Morphometric Analysis of Cod (*Gadus morhua*) Otoliths

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Master's Thesis

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November 2017

Preface

Because of over-exploitation in the fishing grounds in the west Greenland waters, better fishery management for separation of populations is necessary. Currently, the only method for population classification is the use of genetics. However, this is expensive, and a more cost-efficient method is needed. One possible alternative method is using the outline of the otolith for population classification.

This thesis aimed to separate three cod (*Gadus morhua*) populations in the Greenland waters through the use of otolith shape analysis. In addition, the effect of environment, genotype and age on the otolith shape was investigated. This thesis is separated into a review of previous knowledge and an article manuscript of the study conducted.

The review itself is separated into three main parts. The first is about the Atlantic cod, its life-history and population structure along with commercial exploitation. Second part of the review is the description of the otolith, its function, formation and most notably, the factors affecting the otolith shape. The last part covers the shape analysis techniques. This comprehensive part describes the previous use of otolith shape analysis within species and populations, along with the general hardware setup of. Furthermore, different shape analysis methods from simple landmark analysis to the outline analysis, are presented.

The article manuscript details the study conducted on the otoliths of the three Greenland populations and the resulting conclusions. The otoliths were sampled in 2016, and then analysed over a period of three months in spring of 2017 at Greenland Institute for Natural Resources.

This Master's thesis, nominated to 60 ECTS, was conducted in co-operation between Aarhus University and Greenland Institute of Natural Resources.

I would like to thank my supervisor Peter Grønkjær, Associate Professor at the Department of Bioscience, Aarhus University for consistent support to me throughout the study.

Likewise, I wish to thank senior scientist Rasmus Berg Hedeholm and scientists Anja Retzel and Helle Torp Christensen from the Department of Fish and Shellfish at Greenland Institute for Natural Resources, for help in setting up this study, giving me access to the otolith samples and data and providing financial support that enable me to go to Nuuk for three months.

Furthermore, I would like to thank Peter, Rasmus, Anja and Helle for valuable review and comments to the article manuscript and review presented in this thesis.

Sebastian Glindtvad

Contents

The Atlantic Cod

Taxonomy and Population Structure

The Atlantic cod, *Gadus morhua* (Linnaeus), is a demersal predatory fish species indigenous to most of the North Atlantic (Lough 2004). Taxonomically, the species belongs to the family of Gadidae, with in the paracanthopterygii superorder, subfamily *Gadinae,* which includes haddocks and cods. This subfamily includes 12 genera with one of them being *Gadus* (Nelson 2007). The colour of the cod varies and can be on the dorsal side, brownish, greenish or grey. The ventral side is pale white. The body is an asymmetric fusiform, with the caudal fin being truncated. The head is relatively narrow, with an interorbital space. The upper jaw is protruded and a characteristic barbel on the lower jaw. Characteristic for the genus is also the three dorsal fins and the two anal fins (Cohen 1990). Stock wise, 18 areas in the North Atlantic contains isolated to semi-isolated stocks. However, within each area, several smaller sub-stocks exist. In the Northwest areas alone, 12 recognized stocks exist (ICES 2005; Shelton et al. 2006). Areawide, the Atlantic cod is found from the Bay of Biscay to the Barent Sea in the north, and westward around Iceland and further past Greenland to Ungava Bay in the north and Cape Hatteras in the south (Figure 1[1]).

General Life-history

The spawning of the Atlantic cod is dependent on the environment the population inhabits. As such, spawning is observed to vary across the period of early spring to early summer (ICES 2005). The spawning period seems to be triggered by photoperiods, as cod stocks can be reared to spawn earlier, this at the cost of fecundity and egg sizes (Hansen et al. 2001). Migration varies from population to population, as for example, the Norwegian coastal cod will stay within in the Norwegian fjord system, while other populations like the North East Atlantic cod and the Newfoundland populations will migrate for long
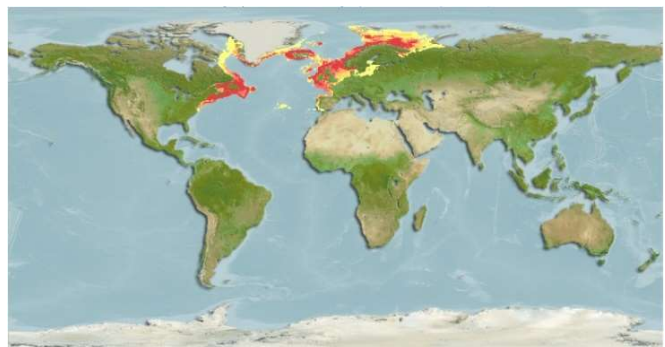
Figure 1. The distribution of the Atlantic cod.

distances (Rose 1993; Stransky et al. 2008). The eggs of Atlantic cod are spherical, with a diameter of 1.2-1.7 mm. The eggs are pelagic and hatch between 8 and 60 days, depending on the temperatures of the environment. The size of the larvae at the moment of hatching can vary between 3.3 and 5.7 mm in length. The juvenile stage starts at 20 mm or greater. By the end of the first year, the mean length varies from stock to

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[1] http://fishbase.org/summary/Gadus-morhua.html

stock, e.g. 16 cm length for arcto-norwegian stocks and 26 cm for North American stocks. The juvenile is benthic in its lifestyle, which is reflected both in its coloration and diet. The weight of the adults varies between stocks and depends on age, with individuals up to 95 kg having been recorded, although individuals above 30 kg are rare (Lough 2004; ICES 2005). 50% maturity is achieved between 2 and 7 years, with lengths between 37cm and 75 cm depending on the stocks (ICES 2005). The diet of the Atlantic cod varies, mostly based on availability, as the specie is an opportunistic feeder. However, it also varies from life history stage and area. Indeed, much like other fish species, an ontogenetic shift in diet is observed as small cods will prey primarily on small pelagic invertebrates, whereas the diet will change towards a more benthic invertebrate and piscivorous diet as they grow. In some areas, the preferred diet of the adult cod is in some areas small pelagic fish such as capelin (Henriette Schack 2005), sand lance and herring but also benthic prey such as crabs (Lough 2004).

Atlantic Cod in Greenland Waters

The Atlantic cod in the Greenlandic waters are separated into 4 different populations, the West Greenland Inshore cod, the West Greenland Offshore cod, the East Greenland Offshore/Icelandic Offshore cod (referred to as the Icelandic Offshore cod in this review) and the Icelandic Inshore cod (Therkildsen et al. 2013; Bonanomi et al. 2016).

The **West Greenland Inshore cod** has been documented in the fjord systems on the west coast from 67°N to 57°N, with documented spawning occurring within the range 67°N to 64°N (ICES 2005, Therkildsen et al. 2013; Bonanomi et al. 2015).

The population inhabits the fjord systems and coastal areas, and surveys suggest they spend their entire life-history in these areas. 50% maturity has been documented to be at the age of 5 to 6 years in the areas of Sisimiut and Nuuk in the 1930s, unfortunately more recent information is not available. Optimal spawning occurs in small sheltered shallow-water fjord branches from February till June with larval appearance from April to early May. Temperatures range between 0.5°C and 4.0°C in these parts. The early eggs and young larvae are mostly restricted to the spawning area. However, later as the larvae becomes larger, they disperse more evenly throughout the fjord, mainly transported by currents to parts of the fjord where prey is more abundant (ICES 2005; Swalethorp et al. 2016).

The inhabited area of the **West Greenland Offshore cod** stretches across several of the common fishing grounds from Store Hellefisk Banke at 67-68°N and south to the tip of Greenland at 59°N. Spawning occurs across the same area and in the fjord systems along the coast. The majority of eggs and larvae being distributed from Fylla Bank and south. Though because of ocean currents, eggs and larvae are transported northwards as far as Store Hellefisk Bank at 67-68°N. The spawning itself peaks in the end of March to the beginning of April, with

completion in June. At the offshore slope of Fylla Bank spawning has been observed at depths down to 120 m and temperatures at 1.5°C, however at more southern fishing banks the spawning was observed down to 350 m, probably due to higher temperatures (ICES 2005).

The adult **Icelandic Offshore cod** inhabits mainly the east Greenlandic waters from 62°N-66°N, even though recaptured cods are frequently caught in Icelandic waters, indicating a migration. 50% maturity is attained at age 8 for this population. Spawning occurs between 62°N and 66°N offshore of the east coast and from March to June, though maybe into July as well. Depths and temperature range from 170 m to 400 m and 3.2°C and 5.2°C at the spawning sites. (ICES 2005).

Not much is known specifically about the **Icelandic inshore** population**,** but Icelandic waters contain genetic distinct populations. As such, this review refers to these populations as the Icelandic stock. The 50% maturity is reach at age 6.6 years for females and 5.8 years for males. The spawning of these stocks occurs along the whole Icelandic coastline. However, the highest concentration of spawning occurs in the south and south-west parts of Iceland (ICES 2005).

Due to ocean currents, eggs and larvae are transported south from the spawning grounds and around the tip of Greenland to the west Greenland waters. Here, both Icelandic Offshore and Icelandic Inshore cod settle on the same nursery grounds as the West

Figure 2. Transportation of eggs and larvae of the Icelandic population from spawning grounds on the Greenlandic east coast to the nursey-grounds on Westcoast.

Greenland Offshore (Figure 2[2]) and the West Greenland Inshore cod. With growth and age, the Icelandic Offshore and Icelandic Inshore cods migrate back to the east coast of Greenland and to Icelandic territorial waters (Bonanomi et al. 2015; Bonanomi et al. 2016).
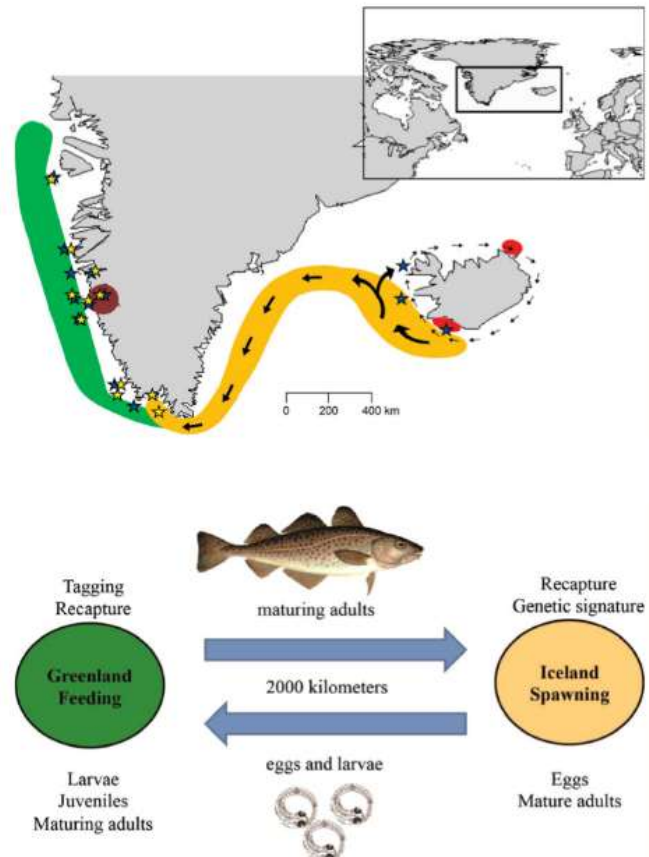
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[2] Bonanomi et al. (2016)

Exploitation

The Atlantic cod has been a popular and important species for numerous countries on the European as well as the North American continent. However, throughout the geographical range of the species, over-exploitation has decimated several stocks (Cook et al. 1997). Most notably are the stocks off the coast of Canada, which collapsed in the 1990's and have yet to be re-established properly in this region (Myers et al. 1997; Hutchings and Rangeley 2011). However, although over-exploitation of the stocks is considered the main reason for the collapse of fish stocks, oceanographic changes and ecological changes can cause population shifts towards other areas along the coast causing decline of local populations within specific areas (Rose et al. 2000).

In Greenland, before 1920, cod fishery consisted mostly of inshore fisheries and catches of cod yielded less than 500 tons annually. Post 1920, the Greenland fishery extended to an offshore fishery. Catches of offshore fishery were considerably higher, frequently above 300,000 tons annually from the mid-1950s to early 1970s. The highest yield was 480,000 tons in 1962. Hereafter, the catch declined as the spawning-stock biomass was reduced. From 1990 and on, the Atlantic cod had almost disappeared from offshore fishing areas. On the east coast, fishery continued up till the mid-1990s, with 1994 seeing catches less than 500 tons.

Today, cod fishery is done on the inshore and Icelandic offshore stock in the east Greenland waters (ICES 2005). The advice offered by the International Council for the Exploration of the Sea (ICES) on the fishing quotas for the Greenlandic fishery in 2017, are no more than 7930 tons of the East Greenland stock (mixed Icelandic populations in east Greenland waters). For the West Greenland fisheries, the quotas for the Inshore cod based on biological advice is no more than 12,379 tons, and 0 tons for the Offshore cod (ICES 2016a; ICES 2016b; ICES 2016c). In 2015 the total catch of all three areas was 45,887 tons.

Otoliths

The first use of otoliths in research was at the end of the 19th century with Reibisch in 1899. Used originally for age estimation, otoliths were quickly used for species determination, stomach content observations and has since been used for stock discrimination (Scott 1903; Frost 1924; Rollefsen 1934; Panfili et al. 2002). In the 1960's and early 1970's there was a breakthrough in the use of otoliths in various ways. Isotopic analysis was used on otoliths in 1966 for the measurement of oxygen ratios to determine the temperature in habitats, which could be used in the field of paleobiology (Devereux 1967). However, it was Pannella with the discovery of formation of daily increments in larvae and juvenile individuals that changed the scientific field of otolithometry (Pannella 1971; Panfili et al. 2002). The late 1970's and 1980's saw the more widely use of isotopic analysis and microchemistry, especially showing how otoliths are records of life histories of the

individual fish (Mulcahy et al. 1979; Secor et al. 1995).

Today, otoliths continue to be of great interest for both marine and aquatic biologists. The international symposium on fish otolith research and application proves this, with the 6th symposium to be held in 2018. In 2005 the 3rd symposium divided the field up into eight main areas, with each area including both application and research topics (Begg et al. 2005). The 5th otolith symposium held in 2014 used otoliths as themes on the environmental, community, population and individual levels (McBride et al. 2015).

The Physiological Function

As most jawed vertebrates, the modern teleost fish has an inner ear. The inner ear consists of three semi-circular canals, which are interconnected by chambers or otherwise known as otic sacs. The whole structure is filled with endolymphic fluid. The three otic sacs are known as the sacculus, utriculus and lagena, with each sac containing the sagitta, lapillus and asteriscus otolith, respectively (Figure 3[3]) (Panfili et al. 2002; Popper et al. 2005). Each otolith is fixed to a sensory epithelium, commonly known as the macula. The macula consists of mechanoreceptor hair sensory cells. On the apical side, the sensory cells contain one kinocilia and several stereocilia which are connected to calcium channels. In bending the cilia, the calcium channels are activated, triggering the eight cranial nerve using a neurotransmitter. Between the macula and the otolith, a thin otolithic membrane separates the two (Figure 4[4]). The otolith

Figure 3. The inner ear. The three otoliths sagitta (Sag), lapillus (Lap) and asteriscus (Ast) in the otic sacs sacculus (Sac), utriculus (Utr) and the lagena (Lag).

membrane contains two zones, a structured gelatinous zone and a sub-cupular zone. The structured gelatinous zone exhibits a reticulated or honeycomb structure while the sub-cupular zone consists of a loose network of fibres. While the gelatinous zone covers the sensory region of the macula, the sub-cupular zone covers both sensory and non-sensory regions of the macula. A second sensory epithelium, known as the crista is found in each of the semi-circular canals. This sensory epithelium is structured much like that of the macula, however, a gelatinous cupula takes the place of the otolith, with the cilia sensory hairs embedded in the cupula.
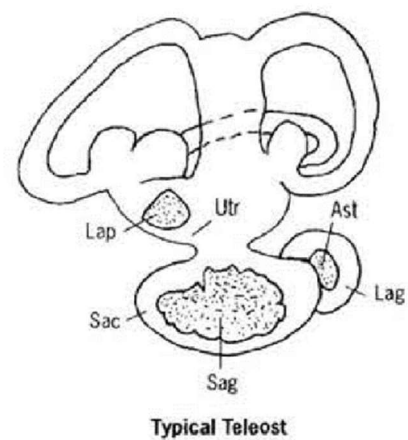
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[3] Panfili et al. (2002)

[4] Popper et al. (2005)

The functions of the labyrinths of the inner ear are both of an auditory and vestibular nature. The auditory part is due to the pars inferior, involving the sacculus and lagena otic sacs. Hence the sacculus and lagena otoliths, the sagitta and asteriscus, are partly sound receptors. The vestibular nature is in the pars superior, involving the semi-circular canals' crista areas and the utriculus otic sac, detecting linear and angular movement, though the sagitta and asteriscus are also involved in this (Panfili et al. 2002; Popper et al. 2005).

Microchemistry

Otoliths are mainly made by calcium, carbon and oxygen, bound into calcium carbonate ($CaCO_3$) crystalizing on an
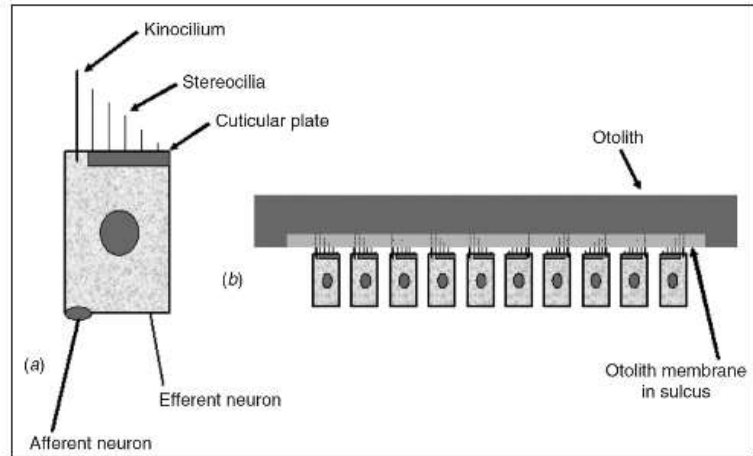

Figure 4. Sensory cell (left) consisting of an efferent neuron with kinocilium and stereocilia. The macula, consisting of several sensory cells with kinocilium and stereocilia connected to the otolith membrane.

organic matrix consisting of proteins. Yet even with a high purity level, several trace elements, more than 31 different ones, can be found in otoliths. The percentage division is usually with below 1% of trace element, around 3-4% of protein and the rest being pure calcium carbonate (Campana 1999; Panfili et al. 2002). The bio-mineralization of the otolith is done through the endolymphic fluid, yet different factors such as pH, alkalinity and temperature affect the rate of the crystallization. Three different crystal polymorphs can be taken by the calcium carbonate, these being the calcite, the aragonite and the vaterite form. Aragonite and vaterite are unstable polymorphs and will transform into the calcite polymorph, though the process from aragonite to calcite is slow. Yet aragonite is the main polymorph observed in the sagitta and lapillus otolith, with most asteriscus consisting of the vaterite polymorph form. Organic molecules in the endolymphic fluid is possibly the cause for the makeup of specific polymorph (Campana 1999). Such organic molecules obtained from an aragonite polymorphed shell layer have been found to promote aragonite formation in mollusc shells (Falini et al. 1996). Additionally, ions such as strontium (Sr) and magnesium (Mg) also favours the formation of aragonite (Campana 1999). However, the complexity of the otolith structure cannot be described by mere inorganic processes alone, and a biological component, proteins, are contributing to process. Two main protein groups consisting of high fraction of acidic amino acids are thought to be highly influential. The first being a water-insoluble protein, known as otolin, which is thought to make up the "structural framework". The second group is water-soluble, and appear to regulate the calcification rate (Campana 1999).

Formationprocess

The nucleus of the otolith is formed from one or more primordial granule or primordia, on the epithelium cells.

The nucleus is often termed as the primordium, often consisting of calcium carbonate in aragonite form, though in some species, the sagitta's primordium may be of the vaterite form. The primordium is developed in the egg stage, and even grows without the addition of rings. Though this varies
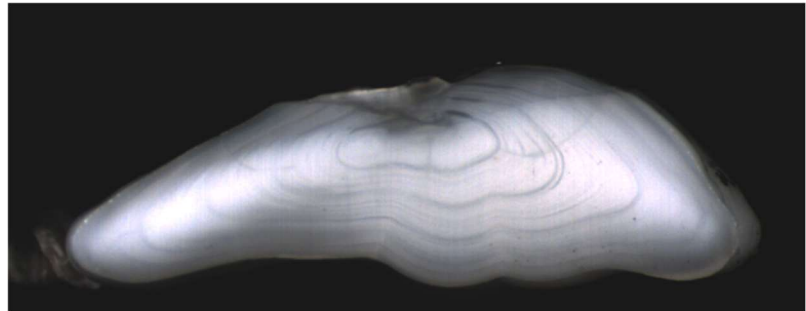
Picture 1. Ventral broken 6-year-old Atlantic cod otolith under reflected light. The seasonal increments are clearly visible, with the light opaque bands areas and the dark translucent bands.

from species to species it has also been noted, that rings can form before hatching. Primary increments, also called daily increments, discovered by Pannella (1971), is the accretion of daily rings around the primordium. These have also been termed as the L and D zones, due to their tendency for forming light bands and dark bands under transmitted light. The L zone is rich in mineral, while the D zones are mineral deficient, yet protein-rich (Panfili et al. 2002). Under scanning electronic microscope (SEM), the ratio of calcium carbonate and protein differs between the zones, as L zones has a higher ratio of calcium carbonate, while the D zones have a higher ratio of protein compared to calcium carbonate. The width of the D zone compared to the L zone is smaller, usually under 1 µm, while L-zones vary from 0.4 µm to 10 µm in width. That primary increments, is formed daily, is not necessarily true and should be treated with great scepticism. In the adult and/or juvenile stage, observation of the daily increments can only be done under extreme magnification. During the first year, the otolith starts to develop annuli, commonly known as seasonal increments. Though the seasonal increments differ from the primary increments in several ways, the zones are still defined based on their mineral rich or protein-rich nature. Seasonal increments are either developed as opaque zones or translucent zones, and much like the light and dark zone of the primary increments, these will also appear either as dark or light zones. However, under reflected light the opaque zone appears light while the translucent zone appears dark, while under transmitted light the opposite is achieved (Picture 1). A year is comprised of two seasonal increments (one opaque zones and a translucent zones) (Panfili et al. 2002). However, both the formation of primary increments and the seasonal increments are not completely understood and factors effecting the formation of these increments varies. For daily increments, temperature, photoperiod and feeding frequency has been attributed as influencing factors. However, while this might seem true for some species, it is not universal for all, as other species have shown not to be affected by certain environmental factors such as temperature. For seasonal increments, much of the same is true. Whether the increments are tied to somatic growth, reproduction or is a physiological response to environment factors are uncertain (Panfili et al. 2002). For most species of fish, the otolith is symmetrical,

however flatfish and flounders have asymmetrical ones. The shape itself is heavily dependent on the species, since great variation is observed from species to species. Being the biggest of the three otoliths in most species, the sagitta is usually used for most research and age-estimation. The traditional otolith itself is often elongated, with an elliptical shape, and laterally compressed. Figure 5[5] gives an overview of the otolith and the terminology used for research purposes.

Effects on Otolith Shape

The overall shape of the otolith is defined genetically from species to species (Leopold et al. 2001). However, this changes as one moves to the population level. How much the environment or genotype affect the shape of the otolith compared to each other is unknown, but both influences the shape of the otolith. While genetic has a direct effect on the shape of the otolith, environment has a direct and indirect effect based on e.g. growth rates which are regulated by both temperature and feeding levels (Cardinale et al. 2004). However, Hüssy did not find a significant effect by temperature on the shape of the otolith (Hussy 2008).
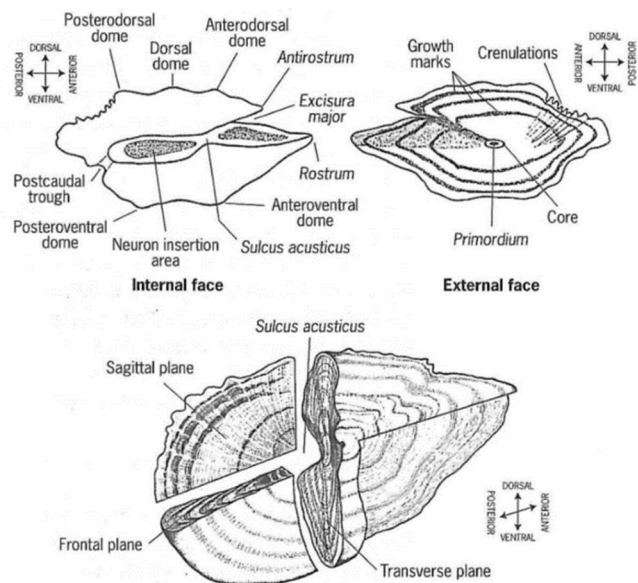
Figure 5. Overview of the terminology used in the description of otoliths.

Feeding levels have been found to have the greatest effect on otolith shape both directly and indirectly (Gagliano and McCormick 2004, Hussy 2008). Directly the feeding levels have been shown to affect the number of lobes being formed (Hussy 2008). Indirectly the shape is thought to be affected through somatic growth. Otolith growth being an acellular process, occurring in the endolymphic fluied of the inner ear, means the environment can to some degree affect the chemistry of the endolymphic fluid, and the transport mechanisms across the endolympatic ephithelium. The lobes seem to contain more protein than interlobe areas, and an increase in protein consumptions seems to increase the growth of lobes. This is thought to be due to an increase in secretion of proteins into the endolymphic fluid, though the specific processes of the accretion of protein in the lobes

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[5] Panfili et al. (2002)

remain to be researched. This affects the dorsal and ventral sides the most, as the lobes are more prominent there, affecting the length-width ratio of the otolith (Hussy 2008).

Mille et al. (2016) supported this to some degree, finding a diet significant influence on the shape of the otoliths of four out of five species of round and flatfish. Based on this, Mille produced two hypotheses. The first being that the consumption of protein influence the shape of the otolith. The higher the amount of protein consumed, the more protein available within the endolymphic fluid for otolith. The second hypothesis was the composition of protein and amino acids within a prey, increased or decreased the otolith growth. This relies on the fact that certain proteins and macromolecules within the endolymphic fluid regulates and controls the structure of otolith. For these proteins and molecules, certain amino acids are essential while others are not, and an increase in essential amino acids may directly affect the otolith shape through variations in crenulation, and the global shape through growth regulation. Mille et al. (2016) did not find a significant effect of the energy of the prey on the shape of the otolith nor a relation between food quantity and otolith shape (Mille et al. 2016). This is contrary to the previous research (Cardinale et al. 2004, Gagliano and McCormick 2004, Hussy 2008) and five species were used with only four giving significant results. Moreover, because the stomach content was only a snapshot of the October month of 2016 diet and an assumption of previous diet, more research is needed to properly support these hypotheses (Mille et al. 2016).

Additionally, ontogenetic shift has been found to affect the shape of an otolith both of a direct and indirect nature. For the Atlantic cod, in the transition phase from larvae to juvenile, secondary growth centers occur along the anterior end. This direct change causes the otolith to change its shape, with the number of lobes being an indirect effect of the ontogenetic shift. Lobe formation has been found to average between 27, 28 and 31 for individuals with lengths of <15 cm, between 15 cm to 20 cm and 20 cm to 30 cm (Hussy 2008).

Shape analysis

The use of otolith shape for the discriminations of different species have been used since the early 20th century (Scott 1903; Frost 1924). Numerous descriptive keys have since been provided for the distinguishing between species based on otolith shapes (Frost 1981, Jones 1988, Lowry 2011). Though this was through recognition of the human eye, the beginning of the 21st century saw the use of otolith shape analysis for species, using mathematical models and computer programs, showing just how the otolith of species differed from each other (Torres et al. 2000, Tuset et al. 2003, Tuset et al. 2006). The use for stock discrimination on the other hand can be traced back to the early 30's. Here Norwegian biologist visually distinguished between the "skrei" and "loddetorsk" using otolith shape (Rollefsen 1934). Assigning individuals based on otolith shape has since been developed further and used in several studies for species and stock separations (Begg and Brown 2000;

Bolles and Begg 2000; Torres et al. 2000; Turan 2000; DeVries et al. 2002). With several of the cod stocks being decimated, different stock assessment tools are needed, and the use of otolith shape analysis for fishery management can potentially be added as a tool. Berg et al. (2005) and Stransky et al. (2008) showed through morphometric analysis of the otolith and genetic analysis that Norwegian waters were inhabited of two distinct populations of cod, the Norwegian Coastal cod and the Northeast Arctic cod, along with a "Svalbard type" though this specific type was not processed. The use of otolith shape analysis and trying to determine the factors affecting the shape of otolith is continually being researched (Vignon 2012; Pavlov 2016; Renan et al. 2016; Thiel and Knebelsberger 2016; Mapp et al. 2017).

Examples of otolith shape analysis used

Otolith shape analysis have been used on numerous species of fish. Tuset et al. (2003) demonstrated how variations in the shape of otoliths could be used in differentiating between three species of the genus *Serranus* in the Canary Islands. Torres et al. (2000) showed in a different approach, how the sulcus acusticus of the sagitta otolith could be used in shape analysis as well, however, this was in a separations of species approach, not separation of stocks. Indeed, this study supported previous studies of genetics, parasites and morphological data about two biogeographical groups of the genus *Merluccius*. Begg and Brown (2000) tried to differentiate between the different stocks of haddock, *Melanogrammus aeglefinus,* off the coast of Canada on the Georges Bank. This was an attempt to differentiate between an eastern and western Georges Bank haddock using area, length, width, and perimeter of the otoliths and circularity and rectangularity to describe the otoliths. The resulting analysis revealed an indication of two separate stocks of haddocks from east to west, however, the classification success varied from 63% to 80%. Bolles and Begg (2000) also demonstrated how two stocks of the species could be differentiated from each other by otolith shape analysis. Indeed, much like the study by Begg and Brown (2000), the use of the whole otolith shape of the sagitta was used. This meant variables such as length, width, area, perimeter, circularity, and rectangularity. The study identified two stocks of silver hake, *Merluccius bilinearis*, within North American waters. Indeed, because of one stocks habitat being in colder waters than the other stocks habitat, the otolith was often larger in the northern stocks when comparing same age individuals of the two stocks. Turan (2000) also showed how otolith shape analysis could be used to distinguish between different populations within one species. In this case, four different stocks of herring *Clupea harengus*, were found. These being the Icelandic summer-spawner, the Baltic, the Trondheimsfjord herring and the British Isles group. DeVries et al. (2002) conducted a study for the purpose of stock assessments on the King Mackrel *Scomberomorus cavalla*. Previously, the King Mackrel had been described as one stock unit, along the U.S. coast to the Gulf of Mexico. However, this changed as genetic

research documented two stocks. DeVries indeed also showed two stocks of King Mackerels, and even showed how otolith shape analysis could be used in stock assessment purposes. Stransky et al. (2008) likewise made a study for stock assessment purposes. Norwegian waters are inhabited by two genetic differentiated cod stocks, the Norwegian coastal cod and the north east Atlantic cod. Much like the King Mackrel in the Atlantic Ocean, the two stocks spawn in the Norwegian fjord systems. Though they have different migration routes, this still creates mixed catches around the spawning season. The study showed 87% and 90% success in differentiating between the Norwegian coastal cod and the north east Atlantic cod respectively.

General Setup for Otolith Shape Analysis

The setup for the image acquiring of otoliths is much the same across several studies. However, it varies, depending on the analysis method and which morphological features are to be analysed. The common approach, is with the otolith placed on a small podium under a binocular microscope or microscope, with ample of light to illuminate the features of the otolith. Through the binocular microscope or microscope, a camera transmits the picture of the otolith to a computer, saving the images for the mathematical analysis. The camera in question, varies from article to article though what they have in common is the ability to produces pictures of high quality (Vieira et al. 2014; Pavlov 2016). Taking high resolution images through binocular microscope or microscope are not the only way of getting a digitized image of an otolith. Acquiring a drawing of the otolith has also been done, and has been done with the analyzation of the sulcus acusticus (Torres et al. 2000). Another approach is the use of a scanning electron microscope (SEM) for image acquiring, and has been done for both the overall morphological features and the sulcus acusticus (Tuset et al. 2003). The standardization of both treatment and positioning of the otolith is quintessential for the shape analysis. Treatment is often in form of the use of a solution, with examples being glycerine-alcohol or glycerol, for cleaning off any organic material and afterwards heating to achieve the dry weight of the otolith (Turan 2000; Tuset et al. 2006; Vieira et al. 2014). Treatment may also include the preparation of the otolith depending on extra requirements of the method. An example is the use of a SEM. Here Tuset et al. (2003) first sonicated the otolith in alcohol, dried it and then coated it with a layer of gold-palladium, as otherwise the SEM could not take a proper image of the otolith.

The position of the otolith varies from approach to approach, depending on which morphological features are to be analysed. Although otoliths are left-right symmetrical, except in certain species (Begg and Brown 2000, Panfili et al. 2002), either the left or right otolith is often specifically used in shape analysis (DeVries et al. 2002; Vieira et al. 2014; Pavlov 2016; Renan et al. 2016). As stated previously, depending on the

shape analysis method, the otolith may be placed with sulcus acusticus down-ward or up-ward (Vieira et al. 2014; Renan et al. 2016). This depends on whether the acusticus is need for the shape analysis, or the general shape is to be analysed. The standardization of positioning of the otolith with the rostrum pointing in a specific direction is more of a personal choice of the authors than for a specific reason (Begg and Brown 2000; Vieira et al. 2014).

Definitions

Defining a shape or form can be difficult. Indeed, the very distinction between a "shape" and a "form" is not very clear, with both words often being used interchangeably. In early works, the "form" of an object was defined as the linear formula, Form = Size + Shape + Structure.

Size: Can be a length in a one-dimension space, while in two-dimensional space it can be an area, a volume in a three-dimensional space.

Shape: Defined as the residuals after size is estimated, being boundary as an example.

Structure: This is defined by external or internal properties, such as smoothness, orientation and patterns.

Taken from Lestrel (1997)

However, some of these definitions such as shape can be difficult to adequately define and measure (Needham 1950; Lestrel 1997). Lestrel redefined the equation in 1997 with a non-linear equation, adding more attributes, while breaking "structure" down and hence removing it.

Form = [State, Size, Shape, Orientation, Surface, Interior, Substance]

State: This attribute defined if the form is being liquid, solid or gas.

Size: Is defined depending on the dimension, that being the volume for 3-dimension, area for 2-dimension and length for 1-dimension.

Shape: Is defined as the outline of a 2D or 3D object.

Orientation: Defines the location in space.

Surface: Is defined by texture and colour.

Interior: Being also texture and colour, though there is a thickness being described as well, while Surface had not.

Substance: Defined as mass, density or other physical properties.

Taken from Lestrel (1997)

Even though the equation consists of seven attributes, these can easily be expanded upon, and more attributes added (Lestrel 1997). However, during otolith shape analysis, the attributes that varies the most are the Shape, Orientation and Surface, while the rest of the attributes remain constant. Though attributes are obtained, they must be compared in the correct fashion. This call for a homologous position or part of the object in question. An example would be comparing shapes of two trees. To make it homologous, the roots are compared with each other and the branches are compared with each other. Comparing branches of one tree with the roots of another tree will gain no useful information in shape analysis. To make it homologous, the use of landmarks is needed. Within biological objects, anatomical landmarks are defined and used. Landmarks are points which are homologous in an interspecific comparison, and must have an origin of either an embryological, anatomical, or historical nature. Surfaces or outlines which have the same anatomical structure can also be used, if these are homological between organisms. This might be the edge of a birdwing, or elbow of an arm. For otoliths, this may be the rostrum, posterior or sulcus acusticus. Three "true" types of anatomical landmarks have been classified, and differs very much from each other. Type I is a small feature, sufficiently defined by a single point. Claude (2008) considers this landmark the only one with a true biological origin. Type II is defined by a maximum in curvature. Lastly, Type III corresponds to a centroid, an end-point of diameter or an intersection between inter-landmark segments. Figure 6[6] illustrates the three different anatomical landmarks on the lower jaw of a mouse (Claude 2008). In addition to the three anatomical landmarks, pseudo-landmarks and mathematical landmarks are also utilized. Pseudo-landmarks are additional
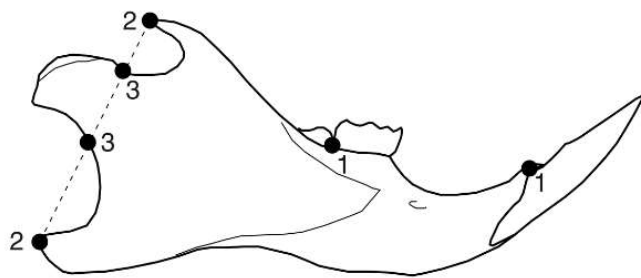
Figure 6. Example of the three anatomical landmark types.

points, which describes construction rather than defining biological homologous features. In figure 1, pseudo-landmarks would describe the pieces of the jaw not described by the anatomical landmarks, i.e. between the upper landmark 2 and upper landmark 3 on the figure 1. Mathematical landmarks are defined by a mathematical property such as a maximum in curvature, but does not contain any biological homological information (Claude 2008). Within the field of otolith shape analysis, two categories of approaches exist. These being landmark analysis and outline analysis. For landmark analysis, the Truss Network approach has been used on otoliths (Turan 2000), while in outline analysis, both Fourier and Wavelet analysis have been used (Stransky et al. 2008; Libungan et al. 2015).

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[6] Claude (2008)

Landmark analysis

Landmark analysis compared to outline analysis, have not seen the widest of application, though it has produced promising results. Due to the high amounts of different analysis methods within landmark analysis, only two methods will be shortly addressed in this review. The **Truss Network** uses specified landmarks, preferably of the type I or type II, and connects these into quadrilaterals, along with two diagonals for each landmark. This method is useful for elongated shapes, and as long as landmarks are positioned along the outline. The method becomes less objective as landmarks within the shape are identified and used in the analysis (Claude 2008). Objects within biology are rarely two dimensional, and to further analyse the reconstructed Truss, one must convert it to a 2-dimensional plane. The flattening process, calls for the distances to be applied to a matrix and the determinant of that matrix to achieve zero. However, reconstructing an object on a coplanar scale, will differ from the original object, which can be measured in strain between landmarks. Figure 7[7] is an example of the Truss Network applied to a herring otolith. The strain can then be used to measure the root sum of squares which in turn can be used for statistical analysis, using for example a principal component analysis (Strauss and Bookstein 1982).
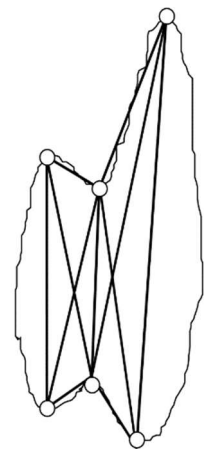
Figure 7. A six landmark Truss Network analysis of a herring otolith.

Superimposition, more commonly known as the **Procrustes method**, is the second method shortly mentioned in this review. It aims to measure through multivariate analysis the differences between landmarks when scale, location and rotation have been adjusted for. Figure 8[8] shows a visual representation of the Procrustes method. Once scale, location and rotation have been adjusted for, the sum of squared distance between landmarks are used to describe the difference between two shapes. Due to this distance measurement, ANOVA test can readily be applied to statistical analysis (Klingenberg 2015).

Using either the Truss Network or the Procrustes analysis in otolith shape analysis demands a shape, where numerous anatomical landmarks can easily be defined. As such, using these methods on cod otoliths where only two anatomical landmarks are available, will provide poor results.

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[7] Turan (2000)
[8] Klingenberg (2015)

Outline analysis

Outline analysis uses both anatomical as well as pseudo-landmarks, sampled along the outline. This means the whole shape is analysed and not just distances between landmarks as in the Truss Network analysis. Outline analysis follows generally four steps. Firstly, a number of pseudo-landmarks are sampled along the outline of the shape. Secondly, superimposition is used as to nullify the scale, rotation and location from further analysis. The third step is the application of a mathematical function that best fits the shape based on the landmark points. Finally, the last step is the statistical analysis of the coefficients acquired from the function. Several methods exist within outline analysis. However, this review focuses on two, being the Fourier and the Wavelet analysis (Crampton 1995; Claude 2008; Nason 2010; Libungan et al. 2015).
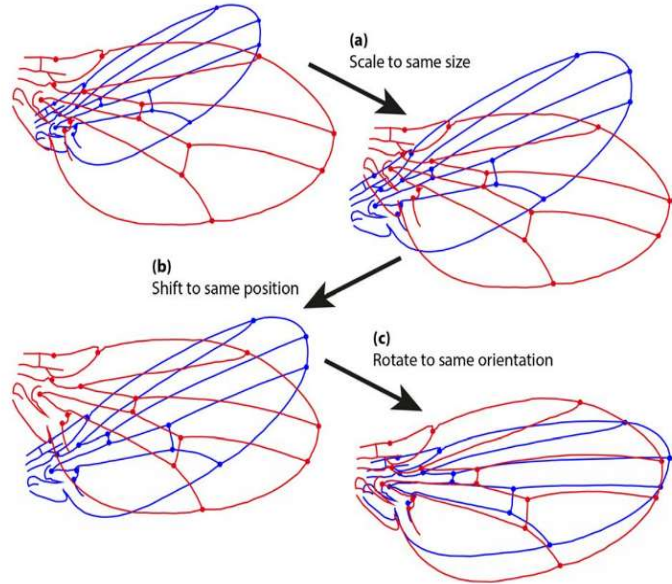
Figure 8. Visual representation of the Procrustes method.

Conventional Fourier

Jean-Baptiste Joseph Fourier (1768 – 1830), a French mathematician, was the father of what is known as Fourier analysis. Having become an essential part of the science of mathematics, physics, acoustics, astronomy, climatology geophysics, the Fourier analysis has since been expanded into the fields of biology (Begg and Brown 2000; DeVries et al. 2002). The complete rigorous proof of the Fourier is not the subject of this review, but some mention of the mathematics behind the Fourier series will be addressed. For a deeper understanding of Fourier series, see (Lestrel 1997; Stein and Shakarchi 2011). Fourier analysis is the fitting of a periodic function to the sampled pseudo-landmarks of the outline, and decomposing it into a weighted sum of sinusoidal component functions.

$$f(t) = \frac{1}{2}a_0 + \sum_{n=1}^{\infty} [a_n \cos(\omega_n t) + b_n \sin(\omega_n t)]$$

These sinusoidal components are termed harmonics, with each harmonic having an even (a_n) coefficient and an odd (b_n) coefficients.

$$a_n = \frac{2}{T} \int_{t_1}^{t_2} f(t)\,cos(\omega_n t)dt$$

$$b_n = \frac{2}{T} \int_{t_1}^{t_2} f(t)\,sin(\omega_n t)dt$$

These coefficients are used for further multivariate analysis and can be used for reconstruction of the original shape. However, as the pseudo-landmarks being sampled as x,y coordinates, the conventional Fourier analysis cannot be used directly. This problem is solved through the three following methods (Claude 2008).

Additionally, the conventional Fourier series suffers under four limitations. The first being that tabulated functions must be single-valued. The second is that the data must be in a polar form. Thirdly, the angles between two vectors must be equal. And lastly for the evaluation of the Fourier coefficient, integration is required. Since shapes within the field of biology require more than single values and equal angles cannot always be reached for enough amounts of information within biology, it makes conventional Fourier series rather unsuited for such analysis (Claude 2008).

Fourier analysis of equally spaced radii

The first method uses the information of equally spaced radii from the centroid to the outline. Figure 9[9] shows the outline of a bee wing turned into intercepting points by equally spaced radii.
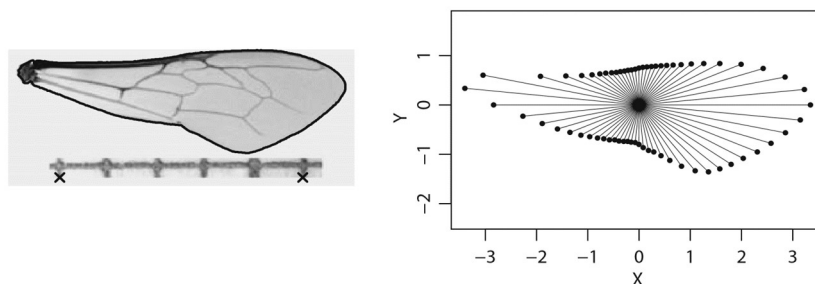
Figure 9. Representation of a bee wing (left) through equally spaced radii (Right).

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[9] Claude (2008)

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The radius can be expressed as a function of the angle, with k being the harmonics 0 to k.

$$r(\theta) = \frac{1}{2}a_0 + \sum_{n=1}^{k}[a_k \cos(\omega_k\theta) + b_k \sin(\omega_k\theta)]$$

However, k cannot be more than half the amount of sampled pseudo-landmarks (p).

$$a_n = \frac{2}{p}\sum_{n=1}^{p} r_i \cos n\theta_i$$

$$b_n = \frac{2}{p}\sum_{n=1}^{p} r_i \sin n\theta_i$$

$$a_0 = \sqrt{\frac{2}{p}\sum_{n=1}^{p} r_i}$$

Successfully using equally spaced radii from a central point of the shape, depends on the shape itself. Using the method on a circular shape will provide a more accurate fitting of the function, due to more equally spaced pseudo-landmarks. The more complex the shape, the more inaccurate the function fitting will be. Figure 9 shows the digitization of a honeybee wing. The middle upper and lower part of the wing contains more detailed information, while this decrease towards the left and right end of the wing. Especially, concavities and convexities can cause problems as well as a given angle that intercepts the outline twice (Claude 2008).

Fourier analysis of the tangent angle to the outline

This is the second method when dealing with x,y coordinates for the pseudo-landmarks, and was first used by Zahn and Roskies in 1972. It solves the problem of pronounced concavities that the previous method was unable to describe. The perimeter of the shape is first scaled to 2π, and the coefficients can then be estimated by the following equations (Claude 2008).

$$a_n = \frac{2}{p}\sum_{n=1}^{p} \emptyset(t) \cos n\theta_i$$

$$b_n = \frac{2}{p}\sum_{n=1}^{p} \emptyset(t) \sin n\theta_i$$

The method is to draw a tangent ((θ(0)), removed later on for standardization), and then describe the cumulative changes to the angle (ɸ(t)) of this tangent along the perimeter (t). θ(t) being the angle at the distance t along the perimeter.
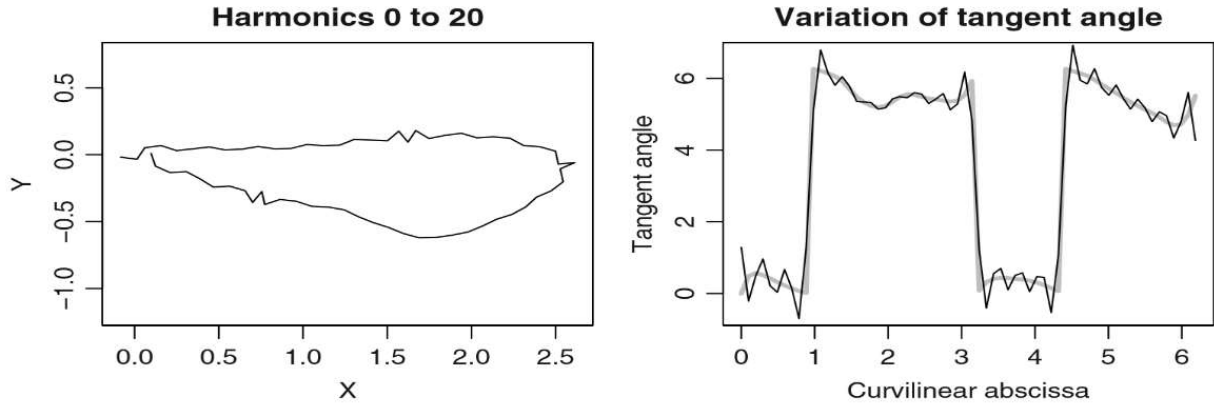
Figure 10. Reconstruction of the bee wing (left) and (Right) the fit of the Fourier (Black line) on the variation of the original shape (Thick Grey Line).

Due to reconstruction problems, the comparison between two groups of shapes using this method are preferably done as in figure 5 (right), as the variation of the tangent angle against the perimeter t, as a curvilinear abscissa (Claude 2008).

Elliptic Fourier analysis

Developed by Kuhl and Giardina (1982), the Elliptic Fourier analysis solved the problems of the conventional Fourier analysis. As such, elliptic Fourier analysis has seen an increase of use in morphometric analysis, compared to conventional Fourier analysis. Instead of x and y being dependent on each other, they were instead separated and described as functions of a third variable, that being the curvilinear abscissa, prescribed as t. Along with this change, the amounts of coefficients are increased with an additional two. The functions of $x(t)$ and $y(t)$ are as follows. Figure 11[10] shows the fitting of elliptic Fourier depending on the amounts of harmonics used.

$$x(t) = \frac{a_0}{2} + \sum_{n=1}^{+\infty} a_n \cos n\omega t + b_n \sin n\omega t$$

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[10] Claude (2008)

$$y(t) = \frac{c_0}{2} + \sum_{n=1}^{+\infty} c_n \cos n\omega t + d_n \sin n\omega t$$

With the coefficients being:

$$a_n = \frac{2}{T} \int_0^T x(t) \cos(n\omega t) \, dt$$

$$b_n = \frac{2}{T} \int_0^T x(t) \sin(n\omega t) \, dt$$

$$c_n = \frac{2}{T} \int_0^T y(t) \cos(n\omega t) \, dt$$

$$d_n = \frac{2}{T} \int_0^T y(t) \sin(n\omega t) \, dt$$

the coefficients:

$$a_o = \frac{2}{T} \sum_{i=1}^{p} x_i$$

$$c_o = \frac{2}{T} \sum_{i=1}^{p} y_i$$

corresponds to the coordinates of the centroid of the outline.

The Elliptic Fourier is superior to the conventional Fourier analysis in four specific ways. 1) Data points on the
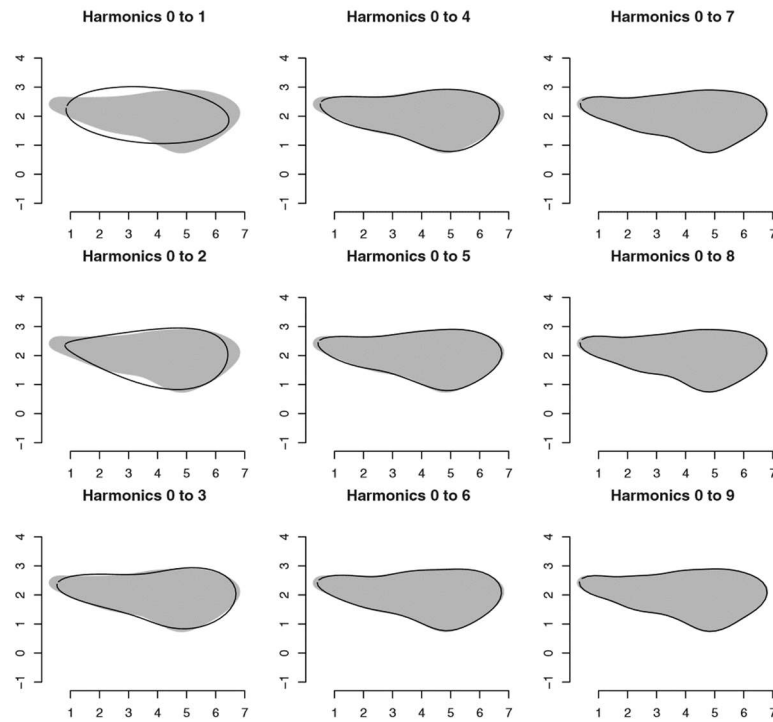
Figure 11. Fitting of the Elliptic Fourier function on the bee wing by amounts of harmonics.

outline does not need to be equally spaced as for the Tangent Angle method does. 2) It does not need a homologous or mathematical centroid of the outline. 3) Possible application for complex structures, where an angle produces two values for outline interception. This contrary to the equally spaced radii approach. 4) The coefficients are independent of outline position, and can be normalized for scale, location and rotation (Crampton 1995; Claude 2008).

Wavelet

The term Wavelets appeared in 1982, coined by Morlet, and is used for signal and image decomposition. Only parts of the mathematics of the wavelets will be reviewed here, and for a more comprehensive demonstration see Nason and Silverman (1994), Graps (1995) and Nason (2010).

Considering a sequence of $y_i = (y_1, y_2, y_3, y_4, y_5, y_6, y_7, y_8)$, for which a multiscale analysis is needed. First to be extracted is the local details, or variations. For this, the letter d is used, with the subscript k, defining the location along the sequence. The equation for extracting the detail is:

$$d_k = y_{2k} - y_{2k-1}$$

A detail sequence of the example y_i would then be:

$$d_1 = y_2 - y_1$$

$$d_2 = y_4 - y_3$$

$$d_3 = y_6 - y_5$$

$$d_4 = y_8 - y_7$$

however, this only describes the detail between half of y_i. To counter this, and obtain more detail information, we obtain a coarser sequence of y_i, named c_k by:

$$c_k = y_{2k} + y_{2k-1}$$

And for the example sequence y_i, it would be:

$$c_1 = y_2 + y_1$$

$$c_2 = y_4 + y_3$$

$$c_3 = y_6 + y_5$$

$$c_4 = y_8 + y_7$$

From c_k, a new detail can be obtained. To keep track of the scaling, the subscript j is used, with *j = J-1*, and *J* being the level of which all original data is present. One can then continue to scale and extract both the detail and coarse values. The designation *J-1* will change to *J-2, J-3… J-n*, depending on the level the data is decomposed to. Both the d and c values are coefficients, known as the wavelet coefficient (d_k^j) and the father wavelet coefficient (c_k^j), and the example above is of a discrete wavelet transform. However, during reconstruction of the sequence y_i from the coefficients d_k^j and c_k^j, some *energy* consists due to the norm

$\|y\|^2 = \sum_{i=1}^{8} y_i^2$, and two filters are therefore needed to be added for adjusting the coefficients d_k^j and c_k^j correctly both input and output wise (Nason, 2010).
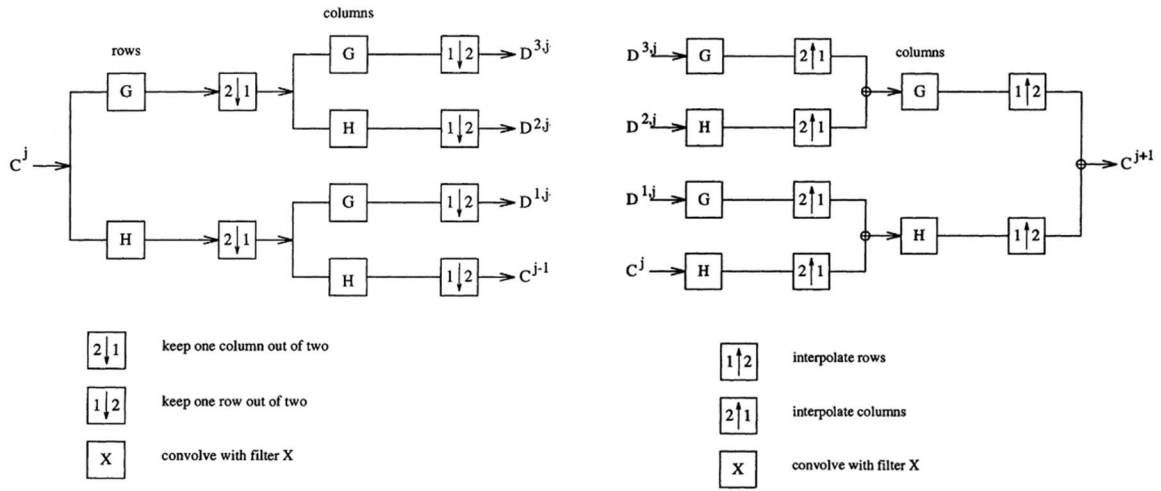
Figure 12. The Wavelet analysis method. Left figure is the decomposition of a signal, while right figure is reconstruction from detail and smoothed signal.

This is basically how the wavelet otolith analysis occurs. The outline of the otolith is used as a signal, and passed through a bandpass filter (g) and a smoothing filter (h). Signal (c^j) which produces a vector of detail coefficients (d^{j-1} by filter g) and a vector of the smoothed signal (c^{j-1} by filter h). Repeating the process on the new smoothed signal (c^{j-1}), creates a new set of vectors, one with the detail coefficients (d^{j-1}) and one with a more smoothed signal (c^{j-2}). This is continued until at level 0, both the c^0 and d^0 is obtained (Nason and Silverman 1994). Figure 12 gives an overview of the wavelet analysis with the decomposing and reconstruction of the signal.

The number of coefficients used for otolith analysis depends on the level at which a 98.5% reconstruction is possible. Figure 13 defines this at level 5 for wavelet, and 12 harmonics for Normalized Elliptic Fourier for the otolith analysis of Atlantic cod off the Greenlandic coast. This produces 64 wavelet coefficients and 45 Fourier coefficients for multivariate analysis, much like in (Libungan et al. 2015; Libungan and Palsson 2015).
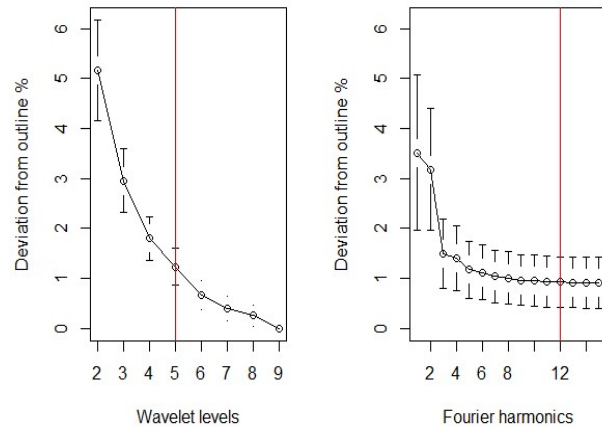
Figure 13. Evaluation of the reconstruction accuracy using Wavelet and Fourier. The red line indicates a 98.5% reconstruction accuracy. From Glindtvad (2017) part of this thesis.

Summary

Due to ocean currents, cod larvae and eggs are transported from the east coast of Greenland to the west coast. This causes a mixing of populations in the nursery grounds along the west coast, and causes mixed catches of these populations in the West Greenland fishery. This is a problem for the over-exploited West Greenland Offshore population.

Analysis of the shape of the otolith can possibly be used as a fishery management tool opposite genetic classification in separating the cod populations from each other. Of the three otoliths in the inner ear, the sagitta is the most used. Both genetic and environment affect the shape of the otolith. With feeding levels having the most effect on the otolith shape. Feeding levels in turn can be expanded as diet composition and amounts, where amounts did not affect the otoliths shape, while diet composition seems to do. However further research is needed to support this hypothesis. For the Atlantic cod, genotype and feeding levels appears to affect the otolith shape the most.

Otolith shape analysis can be done with several methods, but analysis based on the outline seems to be the most promising for the Atlantic cod, as this otolith does not contain many anatomical landmarks due to its shape. The difference between analysis based on Fourier and Wavelet lies in the approach towards achieving the coefficient of a 98,5% reconstruction of the outlines. Where Fourier will add harmonics until this goal is achieved, the Wavelet will decompose the data until the level of accuracy is achieved. As such, Wavelet is better at finding local variations and sharp edges, and is better for evaluating details. Several studies have shown how otolith shapes can be used for stock discrimination to various degrees, and as an alternative to such methods as genetic classifications.

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Separation by Otolith Shape: Discrimination of three Cod (*Gadus morhua*) Populations

Resume

Fire populationer af atlantisk torsk (*Gadus morhua*) findes i de grønlandske farvande hvor der foregår kommercielt fiskeri efter tre af populationerne. Disse fire populationer blander sig i fiskeriområder, hvilket vanskeliggør adskillelse og forvaltning. På nuværende tidspunkt er den eneste mulighed populationsadskillelse at bruge genetisk klassifikation. Dette er dog både tidskrævende og dyrt, hvorfor det ikke anvendes forvaltningsmæssigt. En alternativ metode er populationsadskillelses på basis af torskens ørestensform. Denne metode er blevet anvendt både til arts- og populationsadskillelse, og har vist sig at være et lovende redskab. To matematiske metoder der kan anvendes til formanalysen af øresten er Normalized Elliptic Fourier og Discrete Wavelet Transform, og begge metoder har vist sig at være brugbare. Dette studie forsøgte at adskille tre af de fire populationer i de grønlandske farvande. Begge matematiske metoder blev både anvendt på den overordnede form af ørestenen, samt på formen af første og anden års ringene efter at ørestenene var blevet knækket tværgående. Desuden blev effekten af miljø, genetik og alder på både den overordnede form samt første og anden års ringene undersøgt. Normalized Elliptic Fourier udførte en bedre populationsadskillelse end Discrete Wavelet Transform gjorde. To af de tre populationer kunne klassificeres med en høj gennemsnitlig klassifikationssucces (> 70%) på basis af den overordnede ørestensform. Både første og anden års ringene gav en betydelig lavere klassifikationssucces (<65%) end den overordnede ørestensform. Hverken genetik eller miljø havde en signifikant indflydelse på den overordnede form af ørestenene. Ved undersøgelsen af alder, viste der sig en mulig migration hos en af populationerne fra indenskærs til udenskærs områder. Der bør forskes yderligere i, hvilke metoder og kombinationer af disse, der bedst kan anvendes ved populationsadskillelse baseret på ørestens formanalyse.

Abstract

Four genetically different populations of cod (*Gadus morhua*) inhabit the waters along the coast of Greenland. These populations share habitats on the west coast, and current available fishery management tools for population separation are restricted to Genetic classification. As genetic classification is both time consuming and expensive, alternative classification methods are need. In this study, the use of otolith outline shape analysis as a tool for population separation was investigated. Both Discrete Wavelet Transform and Normalized Elliptic Fourier were used. The whole outline of the otolith, as well as the outline of the 1st and 2nd year increments were used for shape analysis. Normalized Elliptic Fourier showed more potential than the Discrete Wavelet Transform for populations classification. Two out of the three populations could be classified with a high mean classification rate (>70%). The 1st and 2nd year increments provided a lower mean classification success (<65%) than the whole shape of the otolith. Neither genetics nor environment were found to have a significant influence on the whole otolith shape. Age group separated shape analysis indicated a possible migration from inshore areas to offshore areas by the West Greenland Offshore cod population. Further studies should be done to investigate a combination of shape analysis and other population classification methods for fishery management purposes.

Introduction

The Atlantic cod, *Gadus morhua*, inhabits marine waters from the North American east coast to the northern coasts of west Russia, and the species is found in several national and international waters (Figure 14[11]). It is undoubtedly one of the most utilized marine resources in the north Atlantic sea with several million tons caught per year (ICES 2005). Several separate stocks exist, many of them having been exploited heavily for the last 60 years, causing stock decimation. The most prominent example is that of the 7 stocks off the Canadian eastern coast,
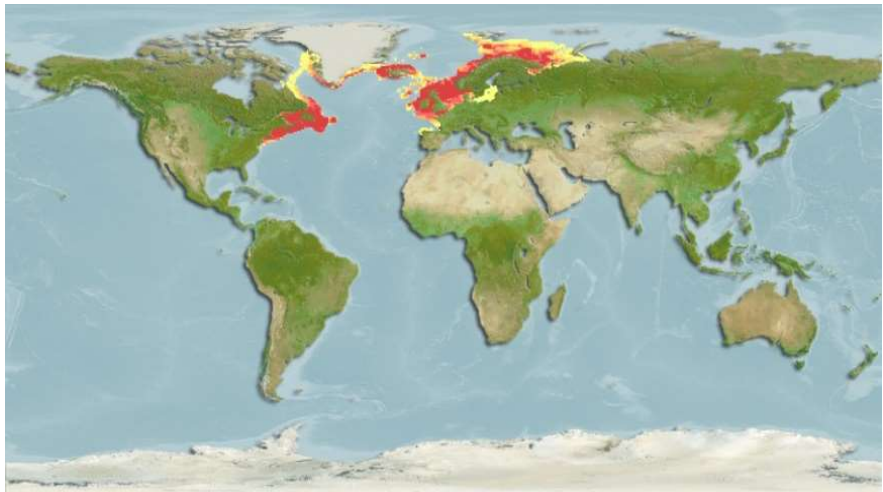

Figure 14. Visual representation of the distribution of North Atlantic Cod.

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[11] Figure from http://www.fishbase.se/summary/Gadus-morhua.html

the decline of which caused a moratorium on the cod industry (Myers et al. 1997; ICES 2005). In Greenland waters, overexploitation occurred on the west coast, with a west offshore stock having been decimated during the 1970's (ICES 2005). Today only minor fishery on an east offshore and west inshore stock exists, with ICES advising against any fishery of the west offshore stock (ICES 2005; ICES 2016a;ICES 2016b; ICES 2016c). To avoid overexploitation, different methods and mathematical models are used for stock assessment and management purposes (King 2013). However, occurrence of stock mixing in spawning grounds (Stransky et al. 2008) or nursing grounds (Therkildsen et al. 2013; Bonanomi et al. 2015; Bonanomi et al. 2016) creates a problem with stock discrimination. Previous shape analysis studies have worked with stocks (Vieira et al. 2014; Hussy et al. 2016; Renan et al. 2016), and a distinction should be kept in mind when reading this study and comparing with others. The Greenland waters are inhabited by four genetically distinct populations which are managed as three stock units (ICES 2016a; ICES 2016b; ICES 2016c). These being the West Greenland Inshore, West Greenland Offshore, the East Greenland Offshore/Icelandic Offshore and the Iceland Inshore cod (Therkildsen et al. 2013). After the spawning periods of the Icelandic populations, their eggs and larvae are transported by currents to the West Greenland coastline which are used as nursing grounds for all four populations (Bonanomi et al. 2016). So far, the best method of population discrimination has been using the genetic method of Therkildsen et al. (2013). However, using genetics to distinguish between the populations is both time consuming and expensive. An alternative for genetic classification is the use of otolith shape analysis, which has been used on several species (Begg and Brown 2000; Bolles and Begg 2000; DeVries et al. 2002; Tuset et al. 2003; Gagliano and McCormick 2004; Tuset et al. 2006; Vieira et al. 2014; Libungan et al. 2015; Pavlov 2016; Renan et al. 2016), and several cod stocks (Cardinale et al. 2004; Galley et al. 2006; Petursdottir et al. 2006; Hussy 2008; Stransky et al. 2008; Paul et al. 2013). The shape of the otolith is genetically determined on species level (Leopold et al. 2001). However, on a population level both environment and genetics have a significant effect on the otolith shape (Cardinale et al. 2004). Feeding levels are known to be the most significant direct factor within environmental effects (Hussy 2008), with some indication that even diet might influence the otolith shape (Mille et al. 2016). Shape analysis methods are divided into two categories. The first is Landmark analysis, were anatomical landmarks (Claude 2008) are used in e.g. the Procrustes (Klingenberg 2015) and the Truss Network method (Strauss and Bookstein 1982). The second category is the Outline analysis. The outline of the otoliths is fitted with a function which yields a number of coefficients for statistical analysis. Fourier analysis has been the most useful for several species (Torres et al. 2000; Pavlov 2016) and stocks (Begg and Brown 2000; DeVries et al. 2002; Stransky et al. 2008; Vieira et al. 2014). Due to limitations of the conventional Fourier analysis, the Elliptic Fourier was developed by Kuhl and Giardina (1982), and solved several of the problems with conventional Fourier analysis (Crampton 1995). Recently, Discrete Wavelet Transform was proposed as an alternative to Fourier analysis in otolith

shape analysis (Libungan et al. 2015; Renan et al. 2016). While Fourier consists of sinusoidal functions, which increase in numbers for better fitting, Wavelet decompose the original data into a dataset of coefficients through filters (Nason and Silverman 1994; Claude 2008; Nason 2010). Due to the sinusoidal functions, Fourier can be poor in the approximations of sharp edges, while the approximating functions of Wavelet are contained in finite domains, and therefore makes them better at approximating sharp edges. Due to this feature, Wavelet is also more suited for determining the areas along the otolith outline where the greatest variation occurs (Graps 1995; Libungan et al. 2015). Shape analysis has mostly been restricted to the outline shape of the otolith with only a few other structures having been analysed (Torres et al. 2000; Tuset et al. 2003). The 1st and 2nd year growth increments have so far only been used once in stock discrimination, with promising results (Berg et al. 2005). Classification success of the otolith outline has been seen to decrease with age, (Mapp et al. 2017). This is caused by exogenous factors influencing the early development and endogenous factors and an ontogenetic shift influencing later otolith shape growth (Lombarte et al. 2003; Hussy 2008; Vignon 2012). The use of 1st and 2nd year increments might therefore provide at higher classification success than the overall shape. However, this has not been investigated before. The overall aim of this study is to investigate the feasibility of separating three of the four cod populations around Greenland from each other based on otolith shape analysis. Specifically, (I) investigate the possible differences of Elliptic Fourier analysis and Discrete Wavelet transformation for population discrimination of the Atlantic cod in Greenland waters, (II) determine the possible use of 1st and 2nd year increments in shape analysis, and (III) assess the effect of genetic, environment and age on the otolith shape.

Method & Materials

A total of 317 sagitta otoliths, sampled in 2016 were collected for analysis. Some of these were only used in parts of the analyses due to deformation or damage. Sampling technique varied. Gillnet survey aimed for Greenland Halibut and adult cods was used on the west coast (NAFO area 1A, 1B & 1D) (Retzel 2017b) and bottom trawl survey was used on the east coast (NAFO area 14B) (Retzel 2017a). Distribution of the sampled otoliths were 45 from 1A (Disco Bay), 61 from 1B (Sisimiut), 88 from 1D (Nuuk) and 120 from 14B (Figure 15**Error! Reference source not found.** for a visual overview). Only otoliths sampled in the NAFO areas 1A (Disco), 1B (Sisimiut) and 1D (Nuuk) were genetically assigned. For more specific details on the genetic method used, this article refers to Therkildsen et al. (2013) and
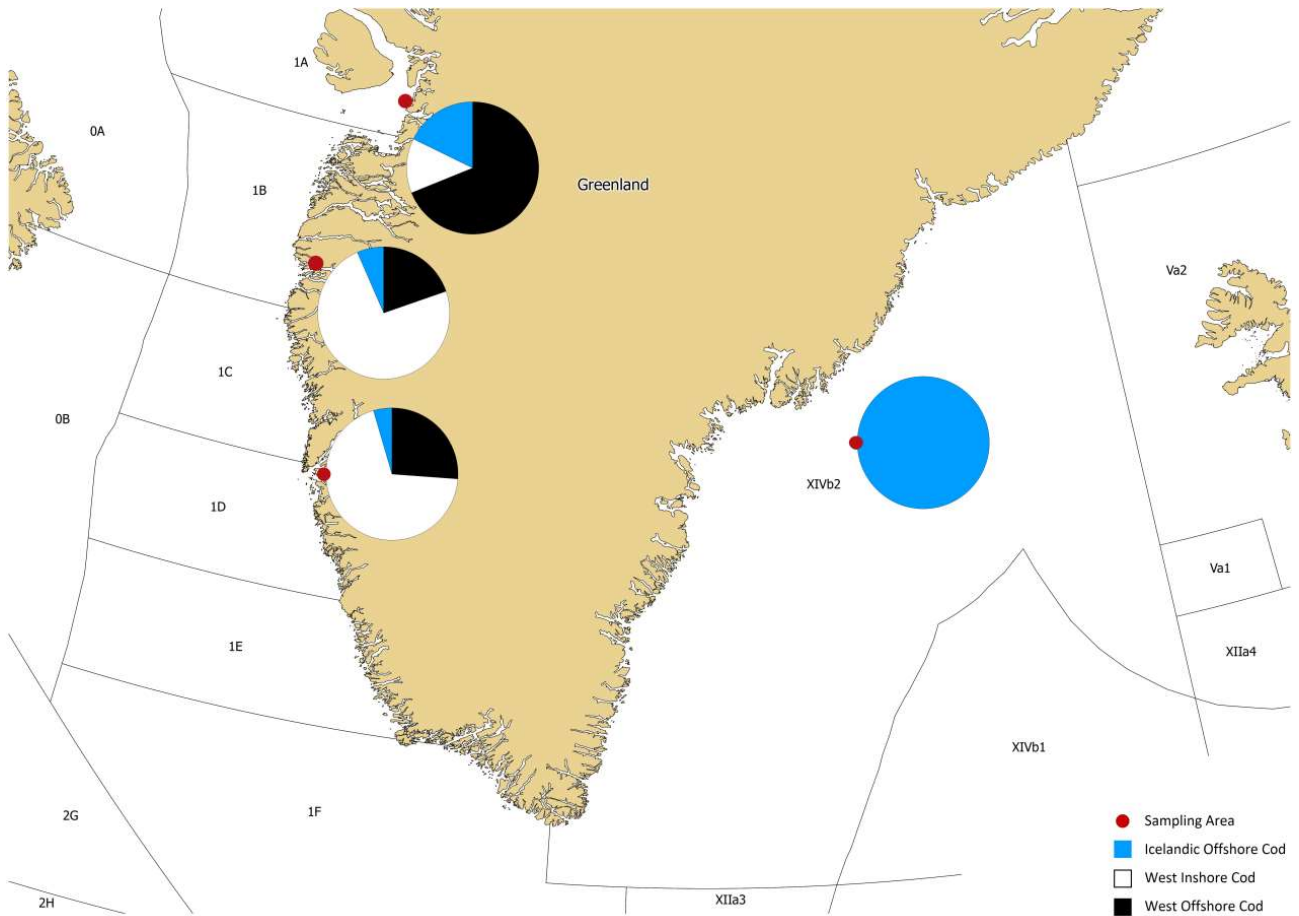
Figure 15. Distribution of the three populations caught in the four NAFO areas.

Henriksen (2015). All the genetic assigned otoliths, had an assignment of at least 80%. With no indication of migration from the west coast to the east coast by West Offshore or West Inshore cods, the otoliths sampled on the east coast were assumed to be Icelandic Offshore individuals. Genetic assigned Icelandic Offshore (west coast sampled) and unassigned Icelandic Offshore (east coast sampled) otoliths were used together. It was not possible to standardize using either the left or the right otolith as these had prior been randomly broken for age estimations. The distribution of otoliths can be seen in Table 1.

Table 1. Total number of otoliths available for use in 1st year, 2nd year and whole shape analysis for each of the three populations.

Population	1st year shape (n)	2nd year shape (n)	Whole shape (n)
Icelandic Offshore	128	85	126
West Greenland Offshore	62	38	53
West Greenland Inshore	98	75	99

For digital image acquisition of each otolith, a Leica MZ6 binocular microscope with an attached Leica DFC280

camera was used, with corresponding software being Leica Application Suite 2.6.0 R1 (build 1192). Images were saved in JPEG format. For image processing, ImageJ 1.51J8 and Artweaver 0.5 were used with an electronic drawing tablet. For the image capture of the whole shape, the otolith was placed with the convex side (sulcus acusticus) facing the camera, and the rostrum facing left. The image was captured on a white transmitted surface, creating a dark shaped otolith as shown in Figure 16.

For the shape of the 1st year and 2nd year rings, the otoliths were broken transversally. Image acquiring was done with the sulcus upward and dorsal side facing left. ImageJ was used for calibration of

Figure 16. The whole shape of a left sagitta otolith used in

pixel/length(mm) ratio for shape analysis. Standardization by centering the otolith in the image was done using Artweaver, along with the manual tracking of the 1st and 2nd year rings using an electronic drawing tablet (Figure 17).

Figure 17. Shapes of the 2nd year (bottom left) and 1st year (bottom right) increments manually tracked using an electronic drawing tablet on the transversally broken otolith (top).

Image and Shape analysis

Image and shape analysis was done in the program R (version 3.3.3) with RStudio (version 1.0.136), using the package ShapeR (Libungan and Palsson 2015) and following the guidelines in (Libungan et al. 2015). Outline was detected and captured from the digital images, using the conte and regularradius function in R (Claude 2008). Polar coordinates of pseudo-landmarks along the outline was then acquired by drawing equidistant angled radials from the centre of the otolith. Discrete Wavelet Transform (DWT) (Nason 2010) and Normalized Elliptic Fourier (NEF) (Kuhl and Giardina 1982; Claude 2008) coefficients were then obtained from the pseudo-landmarks, using a combination of wavethresh package (Nason 2010) and R shape functions (Claude 2008). Ten wavelet levels were used for the DWT, yielded a total of 64 wavelet coefficients while 12 harmonics were used in NEF yielded a total of 45 coefficients. To avoid significant interaction between the length of the fish and population, an evaluation was performed on the coefficients of both DWT and NEF, automatically excluding coefficients with significant interaction. The amount of levels of the DWT and number of harmonics of the NEF was evaluated, ensuring a 98.5% accuracy of reconstruction (Figure 18).
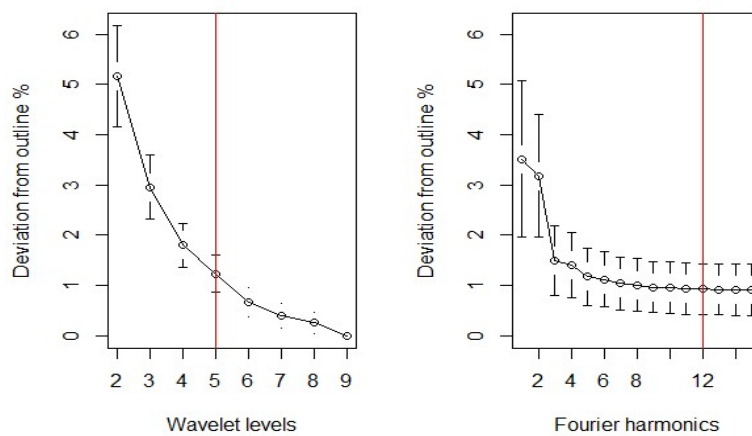
Figure 18. Deviation from the outline for Wavelet and Fourier depending on the amount of levels and harmonics. The red line marks the point of a 98.5% reconstruction accuracy.

Statistical analysis

Display and statistical analysis of the differences between the three populations was done using different functions in the vegan package for R. Plotting the average otolith shapes among populations, the difference between populations were visually evaluated (Figure 19). A Canonical Analysis of Principal Coordinates was performed on the coefficients of the DWT and NEF. An ANOVA with a permutation-like test was then performed

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on the distances between eigenvalues of single otolith samples, and average eigenvalues of the otolith group. Using Ipred and MASS packages in R, a Linear Discriminant Analysis was performed, with a cross-validation function. The function was performed for determining the amount of correct assignment of 1/4 of the otoliths (test) from analysing the other 3/4 otoliths (learning) first.

Analysis

Several analyses were conducted in this study, and Table 2 gives an overview of these.

Table 2. Overall view of the different analyses conducted in this study.

Comparison	Details	Reason
Three-populations analysis	Population discrimination of the three populations based on all the otoliths, regardless of age and environment.	Tested for fishery management purposes, as all three populations will mix in some fishing grounds.
Two-populations analysis	Population discrimination of two populations based on all the otolith of the two populations, regardless of age and environment.	Tested to see which populations will separate more.
Age effect analysis	Population discrimination of two populations based on all the otolith of the two populations, in the age class of 1-3 and 4-6.	Tested to see if development of the otolith through age will affect the classification rate.
Genotype effect analysis	Population discrimination of two populations based on otolith sampled in the same area.	Tested to see if the genetic factors affect the otolith shape.
Environment effect analysis	Population discrimination of one population based on otolith sampled in two separate locations.	Tested to see if the environmental factors affect the otolith shape.

The whole shape of the otoliths was used in the three-population analysis, regardless of age and genetic assignment or lack of. Two-population analysis was performed using all otoliths like in three-population analysis, however the analysis ran the populations against each other in pairs. The analysis of effect of the age on the whole shape of the otolith, was performed like the two-population analysis, but with the otoliths separated in age-groups of 1-3 and 4-7 years old individuals. For the effect of genotype on otoliths, only the otoliths of the West Inshore and the West Offshore sampled in NAFO 1D (Nuuk) was used. For determining the environmental effect on the otolith shape, the West Inshore population was separated into a Nuuk and a Sisimiut group. The whole shape of the otolith was used in all the analysis, whereas the shape of the 1st and 2nd year ring only was used in the three-population, two-population, genetic and environment analysis.

Results

Average shape difference

When reconstructing from coefficients, the average difference in shape between the three populations were negligible on the whole shape for both NEF and DWT (Figure 19). For the 2nd year ring, the average shape showed more visual difference both with NEF and DWT than the whole otolith shape. The visual difference in the 1st year ring between populations was even more visible than when using the shape of the whole otolith and the 2nd year ring.
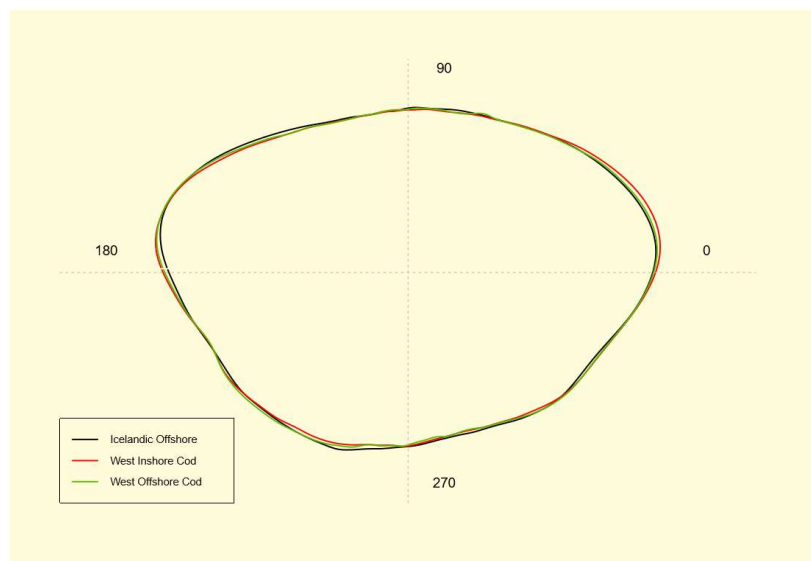
Figure 19. Average shape of the three otoliths reconstructed from Discrete Wavelet Transform.

Multivariate analysis & classification rate

Three population analysis

The Discrete Wavelet Transform (DWT) showed a significant difference ($p < 0.05$) between all three populations, with the 1st ring and the whole shape showing a slightly more significant difference than the 2nd ring (Table 3). The Normalized Elliptic Fourier (NEF) analysis only found a significant difference between the three populations on the whole shape, while there was no difference in neither the 1st or 2nd year rings (Table 4).

Table 3. ANOVA test for differences in shape of the three populations based on DWT.

Shape	Df	Var	F	P-value	WIC (n)	WOC (n)	IC (n)
1st ring	2	0.05501	4.4063	0001***	98	62	126
residuals	285	1.77917					
2nd ring	2	5.184	2.034	0.023 *	75	38	83
residuals	194	247.217					
Whole	2	1.999	4.7195	0.001***	99	53	124
residuals	274	58.013					

d.f., degrees of freedom; Var, variance among populations; F, pseudo F-value; WIC, West Inshore cod; WOC, West Offshore cod; IC, Icelandic Offshore

Table 4. ANOVA test for differences in shape of the three populations based on NEF.

Shape	Df	Var	F	P-value	WIC (n)	WOC (n)	IC (n)
1st ring	2	0.0905	1.0548	0.239	98	62	126
residuals	285	12.2291					
2nd ring	2	0.00852	1.7716	0.063	75	38	83
residuals	194	0.46668					
Whole	2	0.006163	4.6991	0.001***	99	53	124
residuals	274	0.179687					

d.f., degrees of freedom; Var, variance among populations; F, pseudo F-value; WIC, West Inshore cod; WOC, West Offshore cod; IC, Icelandic Offshore

For the LDA, the West Offshore (WOC) showed a poor classification success, regardless when using either the DWT or NEF (mean<20%). Both the West Inshore (WIC) and the Icelandic Offshore (IC) showed a high classification success (mean>60%), with the highest classification success obtained with the NEF approach (mean>70%) (Table 5). Additional, results can be found in the supplemented result sheet.

Table 5. Mean classification success for separation of the three populations, using DWT (top) and NEF (bottom).

Wavelet	WIC	WOC	IC	Error
1st ring	41.2%	12.0%	58.0%	57.6%
2nd ring	56.1%	37.2%	50.9%	49.8%
Whole	51.6%	15.7%	59.6%	51.7%
Fourier	WIC	WOC	IC	Error
1st ring	54.8%	19.0%	62.0%	49.7%
2nd ring	51.3%	28.9%	57.7%	50.3%
Whole	71.4%	19.4%	70.3%	39.1%

Two population analysis

In the two-population analysis where only two populations at a time was compared, the DWT detected slightly more significant differences between the otoliths. Within the population classification for the two-population analysis both the West Inshore and the Icelandic Offshore populations had the highest classification success, while the West Offshore population had the lowest classification success (Table 8 & **Table 9**), though not as low as when all three populations were analyzed at the same time (Table 5).

West Inshore compared to Icelandic Offshore showed significant differences in the whole shape ($p < 0.05$) detected by both DWT and NEF. However, neither DWT nor NEF detected any significant differences between the two populations when using the 1st and 2nd year ring shape (Table 6 & **Table 7**).

Table 6. ANOVA test for differences in shape between West Inshore and Icelandic Offshore based on DWT.

Shape	Df	Var	F	P-value	WIC (n)	IC (n)
1st ring	1	0.01471	1.9509	0.1	98	126
residuals	222	1.67392				
2nd ring	1	2.564	1.8932	0.076	75	83
residuals	155	209.923				
Whole	1	1.407	6.956	0.001***	99	124
residuals	220	44.514				

Table 7. ANOVA test for differences in shape between West Inshore and Icelandic Offshore based on NEF.

Shape	Df	Var	F	P-value	WIC (n)	IC (n)
1st ring	1	0.0615	1.1465	0.405	98	128
residuals	222	11.9108				
2nd ring	1	0.00524	2.1237	0.065	75	85
residuals	155	0.38236				
Whole	1	0.007934	7.8198	0.001***	99	126
residuals	220	0.223223				

For the classification success, the NEF analysis provided a higher mean classification success for both populations and a lower error classification (Table 9) compared to the DWT analysis (Table 8). This was regardless of which shape was used, though the whole shape provided the best results.

Table 8. Mean classification success in the two-population comparisons using DWT. X marks the exclusion of the population from the analysis.

Wavelet	WIC	WOC	IC	Error
1st ring	51.5%	X	65.8%	40.5%
2nd ring	62.5%	X	61.2%	38.2%
Whole	63.4%	X	69.4%	33.2%
1st ring	58.7%	39.8%	X	48.6%
2nd ring	60.4%	48.9%	X	43.5%
Whole	66.5%	39.4%	X	43.0%
1st ring	X	40.5%	69.6%	40.0%
2nd ring	X	48.6%	67.8%	38.2%
Whole	X	38.0%	72.1%	38.1%

Table 9. Mean classification success in the two-population comparisons using NEF. X marks the exclusion of the population from the analysis.

Fourier	WIC	WOC	IC	Error
1st ring	63.0%	X	75.1%	30.2%
2nd ring	61.6%	X	67.3%	35.4%
Whole	79.5%	X	78.4%	21.1%
1st ring	74.9%	59.1%	X	31.2%
2nd ring	66.0%	38.6%	X	43.3%
Whole	77.9%	43.6%	X	34.1%
1st ring	X	35.4%	76.7%	36.9%
2nd ring	X	49.2%	72.4%	34.9%
Whole	X	33.2%	74.0%	38.2%

West Inshore compared to West Offshore showed a more significant difference when using the NEF than using the DWT. For NEF analysis, a significant difference was detected both in 1st year ring and the whole shape, while with DWT, only the 1st year ring showed a significant difference (Table 10 & Table 11).

Table 10. ANOVA test for differences in shape between West Inshore and West Offshore based on DWT.

Shape	Df	Var	F	P-value	WIC (n)	WOC (n)
1st ring	1	0.07101	7.7854	0.001***	98	62
residuals	158	1.441				
2nd ring	1	1.247	0.9674	0.444	75	38
residuals	110	141.76				
Whole	1	0.401	1.6198	0.11	99	53
residuals	149	36.889				

Table 11. ANOVA test for differences in shape between West Inshore and West Offshore based on NEF.

Shape	Df	Var	F	P-value	WIC (n)	WOC (n)
1st ring	1	0.0249	8.6046	0.001***	98	62
residuals	158	0.45724				
2nd ring	1	0.00214	0.9133	0.455	75	38
residuals	110	0.25734				
Whole	1	0.00421	5.9493	0.001***	99	53
residuals	149	0.10533				

The NEF analysis provided once more the best mean classification success for the discrimination of West Inshore and West Offshore (Table 9), however, the DWT did provide some better results for the mean classification success of the West Offshore population (Table 8). Only DWT detected a significant difference between all shapes for the West Offshore compared to the Icelandic Offshore, while NEF only detected a significant difference for the whole shape (Table 12 & Table **13**).

Table 12. ANOVA test for differences in shape between West Offshore and Icelandic Offshore based on DWT.

Shape	Df	Var	F	P-value	WOC (n)	IC (n)
1st ring	1	0.04169	3.8063	0.018 *	62	126
residuals	186	2.0373				
2nd ring	1	3.719	3.2161	0.01 **	38	83
residuals	119	137.595				
Whole	1	1.516	3.8219	0.007 **	53	124
residuals	175	69.411				

Table 13. ANOVA test for differences in shape between West Offshore and Icelandic Offshore based on NEF.

Shape	Df	Var	F	P-value	WOC (n)	IC (n)
1st ring	1	0.0417	0.6498	0.397	62	128
residuals	186	11.9233				
2nd ring	1	0.00476	2.1429	0.066	38	85
residuals	119	0.264533				
Whole	1	0.00835	5.3588	0.002**	53	126
residuals	175	0.272575				

The mean classification success of West Offshore and Icelandic Offshore were mixed. While NEF would classify the Icelandic Offshore otoliths better (Table 9), the DWT provided better results for the West Offshore (Table 8).

Age effect

A significant difference between the West Inshore and Icelandic Offshore was detected for both age groups, regardless of using DWT (Table 16 &Table **17**) or NEF (Table 18Table 19). In classification success, the DWT and NEF provided both varying and close to identical results. The NEF gave the best mean classification success for both age groups (Table 15). The DWT gave a lower mean classification success for 1-3 years old otoliths while giving almost identical mean classification success for 4-7 years old as the NEF (Table 14).

Table 14. Mean classification success in the two-population comparison with age separation in groups of 1-3 (top) and 4-7 (bottom) year old using DWT. X marks the exclusion of population from the specific analysis.

1-3 years old	WIC	WOC	IC	Error
Whole	X	55.71%	65.94%	36.65%
Whole	62.65%	X	59.43%	38.96%
Whole	58.06%	41.18%	X	46.22%
4-7 years old	WIC	WOC	IC	Error
Whole	X	39.69%	60.03%	46.56%
Whole	76.94%	X	76.37%	23.41%
Whole	50.26%	46.04%	X	51.59%

Table 15. Mean classification success in the two-population comparison with age separation in groups of 1-3 (top) and 4-7 (bottom) year old using NEF. X marks the exclusion of population from the specific analysis.

1-3 years old	WIC	WOC	IC	Error
Whole	X	56.37%	74.53%	30.07%
Whole	81.02%	X	76.42%	21.28%
Whole	72.81%	36.83%	X	36.31%
4-7 years old	WIC	WOC	IC	Error
Whole	X	39.39%	63.17%	44.54%
Whole	75.16%	X	76.20%	24.20%
Whole	76.18%	56.53%	X	32.42%

For the comparison between West Inshore and West Offshore, only the age group of 4-7 years old individuals showed a significant difference both for DWT and the NEF (Table 17 &Table **19**). The NEF gave the best mean classification success when using 4-7 years old individuals (Table 15), however for 1-3 years old individuals the DWT gave the best classification success for West Offshore (Table 14), while NEF gave a better mean success for West Inshore (Table 15).

Table 16. ANOVA test for differences in shape of the populations based on 1-3 years old individuals using DWT. X marks the exclusion of population in the specific analysis.

Shape	Df	Var	F	P-value	WIC (n)	WOC (n)	IC (n)
Whole	1	1.0338	6.1785	001 ***	54	X	53
residuals	104	17.4009					
Whole	1	0.1927	0.813	0.583	54	18	X
residuals	69	16.3523					
Whole	1	0.4139	1.5229	0.137	X	18	53
residuals	69	18.7538					

Table 17. ANOVA test for differences in shape of the populations based on 4-7 years old individuals using DWT. X marks the exclusion of population in the specific analysis.

Shape	Df	Var	F	P-value	WIC (n)	WOC (n)	IC (n)
Whole	1	1.182	2.9989	0.06 **	45	X	73
residuals	116	45.708					
Whole	1	1.3156	4.0204	0.002 **	45	35	X
residuals	78	25.5246					
Whole	1	0.632	1.53	0.1	X	35	73
residuals	106	43.799					

With the West Offshore and Icelandic Offshore otoliths, only the age group of 1-3 years old individuals combined with applying the NEF showed a significant difference between populations (Table 18). The NEF provided a little better classification success than the DWT, mostly for the Icelandic Offshore regardless of age, however the West Offshore classification success was almost identical between the two analysis methods for both age groups (Table 14 &Table 15).

Table 18. ANOVA test for differences in shape of the populations based on 1-3 years old individuals using NEF. X marks the exclusion of population in the specific analysis.

Shape	Df	Var	F	P-value	WIC (n)	WOC (n)	IC (n)
Whole	1	0.006798	8.7176	0.001***	54	X	53
residuals	104	0.081099					
Whole	1	0.001018	1.4781	0.164	54	18	X
residuals	69	0.047536					
Whole	1	0.004098	3.4332	0.008 **	X	18	53
residuals	69	0.082371					

Table 19. ANOVA test for differences in shape of the populations based on 4-7 years old individuals using NEF. X marks the exclusion of population in the specific analysis.

Shape	Df	Var	F	P-value	WIC (n)	WOC (n)	IC (n)
Whole	1	0.011257	5.4691	0.001***	45	X	73
residuals	116	0.238761					
Whole	1	0.00922	5.1615	0.002 **	45	35	X
residuals	78	0.13933					
Whole	1	0.003827	1.7221	0.109	X	35	73
residuals	106	0.235578					

Genotype effect

For both the DWT and NEF, no significant difference was detected between the two populations (Table 20 & Table 21). Both methods had a very high p-value on the different shapes (p>0.5), though the NEF produced a lower value for the whole shape (p<0.3).

Table 20. ANOVA test for differences in shape of West Inshore and West Offshore caught in Nuuk, based on DWT.

Shape	Df	Var	F	P-value	WIC (n)	WOC (n)
1st	1	0.01464	0.7746	0.538	51	17
residuals	66	1.24702				
2nd	1	1.008	0.6969	0.635	42	11
residuals	50	72.34				
Whole	1	0.2872	0.8214	0.543	53	18
residuals	69	24.1254				

Table 21. ANOVA test for differences in shape of West Inshore and West Offshore caught in Nuuk, based on NEF.

Shape	Df	Var	F	P-value	WIC (n)	WOC (n)
1st	1	0.001348	0.5707	0.673	51	17
residuals	66	0.15584				
2nd	1	0.0021	0.8189	0.52	42	11
residuals	50	0.12818				
Whole	1	0.001883	1.3233	0.222	53	18
residuals	69	0.098184				

Environment effect

Both the 1st year ring, 2nd year ring and the whole shape was used regardless of fish age. An additional analysis was done using only the whole shape of the population in separated year groups of 1-3 and 4-7 years old individuals. Neither year group showed a significant difference between the Nuuk group and the Sisimiut group regarding the whole shape (Table 24). However, when disregarding the separation of year groups, and comparing them all regardless of age, both DWT and NEF provided significant difference between Nuuk and Sisimiut on the 1st year ring, but not on the 2nd year ring or the whole shape (Table 22 & Table 23).

Table 22. ANOVA test for differences in shape based on DWT between West Inshore individuals caught in Nuuk and Sisimiut.

Shape	Df	Var	F	P-value	Nuuk (n)	Sisimiut (n)
1st	1	0.04457	3.2092	0.016 *	51	43
residuals	92	1.27771				
2nd	1	0.951	0.6572	0.68	42	29
residuals	69	99.814				
Whole	1	0.3666	1.142	0.271	53	40
residuals	90	28.891				

Table 23. ANOVA test for differences in shape based on NEF between West Inshore individuals caught in Nuuk and Sisimiut.

Shape	Df	Var	F	P-value	Nuuk (n)	Sisimiut (n)
1st	1	0.01037	3.5591	0.015 *	51	43
residuals	92	0.26807				
2nd	1	0.002431	0.9401	0.477	42	29
residuals	69	0.178456				
Whole	1	0.002457	1.8436	0.093	53	40
residuals	90	0.119921				

Table 24. ANOVA test for the difference in shape between Nuuk and Sisimiut area separated into age groups of 1-3 and 4-7 years old, with the DWT (top table) and NEF (bottom table).

Shape	Df	Var	F	P-value	Nuuk (n)	Sisimiut (n)
Whole (1-3)	1	0.1743	0.5535	0.859	27	27
residuals	51	16.0608				
Whole (4-7)	1	0.000796	0.6591	0.687	27	27
residuals	51	0.061559				
Shape	Df	Var	F	P-value	Nuuk (n)	Sisimiut (n)
Whole (1-3)	1	0.4681	1.6085	0.12	26	13
residuals	37	10.7686				
Whole (4-7)	1	0.002628	1.7058	0.131	26	13
residuals	37	0.056996				

Discussion

Possibility of population separation

Overall the best classification success rate was achieved using the Normalized Elliptic Fourier (NEF) on the whole shape. Neither 1st nor 2nd year ring performed as well as the whole shape. Mean classification success for the whole shape was >70% for the West Inshore and Icelandic Offshore populations, but with a <20% for West Offshore. Removing the West Offshore from the analysis provided a better mean classification success, close to 80% using the NEF while within 60-70% when using Discrete Wavelet Transform (DWT). Previous stock discrimination within the cod populations have achieved classification rates of >90% between two Baltic populations (Paul et al. 2013), between 60% and 80% for three populations in Norwegian waters (Stransky et al. 2008), and >70% for two populations in Scottish waters (Galley et al. 2006). However, the classification success of each shape analysis was separated, and a combination of all three shapes for population classification was therefore not performed. Combining the three shapes in one analysis may have contributed to a better classification success, than using individual shapes for population classification. Additionally, combining shape analysis with other stock/population classification methods, may provide results both robust and accurate enough for fishery management purposes.

In the comparison of the West Offshore and West Inshore populations in the Nuuk area there was found no significant difference between the two populations overall otolith shape. The obtained results in this study therefore indicates no significant effect on the otolith shape due to genetics, which is in contrast to previous study of the Atlantic cod (Cardinale et al. 2004). Environmental effect must therefore dictate a similar otolith shape between the two populations. However, the individuals used in this study were caught throughout the Nuuk fjord system, which is not abiotic homologous (Mortensen et al. 2014). Previous studies (Hussy 2008) indicate that feeding levels have a significant effect on otolith shape, and West Greenland fjord systems are used as spawning grounds by capelin. This species provides a significant food source for the cods inhabiting the fjords systems of West Greenland (Henriette Schack 2005). Similar feeding ecology for both West Inshore and West Offshore individuals in the Nuuk fjord system might therefore cause a similarity of otolith shape. However, this should be further studied.

Migration, or lack of, between the groups of the West Inshore population inhabiting the Nuuk and Sisimiut fjord systems has yet to be determined, and the groups could possibly be genetically identified as two separate populations. But assuming the two groups are one population, the comparison of otolith shape from the two fjord systems indicated that environment did not have a significant effect on the otolith shape. Both the fjord systems of Nuuk and Sisimiut is host to a spawning period of the capelin which, as previously stated, provides a significant food sources for the inhabiting cods. However, cods of the Sisimiut fjord has a higher

growth rate, possibly due to extended feeding periods compared to the cods of the Nuuk area (Henriette Schack 2005). An increase in feeding levels has been shown to affect the shape of the otolith (Cardinale et al. 2004; Gagliano and McCormick 2004; Hussy 2008), however obtained results in this study contradicts this. A possible answer to the lack of environmental effect on the otolith shape is diet rather than feeding levels (Mille et al. 2016), though this has yet to be thoroughly investigated.

Previous studies indicate a decrease in classification rates with an increase in age (Mapp et al. 2017). With this in mind, the three populations were tested in pairs and in separated age groups. No clear significant influence of age was detected. However, an interesting trend did appear within both classification rates and ANOVA test. Young West Offshore (<4 years) individuals were more prone to be misclassified when analysed with West Inshore individuals, compared to Icelandic Offshore individuals. This switched when using older otoliths (>4 years), where an increase in misclassification of West Offshore occurred when analysed with Icelandic Offshore individuals compared with West Inshore individuals. This was corroborated by the ANOVA tests. If our previous results indicate a lack of genetic influence upon the otolith shape are assumed correct, then environmental factors must be responsible for the change of shape. This could indicate a migratory nature from inshore nursery areas to offshore spawning and feeding grounds by the West Offshore populations. A difference between offshore and inshore diets might exist, as diets in offshore areas consisted mainly of crustaceans (Storr-Paulsen et al. 2006; Hedeholm et al. 2017) while diet of inshore areas consists more of capelin (Henriette Schack 2005). Additional factors such as differences in temperature and salinity may be influencing the otolith shape in inshore and offshore areas. However, the results of comparing West Inshore from Sismiut and Nuuk does not cooperate this. Considering the migratory nature of other populations of cods, the migration of West Offshore cods from inshore areas to offshore areas seems possible (Rose 1993; Bentzen and Bradbury 2016; Bonanomi et al. 2016), however such a hypothesis should be further investigated

Normalized Elliptic Fourier and Discrete Wavelet Transform

Both Discrete Wavelet Transform (DWT) and Normalized Elliptic Fourier (NEF) analysis managed to discriminate between the West Inshore and the Icelandic Offshore populations, while performed with lower classification success when distinguishing between the West Offshore population and the rest. As previously stated, DWT is good at approximating edges and local variations due to functions being in finite domains unlike NEF. This has provided promising results in the separation of two herring populations in the North Atlantic Sea (Libungan et al. 2015) with a classification success rate of >90% for both populations based on >80 otoliths for each population. Using Elliptic Fourier on the Norwegian costal and Northeast Arctic cod scored >80% success rate for the Coastal cod and >70% for the Northeast Arctic cod (Stransky et al. 2008). This indicates how both methods can obtain high classification success. Results obtained in this study on the other hand indicates that

choice of mathematical function to be used should be adjusted towards otolith shape. While the DWT was good at finding local variations in the overall shape of the otoliths and thereby indicate a significant difference between populations, it was less useful in population classification. The NEF performed as well as the DWT in finding significant differences between populations but performed better overall in classification rates than DWT. The rather round shape of the cod otolith is possibly negating the advantage of the DWT, as there's no proper sharp edges. In the case of analysing a herring otolith, which has sharper edges than the cod otolith, the DWT would probably have performed better than the NEF.

Using the 1st and 2nd year ring

Previous work of using the 1st and 2nd year increments for stock separation was done by Berg et al. (2005). However, this was using the growth ratio between the 1st and 2nd year increments and not comparing the shapes. The visual evaluation of the three populations indicates the 1st and 2nd year increments to provide the best results for population classification. The classification results obtained from 1st and 2nd year shape analysis on the other hand did not perform better overall than the use of the whole shape of the otoliths. No answer for this contradiction is clear, and should be investigated more closely in the future. The DWT seemed to be better at finding significant differences between populations in the 1st and 2nd year increments than did the NEF. This may be due to the DWT being able to find local significant variations while NEF focuses more on the global shape. The width of the otolith has been shown to be susceptible to environmental influences for the immature state of several species in the genus *Merluccius* (Lombarte et al. 2003), with a more endogenous effect on the width during the mature state. Early seasonal increments may thus be reflective of spawning and nursery environment, with later seasonal increments being influenced more by population genotype. Further studies should be done to investigate the factors effecting the shape of the seasonal increments compared to the overall shape. Furthermore, a possible alternative to manual tracking should be considered. Only manual tracking through an electronic drawing tablet was possible for isolating the seasonal increments in present study. The DWT was using erroneous variations (through human error), or the actual difference in shape is unknown.

Conclusion

Using the NEF, it was possible to classify two out of the three populations with a mean classification rate around 70%. As such, shape analysis alone, using the Normalized Elliptic Fourier or the Discrete Wavelet Transform, of the over-all otolith shape is not efficient enough for management purposes as of yet. Additionally, the shape of the 1st year and 2nd year increments did not produce a higher mean classification success rate than using the whole shape of the otolith. However, the shape of these increments should be studied further, to understand possible relations between age specific overall shape and corresponding

increments. Furthermore, the use of Discrete Wavelet Transform or Normalized Elliptic Fourier for shape analysis should be considered based on the otolith shape. Neither age of the otoliths or the genetics of the West Greenland populations influenced the overall otolith shape, but rather it seems environmental factors dictates this. This finding combined with a complex migration pattern among populations suggests that otolith shape analysis should be combined with other classification methods, before being used as a management tool for separating cod populations in Greenland waters.

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Supplementary Result sheet