detected to be under positive selection and also exhibited the highest F_{ST} value (0.044). The observed genetic patterns were robust to exclusion of this locus. Herring from Lake Landvik in S-Norway was genetically distinguishable from three fjord populations, Lindås herring, Lusterfjord herring and Trondheimsfjord herring. The study also showed that there was no support for genetic structuring among the Icelandic summer-spawners and the Norwegian spring-spawners, which are known to mix east of Iceland during feeding. It is therefore apparent that genetic markers, in terms of microsatellites, do not seem to be suitable markers for population discrimination of Atlantic herring, especially for populations which mix during feeding.

2.2.2 Phenotypic method

To evaluate otolith shape as a population marker for herring and estimate how accurate the shape is in classifying stocks of different origin, the software shapeR was developed in the programming language R (R Core Team) (**Paper II**, see also the package documentation in the **Appendix**). ShapeR is an open source software package which is specifically designed to study otolith shape variation among fish populations. The package extends previously described software used for otolith shape analysis by allowing the user to automatically extract closed contour outlines (Fig 2.2) from a large number of images and perform quality checks when collecting otolith outlines, perform smoothing to eliminate pixel noise, and choose from two statistical methods to reconstruct the outline by conducting either a Fourier or Wavelet transform to the outlines.

The Wavelet transform provides users with a larger number of variables than the methods hitherto applied and ensures their independence (Graps, 1995; Parisi-Baradad et al., 2005). This provides a powerful alternative to the more commonly known Fourier transform in shape analysis. While the Fourier transform provides functions in the form of sines and cosines, which are non-local and can therefore result in poor

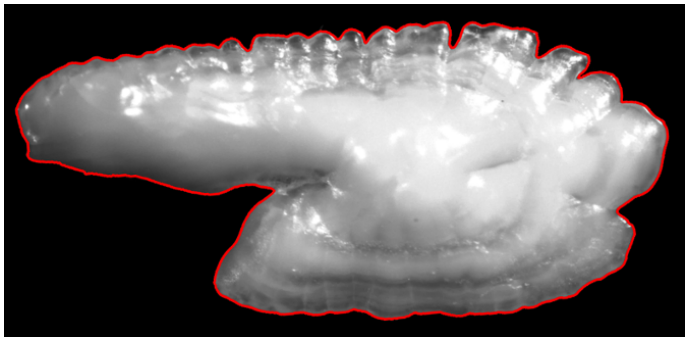

Figure 2.2. Atlantic herring otolith outlined with the R-package shapeR.

approximations of sharp edges, the Wavelet transform uses approximating functions that are contained in finite domains making them well-suited for approximating sharp edges (Graps, 1995). Wavelet is therefore more accurate when more detailed information of the shape differences is needed, e.g. to evaluate which areas of the otolith outline are contributing most towards the variation among populations. Another useful attribute of the shapeR package is that it allows the user to visualize the mean shape of otoliths. The output of the package, i.e. the independent Fourier or Wavelet coefficients, can be directly imported into a wide range of statistical packages in R to further analyse the differences among populations. For example, as demonstrated here, the vegan package (Oksanen et al., 2013) can be used to evaluate Euclidean dissimilarity indices among groups with Canonical Analysis of Principal Coordinates (CAP), a constrained ordination method. The shapeR package might prove useful in studies of variation in any two dimensional objects.

2.3 Population discrimination using otolith shape

To test if otolith shape might be a suitable marker for herring, otolith shape variation was analysed among Atlantic herring populations on three different geographic scales which differ with respect to geographic barriers and environment (oceanic, fjord populations in Norway, and within a brackish lake and connected fjords). And furthermore at the species level between Atlantic and Pacific herring, and at the subspecies level among Pacific herring from three oceans. The geographic scale covered a large proportion of the Atlantic herring distribution's range in the NE- and NW-Atlantic (**Paper III**), the second among 12 local populations and two oceanic ones along the Norwegian coast (**Paper V**), and the third was among three herring populations occupying Lake Landvik and connected fjords in S-Norway (**Paper IV**). At the species level, otolith shape was analysed among Atlantic and Pacific herring from the Atlantic, Pacific and SE-Barents Sea, and among subspecies of Pacific herring (**Paper VI**).

2.4 Otolith shape among oceanic populations in the North Atlantic

Otolith shape was compared among seven Atlantic herring populations in the North Atlantic from Canada, the Faroe Islands, Iceland, Ireland, Norway (Lofoten and Møre) and Scotland (**Paper III**). Significant differences were detected among the seven populations, which could be traced to three morphological structures on the otolith outlines (Fig 2.3). The differentiation in otolith shape between populations was correlated (Pearson, $r = -0.55, p < 0.001$) with their spawning time, indicating a strong environmental effect but could also be due to differing life history strategies (Fig 2.4). A model based on the shape differences discriminates with 94% accuracy between Icelandic summer-spawners and Norwegian spring-spawners, which are known to mix at feeding grounds. This study showed that otolith shape could be used as a marker for herring population discrimination.

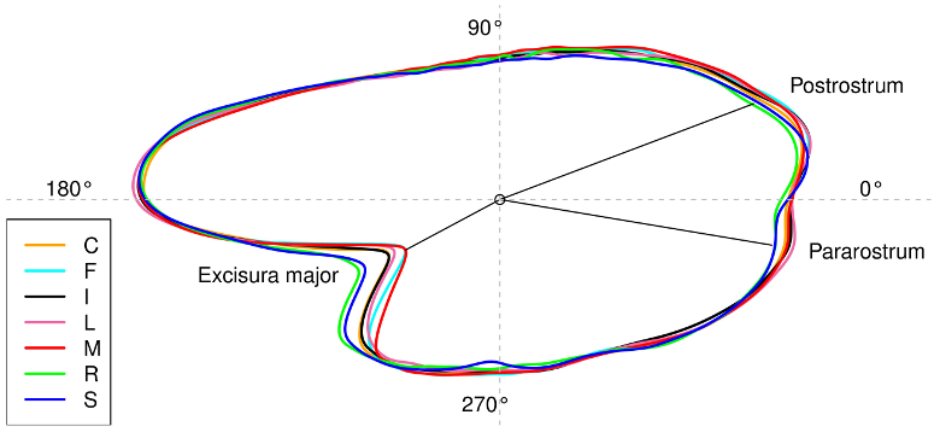

Figure 2.3. Average otolith shape of herring populations in the North Atlantic. Samples were collected from Canada (C), Faroe Islands (F), Iceland (I), Ireland (R), Norway Lofoten (L), Norway Møre (M), and Scotland (S). Lines inside the otolith represent the three radii which are drawn from the otolith centroid towards the excisura major, postrostrum, and pararostrum areas which are the most variable in terms of otolith shape in this study. Degrees refer to angles on the otolith outline.

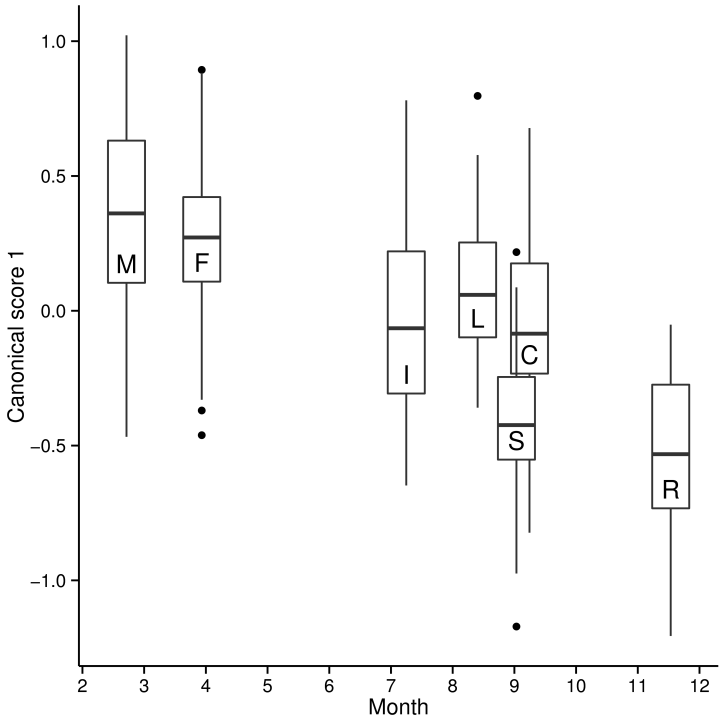

Figure 2.4. The relationship between spawning time and otolith shape for herring. Boxplots of canonical 1 scores (y-axis) derived from otolith shape descriptors with respect to month of spawning in the year (x-axis) for each population: Canada (C), Faroe Islands (F), Iceland (I), Ireland (R), Norway Lofoten (L), Norway Møre (M) and Scotland (S). The boxes are based on the quartiles of the distribution, the straight lines are drawn at variates that are furthest away from the first and third quartile and within a distance of 1.5 interquartile from the upper and lower bounds of the box. Values below and above the lines, representing outliers, are indicated with a dot.

2.5 Otolith shape among local fjord populations in Norway

Otolith shape analysis of Atlantic herring in Norwegian waters (Fig 2.5) showed significant differentiation among fjords and a latitudinal gradient along the coast, where neighbouring populations were more similar to each other than to those sampled at larger distances (Fig 2.6, **Paper V**). There was also temporal stability in otolith shape for the populations that were sampled for more than one year. The local populations from S-Norway were sampled at Kragerø, Risør, Kilsund, Lake Landvik (a brackish lake connected to the ocean), Grimstad, and Høvåg. From W-Norway samples were obtained from Lindåspollene, Lusterfjord (200 km from the coastline), Gloppen (80 km from the coastline), Sykkulven, and Trondheim. The N-Norway representative was sampled in Balsfjord. The oceanic populations were the Norwegian spring-spawners, sampled at their main spawning grounds at Møre, and the Norwegian autumn-spawners from Lofoten (Fig 2.5).

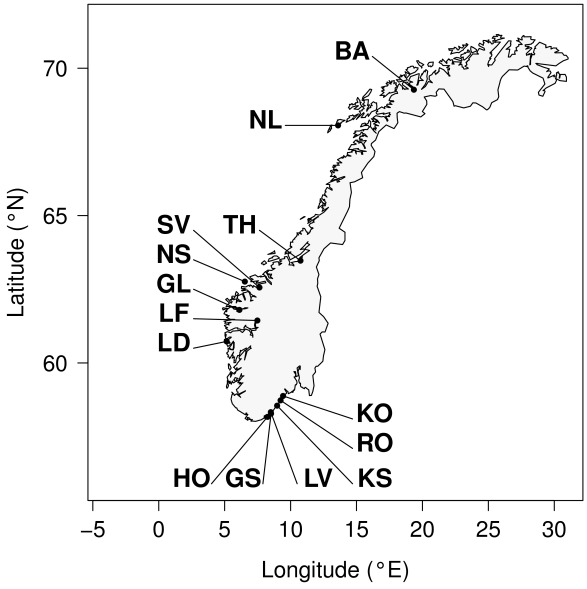

Figure 2.5. Herring sampling areas along the coast of Norway. The local populations from S-Norway are KO: Kragerø, RO: Risør, KS: Kilsund, LV: Lake Landvik, GS: Grimstad, HO: Hovåg. From W-Norway LD: Lindåspollene, LF: Lusterfjord, GL: Gloppen, SV: Sykkulven, TH: Trondheim. From N-Norway BA: Balsfjord. The two oceanic populations, NS: Norwegian spring-spawners and NL: Norwegian-autumn spawners are also shown. Latitude (°N) is shown on the y-axis and longitude (°E) on the x-axis.

The observed differences in shape among the populations most likely reflects environmental differences, but also indicates low dispersal among the populations. This variation also suggests little exchange between the local populations and their oceanic counterparts, which could be due to differences in spawning behaviour. Balsfjord herring, from the most northerly location (69°N), differed in otolith shape from the other populations, and it has also been shown to differ from Atlantic herring in vertebrae number and spawning behaviour (Jørstad and Pedersen, 1986) and show genetic similarity with Pacific herring (*C. pallasii*)(Jørstad et al., 1994; Laakkonen et al., 2013). Our results suggest that the semi-enclosed systems, where the local populations live and breed, are efficient barriers for dispersal. Otolith shape can thus serve as a marker to identify the origin of several herring populations along the coast of Norway.

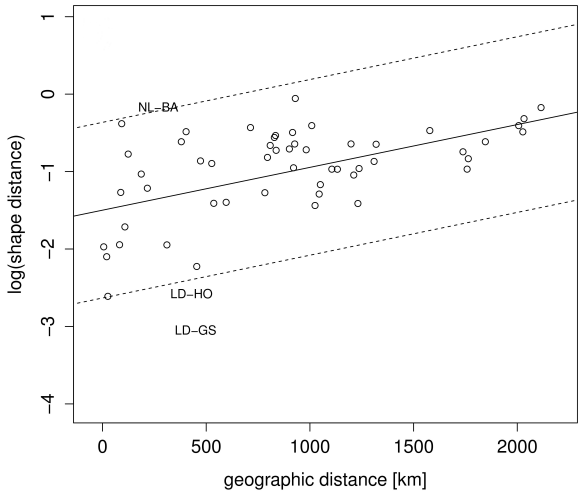

Figure 2.6. The association of otolith shape and distances (in km) in Norway. The geographic distance was measured between sampling areas from Kragerø in S-Norway to Balsfjord in N-Norway (see also Fig 2.5). The correlation of the shape distances with geographical distances was r =0.66 with p<0.001, based on a Mantel test (10.000 permutations). A trend line based on linear regression is shown, and the dotted lines represent two standard deviations of the residuals from the regression line. Population pairs whose distances fall outside of the two standard deviations are presented.

2.6 Otolith shape among herring in S-Norway

Three herring populations, the Norwegian spring-spawners, coastal Skagerrak spring-spawners, and Lake Landvik herring, were analysed in terms of otolith shape, vertebrae count, and growth in the Landvik region in S-Norway (Fig 2.7, **Paper IV**). Lake Landvik is a 1.85 km^2 brackish lake, which was connected to the open sea through a narrow 3 km long artificial channel (Reddalschannel) in 1877. In the study, the lake was observed having oxygen depletion occurring between 2.5 and 5 m depth between March and June, followed by changes in salinity from 1–7% in the 0–1 m surface layer to levels of 20–25% deeper than 10 m. In comparison, no anoxic conditions were found outside the channel that connects the lake with the neighbouring fjord. Salinity in the surface layer increased over the season from 10 to 25%, whereas deeper than 5 m it was stable at around 35%. Temperature at 0–5 m depth increased significantly over the season in both habitats, from 7 to 14°C outside and from 5 to 17°C inside the lake. Despite differences in peak spawning and utilization of the lake habitat between the three putative populations, there was an apparent temporal and spatial overlap in spawning stages suggesting potential interbreeding in accordance with the metapopulation concept.

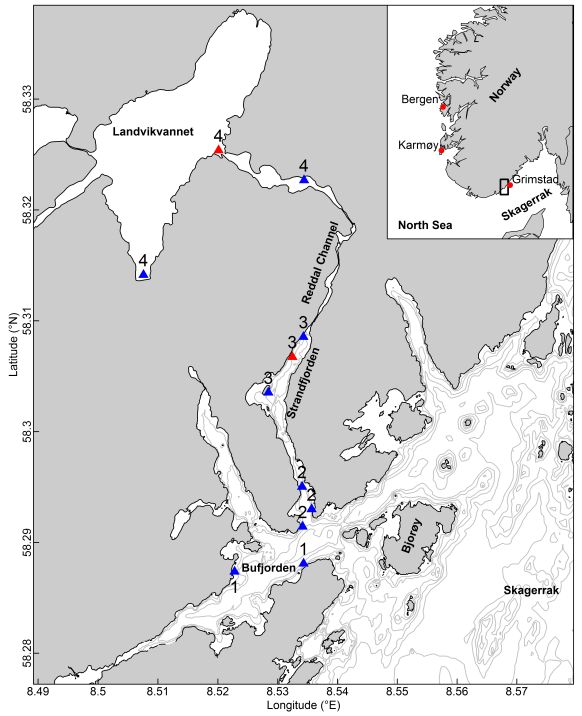

Figure 2.7. Sampling areas of herring in Landvik, Norway. The populations are the coastal Skagerrak herring = 1-3 and Lake Landvik herring = 4. In addition, the Norwegian spring-spawners were sampled both inside and outside the lake.

The results from the otolith shape analysis indicated structuring within this small region with the presence of three putative herring populations which were mixing together over the spawning season from February to June inside and outside Lake Landvik (Fig 2.8). The Norwegian spring-spawners showed a clear divergence in otolith shape along the first canonical axis, whilst the Coastal Skagerrak and Lake Landvik herring were more similar in shape.

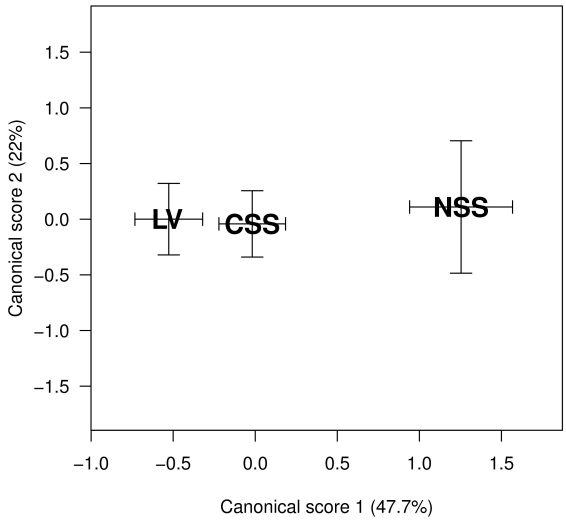

Figure 2.8. Shape differences among herring populations in Landvik, Norway. Canonical scores 1 and 2 representing otolith shape variation among three herring populations in the Landvik region in S-Norway. LV: Lake Landvik herring, CSS: Coastal Skagerrak herring, NSS: Norwegian spring-spawning herring.

2.7 Otolith shape among herring species

Atlantic herring (*C. harengus*) and Pacific herring (*C. pallasii*) have previously been reported to be genetically distinct (Grant, 1986). These species occupy mainly their respective oceans, the Atlantic and Pacific Oceans, whereas subspecies of Pacific herring have been detected in the White Sea (*C. pallasii marisalbi*) and SE-Barents Sea *C. pallasii suworowi*) in Russia and west to Balsfjord (*C. pallasii*) in N-Norway (Laakkonen et al., 2013) which are thought to be early post-glacial colonists from the northwest Pacific (Laakkonen et al., 2013). In previous studies, the Balsfjord population was shown to be closely related to the White Sea population, based on variation in mitochondrial DNA (Jørstad et al., 1994; Laakkonen et al., 2013), and also to the NW-Pacific (Laakkonen et

al., 2013). A mixture of herring populations in Balsfjord have been observed based on genetic studies using allozymes and mitochondrial markers (Jørstad and Nævdal, 1981), and there is evidence for introgressive hybridization from the Atlantic herring into the Pacific herring in Balsfjord (Laakkonen et al., 2015). To study phenotypic variation in terms of otolith shape between the two herring species and among subspecies of Pacific herring, samples were collected from three countries: Norway, Russia, and USA, and five sampling regions: from Balsfjord and Møre in Norway, SE-Barents Sea in Russia, and two populations from USA (Alaska): Kamishak within the Gulf of Alaska and the Bering Sea northwest of the Pacific. The results showed similarity in otolith shape among herring from the Bering Sea in Alaska, SE-Barents Sea in Russia, and Balsfjord in N-Norway (Fig 2.9). Herring from the Gulf of Alaska, sampled at Kamishak, seemed quite different from the other populations at the excisura major area at 200-220° on the outline (Fig 2.9). It seems that the divergence of populations in terms of otolith shape is linked to specific areas on the otolith outline. In the North Atlantic, variation was mainly attributed to three areas, the excisura major, postrostrum and pararostrum (**Paper III**), while the local populations along the Norwegian coast showed differences at the excisura major area, rostrum and excisura minor (**Paper V**).

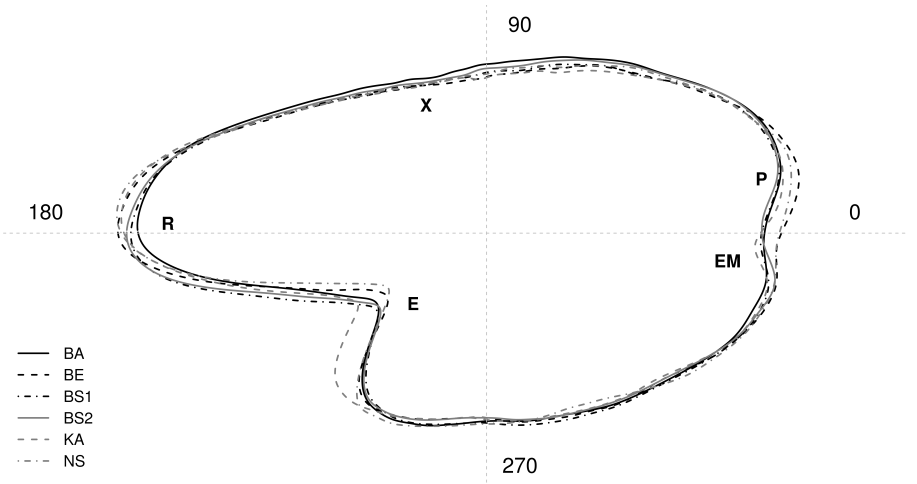

Figure 2.9. Average shape of otoliths for the 5 sampling areas in the study. From Norway: Balsfjord (BA) and Møre (NS), from Russia: Barents Sea (BS1, BS2) and USA: Alaska (Bering Sea (BE) and Kamishak (KA). The most variable areas on the otolith outline, excisura major (E), rostrum (R), excisura minor (EM) and postrostrum (P) are marked. The numbers 0, 90, 180 and 270 represent angles (in degrees) on the outline which correspond to Fig. 3. The area on the outline marked X (at angle 120°) corresponds to the area showing the highest proportion of variance among populations.

Examining along the first canonical axis, Bering Sea and Barents Sea herring show similarity in shape, with the Balsfjord herring positioned close by along the axis. The Norwegian spring-spawners and the population sampled at Kamishak within the Gulf of Alaska showed distinct patterns of divergence from the other populations in their mean canonical scores representing shape (Fig 2.10).

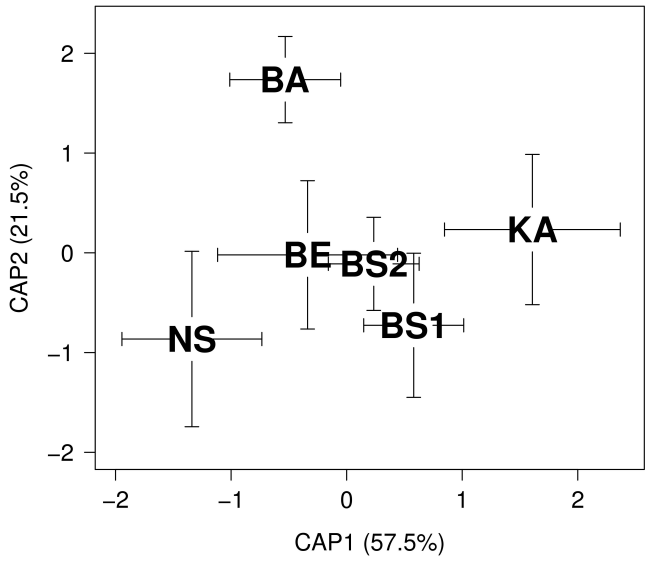

Figure 2.10. Otolith shape variation among Atlantic and Pacific herring. Canonical scores 1 (x-axis) and 2 (y-axis) representing shape differences among Atlantic and Pacific herring from Alaska (BE: Bering Sea, KA: Kamishak in the Gulf of Alaska), Russia (Barents Sea, BS1: sampled in 1996, BS2: sampled 2005-2006) and Norway (BA: Balsfjord, NS: Norwegian spring-spawners from Møre, W-Norway).

3 Discussion

In **Papers I** and **II**, two methods were evaluated in order to find a marker which could be used to identify herring populations of different origin. Six out of the fourteen microsatellite markers developed (**Paper I**) were used in a study by Pampoulie et al (2015) and showed no genetic structuring among the Norwegian spring-spawners in comparison with the Icelandic summer-spawners nor other oceanic populations from the Northeast Atlantic. However, differences were detected among local populations in Norway, in a comparison between Lake Landvik herring and three other populations occupying Lindåspollene, Lusterfjord and Trondheimsfjord. The lack of differentiation among the oceanic populations in the Northeast Atlantic points to high relatedness of the different spawning populations, possibly even gene flow, but might also be due to the population's large effective size, low power of the microsatellite analyses or the sampling design (Ryman and Palm, 2006). A more extensive genetic approach might be needed to detect the low differentiation, for example using variation in Single Nucleotide Polymorphism (SNP) throughout the genome. Next, a phenotypic approach was tested as a marker for herring population discrimination. The R-package shapeR (**Paper II**) was designed to ease the process of analysing a large number of samples within a short amount of time, since no such software existed for otoliths. The shapeR package has proven very useful in otolith shape analysis and gives promising results for future studies in population discrimination and is potentially a useful tool for studying variation in any two dimensional objects.

In **Paper III**, otolith shape was analysed for its usefulness in population discrimination of herring occupying the North Atlantic. Results showed significant differences in otolith shape among populations from Canada, the Faroe Islands, Iceland, Ireland, Norway, and Scotland. Also, by comparing two populations which are known to mix at feeding grounds east of Iceland, the Icelandic summer-spawners and Norwegian spring-spawners, we could classify each population back to their origin of spawning with 94% accuracy based on shape discreteness. This study was the first to confirm that otolith shape can be used as a population marker for Atlantic herring and is able to discriminate between herring populations of different origin that mix at feeding grounds with high accuracy. A comparison among three herring populations occupying the Landvik region in S-Norway (**Paper IV**), the Lake Landvik herring, coastal Skagerrak herring, and the Norwegian spring-spawners, showed variation in otolith shape. This study showed that otolith shape is able to detect small scale structuring among herring on a small geographical scale and among populations which might interbreed, as they were found mixed over the spawning season. This further confirms the usefulness of otolith shape as a population marker. Otolith shape analysis was next conducted among 12 local herring populations occupying semi-enclosed coastal regions, fjords, and a lake

along the Norwegian coast from Kragerø (58°N) to Balsfjord (69°N), and were also compared with the two oceanic herring populations in Norway, the Norwegian spring- and autumn-spawners (**Paper V**). This study serves as a link between **Papers III** and **IV**, where we test whether otolith shape can discriminate between herring populations hindered by geographic barriers as well as how shape is affected by isolation by distance and with respect to latitude. We also tested for temporal stability in otolith shape. The results showed otolith shape to differ among the populations and a latitudinal gradient emerged along the coastline where neighbouring populations were more similar to each other than to those sampled at larger distances. These differences are likely to reflect environmental differences but indicate low dispersal among the populations. There was temporal stability in otolith shape, proving further the usefulness of otolith shape as a population marker. At the species and subspecies level, otolith shape was analysed among Atlantic and Pacific herring from the Atlantic (Norway), the Pacific (Bering Sea and the Gulf of Alaska), and the SE-Barents Sea (Russia) (**Paper VI**). Differences in otolith shape were observed among Atlantic and Pacific herring, but similarity was found among Pacific herring subspecies occupying the NW-Pacific in the Bering Sea, SE-Barents Sea in Russia, and Balsfjord in N-Norway. These results are in accordance with a former genetic study (Laakkonen et al 2013). Given the genetic and phenotypic evidence, a revised classification of Pacific herring subspecies might be warranted.

The studies presented in this thesis show that otolith shape can be used as an accurate population marker for Atlantic herring. Using otolith shape analysis to detect discreteness shows promising results for the management of herring stocks, for example in Icelandic waters where the Icelandic summer-spawners and Norwegian spring-spawners are known to mix during feeding and are currently separated in the catch based on maturity stage since they spawn at different times of the year. Determining stocks solely based on maturity stage lacks precision because the method requires visual examination and relies upon subjective judgment by the sampler, and is also subject to error due to potential overlap in the timing of gonad development between the two stocks. Otolith shape has therefore the potential to aid in the separation of stocks in mixed fisheries. It is important to map the discreteness of herring populations as ocean warming could lead to herring moving to more suitable areas. Studying population structure for fisheries management is important in order to avoid overexploitation of local non-targeted populations. Disregard of population structure might result in loss of genetic variation within species which is vital for adaptation in an ocean that is affected by natural variability and/or climate change. Research and knowledge on population identification is therefore crucial for protecting and maintaining biodiversity within species.

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Paper I

Fourteen new microsatellite markers for Atlantic herring *Clupea harengus*

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Journal of Fish Biology, 81, 1422–1426

Authors contribution: Conceived and designed the experiments: LAL, GÓ, SS, CP, AKD. Analysed the data: LAL, CP, SP. Contributed reagents/materials/analysis tools: LAL, CP, GJÓ, AKD. Wrote the paper: LAL, SP. Reviewed the manuscript: LAL, GÓ, SS, SP, CP, SHB, KÓ, GJÓ, AKD

Journal of FISH BIOLOGY
Published for the Fisheries Society of the British Isles

Journal of Fish Biology (2012) **81**, 1422–1426
doi:10.1111/j.1095-8649.2012.03391.x, available online at wileyonlinelibrary.com

Fourteen new microsatellite markers for Atlantic herring *Clupea harengus*

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(Received 10 January 2012, Accepted 29 May 2012)

Fourteen new microsatellite loci were developed and tested on Atlantic herring *Clupea harengus* with 39 individuals from Iceland and 49 individuals from Norway. The microsatellites, which contain di, tri and tetranucleotide repeats, are polymorphic (7–30 alleles), with observed heterozygosity ranging between 0·69 and 1·00 and expected heterozygosity between 0·55 and 0·97.

Key words: DNA; genetic marker; population genetics.

Studies using microsatellite DNA and allozyme markers have found minor differentiation among spatially discrete populations of Atlantic herring *Clupea harengus* L. 1758. Previous publications focus on the genetic differences among *C. harengus* in the North Sea (Mariani *et al*., 2005), the Baltic Sea (Jørgensen *et al*., 2005), and on comparison of *C. harengus* in the North and Baltic Seas (Bekkevold *et al*., 2005; Gaggiotti *et al*., 2009; André *et al*., 2011) and adjacent waters (Ruzzante *et al*., 2006). These studies have found significant differentiation between populations in the North and the Baltic Seas (Bekkevold *et al*., 2005) as well as correlated genetic and life-history patterns across these regions (Ruzzante *et al*., 2006). In addition, isolation by distance has been observed among populations in the North Sea, determined predominantly by the divergence of the English Channel and Norwegian spring-spawning *C. harengus* (Mariani *et al*., 2005). Many of the microsatellite loci used in these studies, however, experienced technical problems. Deviations from the Hardy–Weinberg equilibrium (HWE) were observed at three out of nine microsatellite loci in Bekkevold *et al*. (2005) and two out of eight microsatellite loci in Gaggiotti *et al*. (2009) showed footprints of selection, one shaped by directional selection associated with salinity, and another by balancing selection or alternatively atypical mutation rate. With an increased number of microsatellite loci, the power to detect putative structure of *C. harengus* can be increased, and in addition it may

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be easier to distinguish between population differentiation driven by genome-wide effects and locus-specific effects caused by selective pressure.

DNA used for the generation of the genomic DNA library was extracted from an Icelandic summer-spawning *C. harengus* (ISSH) caught in Icelandic waters in December 2008. The DNA was isolated from muscle tissue using the NucleoSpin Tissue kit as described by the manufacturer (Macherey-Nagel; www.mn-net.com). The DNA concentration was measured as 92 ng μl^{-1} using the Qubit fluorometer and Quant-iT ds DNA assay kit (Invitrogen; www.invitrogen.com). A single-stranded DNA library was constructed from the isolated DNA and subjected to shotgun sequencing on an FLX Genome Sequencer by using the GS FLX Titanium reagents as described by the manufacturer (Roche; www.454.com). For pyrosequencing, beads containing emulsion-amplified DNA were loaded on one region of a 75 × 75 mm Titanium PicoTiterPlate (www.roche-applied-science.com) equipped with a four-region gasket. The obtained sequence data consisted of 217·117 filter-passed sequence reads that comprised a total of 64 838 985 bases, which cover *c*. 7% of the genome of *C. harengus*, assuming a similar genome size as of *Clupea pallasii* valenciennes 1847, or *c*. 900 million bases (Mb). The obtained sequences, which were on average 300 bp in length, were loaded into Flanker (Matís ltd; www.matis.is) repeat-detecting software. The software uses a suffix array algorithm (Manber & Myers, 1991) to detect simple exact repeats. By setting a specific criterion of minimum sequence length 100 bp, minimum repeat length 40 bp, minimum repeat number 4 bp, left and right flanking length 25 bp, Flanker was able to obtain 269 sequences that fulfilled the criteria out of the 217 117 sequences. Thirty-two primer pairs out of the 269 sequences were designed and tested and 14 of those (Table I) were tested in two samples, one from Iceland and one from Norway. Individuals were collected at spawning grounds in Norway [Landvikvannet near Haneto (58°19′ N; 8°30′ E), Norwegian local spring-spawning *C. harengus* NLSSH, $n = 49$, sampled 12 May, 2010] and Iceland [Faxaflói, south-west Iceland (64°14′; 22°56′ W), ISSH, $n = 39$, sampled 5 July, 2009]. Genomic DNA was isolated from either gill or muscle tissue preserved in 90% ethanol using AGOWA mag Midi DNA Isolation Kit (AGOWA Gmbh; www.bio-equip.cn).

Polymerase chain reactions (PCR) were performed in a 10 µl volume containing 2–3 µl DNA (10–100 ng μl^{-1}), 0·80 µl of deoxynucleotide triphosphate (dNTP; 10 mM), 0·6–1·2 U of Teg polymerase (Matís ltd; Taq comparable; Olafsson *et al*., 2010), 1 µl of 10× buffer (Matís ltd), 0·03–0·25 µl of a 50:50 ratio of labelled forward (100 µM) and reverse (100 µM) primer tagged on the 5′-end with a GTTTCTT PIG-tail (Brownstein *et al*., 1996), adding 1 µl betaine (5 M) when improvement of DNA amplification was needed. PCRs were performed on GeneAmp2700 thermal blocks as follows: initial denaturation step of 4 min at 94° C followed by 30 cycles of 40 s at 94° C, 40 s at 58° C and 1 min at 72° C, and a final elongation step of 7 min at 72° C. Samples were analysed on an ABI PRISM 3730 sequencer using the GeneScan-500 LIZ size standard and genotyping was performed with GeneMapper v4·0 (Applied Biosystems; www.appliedbiosystems.com).

The number of alleles (n_A), the expected (H_E) and observed (H_O) levels of heterozygosity and deviations from HWE calculated by using Genepop v4.1 (Raymond & Rousset, 1995) are given in Table II. The number of alleles per locus and population ranged from 7 to 30, corresponding to markers *msild01* and *msild03*, and observed heterozygosity values from 0·69 to 1·00, for *msild01* and *msild24*,

TABLE I. Primer sequences, repeat motif, size range, amplification conditions (T, annealing temperature) and GenBank accession numbers for 14 microsatellite loci in *Clupea harengus*

Locus	Primer sequence (5′–3′)	Repeat	Size range (bp)	T (° C)	GenBank accession number
msild01	F: CTGAGACTCAGTCAGTCATATC R: TACTGCTGCTCGCATCTG	CA_{21}	93–119	58° C	JQ388198
msild02	F: GCGTATCTTTGCGTAGTTGTG R: ATCTCCCACGGTTCTTTGTC	CA_{22}	105–177	58° C	JQ388199
msild03	F: AGTTGGACATACATGCATTC R: TTTGGTCTGGTCGACATCTG	CA_{22}	107–205	58° C	JQ388200
msild12	F: CCTGAGTTGACTGGGAGTTTAG R: GTCATCTGATGGCCGTGGAG	CTT_{16}	85–121	58° C	JQ388201
msild13	F: TGCAGATCCTGCATGTTC R: TTCGCTTTAGATCAAAGTGTCTG	GAT_{17}	200–233	58° C	JQ388202
msild15	F: CCAGTCATGCCCATCAAATC R: CCAGCAGCATGCAGATTATTC	TTC_{15}	220–283	58° C	JQ388203
msild16	F: GAGAGGGTCAAAGCGTTCTG R: CCATTTCCAATTTCACTCTTAC	$ATGA_{11}$	334–398	58° C	JQ388204
msild17	F: GTTTCTCCTCGGGATTCTGG R: AACTTGCCTACATGTCTATTTGC	$CATA_{19}$	336–396	58° C	JQ388205
msild18	F: AGTTCCATTGCCATGTTAGC R: ATCCATACTCTGCCAGACAC	$GAGT_{16}$	200–256	58° C	JQ388206
msild24	F: GGGTTGTGCTGACCTTTGAC R: GAGTCTGTGAATGCCATGTG	CA_{16}	171–303	58° C	JQ388207
msild27	F: AGAGGCCACAGTGGATCAGAG R: CACTTTGAGCTGCATGAAAGG	GAT_{11}	185–233	58° C	JQ388208
msild29	F: TTTCTGCTCCGGCAAGTG R: CAGTGCTGTGATGCTTATAATG	$ATGA_{13}$	256–319	58° C	JQ388209
msild30	F: GAATATGGCAAGCTGCAACC R: CATTGTAAATGAGGGTCTTATTCC	$ATTG_{8}$	97–137	58° C	JQ388210
msild32	F: GGTCCACCTGGTTCACAATAG R: ACAGGCTTGCTCCAAATCTC	$TAGA_{12}$	172–236	58° C	JQ388211

respectively. Five markers showed deviation from HWE in the two samples with $P < 0{\cdot}05$ (Table II), three markers in the NLSSH samples *msild15* ($P < 0{\cdot}001$), *msild29* ($P < 0{\cdot}001$) and *msild30* ($P < 0{\cdot}001$) showed significant deviation after correcting the P-value with the Bonferroni adjustment (Sokal & Rohlf, 1995). A further analysis using Micro-Checker v2.2.3 (van Oosterhout *et al*., 2004) showed that null alleles may be present at five loci: *msild15* and *msild18* for the ISSH samples, and *msild03*, *msild29* and *msild30* for the NLSSH samples, as suggested by the general excess of homozygotes. Four out of five of these markers showed complex allele peaks and may benefit from primer modification. An inconsistent linkage was found for two marker pairs for the NLSSH samples [*msild01* and *msild29* ($P < 0{\cdot}001$), *msild30* and *msild32* ($P < 0{\cdot}05$)], and three for the ISSH samples [*msild13* and *msild29* ($P < 0{\cdot}01$), *msild16* and *msild27* ($P < 0{\cdot}001$), *msild16* and *msild30* ($P < 0{\cdot}05$)] as tested with Genepop v4·1 (Raymond & Rousset, 1995). None of the pairs, however, was significantly linked after a Bonferroni correction and as they were not consistent across the two populations, they are most likely independent. These 14 new microsatellite loci will be beneficial in studies on population genetics, ecology and conservation of *C. harengus*, and might also prove to be useful for studies on other closely related species such as the *C. pallasii*. A larger number of markers will increase the power

TABLE II. Genetic diversity per locus among sampled populations of Icelandic summer-spawning ($n = 39$) and Norwegian local spring-spawning ($n = 49$) *Clupea harengus*

Population	DI	*msild01*	*msild02*	*msild03*	*msild12*	*msild13*	*msild15*	*msild16*	*msild17*	*msild18*	*msild24*	*msild27*	*msild29*	*msild30*	*msild32*
Iceland	H_O	0·69	0·86	0·93	0·81	0·90	0·79	0·78	0·90	0·77	1·00	0·81	1·00	0·79	0·94
	H_E	0·55	0·92	0·97	0·91	0·90	0·92	0·88	0·89	0·90	0·96	0·84	0·92	0·87	0·92
	n_A	7	18	30	13	12	20	13	11	13	26	12	14	9	14
	P-value	>0·05	>0·05	>0·05	>0·05	>0·05	<0·01	<0·05	>0·05	>0·05	>0·05	>0·05	>0·05	>0·05	>0·05
Norway	H_O	0·74	0·82	0·86	1·00	0·82	0·85	0·82	0·85	0·88	0·98	0·77	0·74	0·63	0·83
	H_E	0·74	0·86	0·95	0·87	0·86	0·92	0·88	0·83	0·90	0·93	0·80	0·89	0·84	0·92
	n_A	8	20	30	7	12	19	11	11	11	25	8	13	11	14
	P-value	>0·05	>0·05	>0·05	>0·05	<0·05	**<0·001**	>0·05	<0·05	>0·05	>0·05	>0·05	**<0·001**	**<0·001**	>0·05

DI, diversity indices; H_o, observed heterozygosity; H_E, expected heterozygosity; n_A, number of alleles per population; P value, probability of excess of homozygotes.
The P-values in bold are significant after Bonferroni adjustment ($P_{adj} < 0.05$).

to detect putative population structure, the ability to distinguish between neutral historical events and effects due to selection and thus the characterization of adaptive variation that can be associated to environmental factors, or pathogens and parasites.

This project was funded by the Working Group for Fisheries Cooperation (AG-Fisk) of the Nordic Council of Ministers, The Fisheries Research Fund of the Faroe Islands, The Fisheries Project Fund of the Icelandic Ministry of Fisheries and Agriculture and The Icelandic Research Fund for Graduate Students. Special thanks goes to A. Slotte at the Institute of Marine Research in Norway for providing the NLSSH samples, A. Klonowski and S. Magnúsdóttir (Matís ltd) for conducting the FLX run on *C. harengus*, S. K. Stefánsson (Matís ltd) for developing the software Flanker that was used during primer design and Sarah Helyar (Matís ltd) for useful comments that improved the manuscript.

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Paper II

ShapeR: An R Package to Study Otolith Shape Variation among Fish Population

Lísa A. Libungan and Snæbjörn Pálsson (2015)
PLoS ONE, 10 (3): e0121102, 1-12

Authors contribution: Conceived and designed the R-package: LAL, SP. Analysed the data: LAL. Wrote the paper: LAL, SP

PLOS ONE

RESEARCH ARTICLE

ShapeR: An R Package to Study Otolith Shape Variation among Fish Populations

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Abstract

ShapeR is an open source software package that runs on the R platform and is specifically designed to study otolith shape variation among fish populations. The package extends previously described software used for otolith shape analysis by allowing the user to automatically extract closed contour outlines from a large number of images, perform smoothing to eliminate pixel noise, choose from conducting either a Fourier or Wavelet transform to the outlines and visualize the mean shape. The output of the package are independent Fourier or Wavelet coefficients which can be directly imported into a wide range of statistical packages in R. The package might prove useful in studies of any two dimensional objects.

Citation: Libungan LA, Pálsson S (2015) ShapeR: An R Package to Study Otolith Shape Variation among Fish Populations. PLoS ONE 10(3): e0121102. doi:10.1371/journal.pone.0121102

Academic Editor: Heather M. Patterson, Department of Agriculture, AUSTRALIA

Received: November 1, 2014

Accepted: February 9, 2015

Published: March 24, 2015

Data Availability Statement: Data from this study are fully available on GitHub (https://github.com/lisalibungan/shapeR) along with the R package and all source code.

Funding: This work was funded by the Icelandic Research Fund for Graduate Students (grant nr. 100710001, www.rannis.is, LAL), the Assistantship Fund of the University of Iceland (www.hi.is, LAL) and by The Entrepreneur Fund of Íslandsbanki (www.islandsbanki.is, LAL, SP). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Introduction

Morphometric analysis of otoliths is a well-established method to delineate fish stocks, characterize population movements and to detect the natal origin of fish. For otolith shape analysis, two main morphometric methods are used: landmark analysis [1] and outline analysis [2–5]. With outline analysis it is possible to quantify boundary shapes so that patterns of shape variation within and among groups can be evaluated based on a large number of independent variables [6]. The advantage of using such methods in population identification is that they are cost effective and only require otolith images from which outlines can be extracted and analysed with statistical software. Here, we present an R package to extract, visualize and generate otolith shape data with a small number of easy-to-use functions. There are built-in functions which allow users to perform automatic processes such as extract the otolith outlines from images, visualize the mean shape, smooth the outline by eliminating pixel noise [7] and transform the outlines into independent coefficients using either Normalized Elliptic Fourier or Discrete Wavelet, which can be entered into a wide range of statistical packages in R. The Wavelet transform provides a powerful alternative to the more commonly applied Fourier transform in shape analysis. While the Fourier transform provides functions in the form of sines and cosines which are non-local and can therefore result in poor approximations of sharp edges, the Wavelet transform uses approximating functions that are contained in finite domains making them well-suited for approximating sharp edges [8].

Competing Interests: The authors have the following interests: they received an award from a commercial source (Íslandsbanki) when developing this R package. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

Methods

The shapeR package is written in the programming language R [9]. The functions are listed in the S1 Table. The package uses commands from the R packages gplots [10], ipred [11], jpeg [12], pixmap [13] and wavethresh [14]. All R source code is publicly available via GitHub (see 'Availability' section).

Images

The first step of the shape analysis is to capture the otolith images (Fig. 1) using a dissection microscope with a digital camera attached. The microscope should be tuned so an otolith on a black background is as clear as possible. When the settings are ready, an image of a calibration measurement stick, in good focus, is taken as a size reference. Images should then be taken and stored in full color, ensuring good focus and high resolution in jpeg format (*.jpg). The otoliths need to be orientated with their rostrum to the left as seen in Fig. 1. For the ease of handling the images, make a folder called 'ShapeAnalysis' and store the images from each sampling unit in a unique area-folder within a folder called 'Original', and make a copy of the whole folder 'Original' and name it 'Fixed'. The folders 'Original' and 'Fixed' need to exist because images in both folders are used when the shapeR package is used to perform quality checks on the otolith outlines. The area-folders in the folders 'Original' and 'Fixed' should be named with two letters of the sampling unit, or country, and the station number of the sample. For example, 'IC' would represent a sample from Iceland. An otolith image name in folder 'IC' should be in the format '403_1', '403_2', '403_3', etc where the first three letters represent the station number and the second number, after the underscore, represents the fish number.

Data files

A data file for each fish specimen (in rows), with information in columns such as population, station nr, sampling date, location, length, maturity stage, etc. (see data file example) is stored in the 'ShapeAnalysis' folder as a text file in a csv format (*.csv). Two columns in the data file are mandatory, 'folder' (consists of folder names such as 'IC') and 'picname' (consists of file names e.g. '403_1'), which are used to link biological information for each fish to the otolith outline. The column 'length_cm' needs to exist so it is possible to remove the allometric growth effect on otolith shape [15,16]. If other measurements are used (fish weight, otolith weight etc.) a column for each parameter needs to be given in the data file. Summary statistics of each otolith (otolith area, length, perimeter and width) can be obtained if the calibration measurements

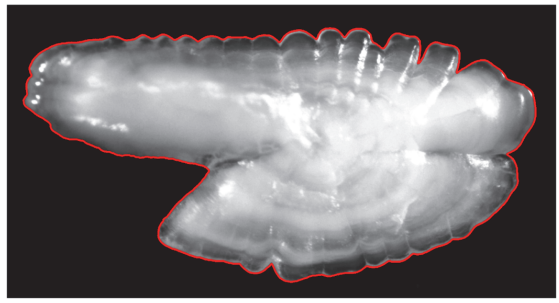

Fig 1. Example of an otolith image. The red outline marks the shape of the otolith which is extracted by shapeR and forms the basis for the analysis of variation within and among populations.

doi:10.1371/journal.pone.0121102.g001

in pixels have been registered in the csv data file in a column labelled 'cal' (see example data file). To get the calibration measurements, use an image manipulation program and measure 1mm on the calibration measurement stick and register how many pixels 1mm is into the column 'cal'. When new images are placed into the area-folders in 'Original' and 'Fixed', the detect.outline function will detect them automatically. The image files are read into R using the functions readJPEG from the jpeg package [12] and pixmapGrey from the pixmap package [13].

Sample Dataset

We present an example of otolith shape analysis on three discrete herring populations in the NE-Atlantic, from Iceland (n = 65), Norway (n = 65) and Scotland (n = 30). Example data set and images can be retrieved from GitHub (see 'Availability' section).

In R, load shapeR and retrieve the example data file with the commands library(shapeR) and data(FISH). To start the analysis, the project path needs to be set to the folder 'ShapeAnalysis' which contains the folders 'Original', 'Fixed' and the data file 'FISH.csv'. If the folder 'ShapeAnalysis' is on your Desktop, read in the data in the following way: > shape = shapeR("C:/Desktop/ShapeAnalysis", "FISH.csv")

Outline extraction

To obtain the outline of each otolith we run the outline detection command detect.outline using the conte and regularradius functions [17]. The outlines are detected by first transforming the images into gray-scale. The images are then binarized using a threshold pixel value (intensity threshold) which can be defined by the user. The outlines are then collected automatically from all images in the folder 'Fixed'. Modification of the outlines are stored in different slots within the shape data object. Different comments will assess data in the different slots as referred to below:

> shape = detect.outline(shape, threshold = 0.2, write.outline.w.org = TRUE)

The threshold argument is used to distinguish the white otolith from the black background. The write.outline.w.org argument determines whether the detected outline should be written on top of the original image (TRUE) or not (FALSE) in the folder 'Original_with_outline' which shapeR makes automatically and places into the folder 'ShapeAnalysis'. It is good practice to run first 10 images and measure the time it takes to extract the outlines so the total run time can be estimated as it varies between computers and image resolution. Extracting each outline from the otolith images with the argument write.outline.w.org = TRUE takes ~5 seconds, while having the argument FALSE takes ~0.6 seconds using a computer with operating system Windows 8.1 and an Intel Core i5–3337U CPU 1.8 GHz Processor. It is recommended to run the images with the write.outline.w.org = TRUE the first time the images are run for quality checking, to see if the outline fits the original image from the microscope. If an error occurs, or the outline is of low quality, the outline can be removed from the shapeR instance:

> shape = remove.outline(shape,"IC","403_54")

Try to run again detect.outline with a different threshold e.g. with a higher threshold of 0.3. Try also mouse.click = TRUE which is added to the detect.outline command arguments and click on the center of the otolith. If that does not work, try to fix the image with an image manipulation program and get a better contrast between the otolith and the background in the

'Fixed' folder and run the detect.outline function again. It will only process again the otoliths which were removed and add them to the list of the other outlines.

It is possible to view one particular outline with:

> show.original.with.outline(shape,"IC","403_54")

Contour smoothing

When the outlines have been captured from the images, the digitized outlines can have high frequency pixel noise around the outlines that can corrupt the Fourier or Wavelet analysis [7]. To eliminate pixel noise, it is possible to calculate a weighted moving average over three successive coordinate points using the function smoothout [17] to smooth multiple outlines. The number of iterations (n = 100 in the example) provided by the user is the maximum number of iterations of smoothing. The run time to smooth one outline takes ~0.03 seconds (see computer specifications in the "Outline extraction" section). To perform smoothing on the outlines:

> shape = smoothout(shape, n = 100)

otherwise omit this step.

Shape coefficients

When all the outlines have been captured with high quality, the shape coefficients can be extracted using the function generateShapeCoefficients. Before the Wavelet transformation, the rotation of all otoliths are positioned horizontally along the longest axis of the otoliths and the area is set equal in all (area = 1). Polar coordinates are then collected by drawing a polar axis (radial) horizontally from the otolith centroid (i.e. the mean of the x and y coordinates of the outline) to the right which corresponds to the 0° angle of the otolith outline (Fig. 2). From the 0° angle, radials are collected counter clockwise towards the 360° angle with equidistant angles between successive radials. The Wavelet coefficients are obtained using the functions wd and wr in the wavethresh package [14]. For Fourier, the Normalized Elliptic Fourier technique is performed using the iefourier and efourier functions [17] which both normalizes the otoliths with regards to size and rotation and collects the coefficients. Ten Wavelet levels give a total of 64 Wavelet coefficients using the Daubechies least-asymmetric Wavelet [18] and 12 harmonics give 45 Normalized Elliptic Fourier coefficients (48−3 = 45, the first three coefficients are omitted due to standardisation in relation to size, rotation and starting point). The coefficients are collected with:

> shape = generateShapeCoefficients(shape)

To connect the data file containing information on origin and size of the fish to the outlines, run:

> shape = enrich.master.list(shape)

It is recommended to save the shape object regularly:

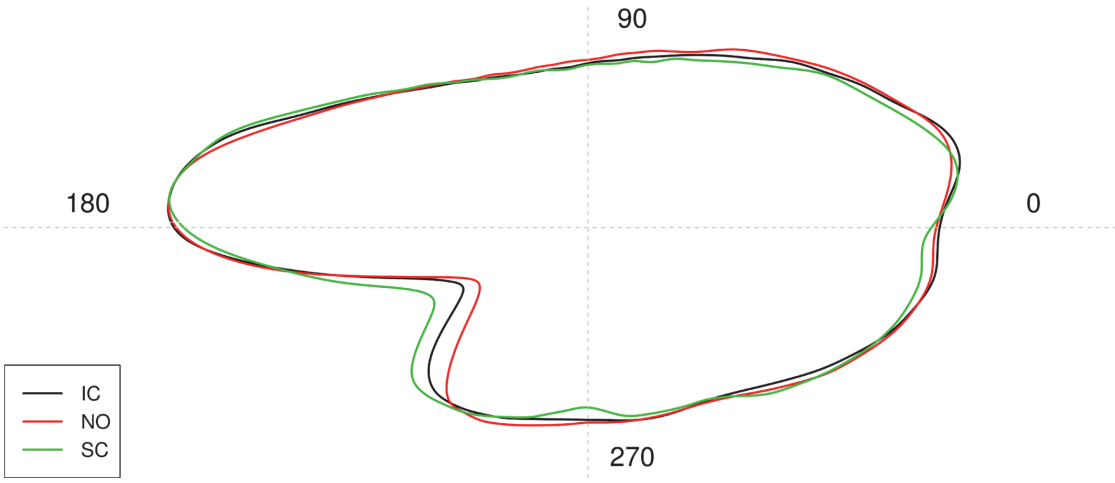

Fig 2. Mean otolith shape based on Wavelet reconstruction for three discrete fish populations from Iceland (IC, n = 65), Norway (NO, n = 65) and Scotland (SC, n = 30). Numbers represent angles in degrees (°) based on polar coordinates (see Fig. 4). The centroid of the otolith (center of the cross) is the center point of the polar coordinates.

doi:10.1371/journal.pone.0121102.g002

```
> save(shape,file = "test.RData")
```

Summary statistics

The maximum or Feret length and width of the otolith, its perimeter and area can all be collected with:

```
> getMeasurements(shape)
```

For each fish population ("pop"), the mean for the variables in the summary statistics (area, length, perimeter, width) can be calculated:

```
> tapply(getMeasurements(shape)$otolith.area, getMasterlist(shape)$pop, mean)
```

If the calibration measurements vary between figures, the area can be adjusted by the appropriate scale for each otolith.

Mean otolith shape

The mean shape using the Wavelet coefficients is plotted in Fig. 2. To base the analyses on the Fourier coefficients instead of Wavelet, replace 'Wavelet' with 'Fourier' in all commands.

```
> plotWaveletShape(shape, "pop", show.angle = TRUE, lwd = 2,lty = 1)
```

Adjusting coefficients for fish length

To adjust the otolith shape with respect to allometric relationships with the fish lengths [15,16], stdCoefs evaluates each Wavelet and Fourier coefficient. Those coefficients which show interaction ($P<0.05$), between population and length, are omitted automatically. In order to account for increased alpha error due to multiple testing of the different coefficients it is possible to conduct the Bonferroni adjustment [19].

```
> shape = stdCoefs(shape, classes = "pop", "length_cm", bonferroni = FALSE)
```

Using the Wavelet coefficients, three coefficients showed an interaction with fish length and were thus omitted, while applying the Bonferroni adjustment they were all included. The inclusion of these three coefficients did not affect the result of the overall analyses presented below.

Reconstruction

The quality of the Wavelet and Fourier reconstruction can be estimated by comparing how it deviates from the otolith outline.

```
> est.list = estimate.outline.reconstruction(shape)
```

```
> outline.reconstruction.plot(est.list, max.num.harmonics = 15)
```

As seen in Fig. 3, the quality increases as expected with the number of Wavelet/Fourier coefficients used.

To inspect how the variation in the Wavelet coefficients is dependent on the position along the outline, the mean and standard deviation of the coefficients can be plotted against the angle (Fig. 4) using plotCI from the gplots package [10]. The proportion of variation among groups,

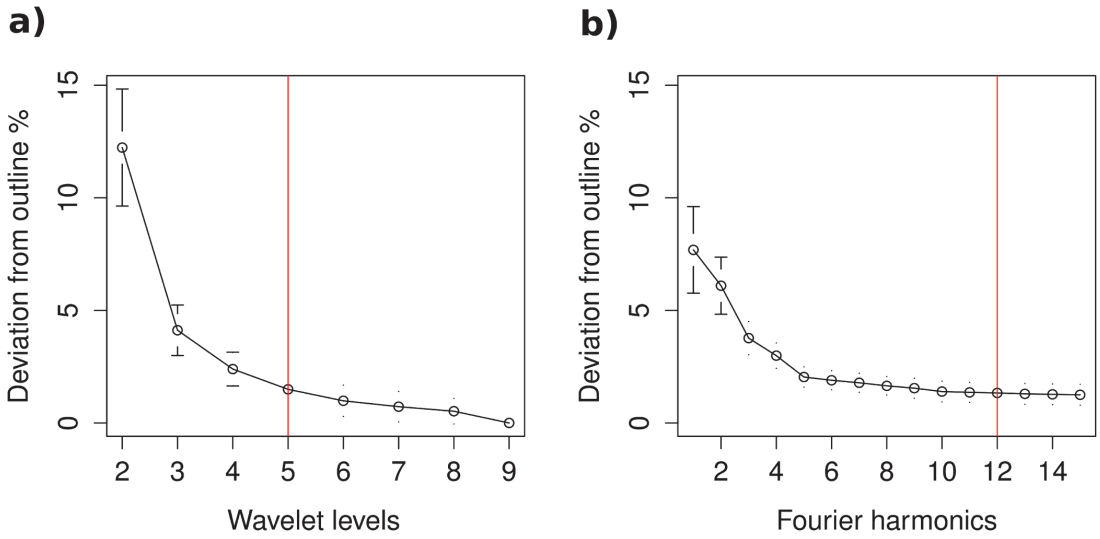

Fig 3. Quality of the a) Wavelet and b) Fourier outline reconstruction. The red vertical lines show the level of Wavelet and number of Fourier harmonics needed for a 98.5% accuracy of the reconstruction.

doi:10.1371/journal.pone.0121102.g003

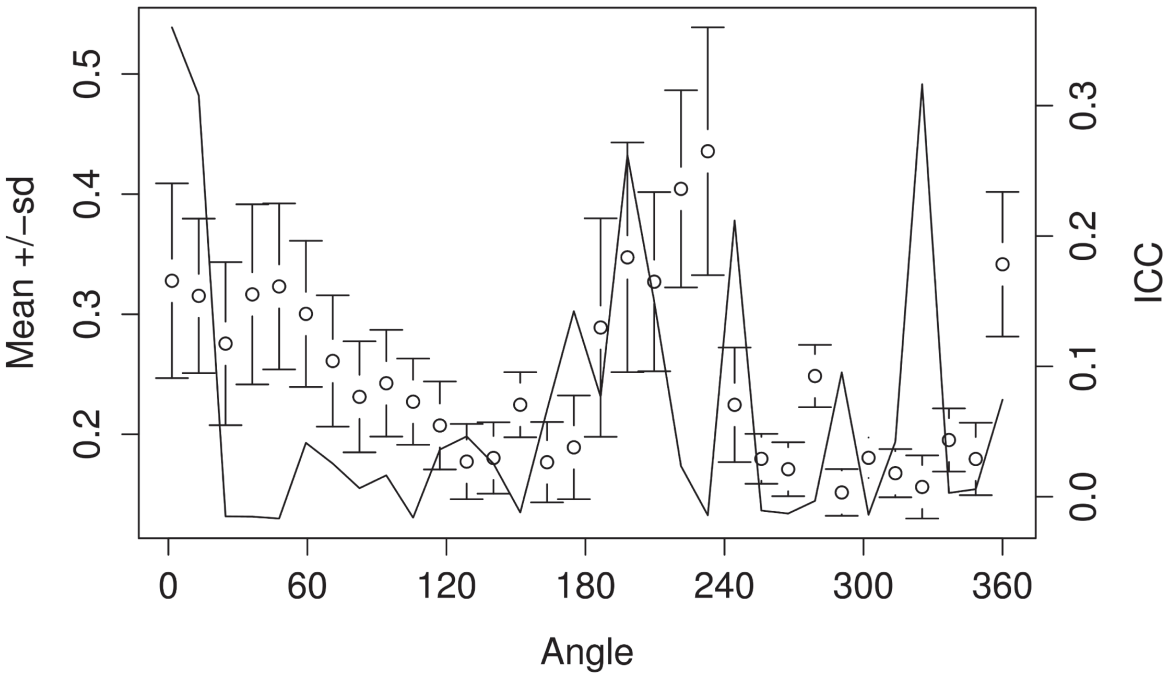

Fig 4. Mean and standard deviation (sd) of the Wavelet coefficients for all combined otoliths and the proportion of variance among groups or the intraclass correlation (ICC, black solid line). The horizontal axis shows angle in degrees (°) based on polar coordinates (see also Fig. 2) where the centroid of the otolith is the center point of the polar coordinates.

doi:10.1371/journal.pone.0121102.g004

the intraclass correlation (ICC), gives further information about the partition of the variation along the outline:

> plotWavelet(shape, level = 5, class.name = "pop", useStdcoef = TRUE)

Based on the patterns in Fig. 4, it is clear that most of the variation among groups can be traced to two areas of the otolith, angles 0–20° and 210–230° (see also Fig. 2) which correspond roughly to the postrostrum and the excisura major [20], respectively.

Results

Using shapeR output in other R packages

Output of the shapeR package can be analysed further using statistical methods implemented in R or other software. Here examples are presented on analyses of smoothed herring otoliths but to ensure a rigorous analysis the user should consider further the requirements of the statistical tests applied such as the number of predictor variables (relative to sample size), their multicollinearity and the independence of sampling units.

To analyse the variation in shape among the populations we apply Canonical Analysis of Principal Coordinates (CAP) [21] using the vegan package [22] on the length standardized Wavelet/Fourier coefficients with smoothed and unsmoothed outlines. The Wavelet coefficients can be analysed in the following way:

> library(vegan)

```
> cap.res = capscale(getStdWavelet(shape) ~ getMasterlist(shape)$pop)
```

Note the number of specimens needs to be larger than the number of coefficients.

The partition of variation among groups in the distance based on ANOVA can be tested using an ANOVA like permutation test (anova.cca), also in vegan [22] (see results in Table 1):

```
> anova(cap.res, by = "terms", step = 1000)
```

Cluster analysis. For visualizing the clustering of the CAP results using the Wavelet coefficients in two dimensions (Fig. 5):

```
> eig = eigenvals(cap.res,constrained = T)

eig.ratio = eig/sum(eig)

cluster.plot(scores(cap.res)$sites[,1:2],getMasterlist(shape)$pop,

xlim = range(scores(cap.res)$sites[,1]),

ylim = range(scores(cap.res)$sites[,2]),

xlab = paste("CAP1 (",round(eig.ratio[1]*100,1),"%)",sep = ""),

ylab = paste("CAP2 (",round(eig.ratio[2]*100,1),"%)",sep = ""), plotCI = TRUE,conf.
level = 0.95,las = 1)
```

The Canonical Analysis of Principal coordinates gives an overview of the differentiation in otolith shape among the three populations which were found significant by the ANOVA (Table 1, Fig. 5, $P = 0.001$). The Scotland sample differs from Norway and Iceland along the first discriminating axis (CAP1) and the Iceland sample shows mainly deviation from the Norwegian sample along the second axis (CAP2). Similar results were observed with separate analyses based on the Wavelet and the Fourier coefficients. Using the Wavelet coefficients, CAP1

Table 1. Comparing otolith shape among three herring populations using an ANOVA like permutation test for smoothed and unsmoothed outlines.

Method	df	Var_{unsm}	Var_{sm}	F_{unsm}	F_{sm}	*P*
Fourier						
Model	2	0.17	0.18	14.83	18.30	0.001
Residual	157	0.88	0.76			
Wavelet						
Model	2	0.25	0.24	19.33	19.34	0.001
Residual	157	1.01	0.99			

Output from the R package shapeR, Fourier and Wavelet coefficients, were entered into the vegan package [22]. Differences among samples were tested by 1000 permutations. Df: degrees of freedom, Var: Variance among populations, *F*: pseudo *F*-value, *P*: proportion of permutations which gave as large or larger *F*-value than the observed one, for each test based on the smoothed and unsmoothed data.

doi:10.1371/journal.pone.0121102.t001

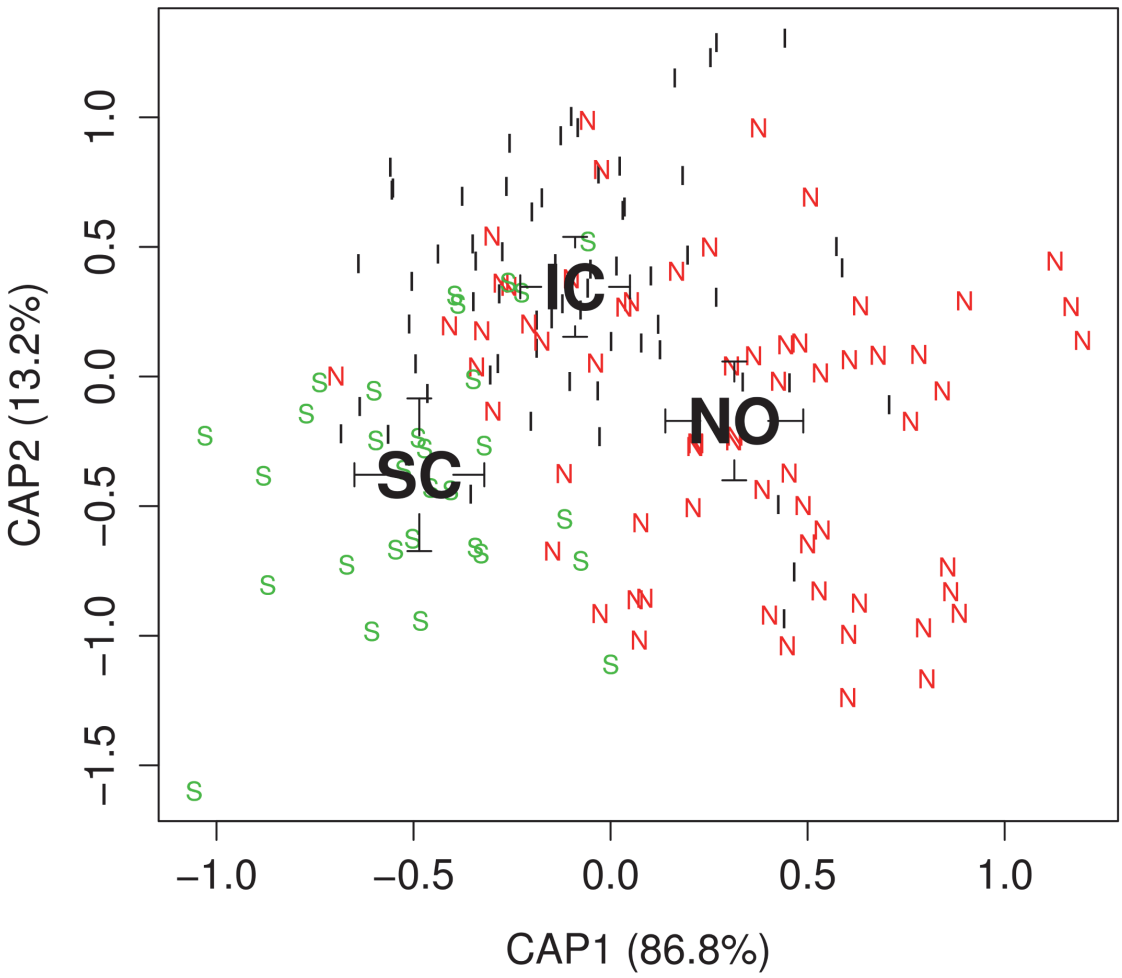

Fig 5. Otolith shape of samples from three herring populations in the NE-Atlantic using Canonical analysis of Principal Coordinates with the Wavelet coefficients. Canonical scores on the first two discriminating axes CAP1 and CAP2 are shown. Black letters represent the mean canonical value for each population, Iceland (IC), Norway (NO) and Scotland (SC) and smaller letters represent individual fish showing the first letter of each population. Interval surrounding the mean canonical values present one standard error (mean +/- 1SE).

doi:10.1371/journal.pone.0121102.g005

explained 86.8% of the variation among populations and CAP2 13.2%. The corresponding values for Fourier were CAP1 89.4% and CAP2 10.6%.

Classification of individuals. To demonstrate classification of individuals to their sampling origin, based on the population variation at the two locations (Iceland and Norway), we apply Linear Discriminant Analysis on the standardized Wavelet coefficients. We start by setting a filter to select which samples (i.e. IC and NO) should be classified:> shape = setFilter (shape, getMasterlist(shape, useFilter = FALSE)$pop %in% c("IC","NO"))

> pop = factor(getMasterlist(shape)$pop)

Estimation of the classifiers success rate based on the Linear Discriminant Analysis can be done with bootstrap or cross-validation using the errorest function in the ipred package [11]. Here we show an example of how to run a cross-validation estimation using the cv estimator:

```
>library(ipred)

>mypredict.lda <- function(object, newdata)

>predict(object, newdata = newdata)$class

> stdw = getStdWavelet(shape)

> pop = factor(getMasterlist(shape)$pop)

> dd = data.frame(stdw = stdw,pop = pop)

>errorest(pop ~., data = dd, model = lda, estimator = "cv", predict = mypredict.lda,est.
para = control.errorest(nboot = 1000))
```

The overall score rate of the classifier based on 65 Icelandic herring and 65 Norwegian herring was 79.2% using cross-validation estimation, but was slightly less using unbiased bootstrap (73.4%) and biased bootstrap (68.1%, sd = 0.002).

Discussion

The shapeR package allows users to easily collect and analyse otolith shape data. Its output can be useful in any comparative study both at the population and species level and might be used in studies of variation on any two dimensional objects. The package allows users to analyse a large number of images in an automatic manner, without the need of selecting data points like in landmark or procrustes analyses, which might be prone to error and may suffer from the Pinocchio effect; where variation at a single landmark might be distributed incorrectly relative to other landmarks [23]. The ability to conduct both Fourier and Wavelet analysis in a single package and compare the results from the two methods is useful because of the variability in otolith shape among fish species. For Atlantic herring, the Fourier and Wavelet methods produced similar results in terms of overall comparison of shape, however the Wavelet method was useful for detecting shape differences at specific regions which could be located at a given angle on the otolith outline. Studying the variability of coefficients at a given angle of the outline is not possible with the Fourier method, because it only provides information about overall differences in otolith shape, not localized differences. Therefore, for some fish species, Wavelet might prove to be better at explaining shape differences, while for others, the Fourier method might be more powerful to distinguish populations. A further evaluation of the applicability of the two transformation methods, Fourier and Wavelet, in otolith shape analysis is warranted.

Otolith shape can be analyzed with standard statistical methods. Here we demonstrated the use of two multivariate methods. The classifier based on linear discriminant analyses gave a high overall score of correct classification when considering two population samples. However a higher score was obtained when samples from the two populations were compared including a larger number of geographic samples [24], and thus different estimates of the linear coefficients. Whether the classifier can be improved by other methods, such as the use of machine learning techniques [25], is a subject of further studies.

Future improvements of the shapeR package would include adding a fine resolution option when plotting the otolith outline, so users are able to zoom in and see the contour with all of its points on a pixel level and on this level see the effect of smoothing as well and adding more options to ease the accessibility of variables. Other scientists are also encouraged to validate and improve the software or send us suggestions for further additions.

Availability

The R package shapeR is available with all source code and test data on GitHub (https://github.com/lisalibungan/shapeR) and will be available on the CRAN repository.

Supporting Information

S1 Table. Functions in the R package shapeR.
(DOCX)

Acknowledgments

Guðmundur J. Óskarsson at the Marine Research Institute in Iceland, Aril Slotte at the Institute of Marine Research in Norway and Emma Hatfield at FRS Marine Laboratory in Scotland are thanked for providing otolith samples and data used in this software. The Editor and three anonymous reviewers are thanked for comments which improved the manuscript and the shapeR package.

Author Contributions

Conceived and designed the experiments: LAL SP. Performed the experiments: LAL. Analyzed the data: LAL. Wrote the paper: LAL SP.

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Paper III

Otolith shape: a population marker for Atlantic herring *Clupea harengus*

Lísa A. Libungan, Guðmundur J. Óskarsson, Aril Slotte, Jan Arge Jacobsen and Snæbjörn Pálsson (2015)

Journal of Fish Biology 86: 1377-1395

Authors contribution: Conceived and designed the experiments: LAL, SP. Analysed the data: LAL, SP. Contributed reagents/materials/analysis tools: LAL, GJOS, AS, JAJ, SP. Wrote the paper: LAL, SP. Reviewed the manuscript: LAL, GJÓ, AS, JAJ

Journal of FISH BIOLOGY

fsbi An International Society for Fish Biology

Journal of Fish Biology (2015) **86**, 1377–1395
doi:10.1111/jfb.12647, available online at wileyonlinelibrary.com

Otolith shape: a population marker for Atlantic herring *Clupea harengus*

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(Received 2 July 2014, Accepted 26 January 2015)

Otolith shape variation of seven Atlantic herring *Clupea harengus* populations from Canada, the Faroe Islands, Iceland, Ireland, Norway and Scotland, U.K., covering a large area of the species' distribution, was studied in order to see if otolith shape can be used to discriminate between populations. The otolith shape was obtained using quantitative shape analysis, transformed with Wavelet and analysed with multivariate methods. Significant differences were detected among the seven populations, which could be traced to three morphological structures in the otoliths. The differentiation in otolith shape between populations was not only correlated with their spawning time, indicating a strong environmental effect, but could also be due to differing life-history strategies. A model based on the shape differences discriminates with 94% accuracy between Icelandic summer spawners and Norwegian spring spawners, which are known to mix at feeding grounds. This study shows that otolith shape could become an accurate marker for *C. harengus* population discrimination.

Key words: mixed fisheries; pelagic fish; population discrimination; shape analysis.

INTRODUCTION

Identifying populations and estimating the contribution of each population in mixed fisheries is important when designing appropriate regulations for effective fisheries management and for the understanding of the distributional range and migration behaviour of species. Disregard of population structure in fisheries management can lead to overexploitation of local non-targeted populations and result in loss of genetic variation (Nelson & Soulé, 1987; Smith *et al.,* 1991), which may be vital for adaptation in an ocean that is affected by climate change.

Atlantic herring *Clupea harengus* L. 1758 may have the most complex population structure of any marine fish species (Iles & Sinclair, 1982; Geffen, 2009), where populations are defined based on where and when they spawn. Nearly 30 separate *C. harengus* populations have been defined in the North Atlantic Ocean (Hay *et al.,* 2001)

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based on the location and timing of spawning. These populations have a wide range of life-history strategies (Geffen, 2009), and some even show sub-population structuring (Broch, 1908; Runnstrøm, 1941; Rasmussen, 1942; Aasen, 1952; Lie *et al.*, 1978; Hognestad, 1994; Husebø *et al.*, 2005; Johannessen *et al.*, 2009; K. E. Jørstad & S. A. Pedersen, unpubl. data). Despite the fact that several *C. harengus* populations have been identified and described in the northern north-east Atlantic, or the Nordic Seas (*i.e.* outside the North Sea and west of Scotland, U.K. and Ireland areas), only two are assessed and managed as stocks today. These two stocks are the Norwegian spring spawners, which spawn off the west coast of Norway (Dragesund *et al.*, 1997), and the Icelandic summer spawners, which spawn off the south and west coast of Iceland (Jakobsson, 1980). Other *C. harengus* populations are to a varying degree caught in the same feeding areas as the two aforementioned stocks. These include the Norwegian autumn spawners that spawn in the Lofoten area in northern Norway (Husebø *et al.*, 2005), which are assessed and managed as a part of the Norwegian spring spawners, and the Faroese spring spawners with spawning grounds in the fjords and east of the Faroe Islands (J. A. Jacobsen, pers. obs.), which have recently appeared in the feeding areas and are not assessed, but managed on a precautionary basis.

The Norwegian spring spawners are highly migratory with feeding grounds across the whole Norwegian Sea and into Icelandic and Faroese shelf waters where they can be mixed with the Icelandic summer spawners and Faroese spring and autumn spawners. As a consequence, a mixed fishery can take place in these areas. Individuals from these stocks not only have the same external characteristics, but also grow to similar sizes, making it often problematic to separate them in mixed fisheries based on body features alone. The spatial distribution of the Norwegian spring and autumn spawners also overlap during most of the year and can thus be caught in the mixed fishery (Husebø *et al.*, 2005). In Icelandic waters, Norwegian spring and Icelandic summer spawners are separated on the basis of maturity stage. Determining stocks solely based on maturity stage lacks precision because the method requires visual examination and relies upon the subjective judgement of the sampler. Stock separation based on maturity is also susceptible to error owing to potential overlap in the timing of gonad development between the two stocks. For example, if maturation and gonad growth of Norwegian spring-spawning individuals starts late and the Icelandic summer-spawning individuals have spawned such that both stocks are in the resting stage simultaneously for some weeks. Another example is in the feeding areas north of the Faroe Islands during late summer when the Norwegian spring spawners have begun to develop gonads after the feeding season and are found mixed with Faroese autumn spawners, which are in a similar or marginally more advanced maturity stage. A tool for confirming the separation would, in these cases, be very beneficial. The economic value of the *C. harengus* fishery is substantial so accurate allocation of catches to the different stocks is important for management of the fishery.

Morphometric analysis of otoliths is a well-established method to delineate fish stocks, characterize population movements and to detect the natal origin of fishes. For otolith shape analysis, two main morphometric methods are used: landmark analysis (Cadrin, 2013) and outline analysis (Bookstein *et al.*, 1985; Rohlf & Bookstein, 1990; Rohlf & Marcus, 1993; Marcus *et al.*, 1996). With outline analysis, it is possible to quantify boundary shapes so that patterns of shape variation within and among groups can be evaluated based on a large number of independent variables (Stransky, 2013). The advantage of using such methods in population identification is that they are

cost effective and only require photography of the otoliths, the outlines of which are extracted in an automated manner with statistical software. Shape analysis has been applied in stock and population identification of several marine fish species, such as cod *Gadus morhua* L. 1758 (Campana & Casselman, 1993; Cardinale *et al.*, 2004; Jonsdóttir *et al.*, 2006), Atlantic salmon *Salmo salar* L. 1758 (Friedland & Reddin, 1994), anglerfish *Lophius piscatorius* L. 1758 (Cañás *et al.*, 2012), comber *Serranus* spp. (Tuset *et al.*, 2003), mackerel *Scomber scombrus* L. 1758 (Turan, 2006; Stransky *et al.*, 2008), anchovy *Engraulis encrasicolus* (L. 1758) (Bacha *et al.*, 2014) and *C. harengus* (Bird *et al.*, 1986; Turan, 2000; Burke *et al.*, 2008*a*; Eggers *et al.*, 2014). Otolith shape is influenced by genetic factors (Cardinale *et al.*, 2004) and ontogenetic processes where otolith size changes in relation to body growth, temperature and food quantity (Einarsson, 1951; Geffen, 1982; Folkvord *et al.*, 2000; Feet *et al.*, 2002; Fox *et al.*, 2003; Vignon, 2012). Higher food rations during early life can lead to a higher number of larger lobes and a more rectangular otolith (Hüssy, 2008). Fishes may thus be marked in their otoliths for life by the environment they were spawned in because as the fishes grow, layers are added to the otoliths and shaped by the initial shape that was formed in their early life stages. Variable spawning time among fish stocks can thus contribute to variation in shape, as it can not only reflect early life temperature, but also be a proxy for ecological differences or variation in seasonal resource availability during the first year of the individual's life (Hempel & Blaxter, 1967; Burke *et al.*, 2008*a*).

For *C. harengus*, otolith analyses have been the subject of several studies. Einarsson (1951) used the structure of the otolith nucleus to discriminate between the Icelandic summer spawners and the Norwegian spring spawners. Several studies have been successful in determining the origin of juveniles using microstructural analyses of the increment width patterns (Moksness & Fossum, 1991; Brophy & Danilowicz, 2002; Clausen *et al.*, 2007), chemical composition (Brophy *et al.*, 2003; Geffen *et al.*, 2011) and otolith shape (Burke *et al.*, 2008*a*, *b*). For mature populations, using outline analysis, shape differences were detected among north-west Atlantic *C. harengus* and its Pacific congener, Pacific herring *Clupea pallasii* Valenciennes 1847 (Bird *et al.*, 1986). Similarly, Turan (2000) could identify four distinct groups of *C. harengus* from Iceland, Norway, the Baltic Sea and the British Isles. In a recent study by Eggers *et al.* (2014), three *C. harengus* populations, which mix over the spawning season in a geographically small region in southern Norway could be identified with otolith shape.

The main aim of this study was to evaluate otolith shape as a population marker for *C. harengus* and estimate how accurate shape is in classifying stocks of different origin which are caught together in the fisheries. This is done by applying a discrete Wavelet transform to analyse otolith shape, which provides a larger number of variables than the methods hitherto applied and ensures their independence (Graps, 1995; Parisi-Baradad *et al.*, 2005). The Wavelet transform provides a powerful alternative to the more commonly known Fourier transform in shape analysis. While the Fourier transform provides functions in the form of sines and cosines, which are non-local and can therefore result in poor approximations of sharp edges, the Wavelet transform uses approximating functions that are contained in finite domains making them well-suited for approximating sharp edges (Graps, 1995). Wavelet is therefore more accurate when more detailed information of the shape differences is needed, for example, to evaluate which areas of the otolith outline are contributing most towards the variation among populations.

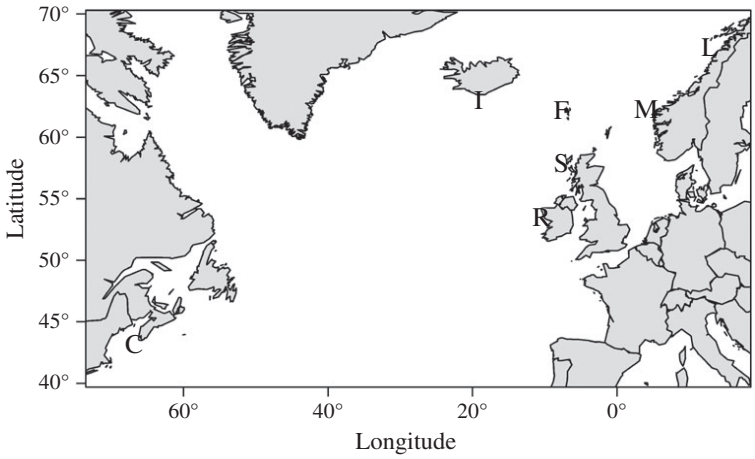

FIG. 1. Sampling areas for *Clupea harengus* in Canada (C), Faroe Islands (F), Iceland (I), Ireland (R), Norway (L and M) and Scotland (S) (see Table I for further details).

Six *C. harengus* populations from the north-east Atlantic Ocean and one from the north-west Atlantic Ocean, which also differ in spawning time, were sampled from spawning aggregations. The populations are: Canadian autumn spawners, Faroese spring spawners, Icelandic summer spawners, Ireland winter spawners, Norwegian autumn spawners, Norwegian spring spawners and Scotland autumn spawners. Otolith shape was compared among populations with univariate and multivariate methods and analysed with respect to spawning time and area. Also, whether otolith shape differences exist between the two management units which co-occur east of Iceland during summer and are harvested in mixed fisheries, the Icelandic summer spawners and the Norwegian spring spawners, were specifically analysed.

MATERIALS AND METHODS

SAMPLING

Spawning *C. harengus*, or those ripe or close to spawning, were sampled on the different spawning grounds with pelagic trawls and purse seines on commercial fishing and research vessels from seven *C. harengus* populations in the North Atlantic Ocean during a 20 month period from 2009 to 2011 in six countries (seven areas): Canada (C), the Faroe Islands (F), Iceland (I), Ireland (R), Norway (Lofoten, L; Møre, M) and Scotland (S) (Fig. 1 and Table I). Sampling areas and time of sampling were selected based on knowledge of spawning behaviour of the *C. harengus* for each population, ensuring that individuals sampled at each locality belonged to the spawning stock of that site. Several samples were obtained in Iceland and Norway (mainly M), to assess variation within these large populations. Biological variables were recorded for each fish: total body length (L_T; cm), mass (M; g), sex and standard maturity stage description applied in *C. harengus* fisheries in the north-east Atlantic Ocean as recommended by ICES (unpubl. data): immature = 1 and 2, maturing = 3–4, ripe = 5, spawning = 6, spent = 7, recovering–resting = 8. The sagittal otoliths were removed, washed in clean water and stored either in paper bags or plastic tubes. All fish were aged, based on otolith increment growth using standard ageing techniques. Ages 6–8 years were selected for the analysis, since there was the best overlap in the dataset for most of the populations at those ages, resulting in otoliths from 400 individuals

TABLE I. A summary of analysed samples of *Clupea harengus* at maturity stages 5–7 and at ages 6–8 years from Canada, Faroe Islands, Iceland, Ireland, Norway and Scotland, U.K. caught from commercial fishing

Country	Stock assessment unit	Stock name	Identity code	Sampling area	Latitude °N	Longitude −ve, °W; +ve, °E	Sampling date	Number of fish	Mean length (range) (cm)
Canada	4Xq	South West Nova Scotia autumn spawners	C	SW Nova Scotia	43·28	−66·20	1 September 2009	112	30·4 (27·7–32·8)*
Faroe Islands	[no code]	Spring spawners in the Faroe Islands	F	Faroe Islands	62·10	−6·75	28 March 2011	25	33·2 (31·5–35·0)
Iceland	her-vasu	Icelandic summer spawners	I	SW Iceland	64·23	−22·94	5 July 2009	30	33·1 (30·0–35·0)
	–	–	–	S Iceland	63·34	−18·89	8 July 2009	18	33·4 (32·0–35·0)
	–	–	–	–	63·75	−16·45	9 July 2009	40	33·6 (31·0–35·0)
Ireland	her-irlw, VIIb	North West of Ireland winter spawners	R	NW Ireland	54·72	−8·67	16 November 2010	16	27·5 (26·5–28·5)
Norway	[no code]	Autumn spawners in N Norway	L	Lofoten	66·64	12·36	10 August 2010	1	33·5
	–	–	–	–	67·24	13·28	11 August 2010	15	33·4 (31·5–36·0)
	–	–	–	–	67·32	11·85	14 August 2010	3	33·0 (32·5–34·0)
	–	–	–	–	67·63	12·31	14 August 2010	1	31·5
	–	–	–	–	69·74	16·94	16 August 2010	3	33·0 (32·5–34·0)
Norway	her-noss	Norwegian spring spawners	M	Møre	62·52	5·23	14 February 2010	12	32·9 (31·5–34·5)
	–	–	–	–	61·88	4·58	19 February 2010	19	32·6 (29·0–34·0)
	–	–	–	–	62·53	5·20	24 February 2010	23	32·6 (30·0–34·5)
	–	–	–	–	62·53	5·25	24 February 2010	29	32·7 (29·0–34·5)
Scotland	her-vian, VIaN	West of Scotland autumn spawners	S	W Scotland	58·73	−5·37	1 September 2010	58	29·4 (27·4–33·6)

*Fork length (total length for others stocks).

TABLE II. Age and sex (F, % of females in the pooled samples) distribution of the *Clupea harengus* populations sampled off Canada, Faroe Islands, Iceland, Ireland, Norway and Scotland

	Age (years)			F
	6	7	8	
Canada	28	36	48	51
Faroe Islands	9	9	7	48
Iceland	34	35	19	44
Ireland	7	8	1	44
Lofoten, Norway	7	7	9	87
Møre, Norway	19	16	48	52
Scotland	40	17	1	41

(Tables I and II). Pairs of the sagittal otoliths were obtained from 155 individuals from: Canada ($n = 10$), the Faroe Islands ($n = 14$), Iceland ($n = 49$), Ireland ($n = 14$), Lofoten, Norway ($n = 7$), Møre, Norway ($n = 20$) and Scotland ($n = 41$). Only fish that were either in spawning condition (stages 5–6) or had just spawned (stage 7) were used to ensure that the fish were from a local population.

IMAGE AND SHAPE ANALYSES

A digital image of each otolith was captured using a Leica MZ95 stereomicroscope (Leica Micro-systems; www.leica-microsystems.com) with an Evolution LC-PL A662 camera (Media-Cybernetics; wwwmediacy.com) using the software PixeLINK 3.2 (www.pixelink.com). Otolith images were read into the programme R (R Core Team; www.r-project.org), ensuring both a correct grey-scale threshold and high image quality. Otolith shape, in terms of outlines of otoliths, was collected from the digital images [Fig. 2(a)] with functions written in the programme R and using the package pixmap (Bivand *et al.*, 2011). Outlines were determined using the conte function in R (Claude, 2008). The shape of each otolith was recorded as a matrix of x and y co-ordinates. To remove size-induced bias, otoliths were normalized so that the otolith area would be equal in all otoliths by dividing the co-ordinates of each otolith with the square root of the otolith area. Equally spaced radii were drawn from the otolith centroid to the otolith outline, using the regular-radius function in R (Claude, 2008). Independent Wavelet shape coefficients were obtained by conducting a discrete Wavelet transform on the equally spaced radii using the wavethresh package in R (Nason, 2012). To determine the number of Wavelet coefficients needed for the analysis, the deviation of the reconstructed Wavelet otolith outline from the original outline was evaluated. By using 64 Wavelet coefficients, an error rate of 1·5% or an accuracy of 98·5% was obtained. To correct for L_T, an analysis of covariance (ANCOVA) was performed on the Wavelet coefficients to determine if there was an interaction between L_T and population and when there was a significant interaction, those coefficients were excluded from the analysis (Begg *et al.*, 2001; Longmore *et al.*, 2010; Agüera & Brophy, 2011). There was one coefficient that showed a significant interaction between L_T and population and was thus excluded from further analysis. To adjust the remaining 63 Wavelet coefficients for allometric growth, a normalization technique based on regression was applied to scale the Wavelet coefficients, which were independent of L_T after normalization (Lleonart *et al.*, 2000).

STATISTICAL ANALYSES

Main shape features

Shape differences among populations were evaluated visually by plotting the average otolith shape for each stock by using means of the reconstructed outlines of the normalized Wavelet

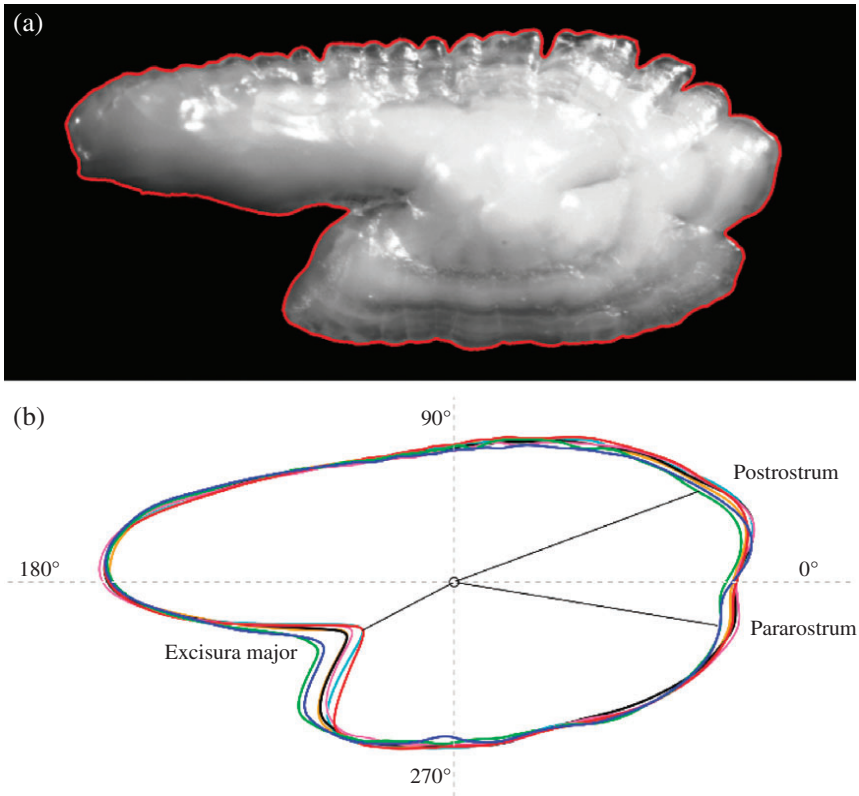

FIG. 2. Otolith shape analysis of *Clupea harengus*. (a) Example of an otolith outlined (—red) that marks the shape used to compare among *C. harengus* populations. (b) Average shape of otoliths for the seven *C. harengus* populations at ages 6–8 years. Lines inside the otolith represent the three radii that are drawn from the otolith centroid towards the excisura major, postrostrum and pararostrum areas that are the most variable in terms of otolith shape in this study. Degrees refer to angles in Fig. 3. Information about the populations codes (—, C, Canada; —, F, Faroe Islands; —, I, Iceland; —, L, Norway Lofoten; —, M, Norway Møre; —, R, Ireland; —, S, Scotland) is provided in Table I.

coefficients [Fig. 2(b)]. To estimate which areas and coefficients on the outline contributed most to the difference between populations, mean shape coefficients and their S.D. were plotted against the angle of the outline from where the coefficients were extracted (Fig. 3). The correlation of the lengths of the three radii [Fig. 2(b)], which contributed most to the difference between populations: excisura major radii, postrostrum radii and pararostrum radii [terminology from Bird *et al.* (1986)], was examined with a Pearson correlation test.

Univariate shape analyses

Radii were drawn from the centroid of the otolith towards the most variable area on the outline, the excisura major area [Fig. 2(b)]. The length of these excisura major radii, serving as a univariate shape descriptor, was compared among populations with analysis of variance (ANOVA).

Multivariate shape analyses

The Wavelet coefficients, which were scaled for L_T, were compared among populations with canonical analysis of principal co-ordinates (CAP) (Anderson & Willis, 2003) using the capscale function in the vegan package in R (Oksanen *et al.*, 2013). Ordination of the population averages

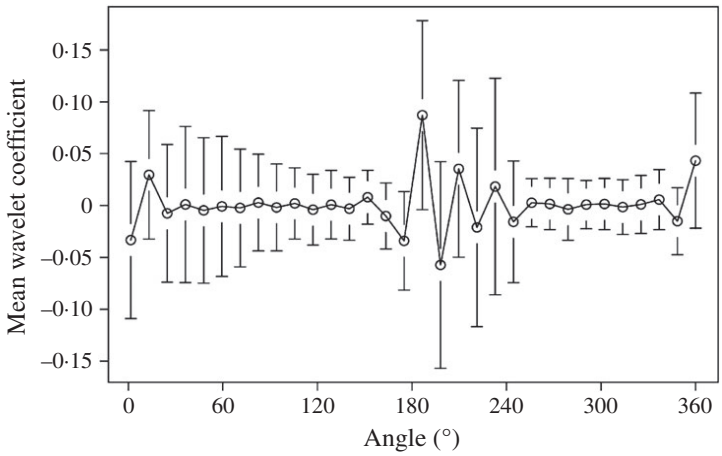

FIG. 3. Mean ± S.D. localized shape coefficients for the seven *Clupea harengus* populations with respect to the angle in clockwise directions from the centroid of the otoliths [Fig. 2(b)].

along the first two canonical axes was examined graphically with shape descriptors for otoliths of fish with ages 6–8 years pooled (Fig. 4). An ANOVA-like permutation test, also implemented with the vegan package, was used to assess the significance of constraints using 1000 permutations. To assess whether there was a significant difference between the left and right otolith of each individual, only individuals with both otoliths were analysed using CAP with respect to population. The first canonical score was tested for normality (Kolmogorov–Smirnov test,

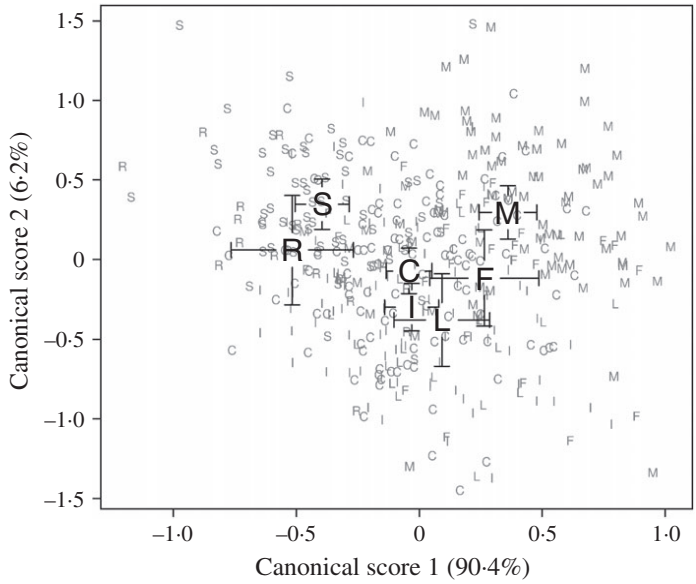

FIG. 4. Canonical scores on discriminating axes 1 and 2 for each *Clupea harengus* population: Canada (C), Faroe Islands (F), Iceland (I), Ireland (R), Norway Lofoten (L), Norway Møre (M) and Scotland (S). Black letters represent the mean canonical value for each stock for ages 6–8 years (see Table I for further details) and grey letters represent individual fish. Values are means ± S.E.

data not normally distributed, $P < 5\%$). As the data were not normally distributed, a Wilcoxon signed-rank paired test was performed to see whether there were differences between the canonical score of the left and right otolith. Variation in otolith shape based on one otolith per individual (if two otoliths existed from the same individual, one was sampled randomly) was analysed with CAP within and among populations with respect to sex, age and the interactions between sex and population as well as between age and population (Table II). Evaluation of the temporal trends and variation among samples within sites was conducted for the sites where multiple samples had been obtained (Table I), this includes three samples from Iceland and four from Møre, Norway. The different samples from Lofoten, Norway, were too small for evaluating the temporal trend and were combined into a single sample. Differences among populations were further analysed applying *a priori* comparisons contrasting the main geographic regions.

Shape differences among populations that are known to mix in the fisheries

Otolith shape was compared among the Norwegian spring spawners and the Icelandic summer spawners with CAP. To demonstrate classification of individuals to their origin based on the population variation at the two locations, linear discriminant analysis (LDA) was applied to the standardized Wavelet coefficients using the lda function in the MASS package in R (Ripley *et al.*, 2014). The LDA is a classification method used to discriminate among predefined groups of individuals based on a sample of observations from each group (Klecka, 1980). To investigate how accurately otolith shape could classify the stocks I and M back to their spawning stocks, the classification success into groups was estimated using a leave-one-out cross-validation procedure using the CV = TRUE argument within the lda function in the MASS package, which returns the posterior probabilities for the groups.

Shape and spawning time

The relationship between otolith shape as expressed by CAN1 and spawning time was analysed with a Pearson correlation test. The association of shape with respect to spawning time was evaluated with Mantel tests (Mantel, 1967), by analysing the association of matrices for the two data sets. Morphological distances were constructed based on average Euclidean distances based on otolith shape (CAN1 and CAN2) for each stock. Distances based on spawning time were firstly calculated as numbers of days separating spawning time for the different stocks within the same year, and secondly finding the shortest time period between two dates including comparison across years. All Mantel tests were conducted using the vegan package in R (Oksanen *et al.*, 2013).

RESULTS

AVERAGE SHAPE DIFFERENCES

Average shape of otoliths differed among the seven populations [Fig. 2(b)]. There were modifications in the shape of otoliths at the excisura major, postrostrum and pararostrum between populations. These three regions, pararostrum at angles 350 to 0°, the postrostrum from 0 to 20° and the excisura major at angles 160 to 240°, show also the largest variation, with excisura major being the most variable (Fig. 3). Further inspection of the mean shapes in [Fig. 2(b)] shows that R and S are similar at the edge, the outermost part of the excisura major, moving inwards towards the otolith centroid, I and L are similar, and closest to them is C, and in the innermost part F and M. At the postrostrum [Fig. 2(b)], populations show a similar pattern as seen at the excisura major, however, the pattern is reversed, where R and S had their otolith outline closest to the centroid, and other populations move outwards from there, with F and M furthest

TABLE III. Analysis of variance (ANOVA)-like permutation test of the otolith shape among the *Clupea harengus* populations in the *a priori* comparisons. Number of permutations was 1000 to assess the significance of constraints

Comparison	Reason for comparison	d.f.	Var	*F*	*P*
All populations		6	0·425	21·375	<0·001
C *v.* IMLFSR	Geographic: far away from the rest	1	0·011	2·649	<0·05
SR *v.* IMLF	Geographic: south and north	1	0·400	76·409	<0·001
I *v.* M	Not geographic, but special case to test the two stocks which mix at feeding grounds	1	0·260	30·785	<0·001
L *v.* M	Not geographic, both are in Norwegian waters, but differ in spawning time	1	0·108	7·447	<0·001
F *v.* M	Not geographic, but both are spring spawners, the only spring spawners in the data	1	3·351	2·185	>0·05
Residual		390	1·294		

Var, variance among populations (see codes in Table I); *F*: pseudo *F*-value (Oksanen *et al.*, 2013). C, Canada; I, Iceland, F, Faroe Islands; L, Norway Lofoten; M Norway Møre; R, Ireland; S Scotland.

away from the otolith centroid. At the pararostrum, R and S also have a similar shape closest to the centroid, while populations I, M, F and C show little variation, and L has a shape farthest from the centroid.

UNIVARIATE AND MULTIVARIATE ANALYSES OF SHAPE

Using univariate analysis, the lengths of the three radii were correlated with each other where correlations within samples C, I, M, R and S ranged from −0·64 to 0·78 (Pearson, $P < 0{\cdot}05$) but not within F and L (Pearson, −0·43 to 0·41, with $P > 0{\cdot}05$). The first discriminating axis (CAN1) from the canonical analysis and the excisura major radii length were correlated (Pearson, $r = -0{\cdot}87$, $P < 0{\cdot}001$) while less correlation was found between the other two radii and CAN1 (Pearson, $r < 0{\cdot}64$, $P < 0{\cdot}001$). Because the radii were correlated, only the excisura major radii length was chosen to test for between-group variation as it showed the most variation between populations. The length of the excisura major radii differed significantly between populations (ANOVA, $P < 0{\cdot}001$).

Using multivariate analysis, no differences were detected between left and right otoliths when the first canonical score of individuals from the seven populations with both otoliths were analysed (CAP, $P > 0{\cdot}05$). Otolith shape did not differ among the samples obtained along the coast of Iceland (CAP, $P > 0{\cdot}05$) and at Møre, Norway (CAP, $P > 0{\cdot}05$), samples within these stocks were thus pooled, and also the samples from Lofoten, which included several small samples (Table I). The effect of sex on the variation in otolith shape and the interaction between population and sex was non-significant (CAP, $P > 0{\cdot}05$). The effect of age and the interaction between population and age was non-significant (CAP, $P > 0{\cdot}05$).

Differences in otolith shape were observed among all populations (CAP, $P < 0{\cdot}01$; Table III and Fig. 4). The first discriminating axis explained 90·4% of the variation between populations and the second axis explained 6·2% (Fig. 4). When examining

the differentiation along the first discriminating axis, the samples from the British Isles, R and S, are clearly different from the rest and are similar on the first axis. There appeared to be no discrimination between C and I, and there was an overlap of the mean ± S.E. of L with both I and F. M appears different from the other populations both on the first and second discriminating axes, although it is closest to F on the first axis.

When testing for shape differences among populations using *a priori* comparisons with CAP (Table III), the Canadian sample (C) differed from the other samples pooled ($P < 0{\cdot}05$), but was similar to Iceland and Lofoten considering the canonical scores (Fig. 4). The samples from west of the British Isles (R and S) differed in shape when compared with the northern populations I, M, L and F ($P < 0{\cdot}01$), which corresponded with the canonical scores (Fig. 4). Among the northern populations, M differed from I ($P < 0{\cdot}01$) and L ($P < 0{\cdot}01$), while the F *v.* M comparison was close to but not significant (Table III; $P > 0{\cdot}05$).

CLASSIFYING ICELANDIC SUMMER AND NORWEGIAN SPRING SPAWNERS BACK TO THEIR SPAWNING STOCK

Otolith shape differed significantly between populations I and M (CAP, $P < 0{\cdot}01$, d.f. $= 1$, Var $= 0{\cdot}260$, $F = 30{\cdot}785$). An LDA classifier was able to classify individuals from stocks I and M back to their spawning stock based on otolith shape with an overall success rate of 93·6%. For I, 96·6% of the 88 individuals were classified correctly and 90·4% of the 83 individuals from M (Fig. 3).

OTOLITH SHAPE IN RELATION TO SPAWNING TIME

There was a significant negative correlation between the CAN1 otolith shape descriptors and spawning time among the seven populations (Pearson, $r = -0{\cdot}55$, $P < 0{\cdot}001$; Fig. 5). Mean CAN1 scores were highest for populations spawning early in the year from February to April (populations M and F), second highest for populations which spawn during late summer and autumn (I, L and C) and lowest for populations spawning in late autumn (S and R). There was a significant correlation between the distance matrices for otolith shape and spawning time (Mantel, $r = 0{\cdot}19$, $P < 0{\cdot}01$).

DISCUSSION

Otolith shape differs among *C. harengus* populations spawning at different locations in the north-east and north-west Atlantic Ocean. Multivariate analysis of the discrete Wavelet transforms successfully distinguished individuals from several of the different spawning grounds tested and could be used to trace the origin of fish that are known to mix at feeding grounds as for the Icelandic summer spawners and the Norwegian spring spawners.

The Wavelet transform proved its usefulness in otolith shape analysis as three interrelated morphological structures were detected on the otolith outline (excisura major, pararostrum and postrostrum) and contributed most towards the overall variation among the populations. These structures were also highly correlated with the first

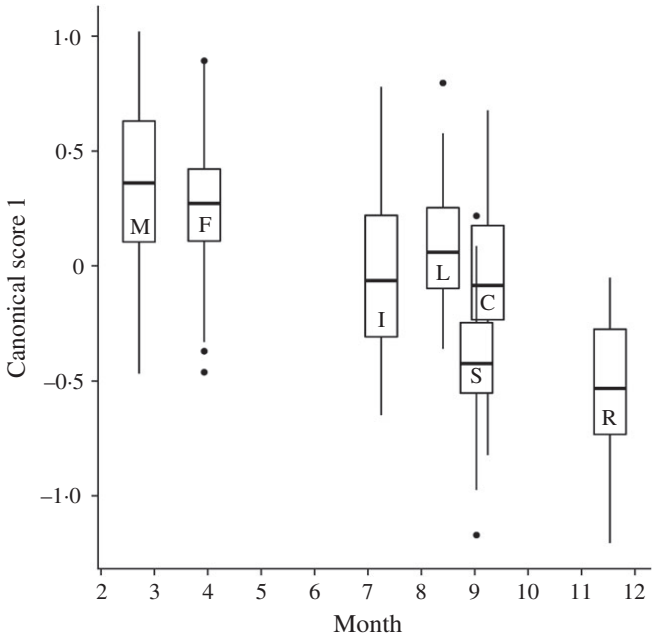

FIG. 5. Box-plots of canonical 1 scores derived from *Clupea harengus* otolith shape descriptors with respect to spawning time for each population: Canada (C), Faroe Islands (F), Iceland (I), Ireland (R), Norway Lofoten (L), Norway Møre (M) and Scotland (S). The □ are based on the quartiles of the distribution, the ⊟ are drawn at variates which are furthest away from the first and third quartile and within a distance of 1·5 interquartile distance from □. Values below and above ⊟, presenting outliers, are indicated (●).

discriminating axis from the canonical analysis. The excisura major area was the most variable, then the postrostrum and finally the pararostrum.

Both univariate and multivariate methods were used to test for shape differences among the populations. The univariate method was based on using radii length as a shape descriptor to compare shape among populations, where radii had been drawn in each otolith from the otolith centroid towards the most variable area on the outline, the excisura major area. The multivariate method used the scaled Wavelet coefficients to compare shape among populations. Interestingly, these two methods yielded the same result from the overall test, which found populations to differ significantly. Although the radii length method may provide sufficient resolution in some areas that can be used to discriminate *C. harengus* populations, a better resolution was obtained using the Wavelet shape descriptors for the *a priori* comparisons.

Different multivariate analyses of the Wavelet shape descriptors, including permutation tests and geographical comparisons indicated clear patterns among the seven populations that correlated with their spawning time. Three major groups of populations were identified: (1) Iceland, Canada and Lofoten Norway, (2) Møre Norway and the Faroe Islands and (3) Ireland and Scotland, where the first two were more similar to each other than to the populations west of the British Isles.

Comparisons among regions in this study are partly confounded by variation in time of sampling and warrant further studies by repeated sampling of the populations, although the general pattern is clear. Two of the *a priori* comparisons, one between

the Canadian sample and the other samples and the second between the Faroe Islands and Møre, Norway, include comparisons over 1 year. Comparison between Lofoten, Norway, and Møre, Norway, and between Iceland and Møre, Norway, are based on samples obtained at different spawning dates, sampled within a year and may thus reflect only the seasonal differences. A relatively large differentiation was observed between the southernmost samples from west of the British Isles (Scotland and Ireland) and the samples from the northern areas (Faroe Islands, Iceland, Lofoten and Møre, Norway), which confirms the results of Turan (2000) who observed high phenotypic discreteness in terms of otolith shape among the Icelandic summer spawners, Norwegian spring spawners and populations west of the British Isles. Populations west of the British Isles mature at a younger age and show considerable size differences and differing growth rates in comparison with the populations in the north (Hay *et al.*, 2001). Variation in growth rates can cause otolith increments to be deposited differently, where faster growth enhances ring deposition and slower growth results in fewer rings, which affects the otolith structure (Geffen, 1982; Folkvord *et al.*, 2000; Feet *et al.*, 2002; Fox *et al.*, 2003). It is therefore likely that differing growth rates are contributing to the shape differences observed among the populations from west of the British Isles and the northern populations.

Otolith shape distinguished between the Icelandic summer and Norwegian spring spawners that mix during feeding east of Iceland (Jakobsson, 1980) with high certainty (94%). The current method used to delineate stocks in mixed fisheries relies on maturity stage, which can be problematic not only due to variation in the onset of gonad growth and spawning, but also because of possible skipped spawners and other abnormalities. Using otolith shape as a marker therefore provides an improvement to population identification of *C. harengus* caught together in mixed fisheries. This research gives large-scale coverage of the existing shape differences among several of the known *C. harengus* populations. Further research is needed, however, incorporating populations from the Baltic Sea and within the North Sea where populations are known to overlap regionally. Also, it is important to target more age classes and over a longer time period to evaluate the generality of the results as a feasible method for population identification in mixed stock fisheries.

The variation in otolith shape has been considered to be affected both by environmental (Campana & Neilson, 1985; Lombarte & Lleonart, 1993; Elsdon & Gillanders, 2004; Teacher *et al.*, 2013) and genetic factors (Cardinale *et al.*, 2004). In accordance with the expectation of adaptive response to similar environmental settings, the two populations in the study that have similar geographical locations and spawning time, the Norwegian spring spawners and Faroese spring spawners, have a similar otolith shape. Given the known clockwise feeding migration route of the Norwegian spring spawners, it is possible that the Faroese spring spawners diverged recently from the Norwegian spring-spawning stock. Jakobsson (1980) reported a similar scenario for the Icelandic spring spawners, a stock that was once found in equal proportion to the Icelandic summer spawners but has not recovered from a collapse in the late 1960s. The Norwegian spring spawners collapsed at the same time and their migration to Iceland did not cease until the population recovered (Jakobsson & Østvedt, 1999). Since the Norwegian spring spawners and Icelandic spring spawners could not be distinguished by spawning time and fecundity, Jakobsson (1980) suggested that they should be considered as one component, with Iceland listed as the outer limits of

the Norwegian spring spawners distribution range. Similarly, the Irish and Scottish populations grouped together and both spawn in autumn and winter. For populations spawning in spring, after the increase in abundance of zooplankton, the larvae hatch under favourable conditions, and therefore their eggs are very small, with little yolk-sac reserves (Hempel & Blaxter, 1967). None of the populations studied here falls into that category. For populations spawning in summer and autumn, the larvae still hatch under favourable conditions, but generally from small eggs and their larvae do not metamorphose until the next spring and overwinter therefore as larvae (Iles & Sinclair, 1982). The Icelandic summer spawners, Norwegian autumn spawners and Canadian autumn spawners belong to this category, spawning from July to September. Even though these populations are separated by large geographical distances, they have a similar otolith shape.

The population shape differences might result from genetic divergence of populations. Hauser *et al.* (2001) found genetic differentiation with mtDNA markers between the Icelandic summer spawners and Norwegian spring spawners and other north-east Atlantic populations. Similarly, by analysing microsatellites, McPherson *et al.* (2004) observed differences between the Icelandic summer spawners and populations from Scotland and Canada. No genetic differentiation was found among the spawning populations (I, F, M, L and S) assessed in this study in an analysis of 24 microsatellite markers (Pampoulie *et al.*, 2015), which suggests that the differentiation among the populations may be recent or environmentally determined. Studies on North Sea and Baltic Sea *C. harengus* populations have been found to be weakly genetically structured (Bekkevold *et al.*, 2005; Gaggiotti *et al.*, 2009; Teacher *et al.*, 2013), but most of the observed patterns were explained by microsatellite loci that were possibly under selection, linked to salinity differences (Gaggiotti *et al.*, 2009; Teacher *et al.*, 2013), or to temperature and oceanographic connectivity (Teacher *et al.*, 2013). Recent studies of genomic variation have revealed that divergence of recently diverged populations may need an assessment of a large number of variable markers, *e.g.* in Baltic Sea *C. harengus* (Corander *et al.*, 2013), the divergence can be restricted to few genomic islands, whose divergence may have been driven by natural selection (Hemmer-Hansen *et al.*, 2013). Whether the variation in otolith shape of *C. harengus* is associated with one or few such variable genetic regions or is mainly affected by environment or the spawning time as suggested by this study remains to be seen and depends upon extensive genomic surveys being conducted. In a study on a species with similar life characteristics, the horse mackerel *Trachurus trachurus* (L. 1758), Atlantic and Mediterranean stocks were also found to be distinct in otolith shape (Stransky *et al.*, 2008) while former genetic studies found only a weak genetic separation (Nefedov *et al.*, 1978; Borges *et al.*, 1993).

The disparity between the differentiation in shape and genetic patterns might reflect the environmental effects on the shape phenotype, which was clearly associated with spawning time in this study. It appears that despite seasonal mixing of some of the *C. harengus* populations in the north-east Atlantic Ocean, natal homing (Ruzzante *et al.*, 2006), discrete retention areas for larvae (Iles & Sinclair, 1982; Stephenson & Power, 1988) and life-history strategies (Hempel & Blaxter, 1967; Geffen, 1982; Folkvord *et al.*, 2000; Feet *et al.*, 2002; Fox *et al.*, 2003) may result in different growth trajectories during early developmental stages that maintains diversity in otolith shape among populations.

Otolith shape analyses, as presented here, could become a valid tool to estimate the contribution of different spawning stocks in mixed fisheries, especially for stocks fished at a time when it is difficult to separate them based on morphological characteristics such as maturity stage. The cost effective method presented in this study may prove to be useful to trace the origin of spawning sites of individuals caught at these areas and thus to the management of important fish stocks, whether caused by genetic or environmental effects. This method may also prove to be valuable where population separation based on genetic markers may not be feasible due to lack of facilities or technological development.

D. J. Knox at Fisheries and Oceans Canada, E. Hatfield at Marine Scotland Science in Scotland and A. Egan at the Marine Institute in Ireland are thanked for providing otolith samples used in this study. S. Bjarnason, student at the University of Iceland, is thanked for taking images of otoliths. The editors and three anonymous reviewers are thanked for constructive comments which improved our manuscript. This project was funded by the Icelandic Research Fund for Graduate Students (grant number 100710001).

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Paper IV

Seasonal Dynamics of Atlantic Herring (*Clupea harengus* L.) Populations Spawning in the Vicinity of Marginal Habitats

Florian Eggers, Aril Slotte, Lísa A. Libungan, Arne Johannessen, Cecilie Kvamme, Even Moland, Esben M. Olsen, Richard D. M. Nash (2014)

PLoS ONE, 9 (11): e111985

Authors contribution: Conceived and designed the experiments: FE, AS, LAL. Analysed the data: FE, AS, LAL. Contributed reagents/materials/analysis tools: FE, AS, LAL, AJ, EMO, EM. Wrote the paper: FE, AS, LAL, RDMN. Reviewed the manuscript: FE, AS, LAL, AJ, EMO, EM, CK, RDMN

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Seasonal Dynamics of Atlantic Herring (*Clupea harengus* L.) Populations Spawning in the Vicinity of Marginal Habitats

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Abstract

Gillnet sampling and analyses of otolith shape, vertebral count and growth indicated the presence of three putative Atlantic herring (*Clupea harengus* L.) populations mixing together over the spawning season February–June inside and outside an inland brackish water lake (Landvikvannet) in southern Norway. Peak spawning of oceanic Norwegian spring spawners and coastal Skagerrak spring spawners occurred in March–April with small proportions of spawners entering the lake. In comparison, spawning of Landvik herring peaked in May–June with high proportions found inside the lake, which could be explained by local adaptations to the environmental conditions and seasonal changes of this marginal habitat. The 1.85 km^2 lake was characterized by oxygen depletion occurring between 2.5 and 5 m depth between March and June. This was followed by changes in salinity from 1–7‰ in the 0–1 m surface layer to levels of 20–25‰ deeper than 10 m. In comparison, outside the 3 km long narrow channel connecting the lake with the neighboring fjord, no anoxic conditions were found. Here salinity in the surface layer increased over the season from 10 to 25‰, whereas deeper than 5 m it was stable at around 35‰. Temperature at 0–5 m depth increased significantly over the season in both habitats, from 7 to 14°C outside and 5 to 17°C inside the lake. Despite differences in peak spawning and utilization of the lake habitat between the three putative populations, there was an apparent temporal and spatial overlap in spawning stages suggesting potential interbreeding in accordance with the metapopulation concept.

Citation: Eggers F, Slotte A, Libungan LA, Johannessen A, Kvamme C, et al. (2014) Seasonal Dynamics of Atlantic Herring (*Clupea harengus* L.) Populations Spawning in the Vicinity of Marginal Habitats. PLoS ONE 9(11): e111985. doi:10.1371/journal.pone.0111985

Editor: Brian R. MacKenzie, Technical University of Denmark, Denmark

Received April 8, 2014; **Accepted** October 10, 2014; **Published** November 5, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data files are available from the DRAYD database (accession number(s) doi:10.5061/dryad.qt984).

Funding: These authors have no support or funding to report.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Typically, fish species may be split into populations based on their degree of reproductive isolation from each other in space and/or time, which could be reflected in genetic or phenotypic differences driven by diverging environmental conditions [1–3]. Under such circumstances exploitation on one population should have little effect on the population dynamics of a neighboring population, and therefore it is also common to assess and manage such populations separately [4,5]. On the other hand, there are also examples where populations are recognized to be separate with diverging spawning season and/or spawning area, but due to mixing in other seasons a separate management of the populations may be difficult [6,7]. The need to identify the different populations, especially where exploitation occurs on mixtures of populations is important for successful management [8,9]. Fisheries biologists therefore often use the term stock instead of population in their fisheries advice; i.e. sometimes a population is harvested and therefore managed as one stock and at other times several separate populations are harvested and managed as one stock. In Begg et al. [10] the concept of a fish stock was simply defined as characteristics of semi-discrete groups of fish with some definable attributes, which are of interest to fishery managers. The definition of ICES [11] for a stock as a part of a fish population usually with a particular migration pattern, specific spawning grounds, and subject to a distinct fishery, will be used hereby. In theory, all individual fish in an area, being part of the same reproductive process, are comprised as a stock. When referring to fisheries management, the term "stock" is used, otherwise the term "population" is preferred.

Atlantic herring (*Clupea harengus* L.) is characterized by highly complex population structure and migration patterns [12]. It is an iteroparous clupeid, becoming sexually mature at two or three years of age, and a total spawner that aggregates at spawning, laying benthic eggs on shells, gravel, coarse sand and small stones at depths down to 250 m [13]. The larvae hatch after 2–4 weeks depending on temperature [14,15]. They drift with currents until metamorphosis [16–18], with vertical migration increasing throughout ontogeny [19,20] and affecting the dispersal trajectories of larvae. The different herring populations are generally

classified according to their spawning grounds, which, due to the specific spawning substratum requirements, are fixed geographically and used at a predictable time of the year. Due to physical and geographical barriers, such as prevailing currents and general location of nursery areas, there is often little mixing of larvae, thus tending to isolate the different populations. However, there are occasions where larvae and juveniles may co-occur. Under these circumstances identification of individuals or groups of individuals is undertaken using otolith or meristic characters [1,21–24] as well as genetic markers [25–28]. In the 1950–60s experimental studies [29–31] demonstrated that myotome counts in herring were influenced by both temperature (negatively) and salinity (positively) experienced during the incubation period. The consequence is that mean vertebral count of adult herring is an indicator of spawning ground and spawning times and in some cases also population.

In Norwegian waters some herring populations occupy marginal habitats along the coastline and deep inside fjords, most of which are thought to be stationary with adaptations to local conditions. Hence, they are often phenotypically and, in some occasions, genotypically different from the nearby oceanic population. Examples of such local herring populations are Trondheimsfjord herring [32,33], Borge Poll herring [34], Lusterfjord herring [35], Lindåspollene herring [36], Balsfjord herring [37], Lake Rossfjord herring [38] and the summer/autumn spawners in northern Norway [39]. Despite the discovery of these local populations, the overall research effort targeting marginal areas along the Norwegian coast has been rather low, and it is therefore expected that a number of additional local populations may exist.

Migratory coastal or oceanic populations may occasionally enter the marginal habitats along the Norwegian coast and mix with local herring. This is in accordance with the metapopulation concept, where two or more distinguished subpopulations have variable but moderate interbreeding and significant gene flow [40]. Temporal and spatial overlap during spawning may allow genetic exchange between subpopulations, which is a prerequisite for the existence of metapopulations. An example of such an overlap was demonstrated by Johannessen et al. [41],[42] in the local Lindåspollene herring, where significant changes in life history traits over a 50 year period were linked to genetic exchange with the oceanic population according to the metapopulation concept.

An important mixing area for herring is the northeastern North Sea and Skagerrak, where three different stocks may occur, Norwegian Spring Spawners (NSS), North Sea Autumn Spawners (NSAS) and Western Baltic Spring Spawners (WBSS). Some of these stocks comprise different herring populations, such as coastal Skagerrak spring spawners or more local herring populations, which are not directly subjected to a distinct fishery. The different populations (stocks) can be distinguished by spawning site, spawning season, meristic characters such as the number of vertebrae (VS) and otolith characteristics [23,41].

Of particular interest in the Skagerrak area is a brackish water environment inside Landvikvannet, an inland lake in southern Norway connected to the open sea through an artificial channel. The Institute of Marine Research (IMR) has been sampling herring in Landvikvannet on regular basis since 1984, mainly in May. Data from these investigations demonstrate that herring inside the lake are normally ripe or with running gonads, with a low mean vertebral number (<56.0), slow growth and high fecundity [43,44]. This has led to the hypothesis that the lake is visited on an annual basis by a herring population with specific adaptations to spawning in these brackish water environments. However, in the coastal areas outside the lake, ripe and spawning herring with higher growth and mean vertebral numbers (56.0–57.5) have occurred in samples over the period February–June [43]. This indicates that there may be a mixture of several populations in the area with some temporal and spatial overlap in spawning, which could be linked to spatial seasonal differences in environmental conditions. Such metapopulation dynamics may be revealed by a more detailed seasonal sampling outside the May period normally focused on in IMR's investigations in Landvikvannet. Hence, the principal objective of the present study was to explore the overlap in time, space and maturation stages of phenotypically different herring appearing in Landvikvannet and neighboring fjord areas and their dependence on seasonal changes in environmental conditions.

Material and Methods

Study area

Landvikvannet is a 1.85 km^2 lake located on the Norwegian Skagerrak coast (Figure 1). In 1877 a 3 km long channel (Reddal channel, Figure 1) was constructed, connecting the lake to the open sea. This narrow 1–4 m deep channel transformed Landvikvannet into a brackish system and in addition lowered the water level in the lake by 3 m. At the entrance of the lake there is a small 25 m deep basin. Further into the lake the bottom depth decreases rapidly to 7–10 m. Most of the shoreline is covered by reeds; otherwise the shore is rocky and steep. There is inflow of saltwater over the tidal cycle, whereas freshwater empties into the lake from streams, resulting in a halocline. Oxygen is depleted in the lower layers whereas the surface layer is oxygen rich. In Landvikvannet, herring have been caught by floating gillnets together with trout (*Salmo trutta*) and other freshwater fish since shortly after the channel was opened.

The Reddal channel drains into Strandfjorden (Figure 1), where conditions are estuarine. The outer Strandfjorden is narrow and shallow (1–7 m), whereas the inner part is deeper (10–13 m). Most herring samples were collected in the inner part, close to the mouth of the Reddal channel. The shore is rocky and steep with sparse macroalgae in the upper few meters. At depths >5–6 m the bottom consists of sand and mud. The outermost fjord (Bufjorden, Figure 1) is small with direct connection to Skagerrak. Strandfjorden is connected to the open ocean via Bufjorden (Figure 1). The entrance of Bufjorden is characterized by a 54 m deep basin. The physical environment is similar to Strandfjorden, only less influenced by fresh water runoff. Access to Bufjorden is from the south or east.

Environmental data

To explore whether potential differences in habitat utilization and timing of peak spawning among herring populations were dependent on seasonal changes in environmental conditions, sampling of environmental data was undertaken between March and June 2012 both inside and outside the lake habitat. Note, that no stations could be sampled in February due to ice cover. Water samples were collected at the site where gillnets were moored in the inner part of Strandfjorden and at the entrance of Landvikvannet in the first basin (Figure 1). We measured temperature and salinity at depth with a CTD (STD/CTD – model SD204, SAIV Ltd. Environmental sensors and Systems, Bergen, Norway), while oxygen and hydrogensulfide concentrations were analyzed in the laboratory at the Institute of Marine Research (IMR). In the lake, water samples were collected each 0.5 meter down to the depth of oxygen depletion (hypoxic depth), which was found using the Winkler test [45], thereafter water samples were taken at 5 m

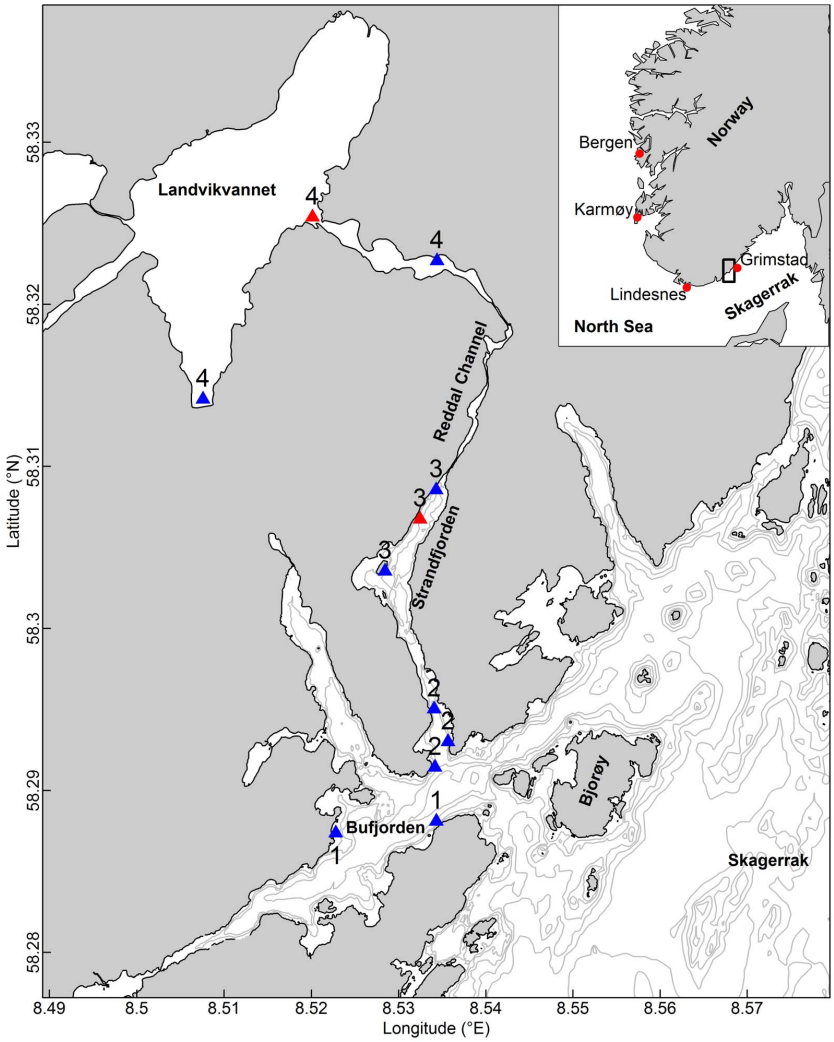

Figure 1. Map of the study area. The map shows CTD-stations (red) and gillnet stations (blue) in 1 = Bufjorden, 2 = Outer part of Strandfjorden, 3 = Inner part of Strandfjorden, 4 = Landvikvannet.
doi:10.1371/journal.pone.0111985.g001

depth intervals. The choice of position for sampling environmental data inside the lake is based on the depth contours of the area. The lake itself is rather shallow, and the bottom depth at most gillnet stations is 2–4 m. However, at the entrance the lake is at its deepest (25 m), which is why this position has been used since investigations started in the area in the 1980s. The environmental conditions at this site between 0 and 10 m have been examined thoroughly over a number of years and are comparable to conditions elsewhere in the lake and as such can be used to characterize the whole lake. These data are therefore representative of all gill net sampling sites.

Biological data

To explore the potential overlap in time, space and maturation stages of phenotypically different herring appearing inside and outside the lake habitat, herring were sampled with gillnet over the full spawning season in 2012 (February–June) concurrently in both habitats (Figure 1, Table 1). In February, due to ice cover both in the lake and inner fjord habitats of Strandfjorden, samples were only taken further out in Bufjorden. The floating gillnets with a mesh size of 26 mm and 29 mm, a depth of 8 m and a length of approximately 10 m were used randomly in all areas. Soak time was 24 hours. This experiment was approved by the Norwegian committee for the use of animals in scientific experiments (FDU). Special permission to fish with floating gillnet inside

Landvikvannet and in the connected fjord system in 2012 was given by the County Governor of Aust-Agder, Department of Climate and Environment, Ragnvald Blakstadsv. 1, Postbox 788 Stoa, 4809 Arendal, Norway. The permission was given to the Institute of Marine Research under the prerequisite that details on the catch were reported when the investigations were finished. The report was delivered to the authorities according to the plan. Our study did not involve endangered or protected species.

Biological samples were analyzed according to IMR standard protocols [46]. The maximum sample size was 100 herring. Biological parameters included in the present study were total length (nearest 0.5 cm below), weight (nearest gram below), sex, stage of maturity, age (otolith readings) and vertebral count (VS). Maturity stages were determined by visual inspection of gonads according to the following scale: immature = 1–2, maturing = 3–4, ripe = 5, spawning/running = 6, spent = 7 and recovering = 8 [46].

Image and shape analyses

Individuals of NSS herring were identified from otoliths, based on a sharper distinction between winter and summer rings compared to local spring spawners (Figure 2). This distinction was also independently tested using image and shape analyses of the otoliths. The rest of the individuals were divided into two populations based on sampling location: local Landvikvannet herring (LV) sampled inside Landvikvannet and coastal Skagerrak spring spawners (CSS) sampled outside Landvikvannet (Table 2). We expected that LV herring would mainly consist of individuals with similar biological characteristics as normally found in May, whereas the CSS herring would mainly consist of spring spawners with characteristics normally found along the Skagerrak coast during February–June. However, some mixture of the two populations would be expected, and this would be evident from results of the biological analyses. To investigate changes in the mixture of NSS, CSS and LV herring in the two habitats, selected biological characters (otolith shape, vertebral count, growth and maturation stage) were analyzed over the full season. The numbers analyzed by month and population are given in Table 2.

Otolith shape was analyzed using the programming language R [47]. Outlines of otoliths were collected from digital images using the package pixmap [48], and applying the conte function [49] to record a matrix of X and Y coordinates (Figure 2a). Mean shape of otoliths differed among the populations, where the modifications in the shape of otoliths mainly were found at the excisura major and antirostrum areas (Figure 2b).

To remove size-induced bias, otolith sizes were standardized to equal area by dividing the coordinates of each otolith with the square root of the otolith area. Equally spaced radiis were drawn from the otolith centroid to the otolith outline, using the regular radius function [49]. Independent Wavelet shape coefficients were obtained by conducting a Discrete Wavelet transform on the

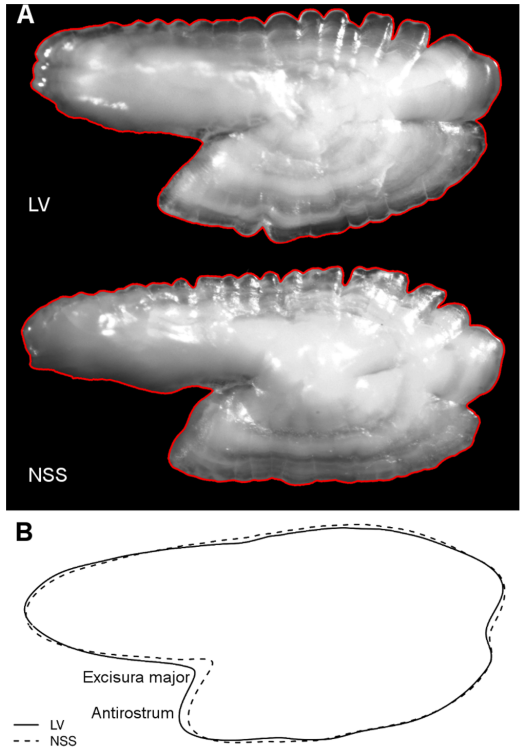

Figure 2. Example of otolith characteristics from two herring populations. A) Example of otoliths used for the shape analysis from Landvikvannet herring (LV) and Norwegian spring-spawning herring (NSS), both at the age of 3 years. Individuals of NSS herring were subjectively identified based on a sharper distinction between winter (dark areas) and summer rings (white areas). Red outline marks the shape of the otolith which was used to compare among populations. B) shows the mean shape of otoliths for the two populations, where the excisura major and antirostrum areas are the most variable areas.
doi:10.1371/journal.pone.0111985.g002

Table 1. Total number of herring caught in the local area for 2012, in brackets number of gillnets; ice = no sampling possible because the area was covered by ice.

Date	Landvikvannet	Inner Strandfjorden	Outer Strandfjorden	Bufjorden
15/2	Ice cover	Ice cover	28 (1)	11 (1)
6/3	4 (3)	129 (1)	119 (1)	
20/3	47 (3)	542 (1)		
26/3	115 (3)	486 (1)		100 (1)
11/4	290 (2)	663 (1)		
14/5	177 (1)	69 (1)		
21/6	82 (1)	66 (1)		
Total	**715**	**1955**	**147**	**111**

doi:10.1371/journal.pone.0111985.t001

Table 2. Total number of herring analyzed in 2012 by month for the three putative herring populations, Norwegian spring spawners (NSS), Coastal Skagerrak spring spawners (CSS) and Landvik herring (LV), in brackets number of NSS inside Landvikvannet.

Month	NSS	CSS	LV
2	7 (0)	32	0
3	108 (38)	440	113
4	32 (14)	68	86
5	8 (5)	61	95
6	0 (0)	66	77
Total	**155 (57)**	**667**	**371**

doi:10.1371/journal.pone.0111985.t002

equally spaced radiuses using the wavethresh package [50]. To determine the number of Wavelet coefficients needed for the analysis, the deviation of the reconstructed Wavelet otolith outline from the original outline was evaluated. To correct for fish length, an ANCOVA was performed on the wavelet coefficients taking fish length as a covariate. Coefficients which could not be adjusted by linear relationships on fish length, due to interaction between the origin and length were excluded from the analysis [51–53]. To adjust the Wavelet coefficients for allometric growth, a normalization technique based on regression was applied to scale the Wavelet coefficients [54].

Data analyses

The number of gillnets varied between Landvikvannet and the neighboring fjord area. Therefore, to estimate the proportions of the LV, CSS and NSS herring, the total catches landed were standardized by catch per unit effort (CPUE), i.e. catch per gillnet.

All statistical analyses were conducted in R (version 3.0.1; [47]). A significance level of $\alpha = 0.05$ was used for all statistical tests. For the plots, mean and standard error (1 SE) are shown. Some samples had very few or no data, and samples with $N<5$ were excluded.

Analysis of Covariance (ANCOVA) was used to test for sex differences in the biological characters (length, age, VS and stage of maturity). Differences in VS among different herring populations were assessed using Analysis of Variance (ANOVA), and a Kruskal-Wallis test for length and age variables as these were not normally distributed. For pairwise comparisons of VS a paired T-test was used, and the Mann-Whitney test for length and age comparisons.

Length-at-age data, used as a proxy for growth of individual herring, were fitted to the von Bertalanffy growth model (VBGM) [55]:

$$L_t = L_\infty(1 - e^{-K(t-t_0)})$$

where L_t is the average length at age t, L_∞ is the asymptotic maximum length, K is the von Bertalanffy growth rate coefficient, i.e. the rate at which length approaches the maximum length asymptote and t_0 is the intercept on the time axis. Growth was compared between the different groups using ANOVA.

Variation in otolith shape, as reflected by the scaled Wavelet coefficients, was analyzed with Canonical Analysis of Principal coordinates (CAP) [56] using the capscale function in the vegan package in R [57]. Using multivariate data to represent otolith shape, an ANOVA like permutation test (vegan package) was used to assess the significance of constraints using 5000 permutations. Variation in otolith shape was analyzed with CAP, while length and VS were compared with ANOVA with respect to herring group: NSS, LV and CSS, the month in which they were caught over the sampling period (Feb–June) and age in years (3–12) using the following models: shape~herring population*month*age, length~herring population*month*age and VS~herring population*month*age. Non-significant interaction terms ($p>0.05$) were excluded from the models. *P*-values for all posteriori comparisons were corrected with the Bonferroni correction [58]. Possible trends of length and VS within herring populations were tested for significance using linear regression, while the stage of maturity was tested with the Spearman's rank correlation coefficient. For the comparisons of environmental data at time of spawning with the VS of herring, measurements from 3 m were used for Landvikvannet due to the depth of oxygen depletion in combination with previous (2010) acoustic observations of school depth [43]. In Strandfjorden, measurements from 5 m were used, based on acoustic observations of herring school depth during tagging experiments and the gillnet sampling [43].

Results

Environmental conditions

The environmental conditions differed considerably between Landvikvannet and the neighboring fjord, and changed over the spawning season in both locations (Figure 3). Anoxic conditions were found in Landvikvannet at increasing depths from 2.5 m in March to 5 m in June. Salinity ILV at 0–1 m increased over the season from 1‰ in March to 7‰ in June, but was stable around 20–25‰ deeper than 10 m. In comparison, there were no anoxic conditions in Strandfjorden, the salinity at 0–1 m increased from 10‰ in March to 25‰ in June and was stable at 35‰ deeper than 5 m. The temperature at 0–5 m depth increased from March to June from 5 to 17°C in Landvikvannet, and from 7 to 14°C in Strandfjorden.

Population structure

A total of 1260 herring were analyzed during the 2012 spawning season. Total length ranged from 22.0–34.5 cm (mean: 28.3 cm) and age from 2–12 years (mean: 4.2 years). None of the biological characters varied between sexes ($p>0.05$). Hence, all further analyzes were carried out with sexes combined.

Mean length, age and vertebral count (VS) differed significantly among the three herring populations ($p<0.001$, Figure 4). For age and length, pairwise comparisons were also significant ($p<0.001$), with the exception of CSS versus LV for age ($p>0.05$). The vertebral count differed significantly ($p<0.001$) for all pairwise comparisons. The main tendency was a significant increase in

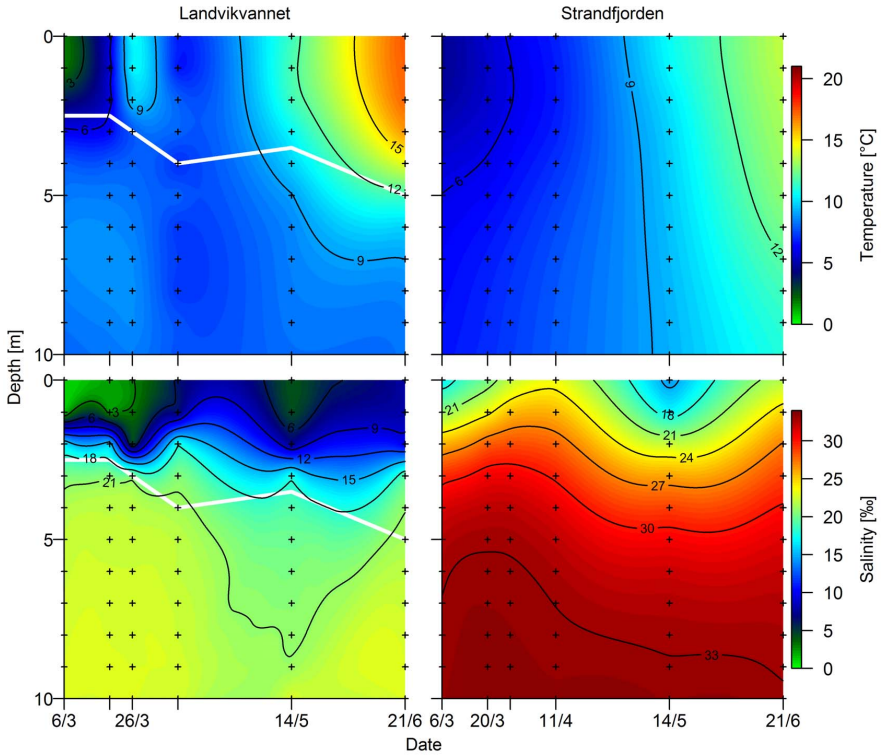

Figure 3. Seasonal change in temperature and salinity by depth. Temperature (upper) and salinity (lower) in Landvikvannet and in Strandfjorden over the study period from March to June. White line indicates the depth of oxygen depletion.
doi:10.1371/journal.pone.0111985.g003

mean body length and VS when moving from LV to CSS to NSS, whereas men age decreased. The most common age was 3 years for NSS, CSS and LV herring. The 4 year olds were also abundant in CSS and LV herring, but hardly present among NSS herring.

Length-at-age data indicated the highest growth for NSS herring, and lowest for LV herring ($p<0.01$) (Figure 5). The von Bertalanffy growth model supported these growth differences (Table 3). Consequently, there were three categories: 'high growth rate' (NSS herring), 'moderate growth rate' (CSS herring) and 'low growth rate' (LV herring).

Between February and June there was a change in the abundance of the different populations (Figure 6). During February–April CPUE was highest for CSS and NSS herring with a low proportion of LV herring (<20%). Also the proportion of NSS herring entering Landvikvannet was insignificant (<10%). The proportion of spawning and spent herring during this period was highest in NSS herring and a little lower for CSS herring, but still indicating peak spawning of two different populations in the fjord habitat during this period. Among the LV herring analyzed in March–April an even lower proportion were in spawning and spent stages than for CSS herring, indicating a later spawning peak for LV herring. This was further demonstrated in the May–June sampling showing a spatial shift in CPUE towards higher abundance of LV than CSS and NSS herring.

Otolith shape differed among the three herring populations ($p<0.001$, Table 4, Figure 7) and also varied though the spawning season ($p<0.001$, Figure 8A). Vertebral count and length differed between the populations ($p<0.001$) and between months ($p<0.001$, Figure 8B, C). Age was a significant factor for all characters ($p<0.001$) and therefore incorporated in the model for all comparisons. Posteriori comparisons showed that LV and CSS differed in otolith shape, VS and length ($p<0.04$, Figure 8, Table 4). NSS and LV ($p<0.001$) as well as NSS and CSS ($p<0.02$) also differed, while no differences were detected for NSS caught inside or outside the lake ($p>0.05$). There was a signifiant ($p<0.001$) negative trend in the mean Canonical scores (CAN1) derrived from the CAP analysis of otolith shape, vertebral count and length for LV and CSS herring at standardized ages over the spawning season, but not for NSS (Figure 8). This indicates that LV herring, characterized by slow growth and low vertebral count, were arriving and mixing with CSS herring.

Maturation and spawning time

Herring in spawning condition were present and overlapped in time for LV, CSS and NSS herring, however, maturation and timing of spawning was delayed in LV compared to NSS and CSS herring (Figure 6). This indicates an adaptation to the environmental conditions and seasonal change in Landvikvannet. Since differences in vertebral count are linked to environmental conditions, the temperature and salinity at depth and time of

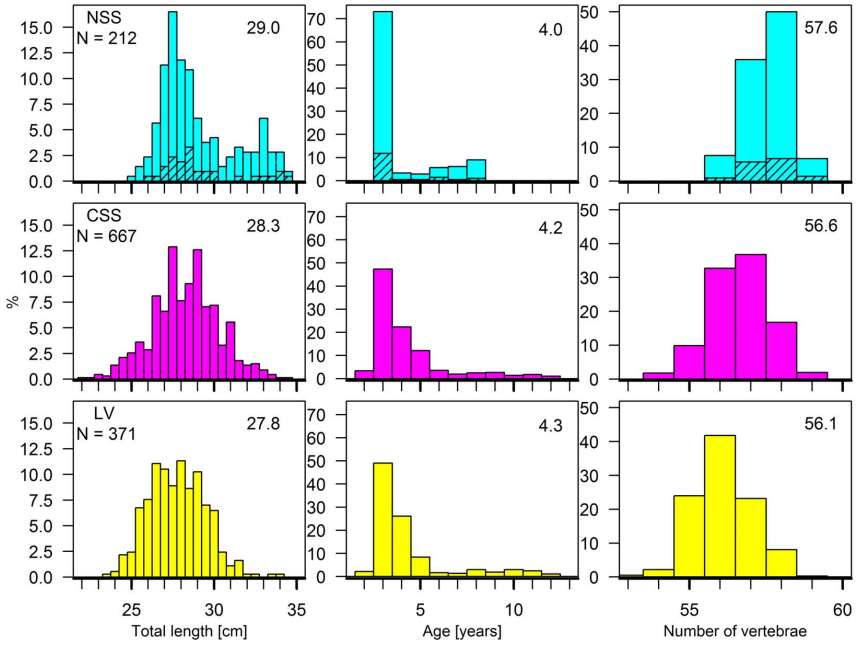

Figure 4. Distribution of length, age and vertebral counts of different herring populations. Comparison between Norwegian spring spawning (NSS), Coastal Skagerrak spring spawning (CSS) and Landvik (LV) herring. Shaded areas are NSS herring inside Landvikvannet. The mean values are included.
doi:10.1371/journal.pone.0111985.g004

spawning affects the vertebral count. The salinity at expected spawning depth in Landvikvannet was distinctly lower (10–15‰) than in the adjacent fjord (>30‰), which could explain the low vertebral count observed in Landvikvannet. The vertebral count was not significantly related to change in salinity over season within habitats; there was negligible change at assumed spawning depth. However, there were significant changes in temperature over season in both habitats, coinciding with a significant decrease in vertebral count at spawning time for both CSS and LV herring ($p<0.05$).

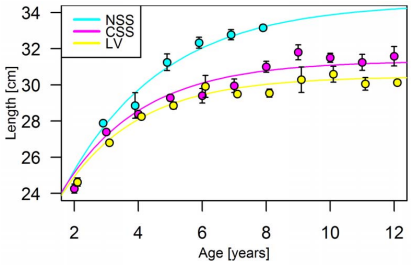

Figure 5. Growth curves of different herring populations. Length-at-age for Norwegian spring spawning (NSS, N = 212), Coastal Skagerrak spring spawning (CSS, N = 667) and Landvik (LV, N = 371) herring in samples pooled over the 2012 spawning season. Means and standard error (1 SE) are given, lines show van Bertalanffy growth models fitted to data.
doi:10.1371/journal.pone.0111985.g005

Discussion

This study reveals strong seasonal dynamics involving three populations of a pelagic migratory fish, the Atlantic herring, in the vicinity of a marginal inland brackish water lake habitat (Landvikvannet) on the Norwegian Skagerrak coast. Gillnet sampling was standardized, implying that the observed differences between herring populations and over season dynamics were not affected by the selectivity normally experienced with gillnet sampling [59]. Three putative herring populations were identified; Norwegian spring spawners (NSS), Landvik herring (LV) and Coastal Skagerrak spring spawners (CSS). Individual NSS herring were identified subjectively based on otolith growth characteristics, and statistically based on otolith shape and mean vertebral count (57.5). NSS herring also had higher growth than the other populations, which is typical for this stock [13,43]. Identification of individual CSS and Landvik herring was not possible. Individuals sampled inside the lake were all classified as LV herring, whereas those sampled outside the channel connecting the lake to the sea were assigned as CSS herring. However, there was a significant decrease in vertebral count over the sampling season in both LV and CSS herring, from levels known as typical for CSS herring (56.5–56.9) in March–April to levels typical for Landvik herring (<56.0) in May–June, again based on historic data [43]. This trend in vertebral count was followed by a decrease in size and change in otolith shape, and a marked change in the relative proportions of the two populations.

The observed seasonal dynamics in biological characters clearly indicate that the assignment of individual fish into CSS and LV herring simply based on sampling location was uncertain, and that

Table 3. Von Bertalanffy growth parameters (L_∞, k, and t_0) of herring populations Norwegian spring spawners (NSS), Coastal Skagerrak spring spawners (CSS) and Landvik herring (LV).

	L_∞	K	t_0
NSS	34.51	0.33	−1.98
CSS	31.31	0.41	−1.98
LV	30.33	0.43	−1.98

doi:10.1371/journal.pone.0111985.t003

the two populations were mixing both inside and outside the lake habitat together with NSS herring showing a different peak occurrence. Early in the season in February–April the biological characteristics indicated that NSS and CSS herring predominated, with only small numbers entering the lake. There was a clear temporal and spatial overlap in spawning individuals from these two populations, although proportions spawning in CSS were comparatively lower than in NSS herring. In May–June there was a significant change with the appearance of a new spawning wave of LV herring, with the highest proportion found inside the lake. Still, the immigration of this population was evident throughout both habitats, where many of the herring found in the fjord would be expected to enter the lake. The data on otolith shape, vertebral count and growth in May tended to differ from the observations in June in both locations, which indicated a spatial and temporal overlap in May between minor proportions of NSS and CSS herring completing their spawning season at the same time as the LV herring was peaking.

All three putative populations were caught at the same location, in the same gillnets, at the same time with running gonads, suggesting that the populations together form a metapopulation [40]. However, there is doubt as to whether interbreeding between distinct populations is occurring despite their proximity in spawning condition. Since breeding was not observed directly,

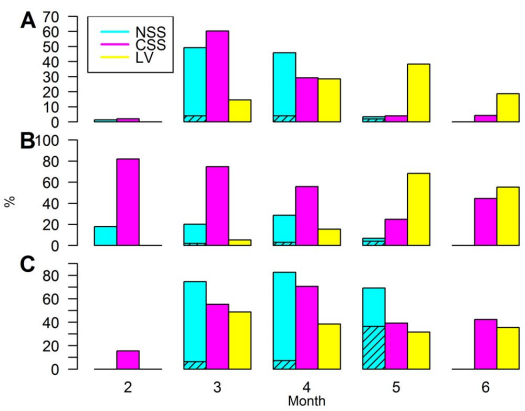

Figure 6. Seasonal change in proportion of different herring populations. Proportion (%), standardized to one gillnet per sample and area, by month of Norwegian spring spawning (NSS), Coastal Skagerrak spring spawning (CSS) and Landvik (LV) herring relative to a) total number analyzed over entire study period (see Table 1 for N), b) total number at month and c) spawning and spent herring (stage of maturity>=6) relative to total number at month (see Table 2 for N). Shaded areas are NSS herring inside Landvikvannet.
doi:10.1371/journal.pone.0111985.g006

one cannot exclude the possibility that the populations separate for spawning events. Such a full separation seems unlikely for NSS and CSS herring because of the high temporal and spatial overlap; whereas it seems more likely for LV herring considering the limited temporal and spatial overlap with the other populations.

The idea that LV herring is reproductively isolated from other populations may be supported by the low vertebral count and concept of natal homing. Differences in vertebral count stem from the incubation phase and thus reflect the origin of the fish at spawning [60]. In general, there is a positive correlation with salinity [31] and negative with temperature [21,29,61] experienced prior to hatching. Hence, the warmer and less saline ambient environment for herring occurring inside Landvikvannet in May–June compared with that experienced by CSS in March–April in the fjord habitat, could result in the observed differences in vertebral count. The low vertebral count of LV herring and the late timing of spawning is an indication of spawning and adaptations to the environmental conditions of the lake habitat. However, this also implies that natal homing [62,63] of Landvik herring occurs on an annual basis. The vertebral number for LV herring in May has been remarkably stable (55.5–55.8) since 1984 [43], supporting natal homing. The principle of natal homing is central to the discrete population concept [12]. Moreover, recent genetic studies support the occurrence of natal homing of herring in the North and Baltic Seas [6,64]. Likewise, Brophy et al. [65] suggested that spawning season and location of Atlantic herring could be predetermined and not learnt from repeated spawning [66]. Support for natal homing and adaptations of Landvik herring to environmental conditions of its marginal habitat also originates from a recent genetic study using 20 microsatellite markers, where Landvikvannet differed from other local herring in Lindåspollene, Lusterfjord and Trondheimsfjord as well as from other herring populations surrounding the Norwegian Sea [67]. Unpublished results on the microsatellite locus Cpa112, which is non-neutral to salinity variability with allele frequencies varying from 45% in the Baltic to 2–4% in the North Sea [27], have shown that Landvik herring is obvious with a frequency of 15% (Carl André, pers. Comm., Department of Biology and Environmental Sciences - Tjärnö, University of Gothenburg, Strömstad, Sweden).

It seems clear from this study that we can refute the hypothesis of a resident local population inside the lake; LV herring definitely migrates into the lake habitat from coastal areas. In this sense the Landvik herring differs from other local herring populations, such as the Trondheimsfjord or Lindås herring, which can be observed throughout the year in their local areas [32,33,36,41]. This may simply be because of the unsuitability of this location as a nursery area for juveniles and feeding grounds for adults. Both CSS and LV herring may still represent more stationary coastal populations not undertaking large scale oceanic migrations. The observed relatively low investment costs in reproduction (low GSI) of NSS compared with that of LV herring supports the assumption that

Table 4. Comparing otolith shape, vertebral count (VS) and length among herring populations Norwegian spring spawners (NSS), Coastal Skagerrak spring spawners (CSS) and Landvik herring (LV).

			Otolith shape				Vertebral count				Fish length			
Comparison		N	df	Var	F	P	df	Mean Sq	F	P	df	Mean Sq	F	P
Overall	NSS vs LV vs CSS	897	2	3.28	5.36	<0.001	2	109.95	136.44	<0.001	2	129.80	102.58	<0.001
	Month		1	1.20	3.91	<0.001	1	71.49	88.71	<0.001	1	690.00	545.44	<0.001
	Age		10	4.49	1.47	0.001	10	3.87	4.80	<0.001	10	178.20	140.90	<0.001
	Residuals		883	270.41			867	0.81			867	1.30		
Posteriori	LV vs CSS	745	1	0.69	2.22	0.04	1	32.10	36.69	<0.001	1	13.10	10.08	0.006
	NSS vs LV	500	1	1.45	4.76	<0.001	1	219.80	276.99	<0.001	1	250.45	196.30	<0.001
	NSS vs CSS	549	1	0.84	2.72	0.02	1	115.53	149.39	<0.001	1	178.20	114.88	<0.001
	NSS-ILV vs NSS-OLV	152	1	0.20	0.65	>0.05	1	0.23	0.47	>0.05	1	1.85	1.65	>0.05

NSS herring were also compared between sampling locations, inside (NSS-ILV) and outside (NSS-OLV) Landvikvannet. ANOVA like permutation tests were used to assess the difference in otolith shape and ANOVA for the vertebral count and fish length comparisons. For otolith shape: df: degrees of freedom, Var: Variance among populations, F: pseudo F-value, P: proportion of permutations which gave as large or larger F-value than the observed one. For the vertebral count and fish length: df: degrees of freedom, Mean Sq: Mean Square, F: F-value, P: P-value. P-values for posteriori comparisons have been corrected with a Bonferroni correction. P<0.05 indicates a significant effect.

doi:10.1371/journal.pone.0111985.t004

Figure 7. Otolith shape compared for different herring populations. Canonical scores for Norwegian spring spawning (NSS, N = 152), Coastal Skagerrak spring spawning (CSS, N = 397) and Landvik (LV, N = 348) herring are shown on discriminating axes 1 and 2. Black letters represent the mean canonical value for each group with standard error of the mean (1 SE).

doi:10.1371/journal.pone.0111985.g007

NSS is more migratory [44]. The fact that growth of CSS was higher than in LV herring, further suggest that these two populations may not overlap much during the nursery period or at adult feeding grounds. In fact, there is probably little or no spatial overlap for most of the year, with overlap only occurring during the spawning season.

The movements of herring between the fjord and Landvikvannet habitats have also been studied with acoustic telemetry [43,68]. The telemetry study showed that some fish moved in and out of the lake habitat, whereas others stayed inside the lake for more than two weeks. Those fish that arrived and only stayed for a short period of time were interpreted as being NSS or CSS, whereas the ones remaining in the area for extended periods of time were thought to be local LV herring. It is likely that some NSS and CSS herring have short visits to the lake as exploratory migrations searching for good habitats cued by the current from the Reddal channel, but migrate out again to spawn in areas which are more characteristic of their normal spawning habitat. Conversely, fish that stay for two weeks inside the lake before leaving is a reasonably good indication of an established adaptation to the lake and to potential spawning within the lake.

The appearance of NSS herring in the habitats within Landvikvannet and adjacent fjords probably does not represent natal homing. The predominance of 3-year-olds among the NSS stock as well as the high stability of growth and meristic characters over the season, suggest independent selection of spawning grounds, as supported by Slotte and Fiksen [69]. In NSS herring specifically, the use of spawning grounds other than their natal ground is common. NSS herring have a tendency to change their spawning ground as they grow older with larger fish tending to migrate further, in this case southward, and thus potentially increase their life time fitness [69–71]. Such straying from natal spawning grounds results in considerable gene flow [72,73]. The predominance of 3-year-old NSS mixing with CSS and Landvik herring in 2012 may be explained by the relatively unusual spawning migrations of NSS herring in 2009–2010. During these two years a significant proportion of the adult NSS migrated from wintering grounds in the northern Norwegian Sea to areas south of 60°N, resulting in the largest fishery in the fjords (e.g. Boknafjorden) east of the traditional spawning grounds off Karmøy since the 1950s [74]. Based on vertebral count and growth data, it was apparent that the fishery was targeting NSS

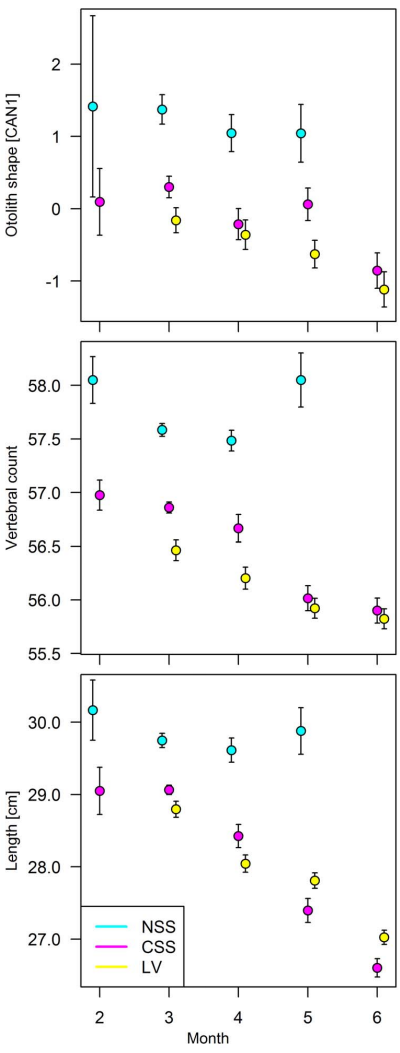

Figure 8. Seasonal changes of otolith shape, vertebral counts and length for different herring populations. For standardized ages. Comparison between Norwegian spring spawning (NSS), Coastal Skagerrak spring spawning (CSS) and Landvik (LV) herring (see Table 2 for N). Values given are means and standard errors (1 SE).
doi:10.1371/journal.pone.0111985.g008

herring [75] and the abundance was high as evaluated by catch levels (Table 5). One hypothesis is that the 3 year old NSS mixing with CSS and Landvik herring in 2012 was a result of this significant spawning at the southern grounds in 2009. Generally, if first time spawners of NSS do not meet older conspecifics and learn to follow their migration towards the spawning grounds then the location of the spawning ground is a chance event [70,71,76,77]. In addition, NSS herring tend to migrate upstream to spawn [69]. Therefore it is not unlikely that NSS from Boknafjorden or further south may have spawned close to their nursery areas or even migrated further south-eastwards against the

Table 5. Commercial catches of herring off Karmøy 2005–2012.

	Year of catch								
Month	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**
1					0.1			
2	21.2				172.0	3302.9	609.1	897.3
3	24.5	32.6	16.5		19052.0	14877.0	6528.4	6283.2
4	129.2	0.7	1.0	4.8	2301.2	1000.3	52.0	13.4
8	1.0							
9					0.9			
10			0.1					
11		72.8				0.5		
12	0.2							
Total	**176.1**	**106.1**	**17.6**	**4.8**	**21526.2**	**19180.7**	**7189.5**	**7193.9**

Live weight (tons) calculated from landed weight to live weight equivalent for Norwegian spring spawning herring in the Norwegian statistical area 08 (SW coastal Norway) by month and year as registered in the Directorate of Fisheries database.
doi:10.1371/journal.pone.0111985.t005

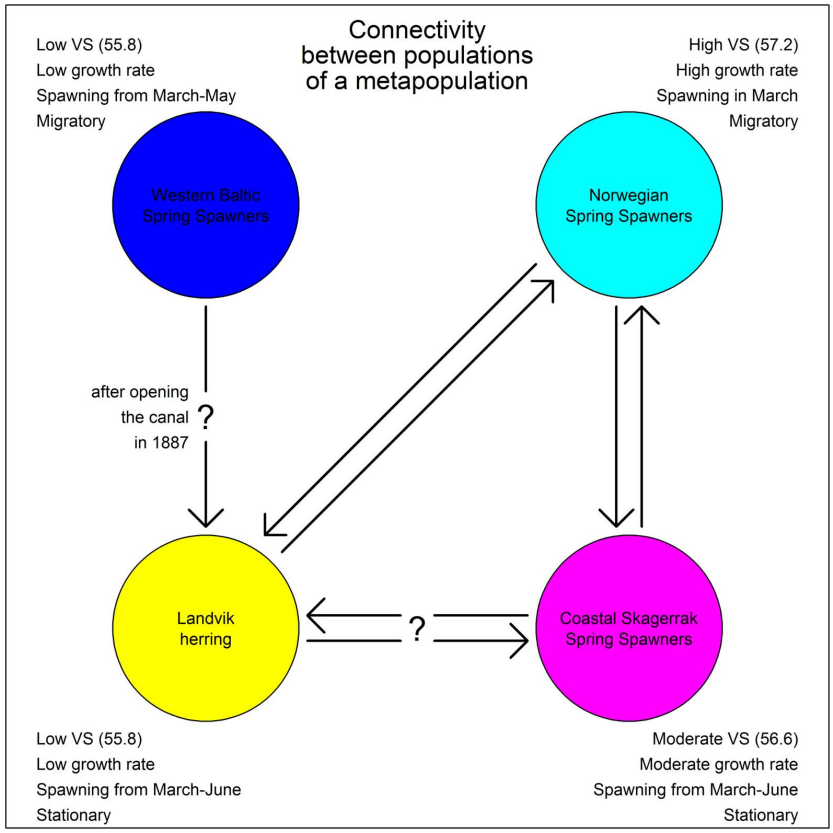

Figure 9. A schematic model of potential metapopulation dynamics in the study area. Potential connectivity between populations of a metapopulation in the study area of Landvikvannet and the connected fjords as hypothesized based on the results of the present study. The biological characteristics (VS = vertebral counts) of the different populations are given.
doi:10.1371/journal.pone.0111985.g009

coastal current to spawn. In addition, school composition tends to involve size-matching among individuals [78], in this case younger, smaller NSS. Three year old NSS (mostly first-time spawners), may have adopted the behavior of the joint local populations with whom they mix during the nursery period as postulated in the adopted-migrant hypothesis [40,79].

From an evolutionary perspective, the Landvikvannet habitat has only been available for marine species for a relatively short period of time. This raises the question of the origin of the herring first colonizing the lake after the opening of the Reddal channel (Figure 9). One possibility is that CSS herring entered the lake sometime after the opening of the channel and successfully spawned there. Due to lower salinity and higher temperature in the lake the offspring developed significantly divergent characters over the years. A strong natal homing effect of herring would lead to the development of a new local population inside Landvikvannet. Hendry and Kinnison [80] concluded that a time span less than 100 years can be sufficient for significant microevolution to develop in response to local agents of selection. Also, Neb [81] demonstrates that such a time interval and differences in salinity are sufficient for herring to diverge in meristic characters. This explanation assumes reproductive isolation during spawning between the original CSS herring and the "new" Landvik herring. A second possibility is that the origin of Landvik herring could be Western Baltic Spring Spawners (WBSS) herring. First time, or even repeated, spawners could have established a new spawning ground in Landvikvannet. The reason for not conducting an annual migration to the original spawning grounds off the island Rügen may be a trade-off between survival of progeny and physiological migration constraints, as shown for NSS by Slotte [70]. WBSS close to their feeding grounds in the Skagerrak could have "discovered" Landvikvannet, cued by similar environmental conditions as those of their original spawning grounds. The continued link to Landvikvannet may have been a result of a fidelity to this site rather than for joining conspecifics in a migration back in to the Baltic region. Huse et al. [76] demonstrate that a high ratio of first-time spawners could lead to the establishment of new wintering grounds. In the case of Landvik herring, it may have led to a new spawning ground.

In conclusion, the present study provides evidence for a distinct small local population of herring associated with Landvikvannet, partly mixing with NSS and CSS herring. This population of LV herring resides, during part of the year in brackish water with many morphometric characteristics indicative of spawning in

warm and low salinity environments. Whilst ripe and spent fish have been found in the area, there is no direct evidence of spawning in the lake. If spawning does occur there are no data to indicate likely survival rates or even the residence time of offspring in the lake. There has been one attempt to find eggs with a diver for 1 hour at one of the many bays in the lake, without success. Also, limited plankton net sampling in selected parts of the lake have failed to capture any larvae. The only evidence of potential spawning in the lake, is from two eels with stomachs full of fertilized herring eggs. There is also no clear evidence of the origin of this population, however, they could have arisen from either WBSS or other local CSS. The presence of mixtures of these and other stocks and populations in the Skagerrak area have been shown previously [6,82]. Recent genetic studies using microsatellite DNA [83] have demonstrated differences between Landvik herring and many other stocks, in addition, unpublished results on one microsatellite locus (Carl André, pers. Comm., Department of Biology and Environmental Sciences - Tjärnö, University of Gothenburg, Strömstad, Sweden) suggesting that Landvikvannet herring has not recently immigrated from the Baltic.

The results of the present study may also have some implications for the official ICES stock assessment of herring in the North Sea and Skagerrak area. The present work demonstrates that there can be a fairly complex population structure in the areas with more than one 'stock' which can be mixed. Whilst this may not be a significant problem for the assessment of NSAS or WBSS due to the relatively small abundances of CSS and LV herring, there is a possibility that these smaller populations could be very vulnerable to overfishing [9]. This is probably not unique for coastal areas as there are a number of relatively small populations bordering the North Sea and Skagerrak area [84].

From management point of view, probably the most striking result of the present study is the conclusive evidence of NSS herring as far southeast as in the Skagerrak. This is the first time that individuals from this historically large herring stock have been studied in the Skagerrak area. By definition this stock is not exploited south of 62°N, with exception of the spawning period when they previously have been found as far south as to Lindesnes (Figure 1). This signifies that migration dynamics and population connectivity among herring in the Northeastern Atlantic may be more dynamic than previously assumed, and this must be taken into account in the future development and implementation of new management strategies.

Acknowledgments

Knut Hansen is thanked for his very valuable contribution with running the sampling program throughout the 2012 spawning season and being in charge of biological analyses. The following are also thanked for their contributions to the research: Åse Husebø undertook the initial work on photographing and outlining otoliths for the shape analyses, Jostein Røttingen and Inger Henriksen contributed to sampling and biological analyses, Terje Jåvold analyzed the water samples and Øystein Paulsen undertook photographing and assisted with the sampling. The two anonymous reviewers are thanked for their very valuable suggestions for improvements to the manuscript.

Author Contributions

Conceived and designed the experiments: FE AS LAL. Performed the experiments: FE AS LAL. Analyzed the data: FE AS LAL. Contributed reagents/materials/analysis tools: FE AS LAL AJ EMO EM. Wrote the paper: FE AS LAL RDMN. Reviewing the manuscript: FE AS LAL AJ EMO EM CK RDMN.

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Paper V

Latitudinal gradient in otolith shape among local populations of Atlantic herring (*Clupea harengus*) in Norway

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Authors contribution: Conceived and designed the experiments: LAL, SP. Analysed the data: LAL, SP. Contributed reagents/materials/analysis tools: LAL, AS, SP. Wrote the paper: LAL, SP. Reviewed the manuscript: LAL, SP, AS, ÅH, JAG

Latitudinal gradient in otolith shape among local populations of Atlantic herring (*Clupea harengus*) in Norway

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Abstract — Otolith shape analysis of Atlantic herring *(Clupea harengus)* in Norwegian waters shows significant differentiation among fjords and a latitudinal gradient along the coast where neighbouring populations are more similar to each other than to those sampled at larger distances. These morphological differences are likely to reflect environmental differences but indicate low dispersal among the local herring populations. Otolith shape variation suggests also limited exchange between the local populations and their oceanic counterparts, which could be due to differences in spawning behaviour. Herring from the most northerly location (69°N) in Balsfjord, which is genetically more similar to Pacific herring *(Clupea pallasii)*, differed in otolith shape from all the other populations. Our results suggest that the semi-enclosed systems, where the local populations live and breed, are efficient barriers for dispersal. Otolith shape can thus serve as a marker to identify the origin of herring along the coast of Norway.

Keywords — Atlantic herring, Norway, local fjord populations, population discrimination, otolith shape

1 INTRODUCTION

Atlantic herring (*Clupea harengus*, Linneaus 1758), being one of the economically most important fish species, has been a subject of several studies on population structure [1-8]. A relatively low level of genetic differentiation has been found among isolated local populations which may overlap geographically during feeding migrations [2-6,9-12]. Genetic markers have shown uniformity among herring occupying the offshore waters of the Northeast Atlantic [13,14] and over large geographical distances [1,15,16]. However, recent studies on population genomics have revealed clear differentiation among Baltic Sea herring [5] and genetic differences have also been found between the geographically isolated local herring populations in Norway, the Lake Landvik herring and herring from Trondheimsfjord, Lindåspollene and Lusterfjord [1] and also within Balsfjord and Trondheimsfjord [17,18]. Studies on Atlantic herring have further revealed the plasticity and high level of adaptability of the species [19] as observed in heterogeneity in life history, morphology and behaviour [20], and reported population differences which have not been detected with genetic markers such as otolith shape [8].

An indented coastline, such as found in Norway, provides an excellent model system for evaluating the effects of geographic barriers on patterns of isolation in marine fish populations. The fjord system presents furthermore different hydrographic conditions than found in the open ocean. Within fjords, the conditions can be uniform and stable due to stratification of the water masses where the upper layers have comparatively low salinity owing to freshwater carried into the sea by rivers [21]. Thermal stratification in the water column is for example known to influence maturation and time of spawning for local Atlantic herring populations in Norway [22]. Several local herring populations in Norway have been identified based on biological characteristics and geographical distribution, such as the Balsfjord, Lysefjord and Østerbø herring [23], Borge poll herring [24], Lindåspollene herring [25], Lusterfjord herring [21], Lake Landvik herring [26], Lake Rossfjord herring [27] and Trondheimsfjord herring [28,29]. The local herring populations are thought to complete their entire life-cycle within fjords [21], lakes [26] and semi-enclosed coastal systems [22] and differ from their oceanic counterparts by having small population sizes, a shorter life cycle, low vertebral number, slower growth rate [21], and smaller size-at-age [30,31], but also in having higher relative fecundity since local populations do not migrate over long distances and therefore invest less energy into growth and more into egg production than oceanic population [27,32-34]. As the herring larvae have limited swimming capabilities, where they can only travel short distances of 14.7-16.1 mm s^{-1} as measured for larvae at the age of 34 days post-hatch [35], and they are not carried passively with the coastal current as most fry of the oceanic populations [36-39], it is likely that they retain close to their site of spawning in semi-enclosed ecosystems. In addition to the local herring populations in Norway, there are two oceanic herring populations: the Norwegian spring-spawners which is highly migratory and disperses all over the Norwegian Sea, and the Norwegian autumn-spawners which is thought to be mainly around Lofoten [40] and is managed as part of the Norwegian spring-spawners. Where the Norwegian spring-spawners overlap geographically with local herring, the first year cohort is known to utilize fjords as an overwintering area and then migrate out of the fjord during the summer to feed [41-43]. The extent of interaction and reproduction between the Norwegian spring-spawners and the local populations is not fully explored. However, the

interaction between the Norwegian spring-spawners and Lindåspollene herring was studied over a 50 year period and results showed the latter population to change in several life-history traits including length-at-age, length at first maturity and longevity when the Norwegian spring-spawners were spawning at the same time and in the same semi-enclosed coastal region [7], confirming that the Norwegian spring-spawners do interbreed at least with some of the local populations.

Otolith shape analysis has been widely used with success in stock identification of various marine fish species with high gene flow, such as cod [44], haddock [45], anchovy [46], mackerel [47,48] and herring [8,49]. Otolith shape is markedly population specific, but also shows intra-specific geographic variation in relation to environmental factors [8,26,50,51]. Since morphometric characters are modified by the environment, they can indicate reproductive isolation if the characters are different between spawning aggregations [52].

The aim of this study was to investigate the structure of local herring populations along the Norwegian coastline using otolith shape, which is a known population marker for Atlantic herring [8], to describe how discrete these smaller populations are and if there were any signs of dispersal or diversification among neighbouring local populations. The northern most population, which was sampled in Balsfjord, is known to be similar to Pacific herring (*Clupea pallasii*, Valenciennes 1847) in vertebrae number, spawning behaviour [17] and genetics [53]. Another aim was to compare otolith shape between local populations and neighbouring oceanic populations as well as among and between the two main geographic regions in the study (southern and northern Norway).

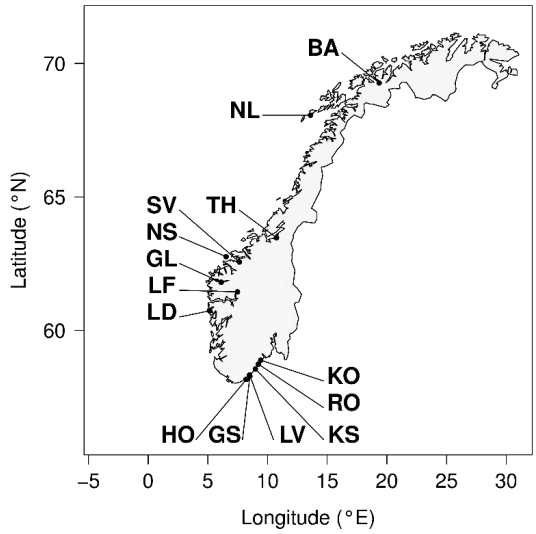


Figure 1. Herring sampling areas along the coast of Norway. Local populations from southern Norway are KO: Kragerø, RO: Risør, KS: Kilsund, LV: Lake Landvik, GS: Grimstad, HO: Hovåg. From western Norway LD: Lindåspollene, LF: Lusterfjord, GL: Gloppen, SV: Sykkulven, TH: Trondheim. From northern Norway BA: Balsfjord. The two oceanic populations, NS: Norwegian spring-spawners and NL: Norwegian-autumn spawners are also shown (see Table 1 for further details). Latitude (°N) is shown on the y-axis and longitude (°E) on the x-axis.

2 MATERIALS AND METHODS

2.1 Sampling

Herring were sampled during the period of 2005-2014 from 14 different spawning grounds with purse-seiners from fjords, semi-enclosed coastal regions, Lake Landvik and the open ocean (oceanic populations) clockwise from southern (Kragerø, 58.88N, 9.43E) to northern Norway (Balsfjord, 69.27N, 19.35E, Fig. 1, Table 1). The local populations from southern Norway were sampled at Kragerø, Risør, Kilsund, Lake Landvik (a brackish lake connected to the ocean), Grimstad and Høvåg. From western Norway samples were obtained from Lindåspollene, Lusterfjord (200 km from the coastline), Gloppen (80 km from the coastline), Sykkulven and Trondheim. The oceanic populations were the Norwegian spring-spawners, sampled at their main spawning grounds at Møre and the Norwegian autumn-spawners from Lofoten [40]. Sampling areas and time of sampling were selected based on knowledge of spawning behaviour of herring at each location, ensuring individuals sampled belonged to the spawning stock of that site.

To test for temporal effects in otolith shape, herring in Balsfjord, Gloppen, Risør and Sykkulven were sampled for 2-4 years (Table 1). Total length (cm) was recorded for each fish and maturity stage according to an 8-point scale: immature = 1 and 2, maturing = 3 to 5, running/spawning = 6, spent = 7, recovering/resting = 8 [54]. The sagittal otoliths were removed, washed in clean water and stored in plastic trays. All fish were aged from their scales using standard ageing techniques [55].

The Institute of Marine Research (IMR), which is responsible for monitoring herring and giving advice to managers in Norway, have permission to sample herring at any location along the Norwegian coast by the Directorate of Fisheries, Bergen, Norway. In addition, any person in Norway has by law permission to conduct recreational fisheries on herring at these sites using gill nets. The samples used in this study stem from both trawl hauls using IMR's research vessel, IMR's gillnet sampling as well as samples collected by recreational fishermen, all sampled within Norwegian regulations and laws. There is, however, one exception from this general permission to sample herring, and that is the Lake Landvik location. Given that this is an inland lake connected to the sea through an artificial channel, other rules are counting. Here, special permission to sample herring with gillnets inside Lake Landvik and the connected fjord system was granted by the County Governor of Aust-Agder, Arendal, Norway. Our study did not involve any endangered or protected species.

2.2 Image and data analysis

A digital image of each otolith was captured using either a Leica M60 stereomicroscope with a Leica DFC450 camera and the software Leica Application Suite (LAS Version 4.5) (Leica Micro-systems, Wetzlar, Germany, www.leica-microsystems .com) or a Leica MZ95 stereomicroscope (Leica Micro-systems) with an Evolution LC-PL A662 camera (MediaCybernetics, Maryland, USA) using the software PixeLINK 3.2 (www.pixelink.com). All statistical analysis were conducted with R [56] using the R packages ade4 [57], shapeR [58] and vegan [59].

2.3 Visualizing the main shape features

The variation in otolith shape was examined by plotting the mean shape of each population using the shapeR package [58]. To inspect how the variation in the Wavelet coefficients is dependent on the position along the outline, the mean and standard deviation of the coefficients was plotted against the angle using the gplots package [60]. To quantify the differences among populations, the proportion of variation among groups (the intraclass correlation, ICC), was calculated along the outline of the otolith.

2.4 Multivariate analysis of shape

Wavelet coefficients, which represent the otolith shape, were obtained from the digital images using the shapeR package [58]. Temporal stability in otolith shape was analysed within sampling areas for the regions with more than one sampling year to see if it was possible to combine the samples (see Table 1) by applying Canonical Analysis of Principal coordinates (CAP) [61] and an ANOVA like permutation test to assess the significance of constraints using 2000 permutations with the vegan package in R [59]. Otolith shape was then compared among populations with overall tests and also by applying a priori comparisons to test for regional differences, also using the CAP and the ANOVA like permutation test and to evaluate differences between age classes and the interaction of age and geographic origin. Age is known to have confounding effects on otolith shape [62] and as interaction between age and geographic origin was significant the dataset was divided into three age groups: 3-5 years, 6-8 years, 9-12 years. Each group was adjusted for fish length and then analysed separately. The CAP values for each population at each age were adjusted by taking age as a covariate in the model. Variation for each age group (3-5, 6-8, 9-12 years) at each location was summarised by calculating the variance (Table 4) in distances among individuals within populations for each age group, high variation could result from admixture of populations or developmental variation. Ordination of the population averages along the first two canonical axes (CAP1 and CAP2) were examined graphically with the shape descriptors.

2.5 The association of shape and distance

To examine the association of otolith shape with respect to geographic distances between sampling areas, matrices with shape distances and geographical distances where calculated. Morphological distances were constructed based on average Euclidean distances based on otolith shape (CAP1 and CAP2) for each population, while the geographical distances between sampling areas where calculated by measuring the distance in km between areas along the coastline from Kragerø in southern Norway to Balsfjord in northern Norway. The association of the distance matrices were evaluated with Mantel tests with 10.000 permutations [63] using the ade4 package in R [57].

3 Results

3.1 Main shape features

Otolith shape differed among all of the populations in the study, mainly at the excisura major area (E), rostrum (R) and excisura minor (EM, Fig. 2) as also seen in the high proportion among groups (ICC) for these regions on the otolith outline at 0-20° (EM) and 170-190° (R and E, Fig. 3).

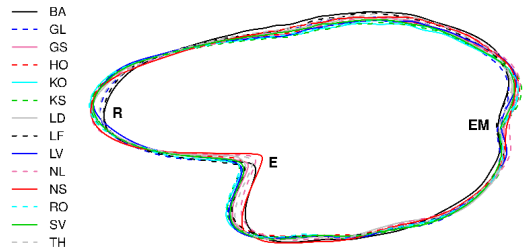


Figure 2. Average shape of all otoliths for fourteen herring populations in Norway. The areas are: BA: Balsfjord, GL: Gloppen, GS: Grimstad, HO: Hovåg, KO: Kragerø, KS: Kilsund, LD: Lindåspollene, LF: Lusterfjord, LV: Lake Landvik, NL: Lofoten, NS: Møre, RO: Risør, SV: Sykkulven and TH: Trondheim for three age groups (see Table 1 for further details). The excisura major (E), rostrum (R) and excisura minor (EM) are marked.

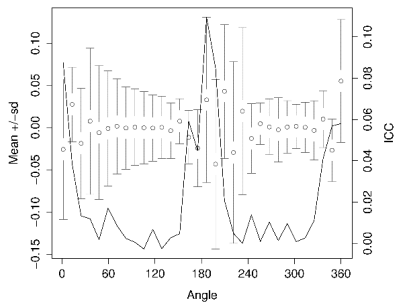


Figure 3. Mean and standard deviation (sd) of the Wavelet coefficients (grey) representing shape for all combined otoliths and the proportion of variance among herring populations or the intraclass correlation (ICC, black solid line). The horizontal axis shows angle in degrees (°) based on polar coordinates where the centroid of the otolith is the center point of the polar coordinates.

Table 1. Samples of Atlantic herring from fourteen stations (Area) shown clockwise from Kragerø in southern Norway to Balsfjord in northern Norway along the coast (see also Fig. 1). ID: Population abbreviation, Lat: latitude (N), Lon: longitude (E), N/W/S: N: populations in northern Norway, W: populations in western Norway, S: populations in southern Norway, System: type of habitat where the herring were sampled, Month: month of sampling, Year: sampling year, Spawn: some in spawning condition (+), none in spawning condition (-), Length: mean length in cm and length range for each age range 3-5 years, 6-8 years, 9-12 years, n: total number of samples for each age range, n_{tot}: total number of samples from each area. Empty cells indicate no data existed.

									Length			n			
Area	**ID**	**Lat**	**Lon**	**N/W/S**	**System**	**Month**	**Year**	**Spawn**	**3-5y**	**6-8y**	**9-12y**	**3-5y**	**6-8y**	**9-12y**	n_{tot}
Kragerø	KO	58.88	9.43	S	Fjord	Mar	2006	+	28.1 [24.5-32.0]	30.1 [27.0-32.5]		42	38		80
Risør	RO	58.73	9.24	S	Fjord	Nov	2005, 2006	+	28.4 [23.0-31.5]			60			60
Kilsund	KS	58.55	8.98	S	Fjord	Jan	2012	+	27.8 [26.0-30.0]			32			32
Lake Landvik	LV	58.33	8.50	S	Lake	June	-	+	26.9 [24.0-30.5]	28.9 [28.0-30.0]	29.8 [28.0-31.5]	132	8	20	160
Grimstad	GS	58.28	8.52	S	Fjord	Feb-May	-	+	28.1 [23-32.5]	31.1 [25.0-34.0]	31.8 [29.0-34.0]	290	66	27	383
Høvåg	HO	58.17	8.25	S	Fjord	Feb	-	+	28.9 [27-31.5]	31.6 [29.5-37.0]	32.7 [31.5-34.5]	15	19	14	48
Lindås	LD	60.73	5.15	W	Fjord	Mar	2010	+	30.0 [28.0-32.5]	32.4 [31.0-34.5]	32.6 [31.0-36.0]	3	10	27	40
Lusterfjord	LF	61.44	7.48	W	Fjord	Nov	2011	-	18.4 [16.0-22.5]	19.5 [19.5-19.5]		89	1		90
Gloppen	GL	61.80	6.12	W	Fjord	Feb	2009, 2010, 2012, 2013	+	22.0 [19.5-24.5]	22.6 [20.5-26.5]	23.8 [21.0-26.0]	34	50	9	93
Møre	NS	62.52	5.23	W	Ocean	Feb	2010	+	30.6 [29.0-32.5]	32.6 [29.0-34.5]		8	78		86
Sykkulven	SV	62.56	7.64	W	Fjord	Nov	2012, 2013	-	27.7 [25.0-33.0]	28.7 [27.5-30.0]	28.0 [28-28]	42	19	1	62
Trondheim	TH	63.47	10.75	W	Fjord	Mar	2010	-	27.1 [23.0-30.0]	26.7 [25.0-28.0]	27.5 [26-30]	8	19	64	91
Lofoten	NL	68.06	13.60	N	Ocean	Aug	-	+	30.6 [27.0-34.5]	33.6 [31.5-36.0]		17	16		33
Balsfjord	BA	69.27	19.35	N	Fjord	Apr	2012, 2014	+	21.8 [17.5-26.5]	26.0 [24.5-27.5]		57	26		83

3.2 Multivariate analysis of otolith shape

Samples obtained from two or more years from the same area did not differ in otolith shape (p>0.05, Table 2) and were therefore pooled. Variation decreased on average with age as analysed with linear regression among populations (b = -0.25, p=6.5x10^{-5}) (Table 4). No interactions were observed for age and populations within age classes 3-5 years, 6-8 years and 9-12 years (p>0.05), however age significant within all three age classes (p<0.05). Significant differences in otolith shape were detected among all herring populations at ages 3-5, 6-8 and 9-12 years (p<0.001, Table 3), although the differences among populations decreased with age as seen with lower F-values (Table 3) and lower CAP values for the older ages (Fig. 4 b-c).

Examining the position of the populations based on shape variation along the first Canonical axis (Fig. 4 a-c), for ages 3-5 years, a pattern emerged with three clusters: the two oceanic populations, Norwegian spring- and autumn spawners, group together (Fig. 4a), Sykkulven from western Norway groups with the populations in southern Norway (Grimstad, Høvåg, Kragerø, Kilsund, Lake Landvik and Risør) while the two populations which occupy the deepest fjords in the study (Lusterfjord and Gloppen) group together. Balsfjord, from the most northerly location, is separate from the rest of the populations. For ages 6-8 years, a similar pattern was observed where the populations from southern Norway (Grimstad, Høvåg, Kragerø, Lake Landvik) group with Lindåspollene from western Norway, the Norwegian spring-spawners and Trondheim which occupy similar latitudes in western Norway group together, while populations from Sykkulven, Gloppen and the Norwegian autumn-spawners seem diverged from the rest. Balsfjord again is quite distinct from the rest as was seen for ages 3-5 years. For ages 9-12 years, populations Grimstad, Høvåg group together, Lake Landvik is rather close along the first axis, while populations Gloppen, Lindåspollene and Trondheim show no sign of grouping and are quite distinct from the other populations.

These results are in accordance with the a priori comparisons (Table 3) where significant differences where found for 3-5 years and 6-8 years in a comparison of Balsfjord vs fjord populations (p<0.001), between populations occupying western and southern Norway for all age groups and also within western Norway (p<0.001). Comparing populations within southern Norway at ages 3-5 and 6-8 years, significant differences in shape where found (p<0.008), while at ages 9-12 populations did not differ (p>0.05).

Table 2. Temporal stability in otolith shape among populations with more than one sampling year. Results from ANOVA like permutation tests based on 2000 permutations, df: degrees of freedom, Var: variance, F: F-value, P: p-value, p<0.05 indicates a significant effect. See Table 1 for further details on the populations.

Area	Df	Var	F	P
Balsfjord	1	1.20	1.54	0.149
Gloppen	3	5.28	1.66	0.051
Risør	1	1.733	1.67	0.114
Sykkulven	1	1.99	1.54	0.152

Table 3. Otolith shape compared among all herring populations in the present study. Results from ANOVA like permutation tests based on 2000 permutations, df: degrees of freedom, Var: variance, F: F-value, P: p-value, p<0.05 indicates a significant effect. Results for the three age groups 3-5 years, 6-8 years and 9-12 years are shown separately. See Fig. 1 and Table 1 for population ID codes. Empty cells indicate data did not exist for these comparisons.

	3-5 years				6-8 years				9-12 years			
	df	Var	F	P	df	Var	F	P	df	Var	F	P
All populations	11	68.25	8.47	0.001	10	0.16	6.43	0.001	5	0.21	5.40	0.001
BA vs rest	1	16.66	21.53	0.001	1	0.05	13.43	0.001				
W vs S-Nor	1	15.00	19.49	0.001	1	0.03	7.98	0.001	1	0.06	7.40	0.001
Within W-Nor	2	8.13	5.90	0.001	2	7.55	5.39	0.001	1	4.82	5.30	0.001
Within S-Nor	5	15.71	4.16	0.001	3	0.05	2.26	0.008	2	0.07	1.36	0.16
NL vs fjord p.	1	7.15	8.95	0.001	1	0.01	4.05	0.003				
NS vs fjord p.	1	5.42	6.77	0.001	1	0.05	16.50	0.001				
Residual	860	590.66			338	0.86			155	1.18		

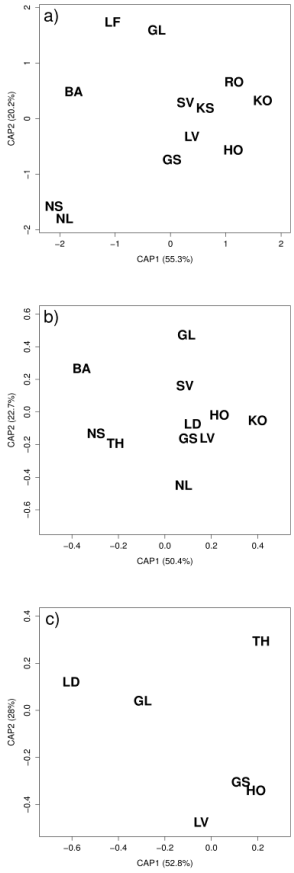


Figure 4. Canonical scores on discriminating axes 1 (CAP1) and 2 (CAP2) for each herring population. BA: Balsfjord, GL: Gloppen, GS: Grimstad, HO: Hovåg, KO: Kragerø, KS: Kilsund, LD: Lindåspollene, LF: Lusterfjord, LV: Lake Landvik, NL: Lofoten, NS: Møre, RO: Risør, SV: Sykkulven and TH: Trondheim in Norway for three age groups: a) 3-5, b) 6-8 and c) 9-12 years (see Table 1 for further details). Black letters represent the mean canonical value for each population, and scores on x- and y-axis show the canonical values which are based on the otolith shape differences among population.

The two oceanic populations, the Norwegian spring- and autumn-spawners, differed each from the fjord populations, both at ages 3-5 and 6-8 (p<0.003).

3.3 Otolith shape and geographical distance

There was a latitudinal gradient along the coastline in otolith shape of the studied populations. Populations found in habitats geographically close to each other were more similar in otolith shape than populations further apart (Fig 5 a-c, $r_{3\text{-}5y}$=0.44, $r_{6\text{-}8y}$=0.66, $r_{9\text{-}12y}$=0.57, p<0.001 for all comparisons based on 10.000 permutations). A few population pairs differed from the overall trend expected by the geographical distance. The oceanic populations were more similar to each other at ages 3-5 years than at the other ages (Fig. 5a). One population from western Norway (Sykkulven), showed similarities with one population from southern Norway (Kilsund) and both these populations had large variance within populations (Table 4).

Table 4. Variance within each population for the three age groups 3-5 years, 6-8 years and 9-12 years shown along the Norwegian coast from south (Kragerø) to north (Balsfjord). Empty cells refer to missing observations.

Area	ID	3-5y	6-8y	9-12y
Kragerø	KO	17.54	5.20	
Risør	RO	19.09		
Kilsund	KS	39.87		
Lake Landvik	LV	17.63	0.67	19.67
Grimstad	GS	20.35	10.07	16.23
Høvåg	HO	18.72	9.79	7.37
Lindåspollene	LD		0.84	19.37
Lusterfjord	LF	12.26		
Gloppen	GL	13.69	5.25	0.67
Møre	NS	14.44	6.45	
Sykkulven	SV	31.51	1.45	
Trondheim	TH		0.25	16.45
Lofoten	NL	32.71	5.99	
Balsfjord	BA	18.04	3.70	

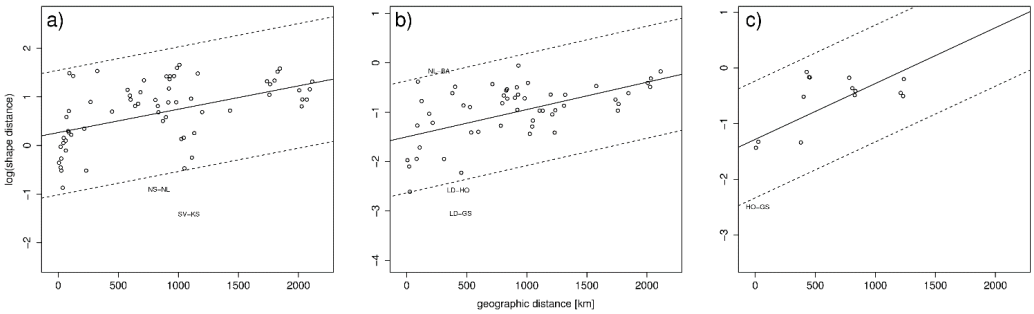
For the age group 6-8 years, Lindåspollene from western Norway showed similarities with Høvåg and Grimstad from southern Norway (Fig. 5b), but Lindåspollene had considerably low sample size at these ages. At the same ages, the neighbouring populations, the Norwegian autumn-spawners and Balsfjord in northern Norway deviated more from each other, when considering the geographic distance, than all pairs from ages 6-8 years. No obvious trend was observed at ages 9-12 years, which might be due to low samples sizes (Fig. 5c).

4 Discussion

Otolith shape analysis of Atlantic herring in Norwegian waters showed significant variation among the locations studied. In addition, isolation by distance emerged with a latitudinal gradient along the coastline. These morphological differences indicate low dispersal and support even a reproductive isolation among the local herring populations [52]. Our results suggest that the semi-enclosed systems, where the local populations live and breed, are efficient barriers for dispersal, which has resulted in diversification of the local fjord populations.

The significant differences in otolith shape points to limited exchange between the local populations and their oceanic counterparts, but to what degree the oceanic populations interbreed with the local populations is not fully known. The oceanic Norwegian spring-spawners have been found to spawn in the same area as Lindåspollene herring for 50 years and to alter the life-history of the resident population [7], but their otolith shape differs. This observed variation between the oceanic and local populations might be due to the environmental differences encountered by the populations during early life. While the local populations are refined in semi-enclosed ecosystems and exhibit relatively stable local conditions, the juveniles of the oceanic populations, which are recruited along the central Norwegian continental shelf, show growth similar to northern populations as they exhibit less growth

Figure 5. The association of otolith shape with respect to geographic distances in km between sampling areas from Kragerø in southern Norway to Balsfjord in northern Norway. The age groups are: a) 3-5, b) 6-8 and c) 9-12 years. The correlation of the shape distances with geographical distances was for the three age classes: $r_{3\text{-}5y}$=0.44, $r_{6\text{-}8y}$=0.66, $r_{9\text{-}12y}$=0.57, with p<0.001 in all cases, based on 10.000 permutations. A trend line based on linear regression is shown, dotted lines represents two standard deviations of the residuals from the regression line. Population pairs which distances fall outside of the two standard deviations are presented (see Area ID codes in Table 1).



with decreasing temperature and increasing latitude as they are carried northwards with the coastal current into the Barents Sea [36-39]. Variation in growth rates can cause otolith increments to be deposited differently, where faster growth enhances ring deposition and slower growth results in fewer rings, which affects the otolith structure [64-67]. It is therefore likely that differing growth rates are contributing to the shape differences observed among the local populations and the oceanic populations. Local populations occupying southern and western Norway were more similar in otolith shape to their neighbouring populations than to the more distant populations. This was observed for all the three age intervals tested, even though the number of samples from the oldest age class was limited. Balsfjord herring, from the most northerly location (69°N), was most different in otolith shape compared to the other local populations. Balsfjord herring is likely to be an outlier in our analysis, not only with regards to their geographic position, but also given their genetic similarity with Pacific herring, based on mtDNA [32,53,68]. Balsfjord herring has also been shown to be more similar to Pacific herring in vertebrae number, spawning behaviour [17] and otolith shape [69] than to both local and oceanic Atlantic herring [17,53]. The oceanic populations, the Norwegian spring- and autumn-spawners, were considerably different in otolith shape compared to the other populations, which might be attributed to their higher dispersal capacity compared to the local populations. At the younger ages (3-5 years, Fig. 4a), the oceanic populations group together but they become different at older ages (6-8 years, Fig. 4b) as previously reported [8].

Deviations from the overall trend include the variability in the results between the 3-5 year olds and the 6-8 years olds as well as the similarity in otolith shape of the population from Sykkulven from western Norway and Kilsund from southern Norway, and Lindås grouping both with Høvåg and Grimstad for ages 6-8 years, and Balsfjord grouping with the Norwegian autumn-spawners from Lofoten. To which extent the overall trend and these deviations can be explained by the particular characteristics of the different populations is unclear. It might be linked to the temperature differences found along the latitudinal gradient along the Norwegian coast [38], or it might be linked to actual different life history strategies as seen in the growth (length-at-age and asymptotic length), maturity ogives and reproductive effort of these local populations (Table 1) [7,21,23,24,26,27,29,33,34]. In general, fish populations are known to be differently constrained by survival and reproduction trade-offs [70], and differ in size at maturity directly influencing the populations growth rates [71]. Also, otolith shape might be influenced by differing food rations [72]. Hence, the observed deviations and variance at particular age groups may result from a single or combined effects of food limitations or temperature differences, even though they may reach their maximum length asymptotically at different ages.

Modifications of the mean otolith shape were detected and differed among populations at three main positions, the excisura major, rostrum and the excisura minor (Fig. 2). An interesting pattern emerged at the excisura major area, moving from the middle of the otolith and outwards, where the Norwegian spring-spawners had the inner most shape which is in line with formers studies both from the Northeast Atlantic [8] and the Landvik region in southern Norway [26]. Next to the Norwegian spring-spawners was the other oceanic population in the study, the Norwegian autumn-spawners from Lofoten, then Trondheim herring and Balsfjord herring. Both at the rostrum and the excisura minor area the same pattern was seen, where Balsfjord herring had the inner most shape, next Lusterfjord and then Gloppen. These populations have in common a considerably shorter body length due to slower growth rates for herring which grow up within the fjord ecosystem [21,30,31] (Table 1), which could be contributing to these differences. Herring populations west of the British Isles which also mature at a younger age, show considerable size differences and differing growth rates in comparison to the populations in the northern part of the NE-Atlantic [73] and variation in otolith shape [8]. As mentioned, the growth rate differences among these populations might be contributing to the shape differences observed [64-67].

The multivariate analysis showed temporal stability in otolith shape among the populations with more than one sampling year from Balsfjord, Gloppen, Risør and Sykkulven. To our knowledge, this study is the first to report temporal stability in otolith shape among herring populations, further proving the usefulness of otolith shape as a marker for population discrimination of herring [8].

For pelagic species with high gene flow, the present results emphasize the importance of not only focusing on genetic variability but also to take into account the identification of phenotypic stocks to ensure sustainable fisheries and conservation of the species. Several of the smaller local populations observed have unique life history characteristics [7,21,23,24,26,27,29,33,34] and therefore differ in their response to exploitation, which needs careful consideration in order to maintain biological diversity of the species.

ACKNOWLEDGMENTS

We thank Knut Hansen at IMR Flødevigen for his involvement with data from southern Norway, sampling, biological analyses and photographing otoliths and also several of the technicians at the IMR for their help in providing biological data and otolith material for the populations in northern Norway. This work was funded by the Assistant teacher's grant of the University of Iceland.

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Paper VI

Classifying Pacific herring (*Clupea pallasii*) subspecies based on otolith shape

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Authors contribution: Conceived and designed the experiments: LAL, SP. Analysed the data: LAL, SP. Contributed reagents/materials/analysis tools: LAL, AS, EOO, SP. Wrote the paper: LAL, SP. Reviewed the manuscript: LAL, SP, AS, EOO

Classifying Pacific herring (*Clupea pallasii*) subspecies based on otolith shape

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Abstract — Otolith shape variation was compared within Pacific herring (*Clupea pallasii*) from the Atlantic, Pacific and Barents Sea, and also with the Atlantic herring (*Clupea harengus*) from western Norway. A clear difference in otolith shape was observed between the genetically differentiated herring species *C. harengus* from the Atlantic and *C. pallasii* from the Pacific, Balsfjord in N-Norway and its subspecies *C. pallasii suworowi* from the Barents Sea. Contrasting regional differences in otolith shape, variation was detected between the *C. pallasii* in N-Norway and *C. p. suworowi* in the Barents Sea and in a comparison between the subspecies *C. p. pallasii* from the Pacific with *C. p. suworowi* in the Barents Sea, which supports the results of genetic studies. Populations of *C. p. pallasii,* sampled east and west of the Alaska Peninsula, which belong to two genetically different clades of the *C. p. pallasii* in the Pacific Ocean, show a clear difference in otolith shape. *C. p. suworowi* and the local *C. pallasii* peripheral population in Balsfjord in N-Norway, are more similar to the NW-Pacific herring (*C. p. pallasii*) than to NE-Pacific herring (*C. p. pallasii*), both genetically and in otolith shape. The Balsfjord population, known to be influenced by introgression of mtDNA from the Atlantic herring does not show any sign of admixture in otolith shape between the two species. A revised classification, considering the observed genetic and morphological evidence, should rather distinguish the NW-Pacific herring in the Bering Sea together with the European populations of *C. pallasii* than with the NE-Pacific herring in the Gulf of Alaska.

Keywords — Pacific herring, Atlantic herring, subspecies, trans-Arctic species, otolith shape

1 INTRODUCTION

Three allopatric species are found within the genus Clupea: Atlantic herring (*Clupea harengus* Linnaeus 1758) distributed throughout the North Atlantic, Pacific herring (*C. pallasii* Valenciennes 1847) with a wide distribution in the North Pacific Ocean, Barents Sea and west to Balsfjord, N-Norway, and the Chilean herring (*C. bentincki* Norman 1936), also known as Araucanian herring, occupying the waters off the west coast of South America. A large variation has been described for *C. pallasii* with three subspecies, the nominate subspecies *C. p. pallasii* in the Pacific, the White Sea herring (*C. pallasii marisalbi* Berg 1923), and the Chesha-Pechora herring (*C. pallasii suworowi* Rabinerson 1927) of the SE-Barents and Kara Seas. The European populations of *C. pallasii* are thought to be early post-glacial colonists from the NW-Pacific (Laakkonen et al. 2013).

The Alaska Peninsula separates the Bering Sea from the NE-Pacific Ocean (Gulf of Alaska) and is an obstacle for marine fauna and connectivity of populations. Genetic divergence in mtDNA and microsatellites has been detected between herring occupying each side of the Alaska Peninsula (O'Connell et al. 1998; Liu et al. 2012). The divergence between the herring in the NW-Pacific and Barents Sea is recent or even after the Weichselian glacial times (Laakkonen et al. 2013), and signs of mixing have been reported to have occurred during the comparatively warm years of the 1930s-1940s at several Arctic Siberian sites (Svetovidov 1952). Analysis of mtDNA variation by Laakkonen et al (2013) on European C. *pallasii,* showed that the European samples clustered within the NW-Pacific lineage ("the trans-Arctic group"). Laakkonen et al (2013) also identified three phylogeographic groups within the European *C. pallasii* characterized by low genetic variation possibly reflecting a colonization of a small group of the Pacific herring: herring in the White Sea, herring in the Pechora Sea east of the White Sea and a strongly bottlenecked peripheral population in Balsfjord in N-Norway. A mixture of local Balsfjord herring and the highly migratory Norwegian spring-spawners based on allozymes and mitochondrial markers has also been observed (Jørstad and Pedersen 1986). Mitochondrial and nuclear introgression has occurred from Atlantic herring into Pacific herring in N-Norway, where 21% of the *C. pallasii* individuals in Balsfjord had variants of mtDNA from Atlantic herring (Laakkonen et al. 2015). Also, a genetic difference was observed between herring in the White Sea versus herring in the Barents and Kara Seas (Semenova et al. 2015). Atlantic herring has been reported to penetrate the Barents Sea from the west, although they have not been found spawning there (Svetovidov 1952; Jørstad 2004).

Otolith shape is a population marker for Atlantic herring (Libungan et al. 2015) and variation in the shape is thought to reflect the developmental conditions during early life (Geffen 1982). For Atlantic herring, it has been shown that populations which spawn at different times of the year and thus experience different conditions during early developmental stages differ in otolith shape (Libungan et al. 2015), despite lack of detectable genetic differentiation (Pampoulie et al. 2015). Otolith shape in Atlantic herring has furthermore been shown to vary among fjord populations along the coast of Norway where neighbouring populations

are more similar in shape than populations separated by larger distances (Libungan et al. *in review*), suggesting that there might also be a genetic basis for the differentiation.

The purpose of this study was to investigate whether the variation in otolith shape of herring in Balsfjord in N-Norway and SE-Barents Sea reflect their taxonomic classification into subspecies or the genetic affinities to the Pacific herring and the split between NW- and NE-Pacific. Furthermore we evaluate whether any signs of hybridization is detectable in the Balsfjord population.

2 MATERIALS AND METHODS

2.1 Sampling

Herring were sampled during the period of 1996-2014 with purse-seiners from Alaska and Møre in W-Norway and research trawl vessels in Balsfjord and the southeast SE-Barents Sea (Fig. 1, Table 1). Sampling areas and time of sampling were selected based on knowledge of spawning behaviour of the *C. harengus* and *C. pallasii* at each location, ensuring individuals sampled belonged to the spawning stock of that site, with the exception of sampling years 2005 and 2006 for Barents Sea herring, which were not sampled during their spawning season (Table 1). Balsfjord herring (*C. pallasii*) were sampled in Balsfjord and distinguished from possible mixture of Norwegian spring-spawning herring based on allozymes according to Jørstad et al (1991). Total length (cm) was recorded for each fish and maturity stage according to an 8-point scale: immature = 1 and 2, maturing = 3 to 5, running/spawning = 6, spent = 7, recovering/resting = 8 (Mjanger et al. 2011). The sagittal otoliths were washed in clean water and stored in paper bags. All fish were aged from their scales using standard ageing techniques.

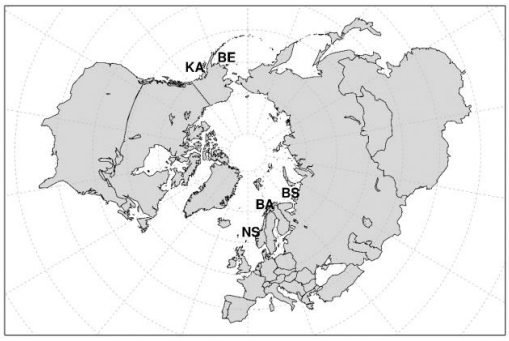

Figure 1. Sampling areas of Atlantic and Pacific herring analysed for variation in otolith shape. NS: Norwegian spring-spawning Atlantic herring and BA: Balsfjord, Norway, an admixture zone of both species, and Pacific herring from BS: SE-Barents Sea, BE: Bering Sea and KA: Kamishak both in Alaska, USA. See further in Table 1.

2.2 Image and data analysis

A digital image of each otolith was captured using either a Leica M60 stereomicroscope with a Leica DFC450 camera and the software Leica Application Suite (LAS Version 4.5) (Leica Microsystems, Wetzlar, Germany, http://www. leica-microsystems.com) and or a Leica MZ95 stereomicroscope (Leica Micro-systems, Wetzlar, Germany) with an Evolution LC-PL A662 camera (MediaCybernetics, Maryland, USA) using the software PixeLINK 3.2 (www.pixelink.com). All statistical analysis were conducted with R (R Core Team 2015) using the R packages ade4 (Dray and Dufour 2007), shapeR (Libungan and Pálsson 2015) and vegan (Oksanen et al. 2013).

2.3 Shape analysis

The variation in otolith shape was examined by plotting the mean shape of each population using the shapeR package (Libungan and Pálsson 2015). To inspect how the variation in the Wavelet coeffi cients, the mean and standard deviation of the coefficients was plotted against the angle (Fig. 4) using plotCI from the gplots package (Warnes et al. 2014). The proportion of variation among groups along the outline was summarized with intraclass correlation (ICC). The Wavelet coefficients, which represent the otolith shape, were obtained from the digital images and scaled for fish length also using the shapeR package (Libungan and Pálsson 2015). Temporal stability in otolith shape was analysed within the Barents Sea sample since there existed samples from three years (see Table 1) by applying Canonical Analysis of Principal coordinates (CAP) (Anderson and Willis 2003) and an ANOVA like permutation test to assess the significance of constraints using 2000

Table 1. Samples of Atlantic and Pacific herring (see also Fig. 1). Area, location: sampling sites, Date: date of sampling, Lat: Latitude (N: north), Lon: Longitude (E: east, W: west), *n*: number of samples, ID: area abbreviation.

Area, location	Date	Lat (N)	Lon (E,W)	*n*	ID
Bering Sea					
Kuskokwin Bay	09, 06, 2006	60°23.0'	165°45.0' (W)	36	BE
Gulf of Alaska					
Kamishak	01, 05, 2014	59°12.0'	154°01.0' (W)	59	KA
Norway					
Balsfjord	08, 08, 2012	69°22.1'	19°15.7' (E)	8	BA
-	23, 01, 2014	69°30.7'	19°36.7'	5	
-	10, 03, 2014	69°52.1'	18°97.5'	9	BA
-	11, 03, 2014	69°30.6'	19°36.6'	16	-
-	23, 04, 2014	69°25.6'	19°28.6'	45	-
Møre	14, 02, 2010	62°51.6'	5°23.3'	12	NS
-	19, 02, 2010	61°88.3'	4°58.3'	19	-
-	24, 02, 2010	62°53.3'	5°20.0'	23	-
-	24, 02, 2010	62°53.3'	5°25.0'	29	-
Barents Sea					
SE-Barents Sea	10, 06, 1996	68°85.8'	45°50.0'	57	BS1
-	11, 06, 1996	69°26.3'	51°83.2'	35	-
-	11, 06, 1996	70°00.0'	46°85.0'	11	-
SE-Barents Sea[a]	19, 02, 2005	71°04.2'	47°05.0'	30	BS2
SE-Barents Sea[a]	19, 02, 2006	70°12.5'	43°59.5'	28	-
SE-Barents Sea[a]	22, 02, 2006	72°13.8'	47°65.5'	14	-

[a]Non-spawning herring

permutations with the vegan package in R (Oksanen et al. 2013). Otolith shape was then compared among populations with an overall test and also by applying comparisons between all populations to test for regional differences, using the CAP and the ANOVA like permutation test. Same analyses were used to evaluate differences between age classes and the interaction of age and geographic origin since age is known to have confounding effects on otolith shape (Castonguay et al. 1991).

Ordination of the population averages along the first two canonical axes (CAP1 and CAP2) were examined graphically with the shape descriptors. Variance within locations was calculated on the shape distances (CAP1 and CAP2) between each individual within each area (Table 1). To compare the fit of the otolith shape variation to the previous taxonomic classification and to the divergence observed by genetic analyses the CAP was conducted by partitioning the variation with respect to classification based firstly on the taxonomic split of species: the Norwegian spring-spawners (*C. harengus*) with herring populations within *C. pallasii*) and secondly between the two subspecies *C. p. pallasii* in the Pacific (Kamishak and Bering Sea herring) with *C. p. suworowi* in the SE-Barents Sea. Thirdly, the NE-Pacific herring (*C. p. pallasii* from Kamishak in the Gulf of Alaska) was compared with the trans-Arctic group as described by Laakkonen et al (2013), comprised of the Bering Sea herring (*C. p. pallasii*) in the NE-Pacific and Bare ts Sea herring (*C. p. suworowi*) in Russia. Lastly, the Balsfjord herring (*C. pallasii*) in N-Norway, known to have introgressed genetic markers from *C. harengus* was compared to its neighbouring populations from *C. harengus* in Norway (NS) and *C. p. suworowi* from the Barents Sea (BS). Euclidean distances were calculated between the coordinates of the averages of the different population samples for the first four axis, weighted by the contribution of each axis to the overall variation and presented with boxplots.

3 RESULTS

3.1 Main shape features

Otolith shape differed among all populations in the study, mainly at the excisura major area (Bird et al. 1986), rostrum, excisura minor and postrostrum (Fig. 2) which was further confirmed by examining variability in the mean Wavelet coefficients and the variation among groups with intraclass correlation (Fig. 3). The area on the outline marked X (at angle ~120°) corresponds to the area showing the highest proportion of variance among populations (Fig. 2 and 3). The population from the NE-Pacific (Kamishak in the Gulf of Alaska) showed a clear separation from all other populations at the excisura major area (Fig. 2).

3.2 Multivariate analysis of otolith shape

No differences in otolith shape were detected within areas with more than one sampling event (p>0.12) with the exception of the Barents Sea sample and samples were thus pooled (Table 1). The samples from the Barents Sea were from three sampling years (1996, 2005 and 2006) the samples from years 2005 and 2006 were similar (p=0.35) and were therefore pooled, however significant differences were observed between the 1996 sample and the 2005 and 2006 samples pooled (p=0.007). The samples from the Barents Sea were therefore divided into two samples, with BS1 representing the 1996 year sample and BS2 representing the 2005 and 2006 samples (Table 1). No interactions were observed for age and populations in an overall test for ages 3-8 years (p=0.82), and samples were thus pooled for those ages and used in all comparisons. Age was not a significant factor and therefore excluded from the model (p=0.49). Significant differences in otolith shape were observed among populations and in tests contrasting different regions (p=5x10^{-4}, Table 2). Also, significant differences (p=5x10^{-4}) were found between all population pairs in the study, even after applying a Bonferroni correction for multiple comparisons (p adjusted=0.008).

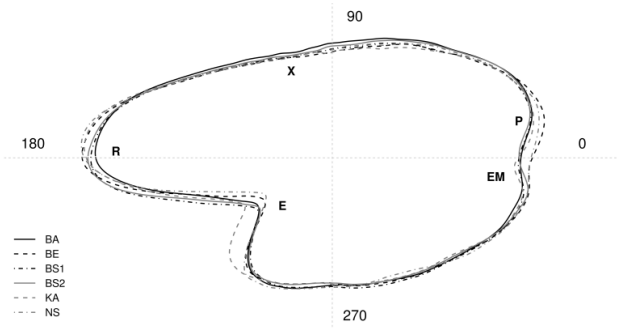

Figure 2. Average shape of otoliths for the five sampling areas in the study. From Norway: Balsfjord (BA) and Møre (NS), from Russia: Barents Sea (BS1, BS2) and USA: Alaska (Bering Sea (BE) and Kamishak (KA). The most variable areas on the otolith outline, excisura major (E), rostrum (R), excisura minor (EM) and postrostrum (P) are marked. The numbers 0, 90, 180 and 270 represent angles (in degrees) on the outline which correspond to Fig. 3. The area on the outline marked X (at angle ~120°) corresponds to the area showing the highest proportion of variance among populations (see Fig. 3).

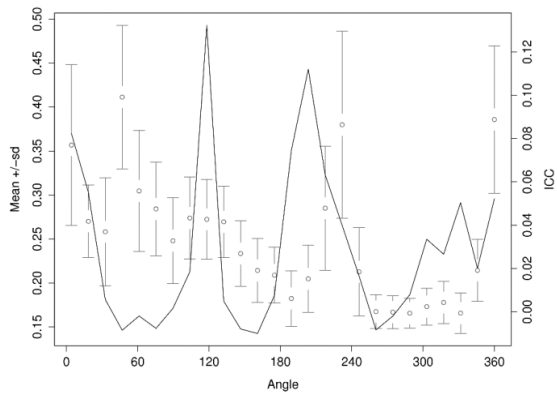

Figure 3. Mean and standard deviation (sd) of the Wavelet coefficients (grey) for all combined otoliths and the proportion of variance among groups or the intraclass correlation (ICC, black solid line). The horizontal axis shows angle in degrees (°) based on polar coordinates where the centroid of the otolith is the center point of the polar coordinates.

Examining the Canonical scores for the populations revealed the largest differences between species (Fig. 4). Barents Sea and Bering Sea herring were similar in otolith shape, although statistically different and showed similarity with Balsfjord herring along the first axis (Fig. 4a), and were intermediate between the distinct Norwegian spring-spawning herring from Møre and the herring from Kamishak in the Gulf of Alaska. The first two Canonical axis explained most of the variation between populations (CAP 1: 57.5%, CAP1: 21.5%) but the third and fourth axis also contributed to the differences observed (CAP3: 12.9% and CAP4: 7.2%). The CAP1 and the CAP3 scores (Fig. 4b) showed that Balsfjord herring were intermediate in shape between the Norwegian spring-spawners and the Pacific herring from the other samples of the trans-Arctic group (Barents Sea and Bering Sea). Otherwise, a similar pattern was observed as with CAP1 and CAP2 (Fig. 4a). The canonical distances representing shape differences between populations showed that the variation in otolith shape between species (*C. harengus* vs. *C. pallasii*) was large but similar differentiation was observed between *C. p. pallasii* in the NE-Pacific (Kamishak in the Gulf of Alaska) and the transarctic group (Bering Sea, Barents Sea and Balsfjord) (Fig. 5). Balsfjord herring (*C. pallasii*) in N-Norway, in comparison with all other *C. pallasii* populations (Barents Sea, Bering Sea, Kamishak in the Gulf of Alaska) revealed large differences, while similar shape was observed among subspecies occupying the Barents Sea (*C. p. suworowi*) and around Alaska (*C. p. pallasii*). The lowest variation among samples was observed within the trans-Arctic group as described by Laakkonen et al (2013). Within group variance based on shape distances between individuals revealed the highest values for Norwegian spring-spawning herring (1.02) and second highest for the population in the NE-Pacific from Kamishak (0.50). For the other populations the values were: Barents Sea (BS1) = 0.43, Balsfjord = 0.29, Bering Sea = 0.27 and Barents Sea (BS2) = 0.21.

Table 2. Otolith shape compared among herring samples in the present study, between species *Clupea harengus* and *Clupea pallasii*, subspecies of *C. p. pallasii* and *C. p. suworowi* and the genetically distinct groups within *C. pallasii*. Results from ANOVA like permutation tests based on 2000 permutations. Df: degrees of freedom, Var: variance, *F*: *F*-value. All tests were highly significant with *p*-values $5x10^{-4}$. See Table 1 for population ID codes.

	df	Var	*F*
All populations	5	0.22	24.03
Residual	433		
Between species			
NS vs (BA+BS1+BS2+BE+KA)	1	0.11	53.83
Residual	437	0.89	
Between subspecies			
KA+BE vs BS1+BS2	1	15.16	21.31
Residual	268	190.67	
Between NE-Pacific and the trans-Arctic lineages			
KA vs (BE+BS1+BS2+BA)	1	19.50	27.17
Residual	378	251.92	

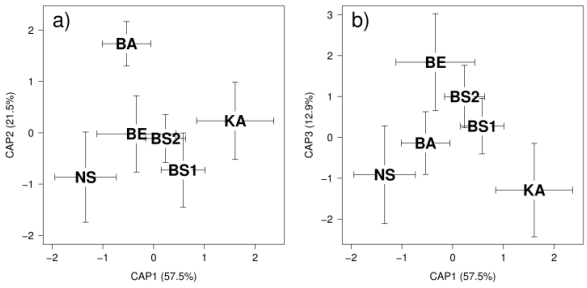

Figure 4. Canonical scores on discriminating axes a) 1 and 2 and b) 1 and 3 for each herring group. The first axis contributed most to the variation observed among the species/populations (57.5%), while the second axis explained 21.5% and third 12.9%. From Norway: Balsfjord (BA) and Møre (NS), from Russia (BS1, BS2) and Alaska USA (BE, KA) (see further details in Table 1). Black letters represent the mean canonical value for each herring population. Intervals represent means ± SE.

4 Discussion

The results of this study showed that otolith shape differed among the Atlantic and Pacific herring species and variation among the species was larger than within Pacific herring. The *C. pallasii* herring occupying Balsfjord in N-Norway, *C. pallasii suworowi* in the Barents Sea and *C. p. pallasii* from the Bering Sea in the NW-Pacific are more similar to each other than to *C. p. pallasii* in the Gulf of Alaska in the NE-Pacific. These results are in accordance with previous studies based on genetic variation (Jørstad and Nævdal 1981; Jørstad and Pedersen 1986; Laakkonen et al. 2013; Laakkonen et al. 2015). The Bering Sea herring and the European branch of the Pacific herring are intermediate between the Atlantic herring and the Pacific herring from the Gulf of Alaska.

Different patterns were observed in the mean otolith shape for the herring populations than in previous studies on Atlantic herring (Eggers et al. 2014; Libungan et al. 2015). At the excisura major area, around the 200° angle, which had the largest variation among Atlantic herring populations (Libungan et al. 2015), the Norwegian spring-spawners at Møre had the inner most shape (closest to the center of the otolith) in this study. A very distinct pattern at the excisura major area, with the outer most shape was observed in the Kamishak population which occupies the Gulf of Alaska. The intraclass correlation, which shows the proportion of variation among populations along the outline, was largest around the 120° angle on the otolith outline (Fig. 2 and 3). The Atlantic and Pacific herring exhibit differences in other areas on the otolith outline than previous comparisons have shown for Atlantic herring (Eggers et al. 2014; Libungan et al. 2015).

The samples from the Barents Sea (*C. p. suworowi*) were sampled in different times of the year, the 1996 sample in June and the 2005-2006 samples were both from February. Shape differences were detected in a comparison between the 1996 sample and the 2005-2006 samples pooled. SE-Barents Sea herring have been reported to spawn on average in July (Semenova et al. 2015). Herring occupying nearby oceans, from the White Sea (*C. p. marisalbi*), southwest of the sampling area in the Barents Sea spawns in spring/early summer in March-June (Semenova et al. 2013; Semenova et al. 2015), while herring occupying the Kara Sea (*C. p.*

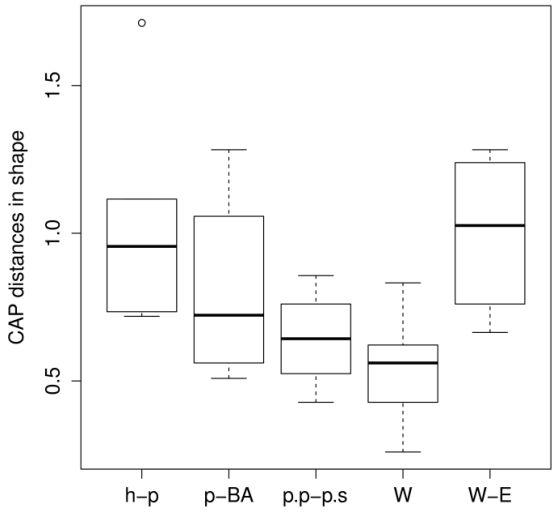

Figure 5. Boxplots of Canonical score distances (see also Fig. 4) with respect to variation among species and subspecies. The comparisons are h-p: *C. harengus* vs *C. pallasii*, p-BA: Balsfjord herring (*C. pallasii*) in N-Norway, in comparison with all other *C. pallasii* populations (Barents Sea, Bering Sea, Kamishak in the Gulf of Alaska). p.p-p.s: *C. p. pallasii* from the Pacific (Kamishak and Bering Sea) vs. *C. pallasii suworowi* from the Barents Sea. W: comparisons within the trans-Arctic group (Laakkonen et al 2013) including the Bering Sea herring *C. p. pallasii*, the Barents Sea herring (*C. p. suworowi*) and Balsfjord herring (*C. pallasii*). W-E: comparisons between *C. p. pallasii* in the NE-Pacific (Kamishak in the Gulf of Alaska) and the transarctic group (Bering Sea, Barents Sea and Balsfjord).

suworowi), east of the Barents Sea spawns in late summer in August (Semenova et al. 2015). Even though the samples from the Barents Sea were sampled in different seasons (February and June), the majority of the herring from each sample were maturing (stage 4), which indicates a mixture of herring populations occupying this region, with one population spawning in spring and the other during late summer. Since the herring were close to spawning, the population sampled in February might have been White Sea herring migrating to their respective spawning grounds during the time of sampling. Since genetic variation exists between spawning groups of White Sea and Barents Sea herring at four allozyme loci (Semenova et al. 2009), further investigations are needed to see if the same pattern of divergence is observed with otolith shape. Comparisons of the species *C. harengus* (Norwegian spring-spawners from W-Norway) and *C. pallasii* from Balsfjord, Barents Sea, Bering Sea and Kamishak in the Gulf of Alaska yielded the highest *F*-value (53.83, Table 2), while a comparison of Kamishak herring in the Gulf of Alaska with the trans-Arctic group of herring from the Barents Sea, Balsfjord and Bering Sea (Laakkonen et al. 2013) had a considerably lower *F*-value (27.17), and thus more divergence in otolith shape, as might be expected, at the species level than intra-species level. Differentiation in otolith shape between the *C. pallasii* subspecies were though less than among populations within *C. pallasii* based on the genetic lineages of the NE- and the trans-Arctic group (Laakkonen et al. 2013).

Studies on genetic variation have shown that the more southerly distributed populations, such as the large Norwegian spring-spawners and the NE-population in the Pacific harbor more genetic variation than the northern populations in accordance with their population sizes and even bottlenecks in populations following the colonization of the Barents Sea and N-Norway (Laakkonen et al. 2013). In otolith variation we observe a similar pattern, where the smallest variation was in the Bering Sea and in *C. p. suworowi* from the Barents Sea. Higher variance could be expected in the Balsfjord population as a result of hybridization (Laakkonen et al. 2015) but this was not the case in the present study.

Several fish species are known to have invaded the Atlantic from the Pacific after the last glacial period of the Ice Age. The discrete geographic distribution may have contributed to the classification but recent molecular studies have revealed closer relationships between the taxa than previously considered. For example Pacific cod (*Gadus macrocephalus* Tilesius 1810) and Greenland cod (*Gadus ogac* Richardson 1836) are closely related, as are Alaska pollock (*Theragra chalcogramma* Pallas 1814) and Norwegian pollock (*Theragra finnmarchica* Koefoed 1956) in the NE-Atlantic, and capelin (*Mallotus villosus* Müller 1776) is now found circumpolar (Laakkonen et al. 2015). For Atlantic and Pacific herring, the diversification between the species is clear both genetically and in the morphology of the otoliths despite introgression. Also, populations of Pacific herring which are separated both by large geographic distances and geographic barriers along the coast of N-Norway and the Alaska Peninsula are clearly distinguishable genetically and in otolith shape.

Further studies are needed to clarify the deviation of the Balsfjord herring from the Barents Sea herring and its similarity to the Atlantic herring. Also, the Barents Sea herring were intermediate in shape between herring in the Bering Sea in the NW-Pacific and herring from Kamishak in the NE-Pacific, which does not reflect the geographic distances between them (Fig. 4ab). Analyses of samples along the coast between Balsfjord and Barents Sea, and from the Pacific could provide information on whether this pattern has resulted from the divergence of the Barents Sea herring or if the Balsfjord population has been shaped by the known genetic introgression and the small effective population size (Laakkonen et al. 2015).

It is apparent, as pointed out by Laakkonen et al (2013), that the pattern does not comply with the current subspecies division within *C. pallasii*. A revised classification, considering the observed genetic and morphological evidence, should rather distinguish the NW-Pacific population occupying the Bering Sea together with the European populations of *C. pallasii* than with the NE-Pacific herring, occupying the Gulf of Alaska.

ACKNOWLEDGMENTS

Torstein Pedersen at the University of Tromsø is thanked for providing the samples from Balsfjord in Norway. Ole Ingar Paulsen at the Institute of Marine Research in Norway is thanked for allozyme analysis, splitting out Norwegian spring-spawning herring (*C. harengus*) from Balsfjord herring (*C. pallasii*) in Balsfjord. This work was funded by the Assistant teacher's grant of the University of Iceland.

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Appendix

Documentation for the R-package shapeR

Lísa A. Libungan and Snæbjörn Pálsson (2015)

Package 'shapeR'

May 3, 2015

Type Package

Title Collection and Analysis of Otolith Shape Data

Version 0.1-4

Date 2015-04-02

Maintainer Lisa Anne Libungan <lisa.libungan@gmail.com>

Depends R (>= 3.0.2)

Imports gplots, jpeg, pixmap, wavethresh, methods, vegan, MASS

Description Studies otolith shape variation among fish populations.
Otoliths are calcified structures found in the inner ear of teleost fish and their shape has been known to vary among several fish populations and stocks, making them very useful in taxonomy,species identification and to study geographic variations. The package extends previously described
software used for otolith shape analysis by allowing the user to automatically extract closed contour outlines from a large number of images, perform smoothing to eliminate pixel noise,choose from conducting either a Fourier or wavelet transform to the outlines and visualize
the mean shape. The output of the package are independent Fourier or wavelet coefficients which can be directly imported into a wide range of statistical packages in R. The package might prove useful in studies of any two dimensional objects.

License GPL(>=2)

URL https://github.com/lisalibungan/shapeR, http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0121102

LazyDataCompression yes

R topics documented:

`cluster.plot` *Plot data clusters*

Description

Plots data clusters

Usage

```
cluster.plot( ddata, classes, main="", col.stock=NULL,
              plotCI = FALSE, conf.level = 0.68, ...)
```

Arguments

`ddata`	Matrix of points
`classes`	A factor including the cluster values
`main`	Title for the plot
`col.stock`	Colors for the plotted classes
`plotCI`	Plot means with confidence intervals
`conf.level`	The confidence interval for the standard error of the mean
`...`	Additional parameters to be passed to 'plot' or 'ldahist' if one dimension

Author(s)

Lisa Anne Libungan

References

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H. and Wagner, H. (2013). vegan: Community Ecology Package. R package version 2.0-10.

Examples

```
data(otoliths)
library(vegan)
cap.res = capscale(getStdWavelet(shape) ~ getMasterlist(shape)$pop)

eig=eigenvals(cap.res,constrained=TRUE)
eig.ratio = eig/sum(eig)

cluster.plot(scores(cap.res)$sites[,1:2],getMasterlist(shape)$pop
,plotCI=TRUE
,xlab=paste("CAP1 (",round(eig.ratio[1]*100,1),"%)",sep="")
,ylab=paste("CAP2 (",round(eig.ratio[2]*100,1),"%)",sep="")
,main="Canonical clustering"
)
```

---

detect.outline *Detect otolith outline*

Description

Determine the outline of otolith images in jpeg format which have been stored in the `Fixed` folder.

Usage

```
detect.outline(object, threshold=0.2, mouse.click=FALSE,
           display.images=FALSE, write.outline.w.org=FALSE)
```

Arguments

`object`	shapeR object
`threshold`	Grayscale threshold. Value between 0 and 1.
`mouse.click`	If TRUE, the user clicks where the starting point for the otolith contour extraction algorithm should start. Default is the center of the image. Could be good to set as TRUE if the otolith detection produces an error.
`display.images`	If TRUE, each image is displayed and the user can visualize how the outline is captured
`write.outline.w.org`	If TRUE, the outline is written on top of the original image using the function `write.image.with.outline`, and can be seen in the `Original_with_outline` folder

Details

Based on the Conte function (Claude 2008)

Value

A shapeR object with otolith outlines in the slot outline.list

Author(s)

Lisa Anne Libungan & Snaebjorn Palsson

References

Claude, J. (2008). Morphometrics with R. Springer. 316 p.

Urbanek, S. (2014). jpeg: Read and write JPEG images. R package version 0.1-8.

Bivand, R., Leisch, F. & Maechler, M. (2011) pixmap: Bitmap Images ("Pixel Maps"). R package version 0.4-11.

Libungan LA and Palsson S (2015) ShapeR: An R Package to Study Otolith Shape Variation among Fish Populations. PLoS ONE 10(3): e0121102.

Examples

```
## Not run:
#Use test data from Libungan and Palsson (2015):
shape = shapeR("ShapeAnalysis/","FISH.csv")
shape = detect.outline(shape, threshold=0.2,write.outline.w.org = TRUE)
## End(Not run)
```

---

enrich.master.list *Link information in the info.file to the coefficients obtained from the otolith images*

Description

Link the original info file to the otolith coefficients

Usage

```
enrich.master.list(object, folder_name = "folder", pic_name = "picname",
                calibration = "cal", include.wavelet = TRUE, include.fourier = TRUE,
                    n.wavelet.levels = 5, n.fourier.freq = 12,...)
```

Arguments

object	A shapeR object
folder_name	Should contain the first letters of the area and the serie or station number of the sample, for example: "IC"
pic_name	Should contain the serie number of a given sample and fish number, for example "403_2" (not including the .jpg extension)
calibration	The name of the column where the pixels to measurement calibration is located
include.wavelet	If TRUE, the wavelet coefficient are included in the master.list

include.fourier
: If TRUE then the Normalized Elliptic Fourier coefficients are included in the master.list

n.wavelet.levels
: Integer saying how many levels of wavelet levels should be included

n.fourier.freq
: Integer saying how many Fourier frequency levels should be included

...
: Additional parameter for `read.csv` for reading the info.file

Value

A shapeR object with values in slots:

- wavelet.coef
- fourier.coef
- shape
- filter
- master.list

Author(s)

Lisa Anne Libungan

Examples

```
## Not run:
data(otoliths)
shape = generateShapeCoefficients(shape)

shape = enrich.master.list(shape)
## End(Not run)
```

---

estimate.outline.reconstruction

Estimate the outline reconstruction based on Fourier/wavelet compared to the outlines that have not been transformed

---

Description

Estimate outline reconstruction using a different number of coefficients of wavelet and Fourier compared to the original otolith

Usage

```
estimate.outline.reconstruction(object, ...)
```

Arguments

object
: shapeR object

...
: Additional parameters to be passed to 'plot' and 'points'

Value

A list containing values

- w.dev.m a list for number of coefficients for mean error of wavelet reconstruction
- w.dev.sd a list for number of coefficients for standard deviation of wavelet reconstruction
- f.power.total Fourier power for number of Fourier harmonics

Author(s)

Lisa Anne Libungan

References

Claude, J. (2008) Morphometrics with R. Springer. 316 p.

Examples

```
## Not run:
data(otoliths)
estimate.outline.reconstruction(shape)
## End(Not run)
```

FISH *An example data file*

Description

The file's columns are:

- country
- station
- pop
- stockID
- day
- month
- year
- lat
- lon
- fishno
- length_cm
- weight_g
- age
- sex
- maturity
- folder
- picname
- cal

Usage

```
data(FISH)
```

Format

An example data file

`generateShapeCoefficients` *Get wavelet/Fourier coefficients and basic shape variables*

Description

Generates shape variables based on Fourier/wavelet reconstruction. Wavelet coefficients for wavelet. Basic shape parameters are also collected (area, length, width, perimeter).

Usage

```
generateShapeCoefficients(object,...)
```

Arguments

`object`	shapeR object
`...`	Additional parameters to be passed to the wd function of the wavethresh package for the wavelet decomposition of the otolith outlines

Value

A shapeR object with values in slots:

- wavelet.coef.raw
- fourier.coef.raw
- shape.coef.raw

Author(s)

Lisa Anne Libungan & Snaebjorn Palsson

References

Nason, G. (2012). wavethresh: Wavelets statistics and transforms. R package, version 4.5.

Claude, J. (2008). Morphometrics with R. Springer. 316 p.

Examples

```
## Not run:
data(otoliths)
shape = generateShapeCoefficients(shape)
## End(Not run)
```

getFourier *Get Fourier coefficients, filtered according to filter*

Description

Returns the Fourier coefficients determined in `stdCoefs`. Returns only values as set in `setFilter`

Usage

```
getFourier(object)
```

Arguments

`object`	`shapeR` object

Value

The Fourier coefficients for all fish as determined by `setFilter`

Author(s)

Lisa Anne Libungan

getMasterlist *Get filtered master.list values*

Description

Returns selected values from `master.list`

Usage

```
getMasterlist(object, useFilter = TRUE)
```

Arguments

`object`	`shapeR` object
`useFilter`	If TRUE, the master.list values are filtered by the slot `filter`. FALSE = no filtering.

Value

The `master.list` is filtered by the slot `filter` if the `useFilter` is TRUE, else no filtering is done.

Author(s)

Lisa Anne Libungan

getMeasurements *Get simple shape variables, filtered according to filter*

Description

Returns shape variables length, width, perimeter and area determined in `generateShapeCoefficients`. Returns only values as set in the slot `filter`. These variables can only be obtained if the calibration measurements in pixels have been registered in the csv data file in a column labelled 'cal' (see example data file). To get the calibration measurements, use a image manipulation program and measure 1mm on the calibration measurement stick (that was taken for that particular dataset) and register how many pixels 1mm is into the column 'cal'.

Usage

```
getMeasurements(object)
```

Arguments

`object`	`shapeR` object

Value

A data frame with all valid fish as determined by the slot `filter` and with columns:

- otolith.area
- otolith.length
- otolith.width
- otolith.perimeter

Author(s)

Lisa Anne Libungan

Examples

```
data(otoliths)
# Calculate the mean otolith area for each fish population
# The results are in square mm since the calibration ('cal') column
# in the data file is in pixels (1 mm/pixel).
tapply(getMeasurements(shape)$otolith.area, getMasterlist(shape)$pop,mean)
```

getStdFourier *Get standardized Fourier coefficients, filtered according to filter*

Description

Returns the standardized Fourier coefficients determined in `stdCoefs`. Returns only values as set in the slot `filter`

Usage

```
getStdFourier(object)
```

Arguments

`object`	shapeR object

Value

The standardized Fourier coefficients for all valid fish as determined by the slot `filter`

Author(s)

Lisa Anne Libungan

getStdMeasurements *Get simple shape variables after standardization, filtered according to filter*

Description

Returns the simple shape variables determined in `stdCoefs`. Returns only values as set in the slot `filter`

Usage

```
getStdMeasurements(object)
```

Arguments

`object`	shapeR object

Value

A data frame with all valid fish as determined by the slot `filter`. Returns only variables that have not been removed after standardization.

Author(s)

Lisa Anne Libungan

Examples

```
data(otoliths)
#Calculate the mean standardized otolith length for each fish population
tapply(getStdMeasurements(shape)$otolith.length,
getMasterlist(shape)$pop,mean)
```

`getStdWavelet` *Get standardized wavelet coefficients, filtered according to filter*

Description

Returns the standardized wavelet coefficients determined in `stdCoefs`. Returns only values as set in the slot `filter`

Usage

```
getStdWavelet(object)
```

Arguments

`object`	shapeR object

Value

The standardized wavelet coefficients for all valid fish as determined by the slot `filter`

Author(s)

Lisa Anne Libungan

`getWavelet` *Get wavelet coefficients, filtered according to filter*

Description

Returns the wavelet coefficients determined in `generateShapeCoefficients`. Returns only values as set in the slot `filter`

Usage

```
getWavelet(object)
```

Arguments

`object`	shapeR object

Value

The wavelet coefficients for all valid fish as determined by the slot `filter`

Author(s)

Lisa Anne Libungan

`outline.reconstruction.plot` *Plot outline reconstruction*

Description

Show graphs of the reconstruction using different number of levels of wavelet reconstruction and Fourier power using different number of Fourier harmonics. Uses the output from estimate.outline.reconstruction

Usage

```
outline.reconstruction.plot(outline.rec.list,ref.w.level=5,
                            ref.f.harmonics=12,max.num.harmonics=32,...)
```

Arguments

`outline.rec.list`	The output from estimate.outline.reconstruction
`ref.w.level`	Reference level for graphical purposes. The default is 5 as is the default of shapeR.
`ref.f.harmonics`	Reference Fourier harmonize. The default is 12 as is the default in shapeR.
`max.num.harmonics`	Maxinum number of Fourier harmonics to be shown
`...`	Additional parameters to be passed to 'plot'

Author(s)

Lisa Anne Libungan

Examples

```
## Not run: data(otoliths)
est.list = estimate.outline.reconstruction(shape)
outline.reconstruction.plot(est.list,panel.first = grid())
## End(Not run)
```

`plotFourier` *Mean and standard deviation of the Fourier coefficients*

Description

The mean and standard deviation of the Fourier coefficients

Usage

```
plotFourier(object, coef.index=NULL,class.name=NULL,useStdcoef=FALSE, ...)
```

Arguments

object	shapeR object
coef.index	An index vector for which fourier coefficents to be shown. Default is NULL and all coefficients are shown.
class.name	Column name in master list for partitioning the data into groups and showing the ratio of variance among to the sum of variance among and variance within.
useStdcoef	Boolean saying if to use the standardized coefficients or not
...	Additional parameters to be passed to 'plot'

Author(s)

Lisa Anne Libungan

Examples

```
data(otoliths)
shape = stdCoefs(shape,classes="pop","length_cm")
plotFourier(shape,class.name= "pop",useStdcoef=TRUE)
```

---

plotFourierShape *Mean otolith shape based on Fourier reconstruction*

Description

A function for showing the mean otolith shape based on Fourier reconstruction

Usage

```
plotFourierShape(object, class.name, show.angle = FALSE,lty=1:5,col=1:6, ...)
```

Arguments

object	A shapeR object
class.name	A string as the column name in the master list
show.angle	If TRUE angles are shown on the plot
lty,col	Vector of line types and colors. Values are used cyclically.
...	Additional parameters to be passed to 'plot'

Author(s)

Lisa Anne Libungan

References

Libungan LA and Palsson S (2015) ShapeR: An R Package to Study Otolith Shape Variation among Fish Populations. PLoS ONE 10(3): e0121102.

Examples

```
data(otoliths)
plotFourierShape(shape, "pop",show.angle = TRUE,lwd=2,lty=1)
```

plotWavelet *Mean and standard deviation of the wavelet coefficients*

Description

The mean and standard deviation of the wavelet coefficients

Usage

```
plotWavelet(object, level, start.angle = 0, class.name=NULL,useStdcoef=FALSE,...)
```

Arguments

object	A shapeR object
level	The wavelet level to be shown
start.angle	The angle in degrees, the x-axis should start on
class.name	Column name in master list for partitioning the data into groups and showing the ratio of variation among groups.
useStdcoef	Choose "TRUE" or "FALSE" if coefficients should be standardized or not
...	Additional parameters to be passed to 'plot'

Author(s)

Lisa Anne Libungan

Examples

```
data(otoliths)
shape = stdCoefs(shape,classes="pop","length_cm")
plotWavelet(shape,level=5,class.name= "pop",useStdcoef=TRUE)
```

plotWaveletShape *Mean otolith shape based on wavelet reconstruction*

Description

A function for showing the mean otolith shape based on wavelet reconstruction

Usage

```
plotWaveletShape(object, class.name,show.angle=FALSE,lty=1:5,col=1:6,...)
```

Arguments

object	A shapeR object
class.name	A string as the column name in the master list
show.angle	If TRUE angles are shown on the plot
lty,col	Vector of line types and colors. Values are used cyclically.
...	Additional parameters to be passed to 'plot'

Author(s)

Lisa Anne Libungan

References

Nason, G. (2012) wavethresh: Wavelets statistics and transforms, version 4.5. R package.

Examples

```
data(otoliths)
plotWaveletShape(shape, "pop",show.angle = TRUE,lwd=2,lty=1)
```

---

read.master.list *Read updated master list*

Description

Reads an updated master list. This is important to run if you want to ensure that a updated master list is used in the analysis.

Usage

```
read.master.list(object, ...)
```

Arguments

object	A shapeR object
...	Additional parameter for read.csv for reading the info.file

Value

shapeR object with values in slots:

- master.list.org

Author(s)

Lisa Anne Libungan

`remove.outline` *Remove otolith outline*

Description

A function for removing an otolith outline from the file 'outline.list'. Typically done if the image is of bad quality and needs to be enhanced in a image processing software

Usage

```
remove.outline(object, folder = "", fname = "")
```

Arguments

`object`	A shapeR object
`folder`	The folder name where the outline that needs to be removed is stored
`fname`	The file name of the outline to be removed

Value

shapeR object

Author(s)

Lisa Anne Libungan

References

Libungan LA and Palsson S (2015) ShapeR: An R Package to Study Otolith Shape Variation among Fish Populations. PLoS ONE 10(3): e0121102.

Examples

```
## Not run:
#Use test data from example in Libungan and Palsson (2015):
shape = shapeR("ShapeAnalysis/","FISH.csv")
shape = detect.outline(shape)
#If otolith outline in folder IC named 403_1 needs to be removed
shape = remove.outline(shape, "IC", "403_1")
## End(Not run)
```

`setFilter` *Set a filter to analyze the shape data*

Description

Sets a filter on `master.list`. Here it is possible to filter the `master.list` by specific ages, maturity stages, areas, etc. If no value is set, all data with shape parameters are used

Usage

```
setFilter(object, filter)
```

Arguments

`object`	A shapeR object
`filter`	A vector restricting the new filter value. Only otoliths having shape parameters are selected.

Value

A shapeR object with the slot `filter` set.

Author(s)

Lisa Anne Libungan

Examples

```
data(otoliths)
#Filter only Icelandic and Norwegian samples
shape = setFilter(shape,
getMasterlist(shape, useFilter = FALSE)$pop %in% c("NO","IC"))
table(getMasterlist(shape)$pop)
#Reset filter
shape = setFilter(shape)
table(getMasterlist(shape)$pop)
```

`shape` *An example shapeR instance including 160 images. The shape coefficients have been generated. The wavelet coefficients have been standardized using pop and length_cm.*

Description

The class slot's are as follows:

- project.path. A path as "ShapeAnalysis/"
- info.file. A file as FISH.csv. The information is stored in the data frame master.list
- outline.list. A list with three elements (IC, NO, SC) which give a list of the otolith outlines
- filter. A logical vector showing which elements of the master list have valid otoliths

- fourier.coef. A matrix of the Normalized Elliptic Fourier coefficients
- wavelet.coef. A matrix of the wavelet coefficients
- shape. A matrix of shape variables after scaling according to calibration otolith.area, otolith.length, otolith.width, otolith.perimeter.
- fourier.coef.std. A matrix which will contain standardized Fourier coefficients
- wavelet.coef.std. A matrix which will contain standardized wavelet coefficients
- shape.coef.raw. A matrix of shape variables before scaling according to calibration otolith.area, otolith.length, otolith.width, otolith.perimeter.
- master.list. The contents of the info.file

Usage

```
data(otoliths)
```

Format

A shapeR class including 160 images

---

shapeR *shapeR*

Description

Collection and analysis of otolith shape data

a shapeR class

Usage

```
shapeR(project.path, info.file, ...)
```

Arguments

project.path	The base project path where the images are stored
info.file	The information file which store the information on the fish and otoliths. This is the base for the master.list
...	Additional parameters to be passed to 'read.csv' for reading the info.file

Value

a shapeR object

Slots

`project.path` Path to the project where the images are stored

`info.file` Info file containing fish and otolith information

`master.list.org` The contents of the `info.file`

`master.list` The contents of the `info.file` with added shape parameters and descriptors

`outline.list.org` A list of all the original otolith outlines

`outline.list` A list of all the otolith outlines. It returns a list of smoothed if contour smoothing (using`smoothout`) has been conducted.

`filter` A logical vector selecting the otoliths used for analysis

`wavelet.coef.raw` The wavelet coefficients for all the otolith outlines

`wavelet.coef` The wavelet coefficients after aligning with the `info.file`. The data is generated when enrich.master.list is run

`wavelet.coef.std` The standardized wavelet coefficients. The data is generated when stdCoefs is run

`wavelet.coef.std.removed` The index of the removed wavelet coefficients after standardization. The data is generated when stdCoefs is run

`fourier.coef.raw` The Fourier coefficients for all the otolith outlines

`fourier.coef` The Fourier coefficients for after aligning with the info file. The data is generated when enrich.master.list is run

`fourier.coef.std` The standardized Fourier coefficients. The data is generated when stdCoefs is run

`fourier.coef.std.removed` The index of the removed Fourier coefficents after standardization. The data is generated when stdCoefs is run

`shape.coef.raw` The uncalibrated shape measurements for all the otoliths. The shape parameters are: otolith.area, otolith.length, otolith.width, otolith.perimeter

`shape.coef` The shape measurements for after aligning with the info file. The shape parameters have been calibrated using the calibration parameter as registered in the datafile as the column 'cal'.

`shape.std` The standardized shape measurements. The data is generated when stdCoefs is run

`shape.std.removed` The index of the removed shape measurements after standardization. The data is generated when stdCoefs is run

Author(s)

Lisa Anne Libungan & Snaebjorn Palsson

References

Libungan LA and Palsson S (2015) ShapeR: An R Package to Study Otolith Shape Variation among Fish Populations. PLoS ONE 10(3): e0121102.

Examples

```
## Not run:

shape = shapeR("ShapeAnalysis/","FISH.csv")
shape = detect.outline(shape,write.outline.w.org = TRUE)
```

```
shape = generateShapeCoefficients(shape)
shape = enrich.master.list(shape)

shape = stdCoefs(shape,"pop","length_cm")

plotWavelet(shape,level=5,class.name= "pop",useStdcoef=TRUE)
plotFourier(shape,class.name= "pop",useStdcoef=TRUE)

#Canonical analysis
library(vegan)
cap.res = capscale(getStdWavelet(shape) ~ getMasterlist(shape)$pop)
anova(cap.res)

#Visualize the canonical scores
eig=eigenvals(cap.res,constrained=TRUE)
eig.ratio = eig/sum(eig)

cluster.plot(scores(cap.res)$sites[,1:2],getMasterlist(shape)$pop
,plotCI=TRUE
,xlab=paste("CAP1 (",round(eig.ratio[1]*100,1),"%)",sep="")
,ylab=paste("CAP2 (",round(eig.ratio[2]*100,1),"%)",sep="")
,main="Canonical clustering"
)

#Only analyze Icelandic and Norwegian samples
shape = setFilter(shape, getMasterlist(shape, useFilter = FALSE)$pop %in% c("NO","IC"))

#Classifier on standardized wavelet
lda.res.w = lda(getStdWavelet(shape),getMasterlist(shape)$pop,CV=TRUE)
ct.w = table(getMasterlist(shape)$pop,lda.res.w$class)

diag(prop.table(ct.w, 1))

# Total percent correct
sum(diag(prop.table(ct.w)))

cap.res = capscale(getStdWavelet(shape) ~ getMasterlist(shape)$pop)
anova(cap.res)

#Classifier on canoncial values
lda.res.w = lda(scores(cap.res)$sites,getMasterlist(shape)$pop,CV=TRUE)
ct.w = table(getMasterlist(shape)$pop,lda.res.w$class)

diag(prop.table(ct.w, 1))

# Total percent correct
sum(diag(prop.table(ct.w)))

## End(Not run)
```

---

show,shapeR-method *Show a shapeR object*

Description

Show the project.path and info.file, the number of outlines that have been read and which fundamental methods have been run.

Usage

```
## S4 method for signature 'shapeR'
show(object)
```

Arguments

object	a shapeR oject

---

show.original.with.outline

Show the extracted outline on top of the original image

Description

A function which displayes the outlines which were extracted from the image in the "Fixed" folder on top of the corresponding image in the "Original" folder.

Usage

```
show.original.with.outline(object, folder, fname)
```

Arguments

object	A shapeR object
folder	The folder name where the image is stored
fname	Image file name. Not including the extension ".jpg"

Author(s)

Lisa Anne Libungan

References

Libungan LA and Palsson S (2015) ShapeR: An R Package to Study Otolith Shape Variation among Fish Populations. PLoS ONE 10(3): e0121102.

Examples

```
## Not run:
#Follow the example in Libungan and Palsson (2015) and run the following lines:
show.original.with.outline(shape,"IC","403_2")
## End(Not run)
```

smoothout *Contour smoothing*

Description

Remove high frequency pixel noise around the otolith outline

Usage

```
smoothout(object, n)
```

Arguments

object	A shapeR object
n	The number of iterations. The default value is 100.

Author(s)

Lisa Anne Libungan

References

Haines, A.J., Crampton, J.S. (2000). Improvements to the method of Fourier shape analysis as applied in morphometric studies. Palaeontology 43: 765-783.

Claude, J. (2008) Morphometrics with R. Springer. 316 p.

Examples

```
## Not run:
data(otoliths)
shape = smoothout(shape,n=100)

# Plot smoothed outline on top of original outline for comparison
outline.org=shape@outline.list.org[["IC"]][["403_2"]]
outline=shape@outline.list[["IC"]][["403_2"]]
plot(outline.org$X,outline.org$Y,type='l',xlab="",ylab="",lwd=2,axes=F)
lines(outline$X,outline$Y,col="red",lwd=2)
legend("bottomleft",c('Original','Smoothed'),lty=1,col=c('black','red'),lwd=2)
## End(Not run)
```

stdCoefs *Standardize coefficients*

Description

Function to standardized the wavelet and Fourier coefficients for a specific parameter such as the fish length. For each country/population a regression coefficient is calculated as a function of fish length. If the slope is significantly different from zero, a correction is made according to Lleonart et al 2000. First ANCOVA is performed: variable ~ pop*length_cm, following a method by Longmore et al 2010. If there is a significant interaction between population and length_cm, then the coefficients are not used and automatically discarded. If there is no interaction, the coefficients are kept and standardized with regards to fish length.

Usage

```
stdCoefs(object, classes=NA, std.by, std.type = "mean", p.crit = 0.05,bonferroni= FALSE)
```

Arguments

object	A shapeR object
classes	The classes to be grouped for standardization. Should be the same as used for the statistical tests
std.by	The parameter to be used for standardization. Typically the length of the fish from the master.list.
std.type	The tuning of the standardization. The standardization can be sensitive to what value all the fishes are standardized to. Possible values are: • min Standardized as the minimum value of std.by • mean Standardized as the mean value of std.by • max Standardized as the maximum value of std.by
p.crit	An argument used to select the threshold critera for omitting coefficients which show interaction with fish length. If p.crit = 0.05, all coefficients which have p<0.05 are omitted. If p.crit = 0.01, only coefficients with p<0.01 are omitted.
bonferroni	A logical parameter for performing Bonferroni for multiple testing

Author(s)

Lisa Anne Libungan

References

Lleonart, J., Salat, J. & Torres, G.J. (2000) Removing allometric effects of body size in morphological analysis. Journal of Theoretical Biology, 205, 85-93.

Longmore, C., Fogarty, K., Neat, F., Brophy, D., Trueman, C., Milton, A. & Mariani, S. (2010) A comparison of otolith microchemistry and otolith shape analysis for the study of spatial variation in a deep-sea teleost, *Coryphaenoides rupestris*. Environmental Biology of Fishes, 89, 591-605.

Reist, J.D. (1985) An Empirical-Evaluation of Several Univariate Methods That Adjust for Size Variation in Morphometric Data. Canadian Journal of Zoology-Revue Canadienne De Zoologie, 63, 1429-1439.

Examples

```
data(otoliths)
shape = stdCoefs(shape,classes="pop","length_cm")
```

`write.image.with.outline` *Write outlines on top of the original images for quality checking*

Description

A function which writes the outlines which were extracted from the images in the folder "Fixed" on top of the corresponding images in the "Original" folder. Viewing the resulted images in the folder "Original_with_outlines" is a good quality check to ensure the correct outline has been extracted. If the outline is not correct, then the image can be fixed in an image software, such as GIMP (www.gimp.org), placed in the "Fixed" folder and then the `detect.outline` step is repeated. The function `detect.outline` calls this function if the parameter `write.outline.w.org` is set to `TRUE`.

Usage

```
write.image.with.outline(object, folder = NA, fname = NA, doProgress = T)
```

Arguments

`object`	A shapeR object
`folder`	The folder name where the image is stored
`fname`	Image file name. Not including the extension ".jpg"
`doProgress`	If TRUE, a progressbar is shown

Author(s)

Lisa Anne Libungan

References

Libungan LA and Palsson S (2015) ShapeR: An R Package to Study Otolith Shape Variation among Fish Populations. PLoS ONE 10(3): e0121102.

Examples

```
## Not run:
#Use test data from Libungan and Palsson (2015) and run the following lines:
shape = shapeR("ShapeAnalysis/","FISH.csv")
shape = detect.outline(shape,write.outline.w.org = FALSE)
write.image.with.outline(shape)
## End(Not run)
```

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