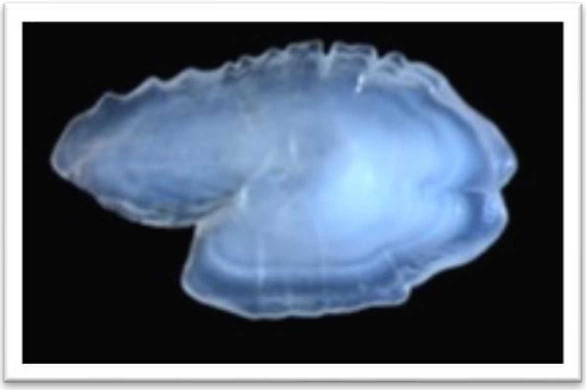
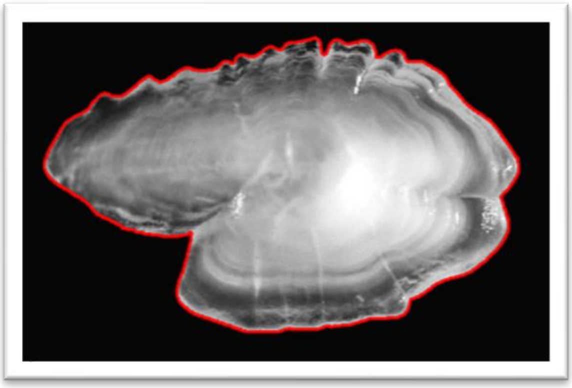
Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Department of Aquatic Resources

Otolith morphology of sprat (*Sprattus sprattus*) along the Swedish west coast

Francesco Saltalamacchia





Independent project in Biology – Master´s thesis • 30 credits

Examensarbete/Master's thesis

Lysekil 2018

Otolith morphology of sprat (*Sprattus sprattus*) along the Swedish west coast

Francesco Saltalamacchia

Supervisor:	Valerio Bartolino, Swedish University of Agricultural Sciences, Department of Aquatic Resources
Assistant supervisor:	Florian Berg, Institute of Marine Research, Bergen, Norway
Examiner:	Michele Casini, Swedish University of Agricultural Sciences, Department of Aquatic Resources

Credits:	30 credits
Level:	Second cycle, A2E
Course title:	Independent project in Biology - Master's thesis
Course code:	EX0565
Course coordinating department:	Department of Aquatic Resources
Place of publication:	Lysekil
Year of publication:	2018
Cover picture:	Francesco Saltalamacchia
Online publication:	https://stud.epsilon.slu.se
Keywords:	European sprat, otolith, shape analysis, wavelet

Swedish University of Agricultural Sciences
Faculty of Natural Resources and Agricultural Sciences
Department of Aquatic Resources

Abstract

The population structure of sprat (*Sprattus sprattus*) in the Swedish Kattegat – Skagerrak has been at present not well investigated, and the boundaries between the management unit in this region and the larger stock occurring in the North Sea are also highly uncertain, posing issues to the successful management of the stock in this area.

In this study, variations in otolith shape among several samples from the Swedish west coast, southern North Sea and Norwegian fjords north of Bergen were studied to investigate the effectiveness of shape analysis for characterise the population structure in the study area, and evaluate whether differences could be used to assign individual fish to their origin.

Otolith sample were partitioned by age, and the shape variations and the relations between shape and size were mapped by means of Wavelet and quantitative shape analysis using digitally acquired otolith profiles. The areas of the otolith showing the highest proportion of variation were identified, and classification techniques were used to investigate the spatial clustering of these variations along the study area.

No differences in otolith shape were observed between left and right otoliths or between male and female individuals of sprat, suggesting that collections of mixed samples collated opportunistically from available collections can be analysed confidently for instance regardless sex in this species. The shape differences among the different geographic locations were observed to occur mainly between the morphological structures of *rostrum* and *excisura major*, and allowed to identify three major clusters. The samples from the Norwegian fjords were the most different in shape among all the groups compared in this study, while the Skagerrak – Kattegat and North Sea samples showed a high degree of similarity. A high differentiation was found for the samples within the Uddevalla fjord system in Sweden, suggesting an isolated unit in this fjord.

A latitudinal gradient of differentiation was also observed in the central – southern Kattegat, but the sample size for this region was limited.

Classification success of the Norwegian samples was rather high, as well as assignment of the southern Kattegat fish in the ages 1 to 3, while values between 31 and 78% were computed for the Uddevalla fjords. The classification success between North Sea and Skagerrak individuals based on otolith shape was particularly low as expected given the low level of differentiation.

Our results are consistent with recent genetic findings and the two methodologies corroborated each other at a recent benchmark of sprat stock assessment. In conclusion, this study shows that otolith shape analysis is a promising tool to identify sprat population structure for management purposes.

Table of Contents

1. Introduction

1.1 The European sprat

The European sprat (*Sprattus sprattus*, Linnaeus, 1758) is a small-bodied, pelagic schooling clupeid inhabiting the Baltic, the Northeast Atlantic from Northern Norway down to Morocco and the northern Mediterranean/Black Sea basins; three sub-species have been defined for each of these areas, named respectively *S. sprattus balticus*, *S. sprattus sprattus* and *S. sprattus phalericus* (ICES-FishMap). Although morphologically similar to juvenile herring *Clupea harengus*, the sprat (fig. 1a) is easily distinguished by the sharply toothed keel on the belly (fig. 1b), the relative positions of dorsal and pelvic fins (fig. 1b, red lines) and the grey coloration on the dorsal side (ICES-FishMap).

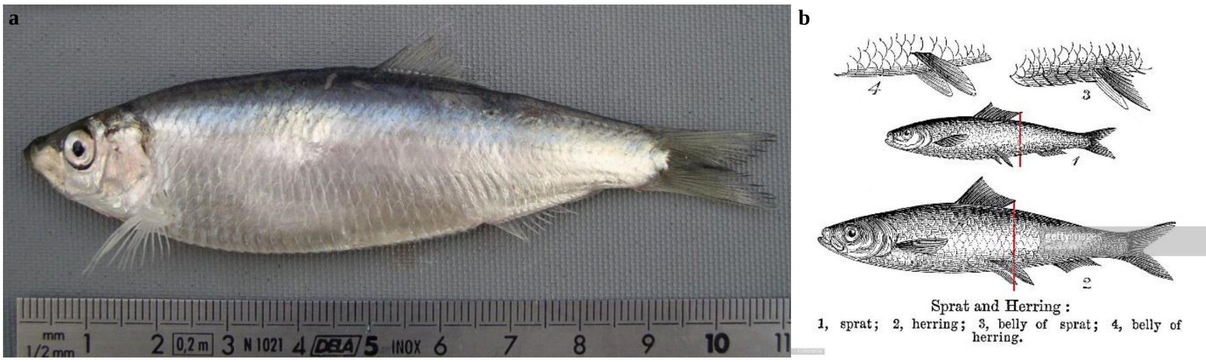


*Figure 1 a. Picture of the studied species, the European sprat (*Sprattus sprattus*). b. Illustration of the main morphological differences between herring and sprat (modified).*

Sprat is commonly diffused in different environmental contexts, including shallow waters as fjords and areas of low salinity such as the Baltic Sea; being ecologically important both as planktivore and as prey, it is one of the main food sources in the diet of numerous species, including demersal fish, elasmobranchs, seabirds and marine mammals (ICES, 2013); annual estimates of sprat consumption by natural predators made for the North Sea stock identify mackerel, horse mackerel, whiting and seabirds as main predators (ICES, 2013). In the Skagerrak and Kattegat, however, the predation mortality is poorly known, as the food webs have not been described as well as for the Baltic and the North Sea, and multispecies models for this area are currently lacking.

The maximum length and age of the species are about 16 cm (Whitehead, 1985) and 5 years (Bailey, 1980); the gonadal maturation normally starts when the fish is 95 – 100 mm long, a size that is usually reached during the first or second year depending on growth conditions (Peck *et al.*, 2012).

Sprat are multiple batch spawners, which means that the females spawn repeatedly throughout the spawning season and produce 100 - 400 eggs per gram body weight (Alheit, 1987). The main spawning area in Skagerrak and Kattegat is located between the northern point of Jutland (fig. 7) and the Swedish coast (Lindquist 1978; Nielsen, 1994; Voss *et al.*, 2009).

Being also a commercially high valuable species, sprat is widely used for canning, fishmeal and oil (ICES 2013). Despite of this, the biological knowledge of this species in Skagerrak-Kattegat is poor, and it is therefore managed as a “Data Limited Stock” (ICES, 2015) within the International Council for the Exploitation of the Sea (ICES).

1.2 Study area and local sprat fishery

The Skagerrak-Kattegat region – labelled by ICES as “Division 3a” – is one of four key areas in the Greater North Sea Ecoregion, (fig. 2) along with the Northern North Sea, the Southern North Sea and the English Channel.

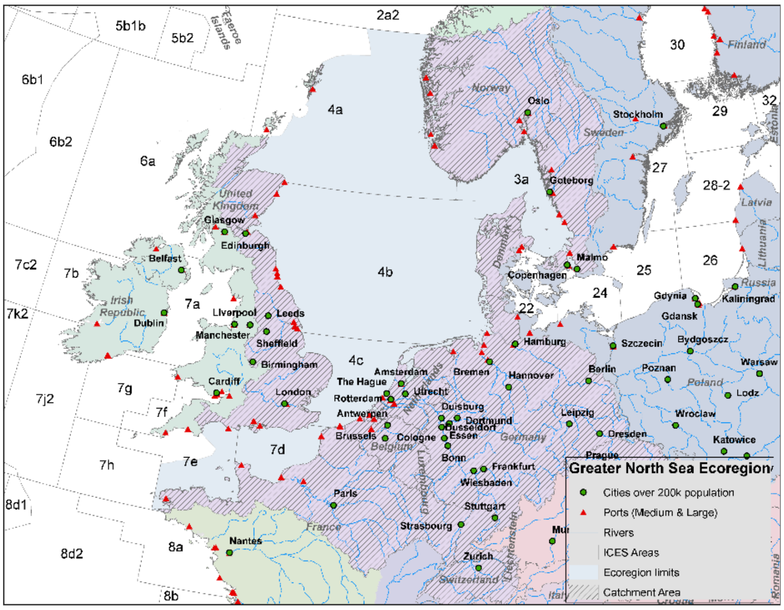


Figure 2 ICES divisions and subdivisions in the Greater North Sea ecoregion (ICES).

The division 3a sustains a high rate of human activities (especially fishing, shipping and wind farms), and with its lower salinity and tide changes it forms the physical and ecological link between the North Sea and the Baltic Sea (ICES, 2017).

Among all the Swedish coastal regions, the west coast marine areas are the most species rich and productive, and provide habitat for a great variety of commercially important fish species: pelagic species like herring, sprat and mackerel are fished on a large scale, as well as demersal fish like cod, haddock and various flatfish, and benthic species as Norway lobster and shrimp (Popescu, 2010).

The local stocks of clupeid fish have a long history of exploitation along all the Scandinavian coasts, and they are an important part of the naturalistic and historical heritage of Sweden (Lindquist, 1978). Among them, herring and sprat play key ecological roles in the food web of the Skagerrak and Kattegat, and they also are the dominating species in the Swedish pelagic fisheries (ICES, 2013).

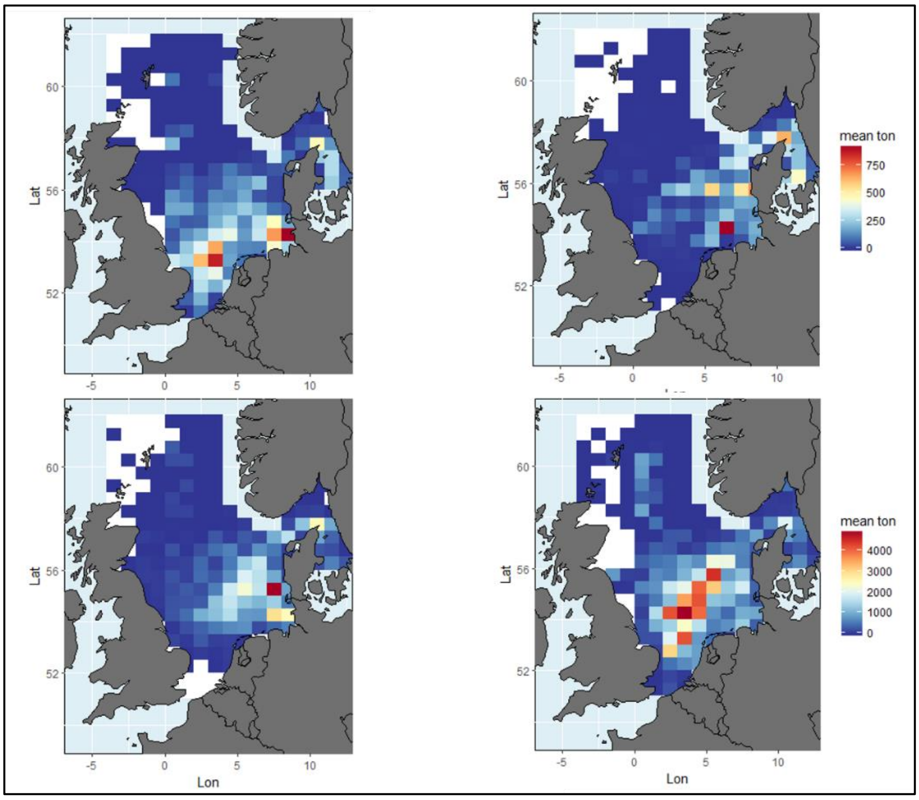


Figure 3   Mean of square-based commercial cathes by quarters from 1974-2017. First row, from left to right: Q1 and Q2; second row, from left to right: Q3 and Q4 (ICES).

Sprat in 3a are mostly fished by Denmark, Sweden and Norway, with Denmark leading the majority of the landings (ICES, 2017). The most exploited area in this region is around the northwesternmost point of the Jutland peninsula (fig. 3).

The Norwegian sprat fishery is seasonal: sprat is protected from 1 January to 31 July, and a traditional, inshore purse seine fishery for human consumption takes place only through the 3rd and 4th quarters of the year. The Danish and Swedish sprat fisheries take place all year round using purse seine, mid-water and bottom trawls. The landings are mainly used for industrial purposes as fishmeal and oil production; sprat for human consumption are caught in Skagerrak during Autumn and Winter by purse seiners.

When the Swedish sprat exploitation started in the late XIX century, it was carried on exclusively in inshore waters with beach seines (Lindquist, 1978); just in the early 1930s this activity was extended to the open sea: in 1929, in fact, the purse seine was used for the first time for taking sprat off the west coasts, and the beginning of trawling in 1933 then increased definitively the importance of open sea fishing (ICES, 2013).

The total landings for sprat between 2015 and 2017 (19770, 11046 and 1413 t respectively) are below the average landings in the last 10 year, and the catches in 2017 were extraordinarily low because of the closure of the Danish sprat fishery due to high herring bycatches (ICES, 2018a).

1.3 Population structure and challenges for management

Despite its high exploitation, the sprat in 3a has not received particular attention in research. The number of publications during the past decades is limited if compared to other species in the area such as herring. At present the species in 3a is treated as a single management stock, although some questions have been recently raised about its geographical distribution and its interaction with the neighbouring stocks and populations (ICES, 2018b).

As a small pelagic species sprat have a potential for high dispersal and gene flow, as found for instance in the Baltic Sea where the stock is composed by a single large population (Shvetsov *et al.*, 1995). However, despite the sea usually offers fewer physical barriers than the terrestrial environments, many marine organisms have been proven to display population structures which reflect barriers to gene flow over relatively small geographic areas (Limborg *et al.*, 2012). This could be due to environmental factors or oceanographic barriers, and the complexity of the Scandinavian fjord system is also a potential source of physical barriers between spatially close populations (Libungan *et al.*, 2015).

Some studies (Limborg *et al.*, 2009) have demonstrated a genetic differentiation which reflects the gradient in mean surface salinity between samples from the Kattegat and the Baltic sea, and also pointed out as the North Sea and 3a stocks may be more similar than what had been considered to date. However, it has not been investigated whether population structures exist within and between the Skagerrak and Kattegat, nor the level of differentiation between the stocks of 3a (Skagerrak-Kattegat) and North Sea. Among all this uncertainty, a recent study on sprat reproductive biology within the Division 3a (Vitale *et al.*, 2016) pointed out that the sprat within the Uddevalla fjords could also be a distinct, well isolated population.

While we have a wide knowledge about other clupeid populations in 3a such as the herring (e.g. Rosenberg & Palmén, 1981; Dahle & Eriksen, 1990), local aggregations of sprat showing morphological or ecological adaptation are poorly reported by few old studies on phenotypic traits from the 1940s-1960s (Molander, 1940; Lindquist, 1968). In fact, even though stock identification is recognised as a starting point for fishery management, no comprehensive mapping of sprat population diversity exists for the area. Moreover, the level of mixing and hybridization of those potential local populations with the larger stocks seasonally occurring off-shore is also unknown, and no quantification on their contribution to the local catches is available. The extent to which sprat in 3a derive from North Sea migrants is also a major source of uncertainty, and its assessment has been defined by ICES (2013) as a priority, and it is currently under evaluation (ICES, 2018b).

The current assessment and management neglect the local populations of sprat along the Swedish west coast, shading uncertainty on their future. In fact, these coastal local units are usually smaller and more vulnerable than their offshore counterparts, and it is of paramount importance, for a successful management, to identify them and take their existence into account.

Clearly, without a comprehensive characterisation of these biological units of local diversity it will be impossible to explore if opportunities for their challenging management may exist. Besides, any strategies based on an erroneous perception of stock structure may lead to overfishing less productive populations and underfishing the more productive ones, impacting the sustainability of the resource (Kerr *et al.*, 2016).

1.4 Morphometrics analysis and fish otoliths

D'Arcy Thompson (1917) in his pioneering studies at the beginning of the 20th century, was the first one to explore the degree to which the differences in the forms of related animals could be described by means of relatively simple mathematical transformations. The term "form" is here intended not just as a function of "shape", but as a quantitatively measurable object which also includes size and their variations. However, the first studies aiming to the quantification of morphometric variations in fish were bivariate allometric analysis, and were often insufficient to discriminate between populations depending on their relative differences. It was only with Teissier (1938) that the multivariate analysis was extended to morphometric researches (Cadrin & Friedland, 1999), when he realised that most of the variability explained by principal component analysis of morphometric data could be interpreted as a multivariate index of size and secondary components (shape indices) not size-related.
Since then, multivariate morphometric analysis have been successfully used to discriminate hundreds of fish stocks (e.g. Burke *et al.* 2008; Sadighzadeh *et al*. 2014), and recent methods of capturing and analysing digital images have extraordinarily increased the power of morphometric research. Quantitative description and comparison of complex shapes is now faster, more accurate and cost-effective than ever.

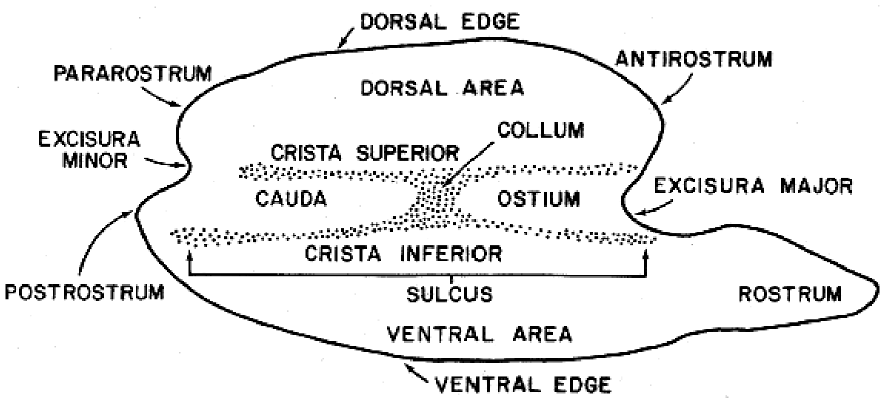


Figure 4 Diagram of inner face of left sagitta showing important characters (modified from Messieh, 1972 & Morrow, 1979); source (McBride et al., 2010)

Fish otoliths (fig. 4) are widely used for morphometric analyses. They are metabolic inert structures located in the inner ear of teleost fish, whose main biological functions are related with balancing and hearing. Otoliths are mostly composed by calcium carbonate ($CaCO3$) in its aragonitic form (>90%), plus minor elements which reflect the environment surrounding the fish and its metabolism (Campana & Thorrold, 2001).

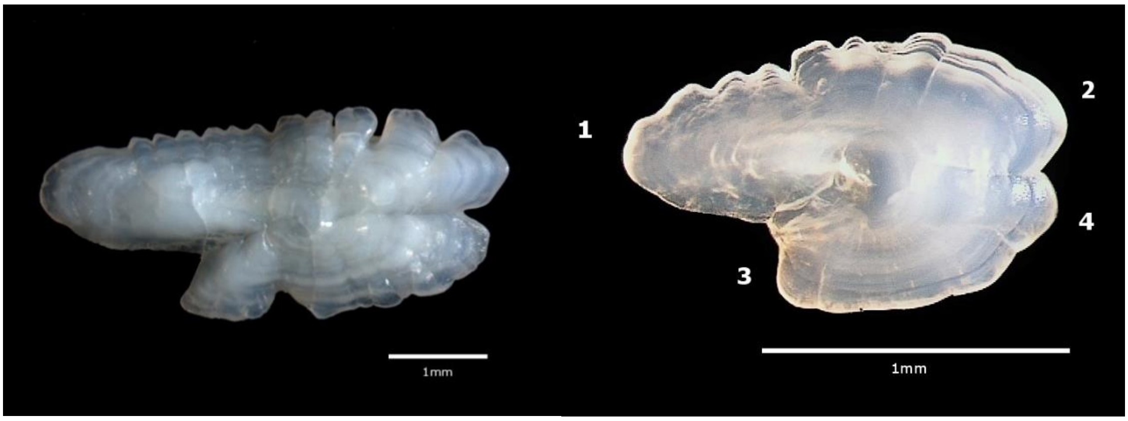


Figure 5 Examples of the diversity of otolith shape and size (white bars = 1mm) among two clupeid species: Left: 6-years-old herring; right: 5-years-old sprat. Labels: 1) Rostrum; 2) Postrostrum; 3) Antirostrum; 4) Pararostrum.

There are three types of fish otoliths: asteriscus, lapillus and sagitta (the bigger in size, used in this study).

The external structure of sprat sagitta otoliths (fig. 5, right picture) is similar among all the three subspecies (Northeast Atlantic, Baltic and Mediterranean Sea; Aps et al., 1991): the ventral edge – between rostrum (1) and postrostrum (2) – is saw-toothed, while dorsal edge – between antirostrum (3) and pararostrum (4) - is usually smooth. Narrow growth rings are formed every winter, in the period before spawning, and at the end of spring the intensive feeding season forms a relatively wide summer growth zone.

The age determination of sprat otolith is performed by recognition and counting of these annual increments, usually by mean of microscope and transmitted light.

These interesting structures have been studied by fishery scientists for long time, revealing themselves useful for several purposes. Otolith's most known and used feature is related to their peculiar pattern of growth consisting in a number of concentric "rings", which reflect the seasonal changes in the environment and can therefore be used to age individuals: this pattern appears in fact like a sequence of opaque and hyaline zones departing from a central nucleus, and reflecting respectively the period of fastest and slowest growth of the organism through the year (Aps *et al.*, 1991).

Otoliths are also characterised by two peculiar features which are not shared with any other known calcified structure. They show, in fact, continuous growth throughout the fish life, and lack of resorption: once the material which forms the otolith has been deposited it will not be used again – not even in case of starvation (Rodríguez Mendoza, 2006).

The otolith shape is species-specific (Tuset *et al.*, 2006), and along with otolith size and growth patterns it varies widely between the different species (fig. 5).

Since genetic variations, fish growth and environmental inputs cause their morphology to vary geographically also within a single species, otolith shape analysis has long been recognized as an efficient tool not only to identify different species (e.g. in studies of predators' diet composition), but even for the discrimination of stocks and populations (Capoccioni *et al.*, 2011).

Environmental variables as water salinity (Berg *et al.*, 2018) and temperature (Gagliano & McCormick, 2004) have been often proved to effect the otolith shape within species, along with biological factors such as size, age, habitat depth (Lombarte & Lleonart, 1993), and diet (Mille *et al.*, 2016).

As the fish grow, the layers added to the otolith are shaped by the initial shape formed at the early life stages, which is strictly depending on genetic factors and the amount of food available to the juveniles: fishes may therefore retain for life the characteristics of the otolith inherited from their spawning places or, if the environmental effects are particularly intense, show a high rate of shape differentiations from the first year of age (Libungan *et al.*, 2015).

Discrimination of fish stocks using shape analysis of otolith contours was developed as a branch of the morphometric techniques, but with recent advances in digital image analysis software it has become a powerful and increasingly popular tool, frequently used to discriminate between fish stocks (Cardinale *et al.*, 2004). Of all the non-genetics methods used for the differentiation of stocks, in fact, otolith shape is the less subject to short-term variability caused by changes in feeding or spawning conditions (Cadrin and Friedland, 1999), and the morphometric analysis of otoliths is therefore considered a well-established method to delineate fish stocks, characterise population movements and detect the natal origin of fishes from mixed samples (Libungan *et al*., 2015).

Moreover, the method is relatively quick and cost effective if compared to other analyses. Otoliths and associated biological data are routinely collected for many commercially relevant fish species as they enable the investigation of age structures, and capturing their digital images is relatively simple with appropriate microscope and camera which are part of the standard equipment in a fishery lab.

1.5 Aims of the project

Given the limited biological knowledge about sprat in Skagerrak-Kattegat, an analytical stock assessment aiming to build a quantitative base for management advice is – at the present moment – hard to be performed (ICES, 2018a): currently, sprat in this area is managed as a single stock unit (ICES, 2013), separated from the North Sea stock; however, even though the boundaries between these two stock management units are highly uncertain (ICES 2018b), and also within 3a there could be several sub-populations so far not identified. The most recent available genetic data (ICES, 2018b) indicated a very uncertain boundary between the two stocks, so an analysis of the differentiation in otolith shape between these two areas could be an effective information to further investigate the matter.

One of the key issues in order to successfully manage fish stocks exploited by different fisheries is to identify the different populations and evaluate their relative contributions to the catches. This study aims to: (a) understand if otolith shape analysis can be used to investigate population structure of sprat and (b) apply otolith shape analysis to infer about population structure of sprat along the Swedish west coast. In order to do so, the otolith shapes of 3a sprat were also compared with the neighbouring populations occurring in the North Sea and from Norwegian fjords in the Bergen area.

2. Materials and methods

2.1 Sampling

The main sample set used in this study is composed by samples collected around the spawning seasons of 2003 and 2004 along the Swedish coast (fig. 6) by both commercial fishing vessels and research surveys as IBTS and Swedish Coastal Monitoring. The range of months (from late January to early July) was selected considering that even though the spawning peak for the sprat in this area is between April and May, and the highest proportion of spawning individuals is between March and July (Vitale *et al*., 2016), spawning individuals may already be found in February.

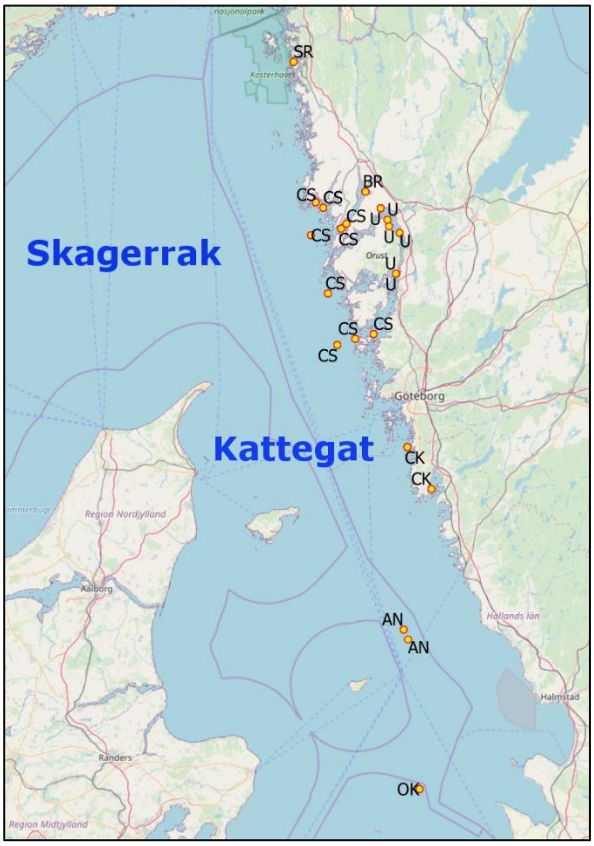


Figure 6 Sampling sites in Division 3a, labelled as in table 2 (according to the groups used in this study).

The sampled locations have been opportunistically chosen among the samples available at the Institute of Marine Research of Lysekil (SLU), giving priority to the Skagerrak as it shows a more diverse coastline compared to the Kattegat.
The southern part of the Division 3a has in fact a less articulated coastline, and a shallow and uniform seafloor with an average depth of 30 m, while the Skagerrak is characterised by a large number of fjords (some of them very isolated, as the Uddevalla fjords system) and a bathymetry varying between 700 m in the centre of the basin and 20-50 m in the inner fjords. Therefore, in order to evaluate the potential variations in otolith shape between both coastal and fjord environments, 16 out of the 21 sampling stations from 3a used in this study are from the Skagerrak basin.
The total number of otoliths examined from this area is 257 for the Kattegat and 866 for the Skagerrak (table 1).

Year	**Month**	**SKA**	***KAT***
2003	February	120	
	April	108	
	May	104	35
	July	58	
2004	January	66	51
	March	92	57
	April	158	
	May	160	10
	July		104
	Tot. (1123)	866	257

Table 1 3a: Number of individuals by year, month and region.

In addition, two datasets from different populations added as “outer groups” contained 281 individuals collected in 2016 by the Norwegian IMR from a system of inner fjords around the city of Bergen, and 73 collected by DTU-Aqua Denmark in 2015-2016 in the North Sea (table 2; fig. 7).

The otoliths were labelled to test several hypotheses of geographical association. This included, for example, merging relatively far points located offshore or along a smooth coastline, when they were known to have similar conditions and no known barriers to the movement of the individuals, or separating two areas within the same – but narrow and articulated – fjord system.

After measuring total length at the nearest millimetre (except for the Norwegian samples, measured in 0.5 cm intervals) and gutted weight, the fishes were sexed and the sagittal otoliths extracted. The otoliths were used independently by two scientists for individual age determination (winter rings counting), and finally stored dry in plastic bags.

Only the otoliths which didn’t show any sign of damage were used in this study.

Region	Position	ID	n. (1477)
Skagerrak	Coastal Skagerrak	**CS**	583
Skagerrak	Uddevalla (Uddevalla fjord)	**U**	208
Skagerrak	Råssö (Northern Skagerrak)	**SR**	17
Skagerrak	Bredungen (Gullmarsfjord)	BR	58
Kattegat	Southern Kattegat	OK	51
Kattegat	Central Kattegat (~ Anholt Island)	**AN**	67
Kattegat	Coastal Kattegat (~ Gothenburg)	CK	139
North Sea	North Sea	NS	73
Norway	Norway	NW	281

Table 2 Sampling locations, related ID labels and total number of individuals per group.

Figure 7 Map showing the regions and the sampling stations examined in this study (yellow dots). Names of the regions are shown in blue, names of the seas in black.

2.2 Image capture and contourline elaboration

Each otolith was photographed with a LEICA DC 500 digital camera mounted on a LEICA MZ16 FA stereomicroscope (reflected light), using the software LEICA Application Suite 4.1.0 (build 1264).

The otoliths were placed sulcus down on a dark microscope plate, with their ventral edge facing upwards and the dorsal edge downwards (terminology as in fig. 4). The digital images were taken in .jpeg format and full colour, ensuring a good focus, a proper contrast between the otoliths and the dark background, and with a fixed resolution of 1044x772 pixels.

Because of the complex three-dimensional structure of the otolith it is not always possible to get an equally good focus on both the anterior and posterior sides. It was found that getting images focused on the post-rostrum provided best overall results with minimum need for post-capture image manipulation.

A photograph of a calibration slide (fig. 8) was taken at the beginning of each microscope session, in order to calibrate the images and derive comparable measurements among the otoliths. Using the software GIMP 2.8.22, 1 millimetre in the calibration photograph was converted into its equivalent in pixels, and used to calibrate pictures taken at the same magnification.

In order to be aged, sprat otoliths are usually mounted on a microscope glass slide and covered by resin. This procedure makes their optical properties more suitable for the observation by transmitted light and the ageing purposes but, unfortunately, it also makes their edges almost impossible to be detected by a digital camera without extensive, prone to error and time-consuming manipulations. Only the right *or* the left otolith of each individual was therefore available for the shape analysis. Almost all the samples used in this study were right-side otoliths but, in a few cases, it was possible to retrieve only the left ones. These photographs of left otoliths were flipped horizontally to have the rostrum facing left as the right.

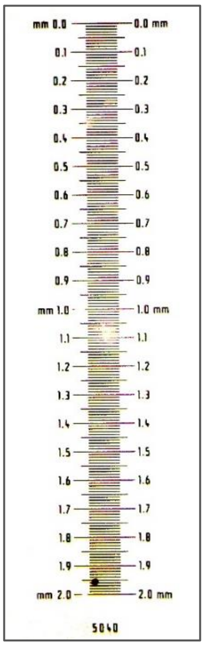


Figure 8 Microscope photograph of the scale used in this study.

The otolith outlines were extracted with of functions written in the R package “shapeR” (Libungan & Palsson, 2015): these functions convert the images into grey-scale and then binarize them using a threshold value defined by the user, in order to detect the edges of the otolith by their contrast against the neighbouring pixels of the dark background.

All the outlines were automatically saved as .png images (fig. 9) and each file was visually evaluated to ensure that the detected outline perfectly traced the edge of the otolith. When the outline was not accurate, the original picture was re-elaborated with a different threshold value, or manually modified with GIMP. The new outlines detected on the fixed file, however, are by default impressed over the original picture, allowing the operator to check that the manual modification didn´t alter the real shape of the otolith.

The main shape measurements (otolith length, width, perimeter and area) based on the scaling information provided with each photograph were also collected.

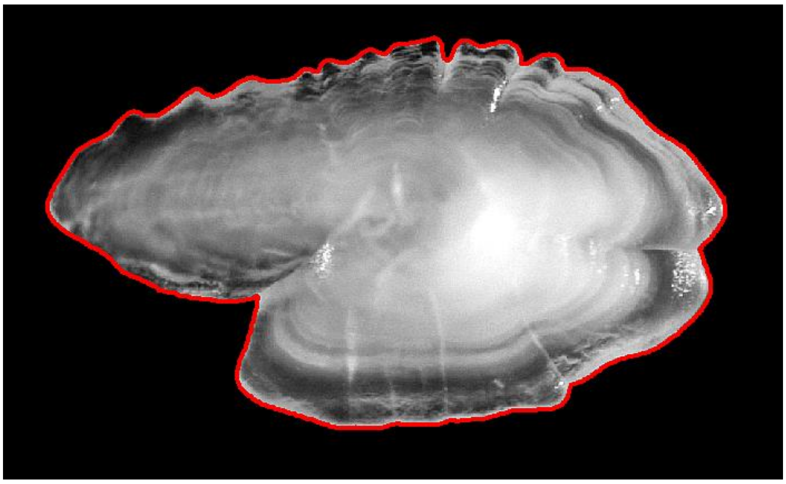


Figure 9 An example of outlined otolith (red line); 3-years-old sprat caught in Skagerrak.

2.3 Shape reconstruction

Conceptually, Wavelet and Fourier analysis can be defined as two different ways of breaking up a signal into the single frequencies which make it up (in our specific case, we aim to describe a complex shape in terms of mathematical waves). This is very useful in many scientific applications, as it allows to decompose different types of signal (e.g. sound, or images) into simple and precisely measurable terms. In otolith shape analysis, radii are drawn at equal angular intervals from a centroid within the contourline to a number of coordinates along it so that, “unrolling” the shape, the radii can be visualized as a series of line segments with different lengths (Campana and Casselman, 1993): this is our *signal*.
Additive levels of waves can then be fitted to the data, in order to replicate as much as possible the pattern described by the top of the radii. Increasing the number of waves increases the amount of coefficients extracted, and therefore the accuracy of the description – better explaining all the variations observed in the shape (fig. 10). However, even though there is virtually no limit to the number of Wavelet levels or Fourier harmonics that can be used, it is usually best to describe the shape with as few levels as possible, thus facilitating the statistical analysis (Campana and Casselman, 1993).
Wavelet and Fourier analysis differ mainly for the type of waves they use: in fact, while Fourier transforms consist of sinusoid waves, which are localised in frequency but lack of a specific time domain, a wavelet has a precise limited duration (is localised in both frequency and time). Wavelet transformations are therefore considered to be better suited to describing the frequencies of highly non-stationary signals with abrupt peaks of discontinuities (Renán *et al.*, 2011), as they not only allow to quantify these changes, but also to determine their exact position. In practice, abrupt transitions in signals result in coefficients with large absolute values and with Wavelet transforms, unlike Fourier, it is possible to examine the variability among the coefficients at a given angle. Each one of the analysed shapes and its parts can then be accurately described and compared against others by the sum of its coefficients.

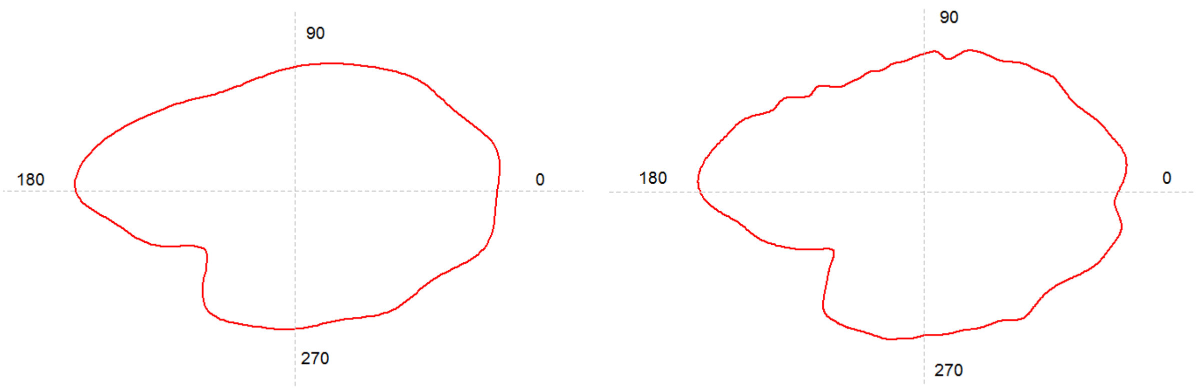


Figure 10 Reconstructed outlines of the otolith shown in fig.9: Fourier (left) and Wavelet (right).

Although the functions implemented in shapeR calculate both Wavelet and Fourier coefficients from the otolith contourlines, only the first method was chosen for the purposes of this study.
By means of the shapeR functions described in Libungan & Palsson, 2015, all the otoliths were set with area = 1 and oriented horizontally along their longest axis prior to the Wavelet transformation; subsequently, starting from a first radial axis drawn from the otolith centroid to the right (defined as the 0° angle of the outline), all the radii were collected clockwise at equidistant angles all along the 360° and the coefficients extracted for further analysis.

2.4 Statistical analysis

Age, as well as size, is known to have confounding effects on otolith shape (Castonguay *et al.*, 1991), and the age coverage of the sample was also not always congruent among the different areas; the dataset was therefore split into age subsets combining groups or single age classes in smaller and more homogeneous sets, thus saving as much information as possible and reducing the age effects which could affect the accuracy of the statistics.
All the analyses were performed within R, using the packages shapeR, vegan, MASS and ade4.

2.4.1 Preliminary tests for differences

The first step of the work was to exclude the effect of any other source of shape variation which was not dependent on genetic or geographical variability.
To check that no significant error was introduced into the analysis during the image capturing process, a dataset of 340 new photos was specifically created by sampling 34 otoliths among all the age classes from random samples, collecting them on a tray and taking photographs with 10 different sets of magnification and lights. After each session the microscope was reset, the settings were changed and the otoliths repositioned. The Wavelet coefficients were then extracted and analysed with Canonical Analysis of Principal Co-ordinates (CAP); the CAP scores among each set of replicates were checked for normality and then compared by means of repeated-measures ANOVA. The canonical scores are very well suited when it comes to evaluate these relative differences, as they shrink most part of the variability into few variables, and the differences among the otoliths can therefore be described by their coordinates in the multivariate space.

As stated in paragraph 2.2, a small amount of individuals (162) were left otoliths. Even though some studies about Baltic sprat (Aps *et al.*, 1991) observed no differences between left and right otoliths, 161 new pairs of unaged otoliths (left-right from the same fish) were used to verify it for 3a sprat; this new

dataset consisted of 73 fishes from Skagerrak and 88 from Kattegat, and was built and used only for the purposes of this check. The shape features were visually evaluated by plotting one against the other the two "average shapes" reconstructed by the mean values of the Wavelet coefficients for the "L" and "R" groups; after checking the coefficients for equal variance (F test) and normality (Kolmogorov-Smirnov test), a paired t-test was performed between the coefficients of each pair of otoliths.
The p-values resulting from the t-tests were plotted with a histogram. The percentage of significant values ($p < 0.05$) was calculated and used to assess whether the otoliths were different or not. Furthermore, following a method implemented in Libungan *et al.*, 2015, the Wavelet coefficients of left and right otoliths were also analysed using CAP: after testing the first canonical score for normality by means of the Kolmogorov-Smirnov test, a paired t-test was performed to see whether there were differences between the canonical scores of the left and right otoliths.

The variation in otolith shape between males and females was tested for the age subsets 1-2, 3-4 and 5-6 on the CAP scores of each otolith, by means of a nested ANOVA with respect to sex and population. Due to insufficient sample size it was not possible to check for temporal stability in the otolith shape among the groups with samples from both 2003 and 2004 (CK, CS, U).

Prior to the univariate analysis, a check for normality and homogeneity of variances was also performed on the otolith measures (length, width, perimeter and area).

2.4.2 Grouping hypotheses

To investigate for geographical differences in otolith shape along the Swedish west coast and between the Skagerrak and its neighbouring areas (North Sea / Uddevalla-fjord / Kattegat) the following groups were considered in the multivariate analyses:

- Coastal Skagerrak – Northern Kattegat – Norway – North Sea [age classes 1-2], in order to evaluate the relative distances between 3a and the neighbouring ICES divisions. The older age classes could not be tested because of insufficient sample size for Norway and North Sea;
- Skagerrak – Northern Kattegat – North Sea [age classes 1-2], to evaluate whether their relative positions in the previous test were influenced by the Norwegian samples;
- Skagerrak – whole Kattegat [age classes 1-3, 4], to check for differences specifically within the division 3a; it was not possible to run this comparison for age classes 1-2 or 3 alone since the sample size for central and southern Kattegat was insufficient;

- Skagerrak – Uddevalla fjord system [age classes 1-2, 3, 4, 5-6]; to specifically test the hypothesis of a sub-population in the fjord.

Many different grouping options were designed and tested for each step of this study. This included, among others, evaluating different ranges of ages (one by one or grouped together) and sample sizes, offshore-inshore and in/out-fjords, as well as wider and smaller-ranged geographical scales.
The combinations of groups and age classes showed in the further paragraphs were chosen as they seemed to best explain the shape variations and to be more significant both visually and statistically. The approach of reducing the analyses to one or few age classes to account for age-related differences is done accordingly to other studies, such as Libungan *et al.,* 2015.

2.4.3 Main shape features

The quality of the Wavelet reconstruction was estimated by calculating the deviation of the otoliths from the reconstructed outlines and the number of Wavelet levels needed for a 98.5% accuracy.
The differences in shape were visually evaluated by plotting the average shape obtained from the reconstructed outlines (rotated and normalised) of each group; in order to inspect which areas of the otoliths contourline and coefficients contribute most to the differences between the groups the mean shape coefficients and their standard deviation were plotted against the angle of the outline from which they were extracted. To further investigate the partition of this variability, the proportion of variation among groups (intraclass correlation, ICC) was also calculated along the outline.

2.4.4 Univariate analysis

The otolith length, width, perimeter and area within the age classes 1 and 2 were used as univariate shape descriptors and compared among the groups. The metrics were first visually compared with box plots, then the differences were evaluated with an analysis of variance (ANOVA). Length and width of an otolith are here intended as the longest horizontal and vertical distances within the outlines.

Since a significant p-value in ANOVA only indicates that *at least* one group differs from the others, a Tukey Honest Significant Differences test (TukeyHSD) was computed on the ANOVA outputs in order to perform multiple pairwise-comparisons between the means of the groups, thus highlighting which populations contributed to the significativity of the values.

As all the post-hoc tests, Tukey´s test is known to be quite conservative; however, it was preferred to less conservative options because this kind of test is less likely to make Type II errors (false positives).

2.4.5 Multivariate shape analysis

Each otolith shape was represented by conducting a discrete Wavelet transformation on the equally space radii drawn from the otolith centroid to the outline, thus extracting the independent Wavelet coefficients.

Since different fish populations are known to have different growth rates, the otolith shape had to be adjusted with respect to allometric relationships with the fish length; an analysis of covariance (ANCOVA) was performed to detect possible interactions between the fish length and the Wavelet coefficients within each population.

The increase in the probability of making one or more type I errors due to the testing of multiple hypotheses simultaneously was taken into account by applying a Bonferroni adjustment during this step of the analysis. In fact, when k independent significance tests are performed, the probability to get no significant differences in all of them (what is called a *Type I error*) is defined as the product of the individual probabilities: $(1 - \alpha)^k$ (Abdi, 2007); this means that the probability of identifying *at least* one significant result by chance increases with the number of hypotheses tested.

The Bonferroni adjustment is a correction made to the p-values to reduce the chances of obtaining false-positive results, by dividing the critical threshold value (α) by the number of tests performed (k):

$$\alpha' = \alpha / k.$$

Every time a significant interaction between a Wavelet coefficient and fish length was detected within a population, the coefficient was discarded and excluded from further analyses. The others were kept and normalised.

The otolith shape was then compared with overall tests and pairwise comparisons to test for regional differences. To investigate the variation in shape among the groups, the standardised Wavelet coefficients were compared with ANOVA-like permutation tests using 2000 permutations (function written in the *vegan* package) and with Canonical Analysis of Principal co-ordinates (CAP) using the *capscale* function (also in *vegan*).

The relative positions of the population centroids along the first two canonical axes were graphically examined. The CAP scores along the first discriminant axis were compared among selected pairs of groups to better investigate their differences.

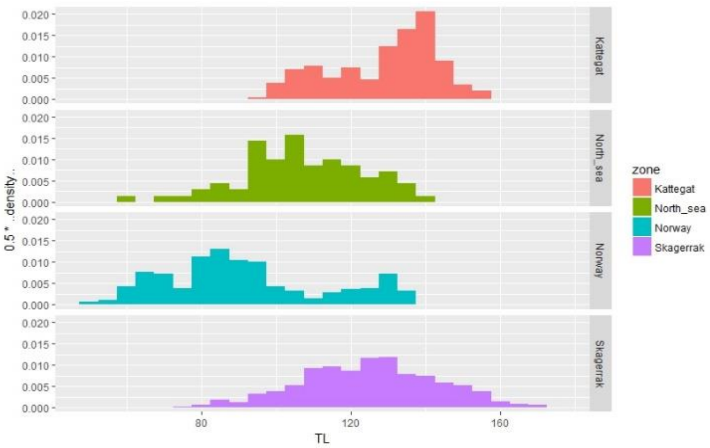
Individual assignment of the otolith geographical origin was evaluated using a Linear Discriminant Analysis (LDA) on the standardised Wavelet coefficients. LDA is a supervised method used to discriminate among predefined groups of individuals based on a sample of observations from each one of the original groups. The classification success rate was estimated using a *leave-one-out* procedure.

3. Results

3.1 Length and age distribution

The total length (T_L) distribution of the sampled sprat (fig. 11a) shows a similar length range for North Sea and Norway. The sampled individuals from these two areas are mainly distributed between age 0 and age 2 (fig. 11b; very few samples are available for North Sea at age 3 and 4, not enough to be included in the analyses), and show a wide length range comparable in width to the length ranges shown for Skagerrak.

a



b

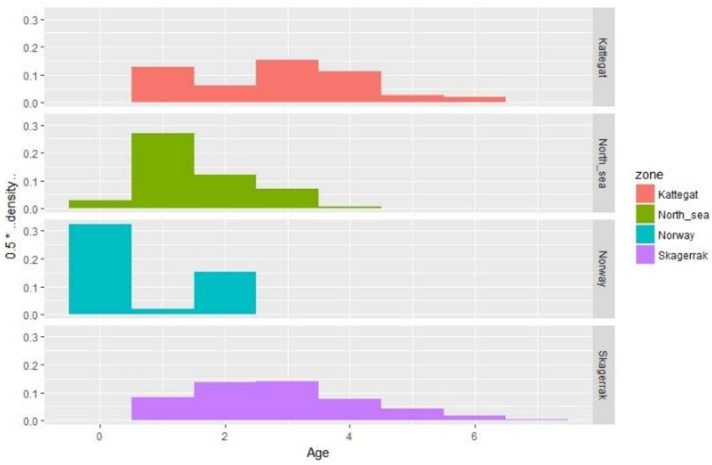


Figure 11 Age (panel a) and length distributions (panel b) of the samples by region. Regions: Kattegat (red); North Sea (green); Skagerrak (blue); Norway (purple).

Kattegat is the area with less variability in length (fig. 12), but the individuals within this area are by average as long as in Skagerrak and no differences can be observed between the overall life span between the two areas (maximum age 7 years; fig. 11b).

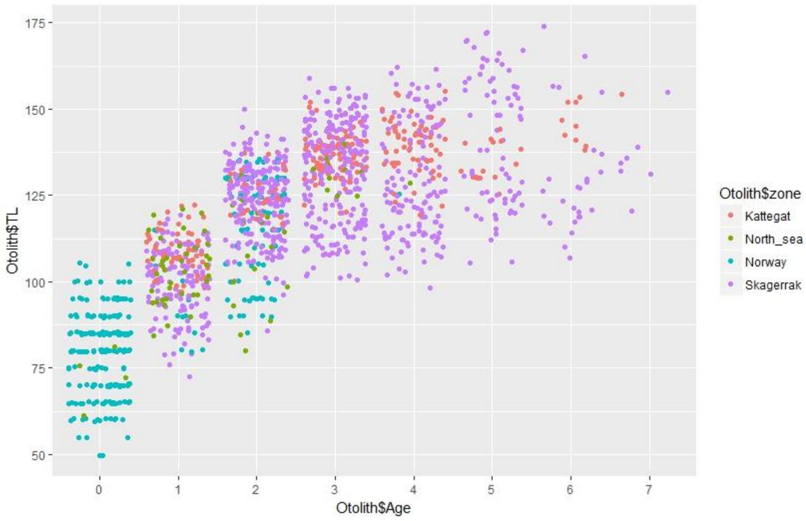


Figure 12 Relations between age and fish length in the data. The observations are grouped by region. Regions: Kattegat (red); North Sea (green); Skagerrak (blue); Norway (purple);

The Norwegian and North Sea samples show in age 2 similar length ranges (fig. 13), respectively of 80-131 and 85-135 mm.

The mean T_{Ls} of the sampled sprat for the age class 2 (fig. 13, left panel) don´t appear to differ when an overall comparison is made between the Kattegat and Skagerrak; however, the Skagerrak area shows a high variability between coastal and fjord regions: the T_{Ls} in the coastal stations range from 86 to 150 mm, with a mean of 128 mm; the fjord populations, on the other hand, are significantly shorter, with T_L ranging 96-119 mm (mean 108.7 mm) in U and 106-131mm (mean 113.6 mm) in BR.

The length range in the whole Kattegat for age 2 is considerably narrower, from a minimum of 115 to a maximum of 138mm. No significative differences in mean length exist between the coastal Skagerrak (CS) and the coastal Kattegat (CK), but both the groups differ from the two Skagerrak fjords. Similar relative patterns are showed in the class 3 (fig. 13, right panel): the range in Skagerrak is 101-159 mm (overall mean 132.4 mm, 139.3 mm with fjords excluded), with a 102-132 mm range in the fjords; in Kattegat, total lengths range 120-152 mm (mean 136.4 mm).

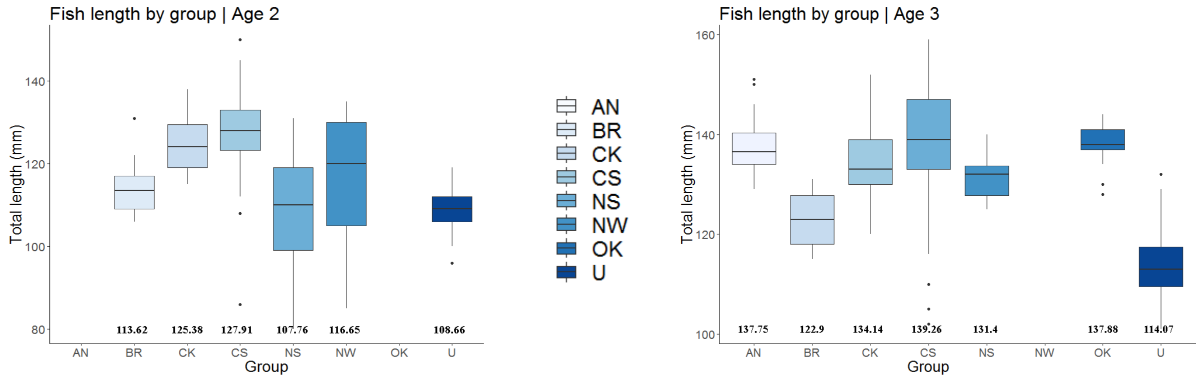


Figure 13 Box plots showing the total length distributions for each group. The mean values are showed as black numbers under each box. Age 2 on the left, age 3 on the right. Groups: AN: central Kattegat; BR: Gullmarnfjord; CK: coastal Kattegat; CS: coastal Skagerrak; NS: North Sea; NW: Norway; OK: southern Kattegat; U: Uddevalla fjords.

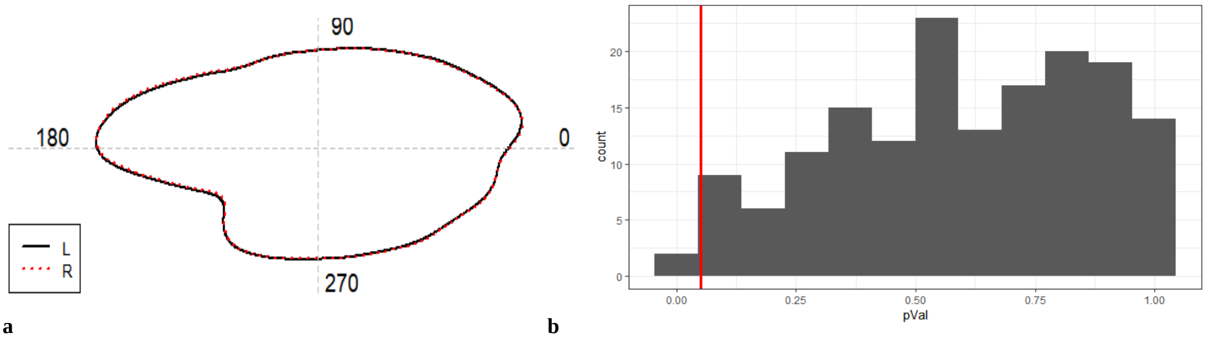
3.2 Preliminary tests

Replicates, left/right side and males/females were checked for differences.

The canonical scores of the 10 sets of replicates were checked by means of a repeated-measures ANOVA (or *within-subjects* ANOVA), which is suitable to detect the effect of different treatments on a set of observations. The ANOVA gave an overall p-value of 0.53, which means that no significant differences were detected between the shapes (in terms of coefficients) of the repeated sample sets.

The reconstructed average-shape plot for left vs right, and the t-tests performed on the coefficients of each pair of otoliths, showed no significant variations between the two groups (fig. 14a); less than 2% of the tested pairs fell below a p-value of 0.05 (which corresponds to 3 pairs out of 161; fig. 14b), with an average value of 0.6.

The t-test performed on the canonical scores of left and right otoliths also showed non-significant differences (P-value > 0.10).



a **b**

Figure 14 a. Average Wavelet-reconstructed shape of left and right otoliths sampled in pairs from the same fishes; b. Bar-plot showing the distribution of the p-values from each t-test (significant values displayed on the right of the red line).

The nested ANOVA performed on the canonical scores with respect to sex and population gave non-significant values for all the age classes tested (table 3).
Consequently, the following analyses were conducted on merged data for males and females, as well as left and right otoliths.

Age	n. males	n. females	p-value
1-2	275	295	0.11
3-4	213	303	0.31
5-6	54	68	0.21

Table 3 P-values of the nested ANOVA performed on 3 groups of age classes (1-2, 3-4, 5-6)to check for variations with respect to sex and sampling areas.

The otolith measures for the age classes 2 and 3 (analysed within univariate analyses) were checked for normality by means of the Kolmogorov-Smirnov test and for homogeneity of variance with Levene´s test (table 4). For the age 2, only the samples from 3a were included in the ANOVA since by including the North Sea and Norwegian samples the data wouldn't respect the assumption of homogeneity of variances.

Age	Otolith variable	K-S	Levene
2	Length	0.12	0.22
	Width	0.95	0.65
	Perimeter	0.43	0.31
	Area	0.95	0.18
3	Length	0.73	0.16
	Width	0.75	0.32
	Perimeter	0.87	0.12
	Area	0.88	0.35

Table 4 P-values of the tests performed to check for the normality (Kolmogorov-Smirnov) and homogeneity of variances (Levene´s test) of the otolith metric variables in age 2 (NW - NS excluded) and age 3.

3.3 Main shape features

The number of Wavelet coefficients extracted increases by the power of 2 for each Wavelet level; 63 coefficients were collected for each outline in this study using 6 Wavelet levels.

The quality of the reconstruction rises with the number of Wavelet levels (fig. 15), and the shape of sprat otolith appears to be precisely described (with a 98.5% accuracy with respect to the original otolith contourline) by the sum of the first 5 Wavelet levels.

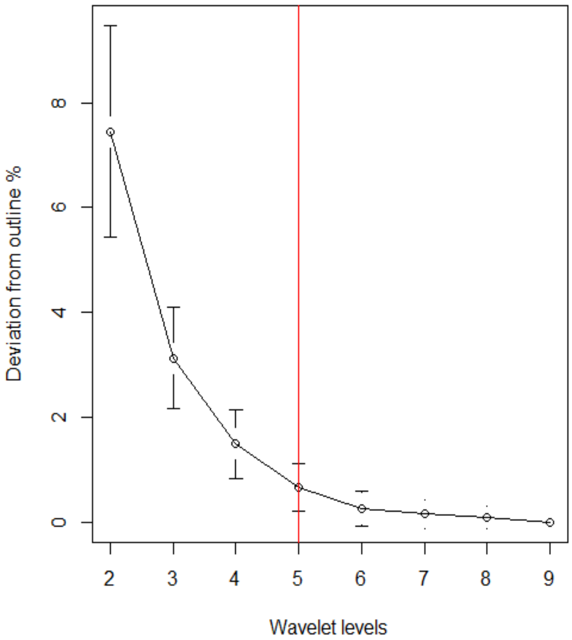


Figure 15 Quality of the Wavelet outline reconstruction. The red vertical line shows the level of Wavelets needed for a 98.5% accuracy of the reconstruction.

<u>Overall differences among populations</u>

The differences among Norwegian, North Sea, Skagerrak and Kattegat for all the sampled were visualised by means of their average shapes (fig. 16).

The strongest differences between 3a and North Sea (fig. 17) are detected at the *excisura major* (EM), while other minor regions of variation are in proximity of *postrostrum* (Po), rostrum (R) and *antirostrum* (A).

The shape of the Norwegian otoliths differs from 3a along all the contourline, except for the *excisura major* and some regions around 150° and 270°; the strongest changes between NW and 3a occur around the rostrum (just past 180°) and between *pararostrum* (Pa) and *postrostrum*. North Sea (NS) and Norway are very similar around the *antirostrum*.

Barely any difference can be observed at this level between Kattegat and Skagerrak (respectively KA and SK, the black and the blue solid lines).

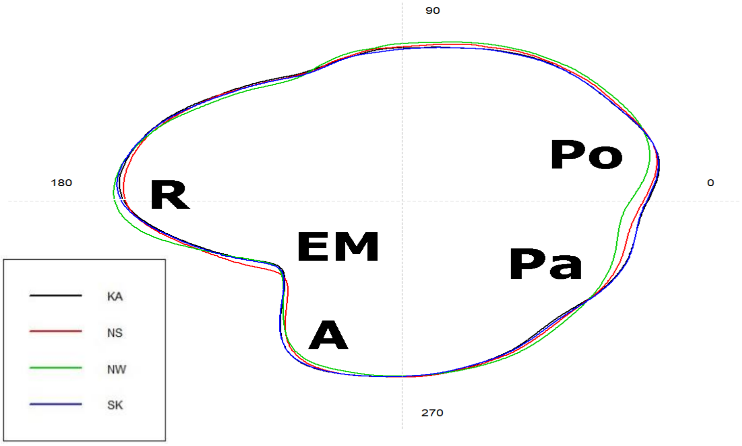


Figure 16 Average shape for all otoliths grouped by sea. The groups are: KA: Kattegat; NS: North Sea; NW: Norway; SK: Skagerrak. Black letters: R: Rostrum; EM: Excisura major; A: Antirostrum; Pa: Pararostrum Po: Postrostrum.

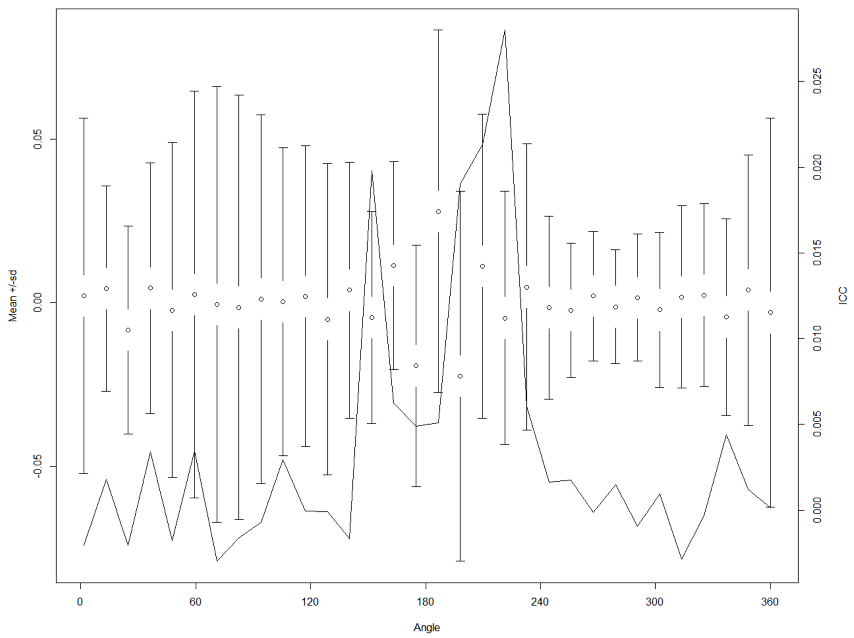


Figure 17 Mean, standard deviation and proportion of variance of the Wavelet coefficients representing the shape differences among Kattegat (KA), Skagerrak (SK), North Sea (NS) and Norway (NW).

This first visual inspection seems to suggest a certain degree of differentiation among all the groups identified in this study, as shown by the differences in the average shapes (fig. 18) and by the high level of intraclass variation in the Wavelet coefficients for the areas of the otolith outline(fig. 19).

The most variable regions of the otolith among different sprat populations appear to be the *rostrum* (R), the *excisura major* (EM) and the *excisura minor* (Em).

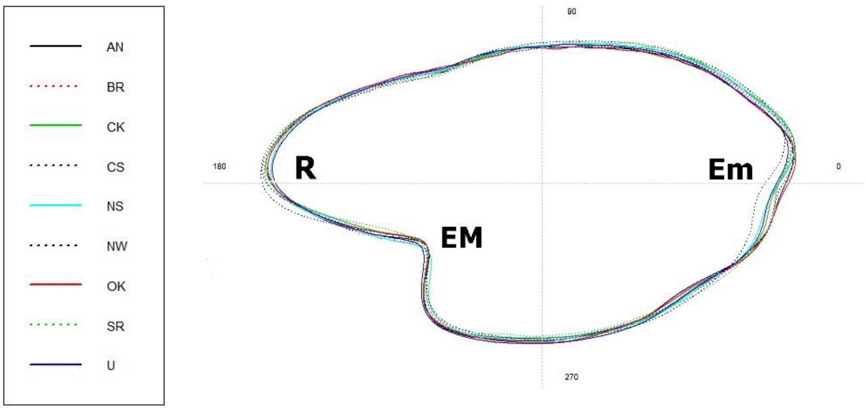


Figure 18 Average shape for all otoliths from the 9 groups tested. The areas are: AN: Central Kattegat; BR: Gullmarnfjord; CK: Coastal Kattegat; CS: Coastal Skagerrak; NS: North Sea; NW: Norway; OK: Southern Kattegat; SR: Northern Skagerrak; U: Uddevalla fjords. Black letters: R: Rostrum; EM: Excisura major; Em: Excisura minor.

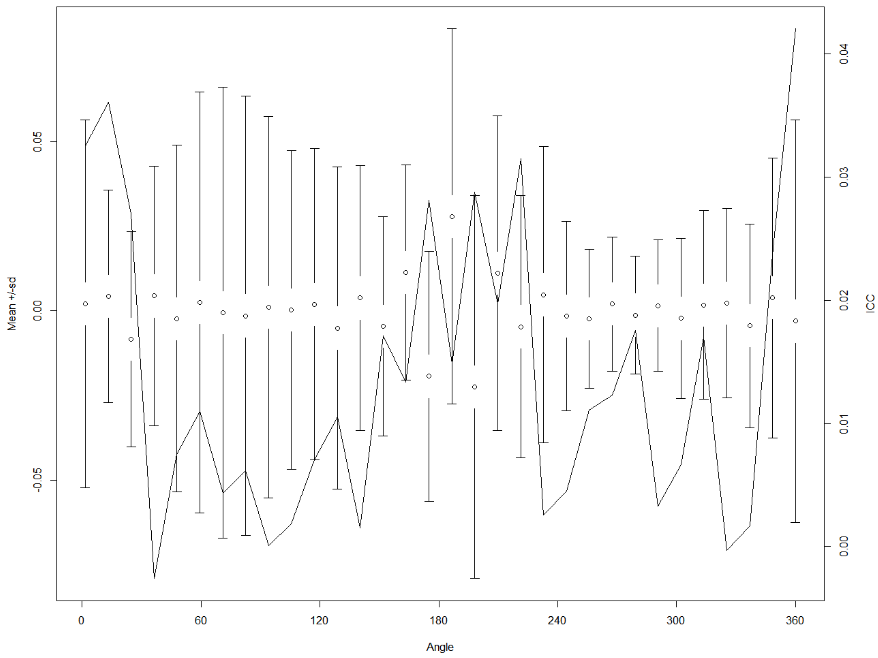


Figure 19 Mean and standard deviation of the Wavelet coefficients representing shape for all the sampled otoliths and groups, and proportion of variance among groups (ICC, black line); the higher coefficients correspond higher variations along the single outlines The X axis shows the angle in degree.

Further inspections of the average shapes show that the morphology of the Norwegian otoliths (NW) is the most divergent of all the samples, especially at *rostrum*, *excisura minor* and on the ventral side.

The first two regions are, respectively, the farthest and the closest to the average centroid among all the shapes.

The two offshore populations from central and southern Kattegat (AN, OK) appear here to be the most similar at shape. OK, AN and CK (coastal Kattegat) are similar at *rostrum*, *excisura major* and *antirostrum*, while CK is highly different from the other Kattegat populations between *pararostrum* and *postrostrum*, where it mimics the shape of the North Sea population.

The coastal Skagerrak samples (CS) seem to be the group with the longest otoliths (maximum distance from the centroid along the X axis), while U is the shortest. Intermediate, similar lengths are showed for the other 3a populations.

By further observations made on age subsets 1-3 and 4 (not shown) similar patterns were observed, and the most variable regions of the otolith identified so far do not seem to change with age.

3.4 Univariate shape analysis

Otolith length, width, perimeter and area were used as univariate shape metrics and were analysed with ANOVA at the age classes 2 and 3 (table 5).

3.4.1 Otolith length

In both age 2 and 3 (fig. 20), the otoliths in 3a are the longest among our samples (mean values: ~1.8 mm ~2 mm respectively); the only exception is Uddevalla, which otoliths are the shortest at both the age classes (age 2: 1.62mm; age 3: 1.79mm); the population of the Gullmarnfjord appear to have slightly longer otoliths than what would be expected from their T_L, and no more significative differences in otolith length were detected among the 3a populations except for U.

The North Sea sprat also presents very short otolith in the age 2 (with mean values similar to Uddevalla, ~1.65mm), but this feature is not showed in the older age class, where the length values are comparable with 3a (~1.9mm; the Uddevalla length values, on the other hand, remain the lowest).

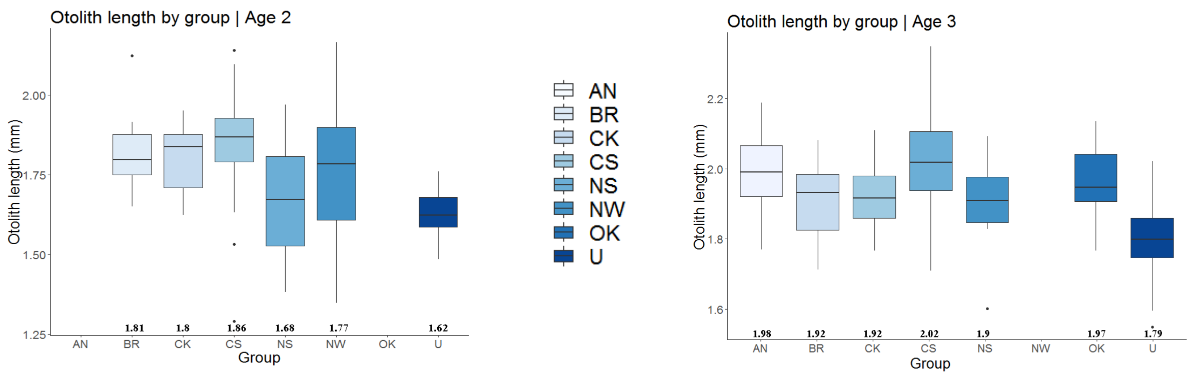


Figure 20 Distributions of otolith lengths among the sampled populations. Left: age 2; right: age 3. Mean values shown in black under each box. Groups: AN: central Kattegat; BR: Gullmarnfjord; CK: coastal Kattegat; CS: coastal Skagerrak; NS: North Sea; NW: Norway; OK: southern Kattegat; U: Uddevalla fjords.

3.4.2 Otolith width

While North Sea and Norwegian samples in age 2 have different otolith lengths, their otoliths don´t differ in width (fig. 21, left panel), with mean values similar to the Uddevalla populations (1.1mm) and smaller than the average for Kattegat and Skagerrak (> 1.15mm). However, as well as for the otolith length, the width of the NS samples grows in age 3 (fig. 21, right panel), to values similar to 3a (1.26 vs 1.28mm), with no statistical differences detectable.

There are no significative differences in width among the 3a otoliths (except for U) for both the age classes.

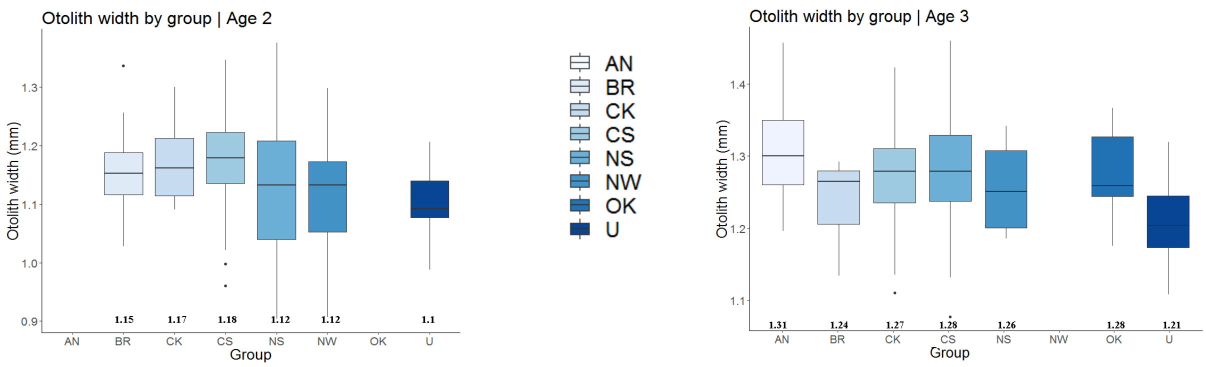


Figure 21 Distributions of otolith widths among the sampled populations. Left: age 2; right: age 3. Mean values shown in black under each box. Groups: AN: central Kattegat; BR: Gullmarnfjord; CK: coastal Kattegat; CS: coastal Skagerrak; NS: North Sea; NW: Norway; OK: southern Kattegat; U: Uddevalla fjords.

3.4.3 Otolith perimeter

As the otolith length, the perimeter values for the age class 2 (fig. 22, left panel) are the lowest in U and NS (means: 4.5 and 4.7mm respectively), while similar – higher – values are shared between NW, CK and CS (~5mm).

The otolith perimeter in age 3 (fig. 22, right panel) is the most variable among all the analysed otolith measures: U and NS have similar, small values as usual (along with BR), but differences are here detected also between CK (~5.4mm) and both CS/AN (~5.6mm).

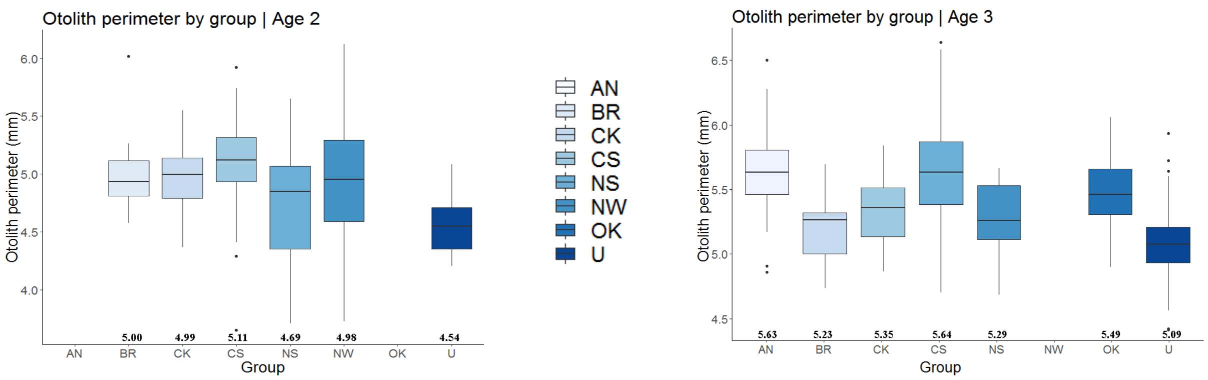


Figure 22 Distributions of otolith perimeters among the sampled populations. Left: age 2; right: age 3. Mean values shown in black under each box. Groups: AN: central Kattegat; BR: Gullmarnfjord; CK: coastal Kattegat; CS: coastal Skagerrak; NS: North Sea; NW: Norway; OK: southern Kattegat; U: Uddevalla fjords.

3.4.4 Otolith area

The only differences in the otolith area (fig. 23) were detected between U and the other groups, even though very low non-significant values (~0.05) are computed between NS and both CS and AN.

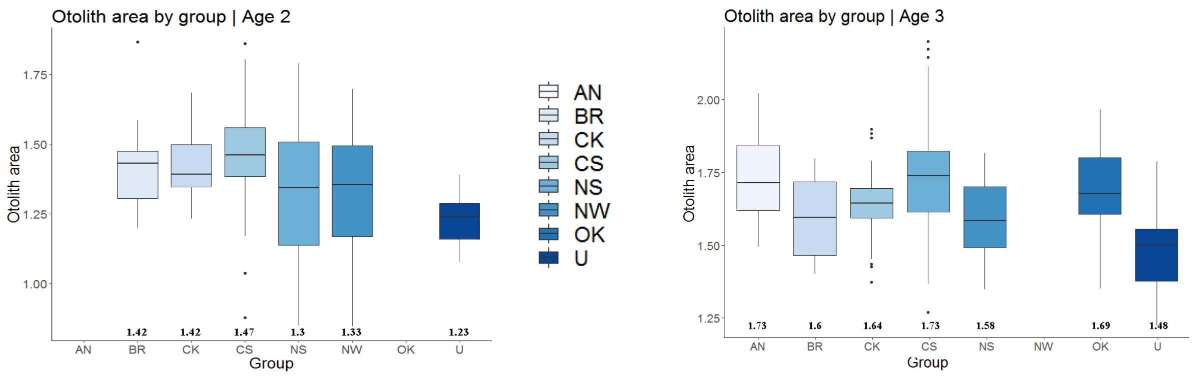


Figure 23 Distributions of otolith areas among the sampled populations. Left: age 2; right: age 3. Mean values shown in black under each box. Groups: AN: central Kattegat; BR: Gullmarnfjord; CK: coastal Kattegat; CS: coastal Skagerrak; NS: North Sea; NW: Norway; OK: southern Kattegat; U: Uddevalla fjords.

	[Age 2]				[Age 3]			
Groups	**length**	**width**	**perimeter**	**area**	**length**	**width**	**perimeter**	**area**
U-BR	< 0.001	0.04	< 0.001	< 0.001	0.04	n/sign	n/sign	n/sign
U-CK	< 0.001	0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.01	< 0.001
U-CS	< 0.001	0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
U-AN	-	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001
U-OK	-	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001
U-NS	-	-	-	-	n/sign	n/sign	n/sign	n/sign
BR-CK	n/sign	n/sign	n/sign	n/sign	n/sign	n/sign	n/sign	n/sign
BR-CS	n/sign	n/sign	n/sign	n/sign	n/sign	n/sign	n/sign	n/sign
BR-AN	-	-	-	-	n/sign	n/sign	0.02	n/sign
BR-OK	-	-	-	-	n/sign	n/sign	n/sign	n/sign
BR-NS	-	-	-	-	n/sign	n/sign	n/sign	n/sign
CK-CS	n/sign	n/sign	n/sign	n/sign	n/sign	n/sign	0.01	n/sign
CK-AN	-	-	-	-	n/sign	n/sign	0.02	n/sign
CK-OK	-	-	-	-	n/sign	n/sign	n/sign	n/sign
CK-NS	-	-	-	-	n/sign	n/sign	n/sign	n/sign
OK-CS	-	-	-	-	n/sign	n/sign	n/sign	n/sign
OK-AN	-	-	-	-	n/sign	n/sign	n/sign	n/sign
OK-NS	-	-	-	-	n/sign	n/sign	n/sign	n/sign
NS-CS	-	-	-	-	0.03	n/sign	0.03	(0.05)
NS-AN	-	-	-	-	n/sign	n/sign	n/sign	(0.05)
CS-AN	-	-	-	-	n/sign	n/sign	n/sign	n/sign

Table 5 Adjusted p-values for the Tukey multiple comparisons of means performed on the ANOVA outputs of age 2 and 3 (empty cells indicate that the comparisons were not made).

3.5 Multivariate otolith shape analysis

When analysing sprat otoliths, the standardisation by T_L of the whole data set causes a remarkable loss of information, as it removes a significant number of Wavelet coefficients: respectively 19 and 13 (out of 63 coefficients) were discarded when the geographic region (Norway – North Sea – Skagerrak - Kattegat) or the area IDs were set as class variable in the ANCOVA – even accounting for the Bonferroni correction.

A number of discarded coefficients > 8 was usually observed to negatively interfere with the detection of differences between the sampled populations; however, usually 0 – and never more than 3 coefficients – were discarded in the ANCOVA performed prior to each one of the multivariate analysis shown in this paragraph on the designed subsets.

The first two of the four grouping hypotheses presented in paragraph 2.4.2 will be analysed in the next paragraph (3.5.1), while the other two will be explored respectively in the paragraphs 3.5.2 and 3.5.3.

3.5.1 Overall differences among populations

Significant differences in shape (table 6) were detected by the ANOVA-like permutation tests among all the sprat populations at ages 1-2, 1-3 and 4, with highly significant p-values (< 0.001). When comparing CS and CK within the age group 1-3 no significant differences are detected between the groups (table 6). The within-Kattegat analysis (CK, AN, OK) detected significant differences for age 1-3, while at age 4 the populations did not differ (but the sample size for CK at this age is very small). Significant differences are also found among the Skagerrak samples for both the age groups, even though the F-values are slightly lower in the analysis performed on age 4; the pairwise comparisons between CS and U also show similar patterns, with highly significant p-values for both age classes 1-3 and 4.

Examining the positions of the populations based on shape variations along the first canonical axis for ages 1-2 in fig. 24, is possible to identify 2 clusters: the 3a and North Sea populations stick together, while the Norwegian samples stand alone. Some differentiation between 3a and North Sea is observed along the second component, but the explained variation on this axis is very low (15.2%) compared with the first one (77%). This result is also supported by the pairwise comparisons between CS and NS and between CS and NW (table 6), where different F-values were computed among the two analyses.

	[	1 - 2	years	]	[	1 - 3	years	]	[	4	years	]
	df	**var**	***F***	***P***	**df**	**var**	***F***	***P***	**df**	**var**	***F***	***P***
All pop.	6	24.49	4.97	0.001	8	39.73	6.61	0.001	5	16.85	3.11	0.001
CS vs NS	1	4.37	5.37	0.001	-	-	-	-	-	-	-	-
CS vs NW	1	8.84	10.45	0.001	-	-	-	-	-	-	-	-
NS vs U	1	2.46	3.30	0.006	-	-	-	-	-	-	-	-
CS vs CK	-	-	-	-	1	1.13	1.52	0.12	-	-	-	-
CS vs U	-	-	-	-	1	10.72	12.25	0.001	1	6.31	6.67	0.001
CS vs OK	-	-	-	-	1	1.42	1.69	0.08	1	1.95	1.92	0.05
OK vs U	-	-	-	-	1	3.31	4.12	0.003	1	3.57	3.32	0.004
within KA	-	-	-	-	2	3.51	2.10	0.007	2	3.40	1.39	0.12
within SK	-	-	-	-	3	13.63	5.20	0.001	2	9.48	4.80	0.001

Table 6 Results from pairwise ANOVA like permutation tests based on 2000 permutations. Results for age groups 1-2 years, 1-3 years, 4 years are shown separately. df: degrees of freedom; var: variance; F: F-value; P: P-value. p<0.05 indicates a significant effect.

The same analysis was performed on the same age group (1-2) with the Norwegian samples excluded (fig. 25); this plot further demonstrates as the short distance detected between the North Sea and 3a populations in fig. 24 was not biased from the fact that an external, highly different population (NW) was included in the same analysis, but is a constant feature: the relative positions of the centroids and their canonical scores along the first canonical axis are unchanged.

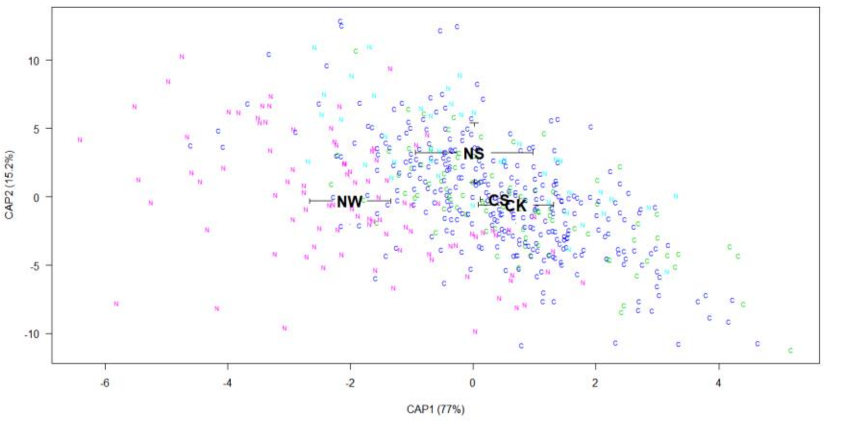


Figure 24 Canonical scores on discriminating axes 1 (CAP1) and 2 (CAP2) for the sprat populations for age group 1-2. CK: Coastal Kattegat; CS: Coastal Skagerrak; NS: North Sea; NW: Norway. The black letters represent the mean canonical value for each population.

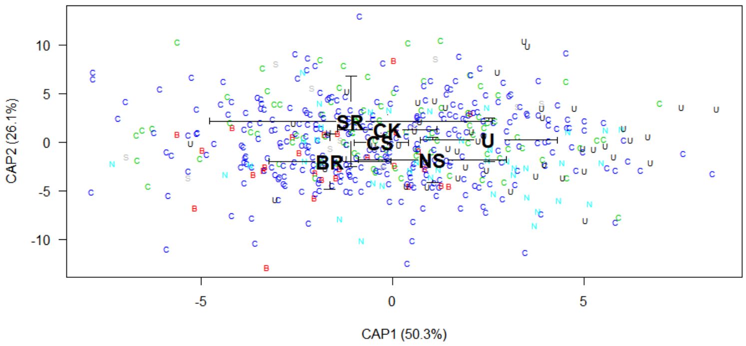


Figure 25 Canonical scores on discriminating axes 1 (CAP1) and 2 (CAP2) for the sprat populations for age group 1-2. BR: Gullmarnfjord; CK: Coastal Kattegat; CS: Coastal Skagerrak; NS: North Sea; SR: Northern Skagerrak; U: Uddevalla fjords. The black letters represent the mean canonical value for each population.

3.5.2 Division 3a

When age 3 is included, it is possible to analyse the relative positions of the central and southern Skagerrak with respect to the rest of 3a (fig. 26, left panel).

A 2-clusters pattern seems to be distinguishable (fig. 26 left panel) along the first discriminant axis, where the fjord population of U is separated from the others. An evaluation of the second axis seems to suggest a slight differentiation between the coastal and fjord samples (CS-CK-U) and the offshore ones (OK-AN); however, the ANOVA-like test gave a slight non-significant p-value of 0.08 when comparing CS with OK in this age group (table 6).

The comparisons between CS and U and between OK and U support the CAP output and the separation of the Uddevalla group.

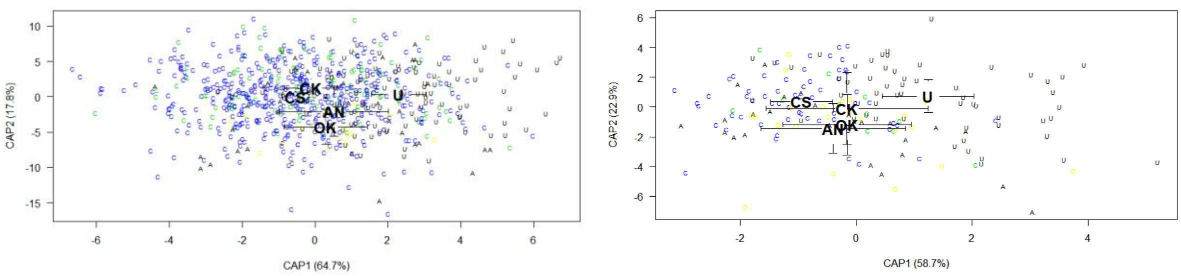


Figure 26 Canonical scores on discriminating axes 1 (CAP1) and 2 (CAP2) for the sprat populations analysed at age classes 1-3 (left) and 4 (right). AN: Central Kattegat; CK: Coastal Kattegat; CS: Coastal Skagerrak; OK: Southern Kattegat; U: Uddevalla fjords. The black letters represent the mean canonical value for each population.

A similar pattern was observed for age 4 (fig. 26 right panel), but as shown in the lower CAP values and by the values of the comparisons in table 6, the differences among populations decrease as the age increases. The Kattegat populations at this age seem to not differ among themselves; along the first axis the 3a populations appear here as a single cluster, while U is again quite distinct, and diverges from the rest. No patterns are shown on the second canonical axis, where all the population centroids have similar scores .

The comparison between CS and OK gives a p-value of 0.05, not in discordance with CAP. The position of U is also confirmed by the ANOVA-like results: in the analysis performed at age 4, in fact, the population remains well isolated from the rest of 3a (comparisons performed on CS-U and OK-U, both with significant p-values).

3.5.3 Skagerrak - Uddevalla

ANOVA-like permutation tests and Canonical Analysis of Principal co-ordinates were performed separately on the Skagerrak samples CS and U, to further investigate the degree of differentiation of the fjord samples from neighbour coastal areas. The sample size per group was here large enough (>40) to perform the analysis on a larger number of separate age classes. The following age groups were investigated: age 1-2, 3, 4, 5-6.

The CAP scores of the otoliths along the first discriminant axis were plotted in a histogram, and the two populations were compared against each other (fig. 27). The differences between the two groups were statistically significant for all age groups (table 7) which motivated further analysis at smaller geographical scale within the region (fig. 28).

Age	**df**	**var**	***F***	***P***
1-2	1	4.26	5.23	0.001
3	1	5.21	5.41	0.001
4	1	6.31	6.67	0.001
5-6	1	7.83	3.16	0.001

Table 7 Results from ANOVA like permutation tests based on 2000 permutations between CS (Coastal Skagerrak) and U (Uddevalla-fjord). Results for age groups 1-2 years, 3 years, 4 years and 5-6 years are shown separately.
df: degrees of freedom; var: variance; F: F-value; P: P-value. p<0.05 indicates a significant effect.

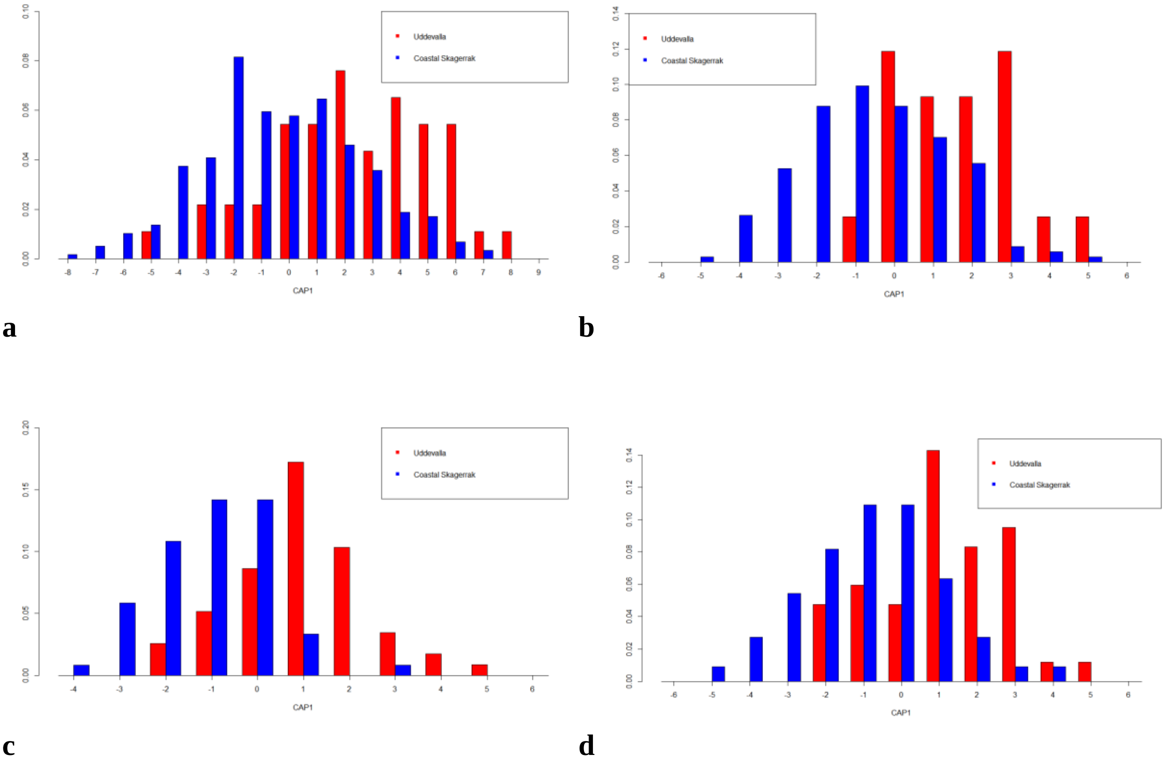


Figure 27 Canonical scores on discriminating axis 1 (CAP1) for the sprat populations CS (Coastal Skagerrak, blu bars) and U (Uddevalla fjords, red bars); (a) Age group 1-2; (b) age 3; (c) age 4; (d) age group 5-6.

For this scope the samples within the Uddevalla fjord system were separated between those in the inner (U*) and intermediate (C1) part of the fjord (fig. 28). Otolith shape of individuals from the two groups showed no significant differences at age 1-3, while significant differences were found for age 4 (table 8). The otolith shapes of age 4 fish from C1 are different, and they appear to be intermediate between the samples of CS and U*, as shown by their distribution along the first axis of the CAP analysis (Fig. 29).

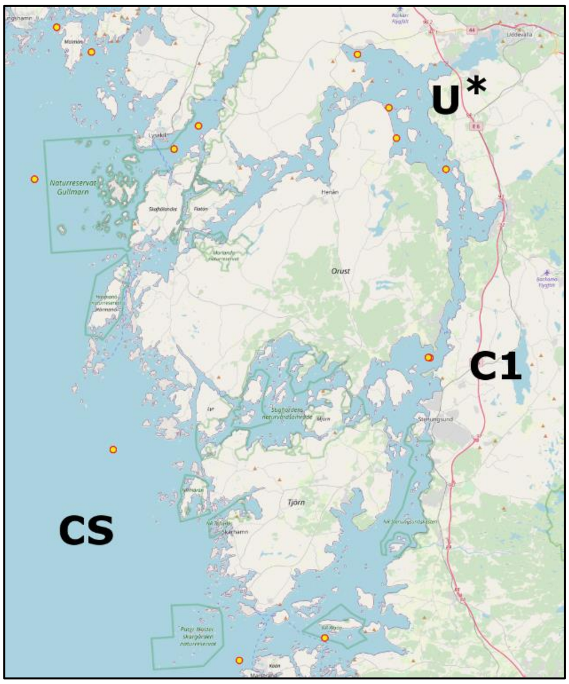


Figure 28 Map showing the fjord system near the city of Uddevalla (in the upper-right corner) on the Swedish west coast North of Gothenburg, and the ID labels used to refine the analysis. Labels: CS: coastal Skagerrak; C1: mid-fjord samples; U: inner fjord samples.*

	[C1 - U*]				[C1 - CS]			
Age	**df**	**var**	***F***	***P***	**df**	**var**	***F***	***P***
1-3	1	0.89	1.11	0.33	1	7.75	8.72	0.001
4	1	2.30	2.39	0.021	1	5.20	5.62	0.001

Table 8 Results from ANOVA like permutation tests based on 2000 permutations between the new labels CS (Coastal Skagerrak), C1 (mid-fjord samples) and U (inner fjord samples). Results for age groups 1- 3 years and 4 years.*
df: degrees of freedom; var: variance; F: F-value; P: P-value. p<0.05 indicates a significant effect.

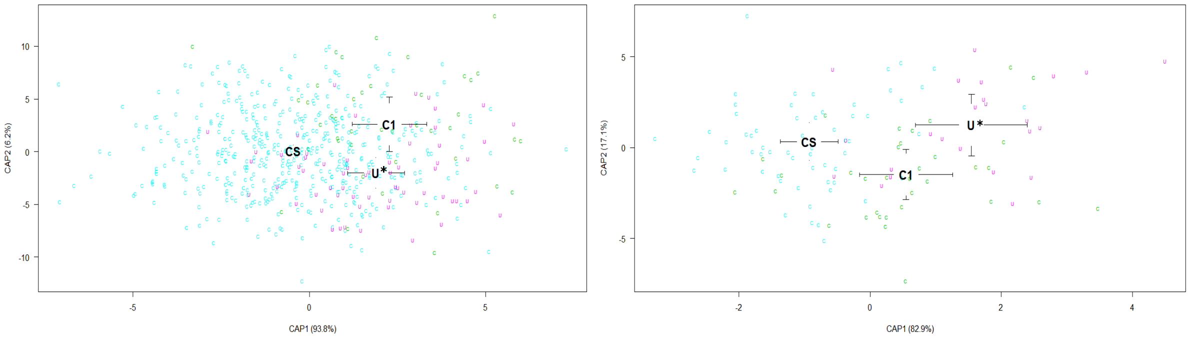


Figure 29 Canonical scores on discriminating axes 1 (CAP1) and 2 (CAP2) for the sampled populations at age 1-3 (left) and 4 (right). Groups: CS: Coastal Skagerrak; C1: mid-fjord samples; U: inner fjord samples. The black letters represent the mean canonical value for each population.*

3.6 Discriminant analysis

To validate whether otolith shape could be used for assigning individuals to their sampling origin, LDA was applied to the standardised Wavelet coefficients (table 9 a/c) with respect to the same samples and groups analysed in the multivariate analysis (age groups 1-2, 1-3, 4). Separate LDA analysis were also performed on CS and the whole Uddevalla fjord system (U) for the age group 1-3 and CS-U*-C1 at age 4 (table 9 d).

Classification success ranged between 20% and 78% among all the ages and geographical groups investigated. NW, CS and AN are the groups with the highest classification success (> 60%) among all the age groups, while NS and CK show the lowest rate of assignment (around 30% or lower). Individuals from OK are well separated at the age group 1-3 (69%), while the classification success decreases to 55% at age 4.

When considering only the assignment of samples to CS or the Uddevalla fjords (table 9 d) the classification success for CS was considerably higher (> 80%) than compared to the overall analysis (table 9 a/c), with a rate of misclassification decreasing from ~30/35% to ~15%.

a

Age		CK	CS	NS	NW	U
1-2	CK	**0.33**	0.45	0.10	0.10	0.02
	CS	0.17	**0.64**	0.06	0.05	0.08
	NS	0.17	0.42	**0.29**	0.04	0.08
	NW	0.03	0.17	-	**0.75**	0.05
	U	0.09	0.33	0.24	0.03	**0.31**

b

Age		AN	CK	CS	OK	U
1-3	AN	**0.68**	-	0.29	-	0.03
	CK	0.04	**0.20**	0.60	-	0.16
	CS	0.03	0.16	**0.70**	0.02	0.09
	OK	-	0.06	0.25	**0.69**	-
	U	0.01	0.13	0.31	0.01	**0.54**

c

Age		AN	CK	CS	OK	U
4	AN	**0.65**	-	0.22	0.09	0.04
	CK	-	**0.25**	0.25	0.25	0.25
	CS	0.10	0.11	**0.64**	0.07	0.08
	OK	-	0.05	0.30	**0.55**	0.10
	U	0.07	-	0.12	0.03	**0.78**

d

Age		CS	U	U*	C1
1-3	CS	**0.88**	0.12	-	-
	U	0.44	**0.56**	-	-
4	CS	**0.84**	-	0.02	0.14
	U*	0.18	-	**0.64**	0.18
	C1	0.21	-	0.23	**0.56**

Table 9 Classification success (bold) of otoliths into their original group based on a linear discriminant analysis. Independent discriminant analysis were conducted on all the populations used in the CAP analysis at age group 1-2 (a), 1-3 (b) and 4 (c). Two separate discriminant analysis were performed on the samples within and outside the fjord of Uddevalla for age group 1-3 and 4 (d). Groups: AN: central Kattegat; CK: coastal Kattegat; CS: Coastal Skagerrak; C1: mid-Uddevalla fjord; NS: North Sea; NW: Norway; U: whole Uddevalla fjords; U: inner Uddevalla fjord.*

4. Discussion

This study characterised the shape of sprat otoliths in three areas of the Greater North Sea ecoregion, focusing particularly on the Swedish west coast. To our knowledge this is the first study to investigate the otolith morphology of this species aiming to identify geographical variations which could be linked to population structure.
All the analysis indicated a pattern among the sampled populations, with three major groups identified:

- Norway;
- North Sea and 3a;
- Uddevalla fjords.

Although the comparisons among regions performed in this study could be partly confounded by the variations in seasons and the long time-gap between the collection of the Swedish samples (2003-2004) and the Norwegian/North Sea samples (2014-2016), results appears consistent with biogeographical considerations, previous knowledge on sprat population structure (Limborg *et al.*, 2009; Limborg *et al.*, 2012) and recent genetic analyses (ICES, 2018b).

The otoliths were tested for size and shape differences with univariate and multivariate analyses. The univariate method was applied to morphometrics measures such as otolith length, width, perimeter and area as descriptors to compare samples of different origin, while the multivariate methods were applied to the scaled Wavelet coefficients.
When looking at phenotypic traits and performing morphometric analysis, it is important to consider that shape is affected by multiple factors: citing the words of the father of morphometrics, " the form of an object is a "diagram of forces" " (D'Arcy Thompson, 1917).
Otolith shape is mainly determined by genetic and environmental factors (Campana and Casselman 1993). Additional factors such as age, sex and size are known to play a role into the otolith shaping process through the fish life. For this reason, when studying spatial variations in otolith shape, the effect of these influential additional variables has to be tested and eventually removed (Burke et al., 2008). When a wide overlap in fish size exists between samples from different regions, a multivariate classification based on size-independent shape variables is likely to be more effective than an analysis based on size alone (Campana & Casselman, 1993). It is also important to consider that differences between left and right otoliths could be significant for some species and should be always evaluated (Ider et al., 2017). In this study the age of the fish was known, so we selected different age groups with

the intent to minimise ontogenetic influences on shape while maintaining sufficiently large sample size for analytical purposes. The effect of sex and side was tested and both were found to be non-significant within the sampled areas. This justified the treatment of mixed samples in our study but most importantly it suggests that otolith collections of sprat which have generally been sampled for other purposes could be analysed regardless sex or using indistinctly left or right otoliths. On the contrary, an important limit to otolith shape analysis was found with the use of samples mounted on glass, which is common practice for age reading but compromised the use of sprat otoliths for the purposes of image analysis of shape morphometrics.

The choice of the Wavelet transform demonstrated its effectiveness in this study since it made possible to detect regions of the contourline that contributed most to the variations in shape among different geographical samples. This could have not been achieved by use of Fourier transforms, as the lack of a time domain which characterise this method over the Wavelet functions does not allow the operator to detect in what regions of the outline the variations occur.

The results from univariate and multivariate analyses show a certain degree of accordance; however, although the otolith length and perimeter were found to be significantly different among some areas (particularly with regard to the samples from Uddevalla), it was not possible to distinguish a clear pattern among these variables.

Otolith shape in the Norwegian samples differentiates most from the others. These samples also showed a high degree of internal variability, which was proved to be related with differences among the four fjords in which the fish were sampled (results not showed as the main focus of this study was the Swedish west coast), suggesting small scale variability among some of these relatively isolated areas. These results are in agreement with recent genetics studies performed on microsatellite DNA loci (e.g. Glover et al. 2011), which pointed out as the Norwegian sprat is highly different from the North Sea, Celtic Sea and Baltic Sea populations, and variable levels of genetic differentiations can also be observed among Norwegian fjords. Nævdal (1968), analysing haemoglobin and serum proteins, suggested the occurrence of reproductively isolated components of sprat among western Norwegian fjords, and similar small-scale patterns of differentiations have already been observed for other fish species. Studies conducted on cod and herring (Knutsen *et al.*, 2007; Bekkevold *et al.*, 2005) revealed demographically independent populations between the fjords of southern Norway, and pointed out that strong natural forces may intervene in the retaining of the early life stages within these areas.

Of particular interest, and unexpected at the beginning of the project, was the low level of differentiation between the North Sea and the 3a samples. The species, in fact, is currently assessed and managed in these two areas as two separate stocks (ICES, 2018b), approach which appear in contrast with the results provided in our study. However, these results sum themselves with many other evidences arising from different methods and presented at the last ICES Benchmark Workshop on Sprat Stocks

(November 2018), where the merging of the two management units was finally discussed (ICES, 2018b).

The level of differentiation found between the samples from North Sea and Skagerrak appears considerably lower than the variations estimated within the Skagerrak region when fjord samples are included. An environmental gradient of differentiation between North Sea, Northern Kattegat, Belt Sea and Baltic Sea was previously reported by Limborg *et al.* (2009) based on genetic analyses, but the differentiation between North Sea and 3a was not investigated. In this respect, and considering the latest scientific advices (ICES, 2018b), corroborating evidences from independent methodologies is highly valuable to support revision of boundaries among assessment units which should mirror as much as possible biological units. However, in this study it was possible to assess the similarities between North Sea and 3a only within the age classes 1-2 (which were the only classes from North Sea available in this study). It would be therefore advisable to perform further studies on otolith shape variations between the two regions by sampling older age classes in North Sea and more offshore locations in Northern Skagerrak.

As expected, given the lack of known physical or environmental barrier that could prevent gene flow, no differences is found among samples within the Skagerrak (excluded samples from the fjords) and part of the Kattegat region. A certain degree of differentiation is found in the central and even more in the southernmost samples from the Kattegat for the age group 1-3 even though, when comparing the fish lengths of the two areas for the same ages, no differences in growth rates seemed to emerge. Such gradient would support the hypothesis of a mixing zone throughout the southern Kattegat in agreement with genetic studies (Limborg *et al*., 2009; ICES, 2018b) where a high rate of differentiation between samples from Skagerrak /southern Kattegat and the Belt/Baltic sea has emerged approximately from the same area. Some lower levels of differentiation, although with much smaller differences between the relative CAP scores, was detected also at age 4. However, the ANOVA-like permutation tests gave slightly non-significant p-values (respectively 0.08 and 0.05) while comparing OK and CS at both the age groups (the number of sampled individuals, unfortunately, did not allow the comparisons with CK at age 4). Further research is needed to better characterise this area of mixing between adjacent populations also in relation to seasonal variability in the level and extent of mixing. Larger sample size, and also a better age and spatial coverage of the southern Kattegat would be necessary, possibly including samples from the Belt Sea and western Baltic Sea to cover the full geographical range of potential overlap.

The variations in the shape of fish otolith has been proved to be a function of both genetics and environmental inputs (Castonguay *et al.*, 1991; Berg *et al.*, 2018). Our results (with few exceptions such as in the Uddevalla fjords) also suggest that differences in the shape of sprat otoliths tend to decrease with age, similarly as it was found for other clupeid species such as herring (Libungan *et al.*, 2015).

This phenomenon could be due to a shift from the genetic imprinting of shape at the early stages of fish life to a proportionally higher effect of environment as fish grow.
Extensive studies have examined the influence of several environmental factors on the shape and structure of fish otoliths: physical and trophic conditions of the water such as salinity (Berg *et al.*, 2018), temperature (Gagliano & McCormick, 2004), habitat depth (Lombarte & Lleonart, 1993), pH (Réveillac *et al.*, 2015; Maneja *et al.*, 2013), availability of food (Massou *et al.*, 2002) and diet (Mille *et al.*, 2016) are well known environmental factors affecting the shape of fish otoliths in many fish species.

Otolith shape observed in samples within the Uddevalla fjord system was somehow different from this general pattern, as old sprat (age 4) tend to maintain their level of differentiation the coastal samples outside the fjord. High level of isolation in the inner part of the fjord from the rest of the coastal areas may represent a possible explanation. It must be pointed out, however, that the dispersal of larvae among these regions is still possible, and it would be undetectable on the otolith shape in case of a quick otolith diversification induced by the peculiar environmental factors within semi-enclosed systems.
The lower body growth of the Uddevalla sprat was already observed by Lindquist (1968); the existence of a potentially isolated biological unit within the area was also suggested by a recent study on sprat growth and reproduction (Vitale et al. 2016) and seems supported by an on going genetic study (ICES, 2018b). The analysis of small scale variability within the different sections of the fjord showed that otolith shape is less differentiated for the older ages along a possible gradient within the fjord. On the contrary, no differentiations in otolith shape could be detected in the younger age classes within the fjord. This change could be due to the dispersal of some older individuals from the coastal region into the fjord; when it comes to the inner sampling stations, however, the results indicate low mixing with the external populations occur so deep within the fjord.
Fjords are semi-enclosed systems, offering potential physical limits to dispersal and hosting peculiar environments where the water conditions can vary over very small geographical scales. Interestingly, the other fjord sample from the Gullmarnfjord (BR) appears also differentiated from the rest of the 3a samples but on the opposite side of the multivariate domain described by the analyses. The Gullmarnfjord is geographically less isolated from the Skagerrak than the Uddevallafjord but mechanisms of separation may still exist, as demonstrated for instance by the presence of local cod spawning in the inner part of the fjord (Øresland & André, 2008). Also in this case the small sample size represents a limit to further inference on the level of differentiation of the Gullmarnfjord sample from the rest of the Skagerrak, and it calls for the need of a dedicated data collection especially considering that most of the fjord is closed to fishing activities.

The success of the assignment to the right group based on the otolith shape seems to reflect the genetic distance between the samples, as proven by the fact that the Norwegian and southern Kattegat samples are better classified than other groups. When the North Sea samples are excluded from the analysis (as for the analysis with age group 1-3), the classification success within 3a is higher. The degree of similarity between groups make the classification less accurate, and the high rate of misclassification shown for CK and NS could be due to their shape proximity to CS. the latter, moreover, is characterised by a high internal variance, and may therefore be advantaged in the assignment of individuals to its domain.

The results produced by this study suggest that the otolith shape analysis can characterise population structure of sprat throughout the North Sea and Kattegat-Skagerrak region, as also supported by the relatively good level of agreement with results from other studies and recent genetics findings. Some studies (Harbitz & Albert, 2015) suggest that a rate of classification success > 70% could support a hypothesis of separate stocks, which is indeed what we found for the Norwegian and Uddevalla samples. The results for the North Sea, on the other hand, seem to disagree with the boundary between the current assessment and management units of North Sea and 3a, and it would be advisable to further investigate the matter. The method, in fact, could not well discriminate between the sprat from the two areas, which is undoubtedly an operative limit since it would be of great interest for management purposes to assess the relative contribution of the North Sea sprat to the catches in 3a. The good discriminatory power against the separate components of Norwegian and Uddevalla fjords, however, support the hypothesis that this method can actually be used to discriminate between different stocks of sprat, and that the current management strategies in North Sea and 3a should be re-evaluated.

These results mirror some other studies which proved the effectiveness of otolith shape analysis as a tool for identifying stock structure in many species, such as herring (Libungan *et al*., 2015), anchovy (Zengin *et al.*, 2015), mackerel (Turan, 2006) and red snapper (Sadighzadeh *et al*., 2014).
This is the first study to focus on shape analysis of sprat otoliths, and we reckon that the discriminatory power could be considerably improved by extending the analyses on a larger sample set with a better coverage in time, space and age. Including additional variables such as biological and environmental information (especially maturity and average water temperature and salinity) could allow to disentangle the relative contributions of genetics and environmental forces on the otolith shape through the ontogenesis.

5. Conclusions

The shape of sprat otoliths was observed to vary between different groups of samples according to geographical patterns which are interpreted to reflect a population structure in the study area which is supported by both biogeographical considerations and genetic results.

The otoliths from the Norwegian fjords were found to be the most different in shape, mirroring their genetic distance with the neighbouring stocks and also showing a high degree of internal differentiations due to the morphological complexity of the area. The samples from North Sea, Skagerrak and Kattegat, on the other hand were relatively homogeneous, except for the fjords populations which showed a high degree of differentiation. These results contrast with the current management of sprat in the North Sea and Kattegat-Skagerrak but agree and add to the results obtained from other different methodologies lately applied to evaluate the consistency of boundaries between the current management units of North Sea and 3a. Further investigations are needed on the otolith shape to evaluate whether the patterns observed in this study can be confirmed also for the older age classes which were poorly represented in our samples.

Relative patterns of differentiations, consistent with a shift towards the transition region of the Belt Sea, appear to emerge with a latitudinal gradient in the southernmost part of the Kattegat, but given the small sample size available in this study it is hard to make further considerations. It will also be of great interest to extend the methodology applied here beyond the geographical limits of the present study, through the Belts and towards the Baltic Sea. This is, in fact, a peculiar region characterised by steep environmental and genetic gradients which could be effectively mirrored by the otolith shape.

The results of this study can provide support to the genetic information for the assessment of the stock in the North Sea and 3a. This is, to our knowledge, the first study applying shape analysis on sprat otoliths, and it could be affected by a number of limits discussed in the previous section. It is advisable to further extend the investigation, but this preliminary analysis is highly promising and it allowed to detect a population structure along the Swedish west coast and discriminate the stock structure of the area.

Integrating this cost effective and relatively fast method in a multidisciplinary framework of stock discrimination studies could be easily done, and it would definitely prove itself very useful to the management of the stock.

Bibliography

Abdi, H. (2007). Bonferroni and Šidák corrections for multiple comparisons. *Encyclopedia of measurement and statistics,* 3, pp. 103-107.

Alheit, J. (1987). Variation of batch fecundity of sprat, Sprattus sprattus, during spawning season. *ICES CM,* 44, p. 9.

Aps, R., Ustinova, L., Gentzen, B., Grygiel, V., Paat, A. & Uder, Y. (1991). Guide for the use of Baltic sprat otoliths in fisheries studies. *Fisherei-Forshung,* 29, pp. 5-17.

Bailey, R. (1980). Problems in the management of short-lived pelagic fish as exemplified by North Sea sprat. *Rapports et Procès-Verbaux des Réunions du Conseil Permanent International pour l'Exploration de la Mer.*

Bekkevold, D., Aandré, C., Dahlgren, T.G., Clausen, L.A., Torstensen, E., Mosegaard, H., Carvalho, G.R., Christensen, T.B., Norlinder, E. & Ruzzante, D.E. (2005). Environmental correlates of population differentiation in Atlantic herring. *Evolution,* 59(12), pp. 2656-2668.

Berg, F., Almeland, O.W., Skadal, J., Slotte, A., Andersson, L. & Folkvord, A. (2018). Genetic factors have a major effect on growth, number of vertebrae and otolith shape in Atlantic herring (Clupea harengus). *PLoS One,* 13(1), p. e0190995.

Burke, N., Brophy, D. & King, P.A. (2008). Shape analysis of otolith annuli in Atlantic herring (Clupea harengus); a new method for tracking fish populations. *Fisheries Research,* 91(2-3), pp. 133-143.

Cadrin, S.X. & Friedland, K.D. (1999). The utility of image processing techniques for morphometric analysis and stock identification. *Fisheries Research,* 43(1-3), pp. 129-139.

Campana, S.E. & Thorrold, S.R. (2001). Otoliths, increments, and elements: keys to a comprehensive understanding of fish populations? *Canadian Journal of Fisheries and Aquatic Sciences,* 58(1), pp. 30-38.

Capoccioni, F., Costa, C., Aguzzi, J., Menesatti, P., Lombarte, A. & Ciccotti, E. (2011). Ontogenetic and environmental effects on otolith shape variability in three Mediterranean European eel (Anguilla anguilla, L.) local stocks. *Journal of Experimental Marine Biology and Ecology,* 397(1), pp. 1-7.

Cardinale, M., Doering-Arjes, P., Kastowsky, M. & Mosegaard, H. (2004). Effects of sex, stock, and environment on the shape of known-age Atlantic cod (Gadus morhua) otoliths. *Canadian Journal of Fisheries and Aquatic Sciences,* 61(2), pp. 158-167.

Castonguay, M., Simard, P. & Gagnon, P. (1991). Usefulness of Fourier analysis of otolith shape for Atlantic mackerel (Scomber scombrus) stock discrimination. *Canadian Journal of Fisheries and Aquatic Sciences,* 48(2), pp. 296-302.

D'Arcy Thompson, W. (1917). On growth and forme. Cambridge Univ. pr.

Dahle, G. & Eriksen, A. (1990). Spring and autumn spawners of herring (Clupea harengus) in the North Sea, Skagerrak and Kattegat; population genetic analysis. *Fisheries Research,* 9(2), pp. 131-141.

Gagliano, M. & McCormick, M.I. (2004). Feeding history influences otolith shape in tropical fish. *Marine Ecology Progress Series,* 278, pp. 291-296.

Harbitz, A. & Albert, O.T. (2015). Pitfalls in stock discrimination by shape analysis of otolith contours. *ICES Journal of Marine Science*, 72(7), pp. 2090-2097.

ICES-FishMap ICES FishMap species factsheet-sprat.

ICES (2013). Stock Annex: Sprat (Sprattus sprattus) in division 3.a (Skagerrak and Kattegat).

ICES (2015). Report of Herring assessment working group (HAWG) 2015.

ICES (2017). ICES HAWG Report 2017 - Sec 10 Sprat in Division 3.a (Skagerrak and Kattegat).

ICES (2018a). ICES HAWG Report 2018 - Sec 10 Sprat in Division 3.a (Skagerrak and Kattegat).

ICES (2018b). Report of the Benchmark Workshop on Sprat (WKSPRAT 2018). 5-9 November 2018, ICES HQ, Copenhagen, Denmark. ICES CM 2018/ACOM: in press.

Kerr, L.A., Hintzen, N.T., Cadrin, S.X., Clausen, L.W., Dickey-Collas, M., Goethel, D.R., Hatfield, E.M.C., Kritzer, J.P. & Nash, R.D.M. (2016). Lessons learned from practical approaches to reconcile mismatches between biological population structure and stock units of marine fish. *ICES Journal of Marine Science: Journal du Conseil*.

Knutsen, H., Olsen, E.M., Ciannelli, L., Espeland, S.H., Knutsen, J.A., Simonsen, J.H., Skreslet, S. & Stenseth, N.C. (2007). Egg distribution, bottom topography and small-scale cod population structure in a coastal marine system. *Marine Ecology Progress Series,* 333, pp. 249-255.

Libungan, L.A., Oskarsson, G.J., Slotte, A., Jacobsen, J.A. & Palsson, S. (2015). Otolith shape: a population marker for Atlantic herring Clupea harengus. *J Fish Biol,* 86(4), pp. 1377-95.

Libungan, L.A. & Palsson, S. (2015). ShapeR: an R package to study otolith shape variation among fish populations. *PLoS One,* 10(3), p. e0121102.

Limborg, M., Hanel, R., Debes, P., Ring, A., André, C., Tsigenopoulos, C. & Bekkevold, D. (2012). Imprints from genetic drift and mutation imply relative divergence times across marine transition zones in a pan-European small pelagic fish (Sprattus sprattus). *Heredity,* 109(2), p. 96.

Limborg, M.T., Pedersen, J.S., Hemmer-Hansen, J., Tomkiewicz, J. & Bekkevold, D. (2009). Genetic population structure of European sprat Sprattus sprattus: differentiation across a steep environmental gradient in a small pelagic fish. *Marine Ecology Progress Series,* 379, pp. 213-224.

Lindquist, A. (1968). Meristic and Morphometric Characters, Year-classes and “Races" of the Sprat (Sprattus sprattus) In Series Biology. *Lyskekil: Institute of Marine Research*.

Lindquist, A. (1978). A century of observations on sprat in the Skagerak and the Kattegat [North Sea]. *Rapports et Proces Verbaux des Reunions*.

Lombarte, A. & Lleonart, J. (1993). Otolith size changes related with body growth, habitat depth and temperature. *Environmental biology of fishes,* 37(3), pp. 297-306.

Maneja, R., Frommel, A., Geffen, A., Folkvord, A., Piatkowski, U., Chang, M. & Clemmesen, C. (2013). Effects of ocean acidification on the calcification of otoliths of larval Atlantic cod Gadus morhua. *Marine Ecology Progress Series,* 477, pp. 251-258.

Massou, A., Panfili, J., Laë, R., Baroiller, J.-F., Mikolasek, O., Fontenelle, G. & Le Bail, P.Y. (2002). Effects of different food restrictions on somatic and otolith growth in Nile tilapia reared under controlled conditions. *Journal of Fish Biology,* 60(5), pp. 1093-1104.

McBride, R.S., Hauser, J.W. & Sutherland, S.J. (2010). Brodeurs Guide to Otoliths of Some Northwest Atlantic Fishes. *Northeast Fisheries Science Center Reference Document*, pp. 10-04.

Mille, T., Mahe, K., Cachera, M., Villanueva, M., De Pontual, H. & Ernande, B. (2016). Diet is correlated with otolith shape in marine fish. *Marine Ecology Progress Series*, 555, pp. 167-184.

Molander, A.R. (1940). *A research upon the sprat of the west coast of Sweden*: Statens Reproduktionsanstalt.

Nævdal, G. (1968). Studies on hemoglobins and serum proteins in sprat from Norwegian waters.

Nielsen, R. (1994). *Fish and shellfish: Shared Nordic marine stocks*: Nordic Council of Ministers.

Øresland, V. & André, C. (2008). Larval group differentiation in Atlantic cod (Gadus morhua) inside and outside the Gullmar Fjord. *Fisheries Research,* 90(1-3), pp. 9-16.

Peck, M.A., Baumann, H., Bernreuther, M., Clemmesen, C., Herrmann, J.-P., Haslob, H., Huwer, B., Kanstinger, P., Köster, F.W. & Petereit, C. (2012). Reprint of: The ecophysiology of Sprattus sprattus in the Baltic and North Seas. *Progress in Oceanography,* 107, pp. 31-46.

Renán, X., Pérez-Díaz, E., Colás-Morrufo, T., Garza-Pérez, J. & Brule, T. (2011). Using otolith shape analysis to identify different stocks of Epinephelus morio from the Campeche Bank.

Réveillac, E., Lacoue-Labarthe, T., Oberhänsli, F., Teyssié, J.-L., Jeffree, R., Gattuso, J.-P. & Martin, S. (2015). Ocean acidification reshapes the otolith-body allometry of growth in juvenile sea bream. *Journal of Experimental Marine Biology and Ecology,* 463, pp. 87-94.

Rodríguez Mendoza, R. (2006). Otoliths and their applications in fishery science. *Croatian Journal of Fisheries: Ribarstvo,* 64(3), pp. 89-102.

Rosenberg, R. & Palmén, L.-E. (1981). Composition of herring stocks in the Skagerrak-Kattegat and the relations of these stocks with those of the North Sea and adjacent waters. *Fisheries Research,* 1, pp. 83-104.

Sadighzadeh, Z., Valinassab, T., Vosugi, G., Motallebi, A.A., Fatemi, M.R., Lombarte, A. & Tuset, V.M. (2014). Use of otolith shape for stock identification of John's snapper, Lutjanus johnii (Pisces: Lutjanidae), from the Persian Gulf and the Oman Sea. *Fisheries Research,* 155, pp. 59-63.

Shvetsov, F., Kozlovsky, A. & Rudneva, A. (1995). The structure of Baltic sprat population as a base for the assessment of the stock. *ICES CM*.

Teissier, G. (1938). Un essai d'analyse factorielle. Les variants sexuels de Maia squinada. *Biotypologie,* 7, pp. 73-96.

Turan, C. (2006). The use of otolith shape and chemistry to determine stock structure of Mediterranean horse mackerel Trachurus mediterraneus (Steindachner). *Journal of Fish Biology,* 69, pp. 165-180.

Tuset, V.M., Rosin, P.L. & Lombarte, A. (2006). Sagittal otolith shape used in the identification of fishes of the genus Serranus. *Fisheries Research,* 81(2-3), pp. 316-325.

Vitale, F., Mittermayer, F., Krischansson, B., Johansson, M. & Casini, M. (2016). Growth and maturity of sprat (Sprattus sprattus) in the Kattegat and Skagerrak, eastern North Sea. *Aquatic Living Resources,* 28(2-4), pp. 127-137.

Voss, R., Dickmann, M. & Schmidt, J.O. (2009). Feeding ecology of sprat (Sprattus sprattus L.) and sardine (Sardina pilchardus W.) larvae in the German Bight, North Sea. *Oceanologia*, 51(1), pp. 117-138.

Whitehead, P.J. (1985). FAO species catalogue, Vol. 7. Clupeoid fishes of the world. An annotated and illustrated catalogue of the herrings, sardines, pilchards, sprats, anchovies and wolf herrings. Part 1-Chirocentridae, Clupeidae and Pristigasteridae. *FAO Fish. Synop.,* 125, p. 303.

Zengin, M., Saygin, S. & Polat, N. (2015). Otolith shape analyses and dimensions of the anchovy Engraulis encrasicolus L. in the Black and Marmara Seas. *Sains Malaysiana*, 44(5), pp. 657-662.

Popular summary

Developing a good knowledge of the different populations composing a fish stock is important since the fishing effort a stock can sustain highly depends on the relations between its different components. The shape of otoliths – small bones in the inner ear of fishes – is widely used to identify different fish populations within species. In this study we analysed for the first time the otolith shape of sprat to investigate its population diversity along the Swedish west coast (Skagerrak and Kattegat seas).

Sprat is a small fish mostly used for fishmeal, oil or canning, and it is also highly appreciated in Sweden as a traditional food during the Christmas season. By comparing samples collected within the Kattegat – Skagerrak and also with samples from the North Sea and the western Norwegian fjords, we found that the shape of sprat otoliths varies between the different regions, and can be used to identify a population structure. At present, sprat from the North Sea and Kattegat – Skagerrak is managed as two separate units; however, our results showed that the otolith shape of sprat from these two areas is very similar, and limited differentiation is also supported by recent genetic analyses. Together, these methods have recently informed a proposal for joining the two areas under a single stock assessment unit.

Interestingly, the samples from the Gullmarnfjord and even more from the Uddevalla fjord system were found to be significantly different in otolith shape from the other samples in the Kattegat – Skagerrak, supporting the hypothesis of existence of local fjord populations along the Swedish Skagerrak.

The results here presented are promising, possibly opening the door for more extensive future application of shape analysis to the study of sprat populations in the region. The relatively low costs of otolith shape analysis compared to other techniques (i.e., genetics, otolith microchemistry) make it an attractive method for operational applications of stock discrimination on mixed catches.

Acknowledgments

The master thesis marks, in my opinion, the end of an age and the beginning of a new one: it's the sum of many years of studying, and the first taste of the career I have chosen to undertake. I am therefore glad I had the opportunity to share these important months with many inspiring people.

I would first like to thank my supervisors prof. Valerio Bartolino of the *Swedish University of Agricultural Sciences* and Florian Berg of the Norwegian *Institute of Marine Research*., who guided and supported me with participation, a lot of patience and the right dose of irony. It didn't matter whether it was early morning or late night, their doors were always open for me.
I want to express my sincere thanks to my examiner prof. Michele Casini of the *Swedish University of Agricultural Science* for his very valuable comments on this thesis, and to Julie Olivia Davies of the *Technical University of Denmark* for her precious samples from the North Sea.
I take this opportunity to acknowledge all the great people working at the Havsfiskelaboratoriet in Lysekil, for having collected the samples used in this work and made me feel welcome and part of the crew. I also want to thank prof. Giandomenico Ardizzone of *Sapienza Università di Roma* who fed my love for the sea with his lectures and introduced me to this lab.

Last but not least, I must express my gratitude to my family and to my girlfriend Juliana, for having provided me with love and encouragement during my years of studying and through this thesis project, standing by my side even when my mood was dark.