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Cod reproductive ecology

Effect of dietary fatty acids on ovarian maturation, spawning time and quality of eggs and larvae

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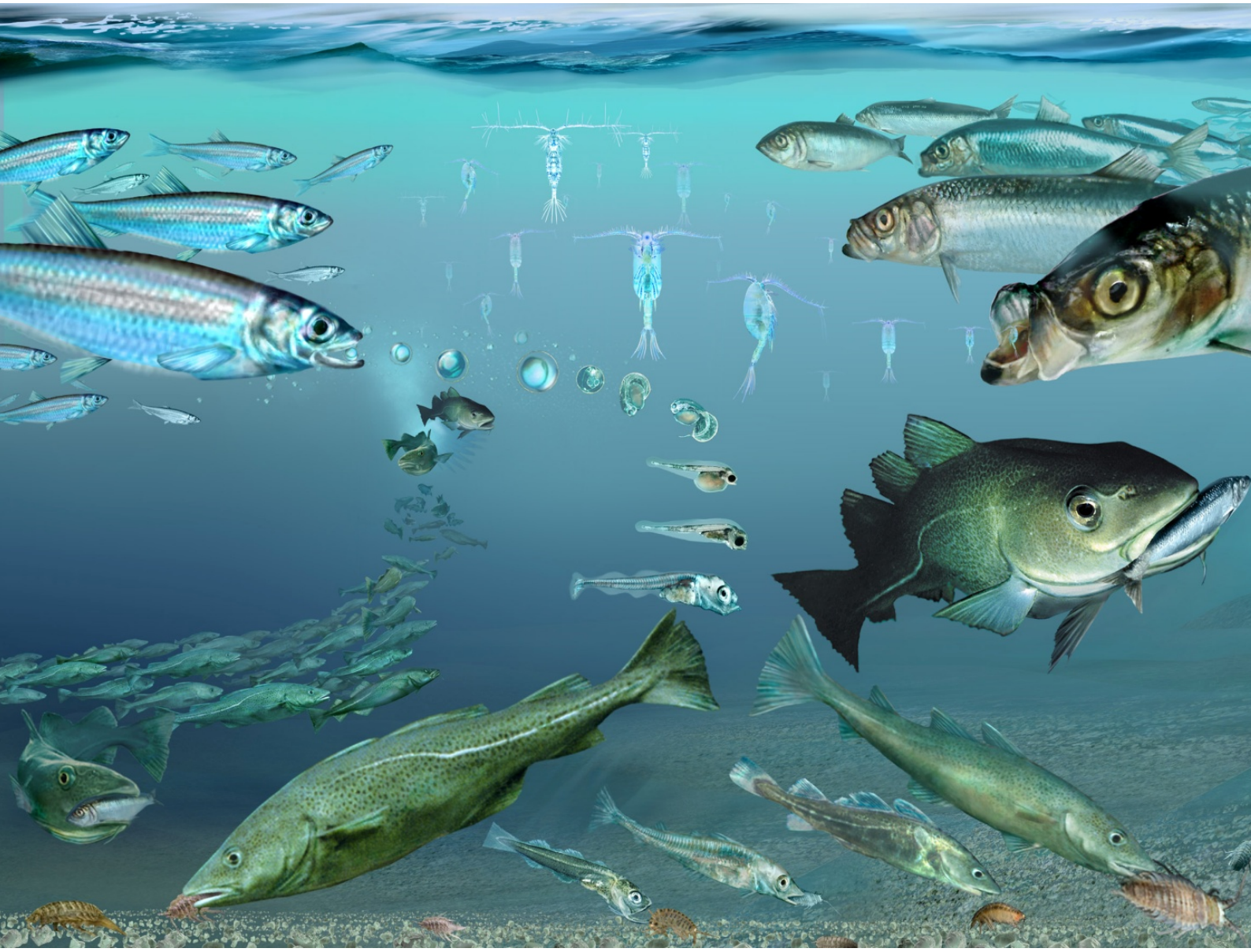
Cod Reproductive Ecology:
Effect of Dietary Fatty Acids on Ovarian Maturation, Spawning Time and Quality of Eggs and Larvae



PhD Thesis
Maria Christina Røjbek
March 2012

DTU Aqua
National Institute of Aquatic Resources

Main supervisor: Josianne Støttrup, Senior Research Scientist, DTU Aqua

Co supervisor: Jonna Tomkiewicz, Senior Research Scientist, DTU Aqua

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Front page image by Glynn Gorick for the ICES/GLOBEC project: Cod and Climate Change

PREFACE

This Ph.D. study was carried out at the National Institute of Aquatic Resources, Technical University of Denmark (DTU Aqua). This research was supported financially by the European Commission through the 6th and 7th Framework Programme projects *Understanding the Mechanisms of Stock Recovery* (UNCOVER) Grant Agreement no. 022717 and *Forage Fish Interactions* (FACTS) Grant Agreement no. 244966. The Cost Action Fish Reproduction and Fisheries, FRESH (F&A0601) supported the study financially through a Short-Term Scientific Mission (STSM) grant. I am grateful for the provision of facilities by "Bornholms Lakseklækkeri" during the entire study. Originally we planned to carry out experiments with Baltic cod at Bornholms Lakseklækkeri, but it was not possible due to the lack of funding.

Thanks to the artist Glynn Gorick and ICES/GLOBEC for the permission of using the fantastic illustration of cod life history and feeding from the ICES/GLOBEC project: Cod and Climate. Thanks to Sune Riis Sørensen for excellent help with layout and illustrations.

I am deeply thankful for the great support of my main supervisor Josianne Støttrup during the entire study. She has always been a great inspiration and helped my keeping focus and developing my writing skills. My PhD study covers a relatively broad field and I have been lucky to have three supervisors with expertise in different areas. Josianne Støttrup contributed with her broad knowledge about lipid and nutrition in aquaculture, Jonna Tomkiewicz with great knowledge in fish reproduction and Charlotte with excellent expertise in lipids. Thanks for the synergistic discussions, your encouragement and pleasant company.

I would like to thank the crews and scientific teams of *Dana* (DTU Aqua, Denmark), RV *Alkor* (IFM-GEOMAR, Germany) and *Heincke* (AWI, Germany) for helping with the collections. Especially I would like to thank Marie Storr-Paulsen, Birgitte Nielsen, Bastian Huwer, Viola Neumann, Holger Haslob, Jörn Smidt, Hans Harald Hinrichsen. Help from Inger Hornum in performing the ovarian histology and Inger Holmblad and Trang Vu in performing the lipid analysis was highly appreciated. Thanks to Henna Lu and Eiríkur Kristinsson for help with Iatroscanner. Thanks to Kirsten Birch Håkansson for retrieving DATRAS data, Morten Aabrink and Kerstin Geitner for help with GIS plots. Thanks to Anders Nielsen for excellent help with statistical models and analysis. Thanks to colleagues for inspiring conversations and interest in my work and for the

support from Viola Neumann and Stefan Neuenfeldt. Thanks to Fritz Köster for comments on one of my manuscripts.

It was a privilege working with Edward A. Trippel at St. Andrews Biological Station, Fisheries and Oceans Canada for making the experiment possible. Thanks for your excellent support and enthusiasm. Help from Steve Neil with broodstock husbandry and coating of diets, help from Steve Neil, Michael Doon, Nathaniel Feindel and Geoffry Harrison with gamete collection, Tammy Blair with developing method of lipid sampling and dry weights were highly appreciated. Thanks to Huntsman Marine Science Center for lending out their technical personal and special thanks to Janie Jones for her indispensable help and expertise during the entire experiment and for working odd hours. Thanks to the big group of fantastic people from the Biological Station that welcomed me warmly and taught me meaning “hot top”, “pot luck” and “the red herring”. Staying in Canada during winter was a great experience and you all contributed to make my stay very pleasant and I am sure I will see you all again.

Finally I would never have succeeded this PhD, without the unfailing love and support from my boyfriend Sune and my kids Asger and Mille. You encourage me to carry out my studies even when I had to be away in a long period. I love you.

LIST OF ORIGINAL PAPERS 7
SUMMARY 8
DANSK RESUMÉ 10
THESIS OBJECTIVES 12
INTRODUCTION 15
Baltic Sea ecosystem 15
Topography and hydrographic conditions 15
Biodiversity 17
Hydrographic and climatic changes 17
Environmental changes 18
Food web of Baltic cod and trophic interactions 19
Recent population dynamics in the Baltic Sea 20
Fish reproduction in Baltic Sea 22
Herring 22
Sprat 22
Cod 22
Dietary lipids and fish reproduction 24
Origin of polyunsaturated fatty acids in fish 24
The role of dietary lipid in parent fish 25
The role of PUFA for fecundity and egg quality 26
Biosynthesis of fatty acids 28
Incorporation and mobilisation of dietary fatty acids 29
Modifications of dietary fatty acids 29
Antioxidants 30
THESIS RATIONALE 32
Delay in spawning time of Baltic cod 32
Recruitment and egg and larvae quality of Baltic cod 33
Consequences of ecosystem alterations for diet of clupeids and cod 34
STUDY APPROACH 38
Field work 38
Determination of gonadal maturity stage 38
Experimental work 39

Analytical work 40
Fatty acids as trophic markers in marine ecosystems 40
Statistical analyses 41
MAIN RESULTS AND DISCUSSION 42
Lipid composition of Baltic clupeids and Saduria entomon 42
Lipid dynamics in Baltic cod 44
Trophic interactions and ecosystem changes 46
Effect of EFA on cod spawning pattern 48
Effects of dietary fatty acids on realized fecundity 50
Effects of dietary fatty acids on egg and larval quality 50
CONCLUSION AND PERSPECTIVES 53
REFERENCES CITED 55
PAPER I 76
PAPER II 107
PAPER III 140
PAPER IV 176

LIST OF ORIGINAL PAPERS

PAPER I. Lipid dynamics of herring (*Clupea harengus* L.) and sprat (*Sprattus sprattus* L.) as major prey species in the Baltic Sea

Maria C. Røjbek, Jonna Tomkiewicz, Charlotte Jacobsen & Josianne G. Støttrup

Submitted to Marine Ecology Progress Series in August 2011 and resubmitted in December 2011

PAPER II. Linking lipid dynamics with reproductive cycle in Baltic cod (*Gadus morhua* L.)

Maria C. Røjbek, Charlotte Jacobsen, Jonna Tomkiewicz & Josianne G. Støttrup

Submitted to Marine Ecology Progress Series in November 2011, accepted with minor revisions.

PAPER III. Lipid energy reserves and fatty acids in reproduction of Baltic and Atlantic cod (*Gadus morhua* L.): Imprint of trophic regime

Jonna Tomkiewicz, Maria C. Røjbek, Josianne G. Støttrup, Charlotte Jacobsen & Friedrich W. Köster

Manuscript

PAPER IV. Effects of dietary fatty acids on production and quality of eggs and larvae of Atlantic cod (*Gadus morhua* L.)

Maria C. Røjbek, Josianne G. Støttrup, Charlotte Jacobsen, Jonna Tomkiewicz & Edward A. Trippel

Manuscript

SUMMARY

In recent decades, Baltic cod has experienced a period of low recruitment. In the same period the pelagic Baltic Sea ecosystem experienced a regime shift, due to hydrographic changes, affecting all trophic levels. The rationale for the thesis is built on the hypothesis that the regime shift has resulted in decreased dietary value of clupeids in terms of lipid content and essential fatty acids (EFA) which originates from phytoplankton and is transferred up through the food web. Clupeids are main prey for cod in the Central Baltic Sea and a decreased dietary value is hypothesised to affect cod reproduction.

The overall objective of the thesis is to investigate the role of lipids in reproduction of cod (*Gadus morhua*) in the Central Baltic Sea. The first objective is to examine the seasonal variation in content of lipid and EFA in whole prey species of cod (Paper I). The second objective is to investigate the variation in lipid content, EFA and antioxidants of female Baltic cod gonads and livers during the reproductive cycle (Paper II) and to examine whether there is a deficiency in lipid energy and dietary EFA that could explain the delayed spawning time observed in the Baltic cod (Paper III). The third objective is to investigate experimentally if EFA levels, comparable to those observed in Baltic clupeids, delayed timing of spawning and influenced egg production and quality in cod (Paper IV). The study combines field data of cod and its main prey species; sprat (*Sprattus sprattus*), herring (*Clupea harengus*) and the isopod *Saduria entomon* from the Baltic Sea sampled during 2002-2004 and 2008-2009 in different seasons and experimental data of farmed broodstock cod. Lipid composition of whole prey species and ovaries and livers of cod in different maturity stages were analyzed and lipid composition in samples of mature cod was compared with samples of North Sea cod with no delay in spawning time. A feeding experiment was carried out to test the dietary effect of different levels of the essential polyunsaturated n-6 arachidonic acid (ARA) and n-3 eicosapentaenoic acid (EPA) on spawning period, realized fecundity, and egg and larval quality under controlled conditions.

Lipid content and fatty acid composition (FAC) differs significantly between sprat, herring and *S. entomon* (Paper I). Sprat has in general high lipid content and proportion of the monounsaturated fatty acid, oleic acid, compared to herring, which in contrast, has high proportion of the polyunsaturated fatty acid, docosahexaenoic acid (DHA). This suggests that sprat feeds more on the copepods *Pseudocalanus* sp. than herring which mainly feeds on *Temora longicornis*. Hence, altered ratios of sprat and herring abundance available for cod may impact lipid content and FAC in cod. High proportions of ARA, EPA and the antioxidant, astaxanthin, in *S. Entomon*, compared to

clupeids, render it a valuable constituent in the diet of Baltic cod. However, the abundance of *S. Entomon* has declined in recent decades in the Baltic. FAC of ovary and liver varies with maturity stage in Baltic cod (Paper II and III). Low content of ARA in sprat and herring in spring and summer is reflected in cod ovaries and coincide with the timing of the maturation period of cod in the Central Baltic Sea (Paper I and II). Fatty acids trophic markers indicates that dinoflagellates dominated during the years examined which may have resulted in a reduction of ARA level in both sprat and herring compared to periods with domination of diatoms (Paper I).

No limitation in lipid energy is evident in Baltic cod but a deficiency in ARA is indicated (Paper II). Selective retention of ARA in ovaries during ovarian maturation is evident (Paper II) but despite mobilization of ARA from liver, the level is not sufficient to keep up with the requirement in ovaries and ARA decreases in late maturation and during spawning. The antioxidants α-tocopherol and astaxanthin accumulates in cod ovaries compared to prey and decreases in late maturation and spawning due to antioxidant protection activity (Paper II). ARA level, important for eicosanoid activity, is lower in ovaries of Baltic Sea cod than in North Sea cod (Paper III), indicating that this fatty acid may be important for spawning time. However the spawning period is not influenced by different ARA levels and EPA/ARA ratios in farmed cod (Paper IV). Uptake of DHA, EPA and ARA into cod eggs from broodstock diet is highly efficient (Paper IV). Diet with low EPA/ARA ratio has significantly higher realized fecundity and eggs from fish fed a diet with high ARA level has higher fertilization success and survival to 8 days post hatch compared to fish fed low level of ARA. The combined results strongly indicate that the low ARA levels in Baltic cod ovaries, reflecting ARA levels in prey, result in low fertilization success and survival of eggs and larvae.

This PhD provides novel information about lipid dynamics in Baltic sprat and herring which is important because these species occupy a central position in the Baltic ecosystem. The results contribute to a better understanding of the lipid requirements and fatty acid mobilization during maturation in cod but do not explain the delayed spawning time in Baltic cod. The improved knowledge of the effect of dietary EFA on realized fecundity and egg and larval quality in cod is of great importance for estimating recruitment but also to cod farming because egg quality in cultured cod is one of the limiting factors for successful mass production of fish fry. EFA proved a useful tool as trophic markers in the Baltic Sea ecosystem and the results indicate that EFA may also be useful as ecosystem state indicators.

DANSK RESUMÉ

I de seneste årtier har Østersøtorsk haft en periode med lav rekruttering. I den samme periode har det pelagiske økosystem i Østersøen oplevet et regime skift som følge af nogle hydrografiske ændringer, der har påvirket alle trofiske niveauer. Rationalet for afhandlingen bygger på hypotesen, at dette regime skift, har resulteret i en reduceret næringsværdi i clupeider, med hensyn til fedtindhold og essentielle fedtsyre, der dannes i alger og transporteres op igennem fødekæden. Clupeider er den primære fødekilde for torsk i den central Østersø og dette fald i næringsværdi, kan have påvirket torskens reproduktion.

Det overordnede formål med denne Ph.d. er, at undersøge lipiders rolle i reproduktion hos torsk (*Gadus morhua*) fra den central Østersø. Første delmål er, at undersøge om sæsonvariationen in fedtindhold og essentielle fedtsyre i hele byttedyr af torsk (Artikel I). Det andet delmål er, at undersøge om torskens ovarie og lever ændrer indholdet af fedt, essentielle fedtsyre og antioxidanter gennem den reproduktive cyklus (Artikel II), samt hvorvidt mangel på fedt og essentielle fedtsyre kan forklare det observerede forsinkede gydetidspunkt observeret hos torsk i Østersøen (Artikel III). Det tredje delmål er, at undersøge om et niveauet af essentielle fedtsyre, tilsvarende det i clupeider fra Østersøen, forsinker gydning og påvirker produktion og kvalitet af afkom i torsk (Artikel IV). Afhandlingen kombinerer feltdata af torsk og dens primære byttedyr; brisling (*Sprattus sprattus*), sild (*Clupea harengus*) og østersøkrebs (*Saduria entomon*) fra Østersøen indsamlet i 2002-2004 og 2008-2009 på forskellige årstider samt eksperimentelle forsøg på opdrættede moderfisk af torsk. Lipidsammensætningen i hele byttedyr og i ovarier og lever af torsk med forskellige modenhedsstadier er analyseret og lipidsammensætningen i de gydemodne torsk er sammenlignet med prøver af vildfisk fra Nordsøen, der ikke udviser en forsinket gydeperiode. Foderforsøg på opdrættede moderfisk er udført for at undersøge gydetidspunkt, realiseret fekunditet og æg- og larvekvalitet ved forskellige foderniveauer af den essentielle flerumættede n-6 fedtsyre, Arichidonsyre (ARA), og n-3 fedtsyren, Icosapentaensyre (EPA).

Fedtindholdet og fedtsyresammensætningen er signifikant forskellig mellem brisling, sild og østersøkrebs (Artikel I). I forhold til sild har brisling generelt et højere fedtindhold og indhold af den enkeltumættede fedtsyre, Oliesyre. Sild har til gengæld et højt indhold af den flerumættede fedtsyre, Docosahexaensyre (DHA). Dette indikerer at vandloppen *Pseudocalanus* sp. udgør en større andel af føden i brisling end i sild, der i stedet synes at leve af *Temora longicornis.* Derfor kan ændrede forhold i forekomsten af sild og brisling i Østersøen påvirke fedtindholdet og fedtsyresammensætningen i torsk. Østersøkrebs har et højt indhold af ARA og EPA i forhold til

clupeider og den er derfor et vigtigt fødeemne for Østersøtorsk. Men tætheden af Østersøkrebs Østersøen er faldet markant i de seneste årtier. Fedtsyresammensætningen i ovarie og lever varierer med modenhedsstadie i Østersøtorsk (Artikel II og III). Et lavt indhold af ARA i sild og brisling om foråret og sommeren, er reflekteret i torskens ovarier og er tidsmæssigt sammenfaldende med modning af torskens ovarier i den central Østersø (Artikel I og II). Trofiske fedtsyre markører indikerer, at dinoflagellater dominerede i undersøgelsesperioden, hvilket kan have resulteret i et fald i ARA-niveauet i både sild og brisling, i forhold til perioder domineret af diatomeer (Artikel I).

Der var ingen tegn på, at Østersøtorsken er energibegrænset, men der er indikationer på ARA-mangel (Artikel II). En selektiv retention af ARA i ovarierne er tydelig (Artikel II), men på trods af mobilisering af ARA fra leveren, er indholdet ikke nok til at dække behovet i ovarierne. Som følge heraf, ses et faldende niveau af ARA i den sene modning og under gydningen. Antioxidanterne, α-tocopherol og astaxanthin, ophobes i torskens ovarier, i forhold til niveauet i byttedyrene, og niveauet falder i den sene modning og under gydningen på grund af antioxidanternes beskyttende effekt (Artikel II). ARA niveauet, der er vigtig for aktiviteten af eicosanoider, er lavere i ovarierne hos østersøtorsk end i nordsøtorsk (Artikel III) og indikerer, at denne fedtsyre er vigtig for gydetidspunktet. Gydetidspunktet påvirkes dog ikke af forskellige ARA-niveauer og EPA/ARA ratioer i opdrætstorsk (Artikel IV). Optag af DHA, EPA og ARA i torskens æg fra moderfiskenes foder er meget effektiv (Artikel IV). Foderet med lav EPA/ARA ratio har signifikant størst realiseret fekunditet og æggene fra fisk, fodret med et højt ARA-indhold har en højere befrugtningssucces og overlevelse indtil dag 8 efter klækning sammenlignet med fisk fodret med lavere ARA-indhold. Samlet set peger resultaterne på, at de lave ARA-niveauer i ovarierne af østersøtorsk, der reflekterer ARA niveauet i byttedyrene, resulterer i en lav befrugtningssucces og overlevelse af æg og larver.

Denne afhandling giver ny viden om lipiddynamikken i Østersøens sild og brisling, der er vigtig på grund af deres centrale placering i Østersøens. Resultaterne bidrager til en bedre forståelse af lipidbehovet og fedtsyremobilisering i modningsperioden hos torsk, men forklarer ikke det forsinkede gydetidspunkt i østersøtorsk. Den øgede forståelse for effekterne af de essentielle fedtsyrer kan bidrage til en forbedret estimering af rekruttering i bestandsanalyser, men er også gavnlig for torskeopdræt, hvor optimering af ægkvalitet er vigtig, for at opnå succesfuld masseproduktion. Essentielle fedtsyre synes, at være et brugbart redskab som trofiske markører i Østersøen økosystem og resultaterne indikere at EFA også kan være anvendelige som økosystems tilstandsindikator.

THESIS OBJECTIVES

The overall objective of the thesis is to investigate fatty acid dynamics in cod (*Gadus morhua*) from the Central Baltic Sea in relation to dietary lipids and maturation, the identification of potential essential fatty acid (EFA) deficiencies and possible impacts on spawning time, egg production and the quality of egg and larvae. The aim is to gain a better understanding of lipid requirements in cod during maturation and to combine knowledge from aquaculture and population ecology to create interdisciplinary knowledge which can be used for ecosystem orientated stock management of Eastern Baltic cod. A better understanding of the underlying reasons for the observed prolongation of maturation process in the Baltic would improve decision making in management of this Baltic cod. The improved knowledge of the effect of dietary EFA on realized fecundity and egg and larval quality in cod is of great importance for estimating recruitment but also to cod farming because egg quality in cultured cod is one of the limiting factors for successful mass production of fish fry (Kjørsvik et al. 1990, Penney et al. 2006).

OBJECTIVE 1

The first objective is to examine the seasonal variation in content of lipid and EFA in different sizes of whole sprat and herring and test whether the seasonal variation is linked to gonadal maturation in sprat and herring of different sex and size (Paper I). The major prey species of Baltic cod (Fig. 1); sprat *(Sprattus sprattus*) and herring (*Clupea harengus*) are believed to be affected by changes in the lower trophic levels due to changes in hydrographical conditions in the recent decades. These effects are expected to be expressed in altered fatty acid composition (FAC) as EFA originates from phytoplankton and is transferred up through the food web. This may affect clupeid condition and recruitment as well the nutritional quality for Baltic top-predators, mainly cod. The crustacean S*aduria entomon* (Fig. 1) is also investigated as it is at times an important alternative prey species for cod. We furthermore investigate whether the lipid content in sprat and herring is lower today than in the past decades by comparing with previous studies.

OBJECTIVE 2

The second objective is to investigate the variation in lipid content and EFA of female Baltic cod gonads and livers and to examine whether deficiencies in lipid energy and dietary EFA resulted in a

prolongation of the ripening period causing the delayed spawning time (Paper II and III). We investigate the variation in lipid content as well as proportions and absolute values of EFA of female Baltic cod gonads and livers during a complete reproductive cycle to test if ARA is critically low in the period of ovarian maturation (Paper II). Variation in levels of α-tocopherol and astaxanthin in gonads with ovarian maturation is investigated to see if there was sign of antioxidant protection deficiencies. It is examined whether the lipid composition of cod liver reflected its major prey; sprat and herring and if it was possible to detect fatty acid "finger prints" of *Saduria entomon* (Paper II). The spawning season has shifted in the Baltic Sea but not in the North Sea and low levels of PUFA in the Baltic Sea could indicate that the shift in peak spawning time of Baltic is related to diet. We therefore examine whether the lipid content and FAC of cod ovaries and liver differ between mature female cod originating from either the Baltic Sea or the North Sea (Paper III).

OBJECTIVE 3

Objective 3 is to investigate if the content of ARA and EPA/ARA ratio level observed in Baltic clupeids is critical for fecundity and egg and larval quality in the Baltic Sea. We investigate the effect of different ratios of ARA and EPA in broodstock diet on realized fecundity and egg and larval quality of individual females in farmed broodstock cod (Paper IV). We examine whether long-term low dietary ARA and/or high EPA/ARA ratio, identical to Baltic clupeids, delay the spawning time in Atlantic cod compared to high ARA level and low EPA/ARA ratio. Even though no delay in spawning time is observed in the Atlantic cod stock, it is believed that the same processes control maturation in both cod stocks.

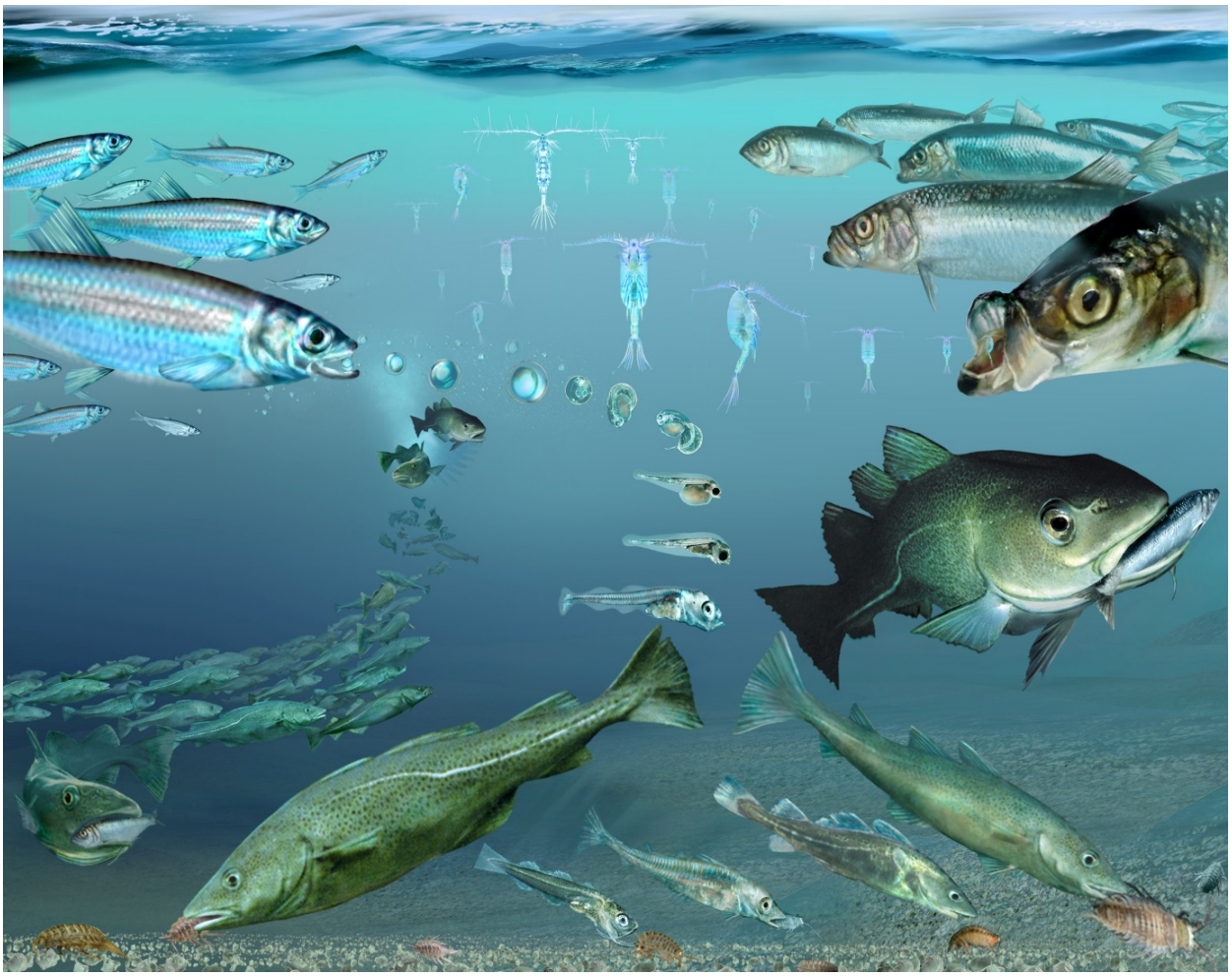


Fig. 1. Illustration showing main groups examined in this PhD thesis: cod (*Gadus morh*ua) and their main prey items planktivorous sprat (*Sprattus sprattus*), herring (*Clupea harengus*) and the bentic crustacean *Saduria entomon* as well as eggs and larvae of cod. Image by Glynn Gorick for the ICES/GLOBEC project: Cod and Climate Change.

INTRODUCTION

BALTIC SEA ECOSYSTEM

Topography and hydrographic conditions

The Baltic Sea is a landlocked intracontinental sea and one of the largest brackish seas in the world (Lass & Matthäus 2008). It was covered by ice sheets during the ice ages and filled with water in the warmer periods and has in the course of history undergone several different freshwater and brackish water phases (Leppäranta & Myrberg 2009). The transition period ended approximately 7500 years ago and for the last 2000 years the water body has been the present Baltic Sea. The Baltic Sea is a semi-enclosed sea composed of a series of connected deep basins separated by shallow silts (Stigebrandt 2001, Leppäranta & Myrberg 2009). It is a shallow sea with a mean depth of 54 meter and the greatest depth is 459 meter in the Gotland area (Fig. 2). The connection between the North Sea and Baltic Sea is through the shallow Kattegat, with an average depth of ~20 m, and through the narrow and shallow Danish Straits (Winsor et al. 2001). This reduces the exchange of water with the ocean and horizontal and vertical salinity gradients exist as a result of the large freshwater supply from rivers and net precipitation (Meier 2007). The surface salinity of the Baltic Sea increases from ~3‰ in the northernmost part of the Gulf of Bothnia to just below 10‰ inside the Danish Straits to 18-26‰ in Kattegat (Leppäranta & Myrberg 2009) with a overall mean salinity on 7.7‰ (Winsor et al. 2003). A permanent halocline separates the upper layer of fresh water from the dense saline bottom water. In the Bornholm Basin the upper layer is 50-60 meter deep with an average salinity of 7.5‰ and 17‰ in the bottom layer below the halocline (Meier 2007, Eilola et al. 2011). The halocline prevents vertical mixing of the water column and transport of more oxygenated waters to the bottom (Conley et al. 2009). The seasonal cycle of average sea surface temperature (SST) varies from a minimum in February and March around 1°C to a maximum in August around 17°C with a temperature difference of 3-8°C between the northern and southern parts of the Baltic Sea (Siegel et al. 2008). During spring, a vertical thermocline develops at 25-30 m depth separating the warm upper layer from the cold intermediate water (Lass & Matthäus 2008). This thermocline restricts additionally vertical exchange within the upper layer until late autumn.

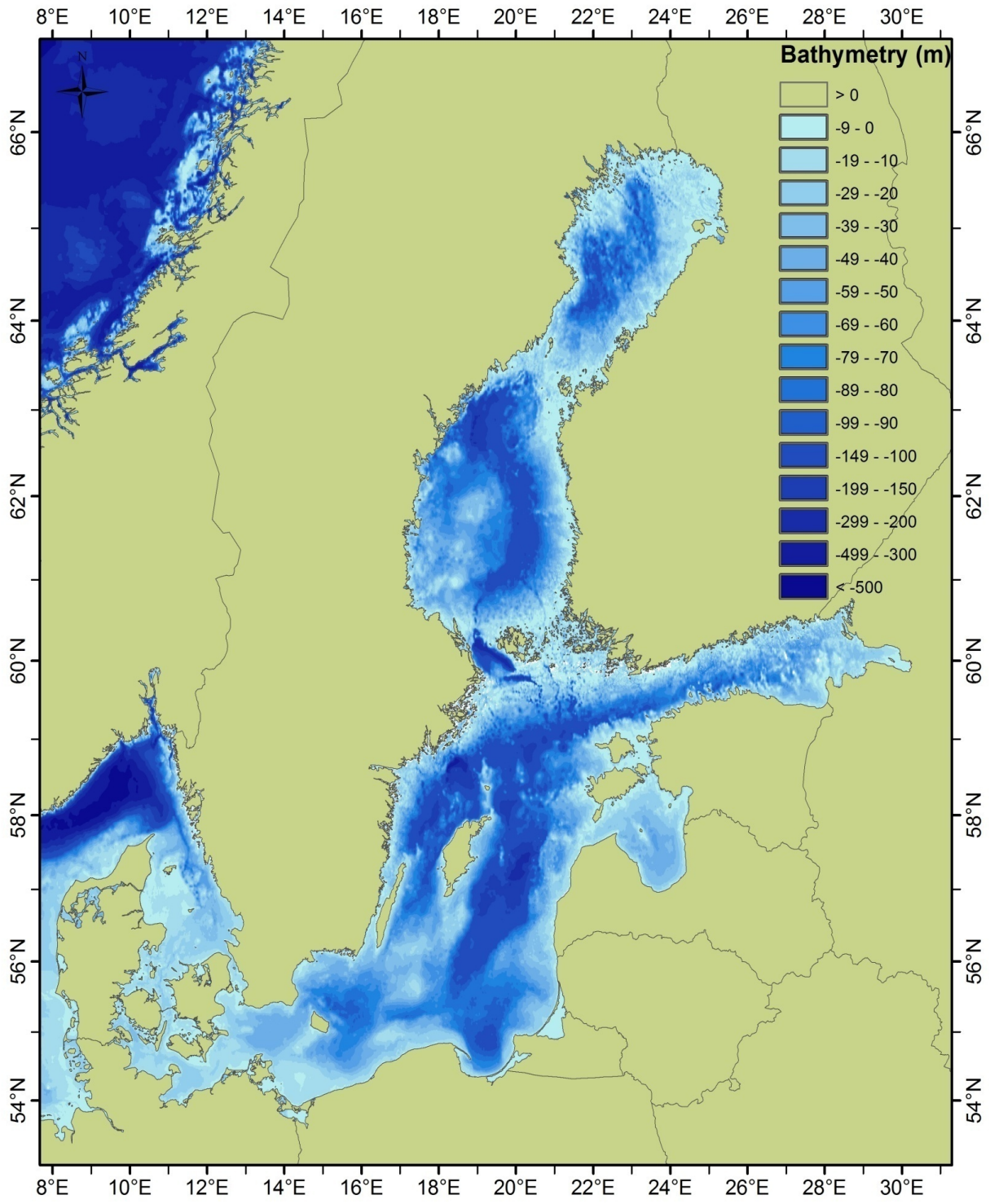


Fig. 2. Map and bottom topograhpy of the Baltic Sea. The connection between the North Sea and Baltic Sea is through the shallow Kattegat, with an average depth of ~20 m, and through the narrow and shallow Danish Straits. This reduces the exchange of water with the ocean and as a consequence horizontal and vertical salinity gradients exist as a result of the large freshwater supply from rivers and net precipitation.

Biodiversity

The Baltic Sea supports fewer species than fully marine areas due to the low salinity that stresses both marine species immigrating from the North Sea and freshwater species immigrating from freshwater and the species coping with the brackish water of the Baltic Sea have various origins and environmental tolerances (Ojaveer et al. 2010). The species richness is also low because the Baltic Sea is geologically young and species had relatively little time to invade and adapt to its environment (Hammer et al. 2008). The Baltic Sea, incl. Kattegat, hosts altogether at least 6,065 species, including at least 1,700 phytoplankton, 442 phytobenthos, 1,199 zooplankton, 569 meiozoobenthos, 1,476 macrozoobenthos 380 vertebrate parasitesand about 200 fish, 4 marine mammals and 83 bird species (Ojaveer et al. 2010). The species richness decreases from over 773 marine species in the open Skagerrak to 187-254 species in the Belt Sea, approximately 127 at the Bornholm Basin (ICES sub division 25), to around 29 marine species in the Bothnian Bay. On the other hand, limnic species increase diversity from 1 species in Skagerrak to 18 at the Bornholm Basin to 161 in the Gulf of Finland.

Hydrographic and climatic changes

The inflows of saline water across the sills in the Danish Straits are in part forced by regional winds and are, therefore, quite variable (Gustafsson & Stigebrandt 2007). In the period from 1897 to 1976 (excluding the two world wars) a total of 90 major inflow events, mainly between October and February, have been identified at the Dars Sill (Matthäus & Franck 1992). From the 1980s to 1990s there were exceptionally long stagnation periods (Fig. 3), during which, precipitation, runoff, and westerly winds were stronger, and salt transports into the Baltic were smaller than normal (Meier & Kauker 2003). The only major inflows was in 1993 and subsequently there has been one major inflow in 2003 (Meier et al. 2004, Matthäus et al. 2008). Due to the reduced frequency of large inflows during the 1980s, the deep water ventilation decreased and salinity decreased in all sub basins (Meier et al. 2006, Meier 2007). During low saline phases on a decadal time scale the deep water is poorly ventilated, however even during highly saline phases reduced deep water ventilation can occur due to increased stratification causing oxygen depletion (Conley et al. 2002, Meier 2005). Small and medium-strength inflow events are also important for ventilating intermediate layers of the Baltic proper halocline (Meier et al. 2006). This includes warm summer inflows which were rare events in the last 50 years, but their frequency has increased since 1990 likely due to climate change (Feistel et al. 2004, Mohrholz et al. 2006) as annual temperature minimum of the

intermediate water at Bornholm Deep has increased by about 1.5°C (Meier et al. 2006). During the period 1871–2004 there were significant positive trends in the annual mean temperature for the Baltic Sea Basin, being 0.10˚C/decade on average in the northern part and 0.07˚C/decade in the southern part (The BACC Author Team 2008). These trends are larger than the trend for the entire globe, which amounts to 0.05˚C/decade (1861–2000). Combined results of projected future warming in the Baltic Sea Basin show a warming of the mean annual temperature by some 3 to 5˚C for the total basin in the late 21st century (The BACC Author Team 2008).

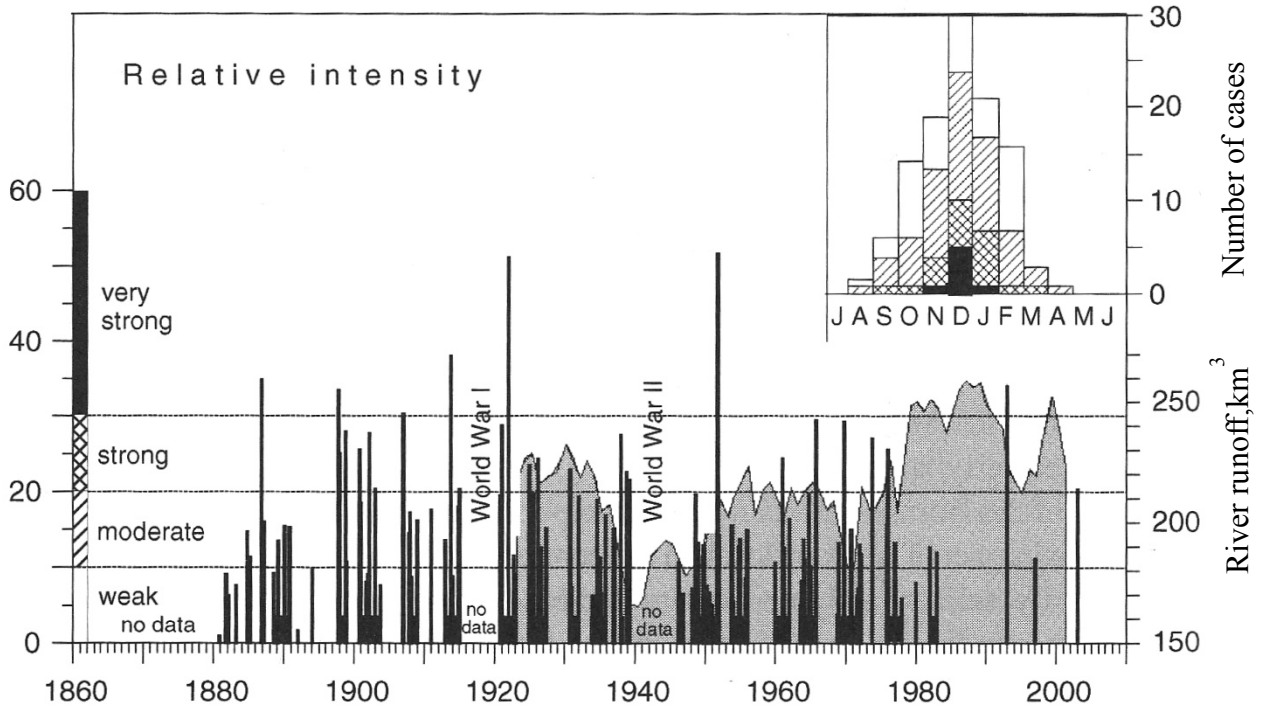


Fig. 3. Major Baltic inflows between 1880 and 2007 and their seasonal distribution (upper right) shown in terms of their relative intensity. And 5-year running means of river runoff to the Baltic Sea (inside the entrance sills) averaged from September to March (shaded). From Matthäus, W. et al. 2008.

Environmental changes

The deep parts of the Baltic Sea have experienced intermittent hypoxia during most of the Holocene and regular laminations started to form c. 8500–7800 cal. yr BP ago, in association with the formation of a permanent halocline (Zillen et al. 2008). Large areas of the Baltic Sea were hypoxic around AD 1900, which coincides with the beginning of the industrial revolution at AD1850 in Northwestern Europe. In the last century oxygen concentrations decreased in the deep waters of the Baltic Proper while hydrogen sulfide increased (Leppäranta & Myrberg 2009, Hansson & Gustafsson 2011) During the last century nutrient loads have increased by ca. 2.5 for nitrogen and

3.7 times for phosphorus and the area of hypoxia has increased more than 10-fold during the last century (Savchuk et al. 2008) and over the time period 1970–2000 average hypoxic water area covered 41,000 km^2 (Conley et al. 2009).

Food web of Baltic cod and trophic interactions

The fish community in the central Baltic Sea is dominated by three closely interlinked species: cod (*Gadus morh*ua L.), sprat (*Sprattus sprattus* L.) and herring (*Clupea harengus*) which constitute about 95% of the commercial catch of fish in weight in the Baltic (Sparholt 1994). Cod is the top piscivore predator in the Baltic Sea ecosystem as the biomass of other piscivorous fish; salmon, trout, pike, pikeperch and perch is low compared to cod. Cod is a generalist carnivorous species which feeds on a variety of fish, crustaceans, polychaetes and bivalves. The prey composition in cod stomachs depends on prey density, age and size of cod as well as geography, and season (Schulz 1987, Dos Santos & Falk-Petersen 1989, Daan et al. 1990, Hop et al. 1992, Dalpadado & Bogstad 2004). Due to the low biodiversity in the central Baltic Sea food choice for aquatic animals is limited. Three prey species; sprat, herring and the crustacean *Saduria entomon* constitute the main bulk of stomach content in adult cod in the Eastern Baltic Sea (Bagge et al. 1994, Uzars 1994, Neuenfeldt & Beyer 2003). The proportion of sprat and herring consumed by cod depends on the demography of cod and age structure and spatial distribution of prey stock. Cod prey on all age classes of sprat but predation mortality in herring is only high in the young age classes of herring (Bagge et al. 1994) so the age structure of clupeids is also important for the diet composition of cod. The proportion of herring in cod stomachs increases with size of cod (Neuenfeldt & Beyer 2003). Seals prey on cod (Lundström et al. 2010) and historically seal predation had a substantial impact on cod biomass (Eero et al. 2011). However the predation level today has much lower impact on cod recovery, compared to the effects of exploitation and salinity (Mackenzie et al. 2011). Furthermore cod cannibalism may reduce initial number of juveniles between age 0 and age 2 year class, particular at high juvenile abundance and large adult stock size (Neuenfeldt & Koster 2000). Cod larvae of Baltic cod preys primarily on copepods (Zuzarte et al. 1996).

Small herring and all sizes of sprat are strictly zooplantivorous (Möllmann et al. 2004, Casini et al. 2004). The preferred prey of Baltic sprat and herring are the calanoid copepods; *Pseudocalanus* sp., *Temora longicornis* and *Acartia* spp (Möllmann et al. 2004). In addition, sprat preys on cladocerans and larger herring favour mysids, amphipods and polychaetes in autumn and winter (Casini et al. 2004). A substantial predation on cod eggs by both herring and sprat also occurs (Köster &

Möllmann 2000) as well as egg cannibalism in sprat (Köster & Mollmann 2000). Zooplankton biomass is highest in spring and summer in the south eastern Baltic Sea (Aleksandrov et al. 2009). This is simultaneous with peaks of phytoplankton and protist production in the Baltic Proper (Wasmund & Siegel 2008). The phytoplankton composition is characterized by a spring bloom in April with a succession from diatoms to dinoflagellates followed by a summer bloom of nitrogen-fixing cyanobacteria and an autumn bloom dominated by diatoms (Wasmund & Siegel 2008). *S. entomon* is a benthic crustacean and in the Northern Baltic its diet has been found to consist mainly of deposit-feeding amphipods *Monoporeia affinis* (Haahtela 1990b, Englund et al. 2008).

Recent population dynamics in the Baltic Sea

The Baltic Sea has recently experienced shifting regimes (Köster et al. 2003b, Möllmann et al. 2008, Casini et al. 2008, Möllmann et al. 2009, Casini et al. 2010, Casini et al. 2011). Climate induced changes in the phyto- and zooplankton assemblage have been observed (Möllmann et al. 2000, Wasmund & Uhlig 2003). Due to reduced salinity the abundance of *Pseudocalanus* sp. reduced substantially (Möllmann et al. 2000, Möllmann et al. 2005, Aleksandrov et al. 2009) while the abundance of *Temora longicornis* and *Acartia* spp. increased due to increased sea temperature (Möllmann et al. 2005). Phytoplankton composition varied in the Baltic Sea between 1979 and 2005 due to an increase in temperature and decrease in salinity and inorganic nitrogen concentrations (Wasmund & Uhlig 2003, Wasmund et al. 2011). The main trend observed was a significant reduction in the share of diatoms in the spring bloom, which is most likely, related to the mild winters that commenced in 1988. During mild winters, surface water temperature does not fall below the temperature at which the water has its highest density and the water column remains stratified preventing deep mixing (Wasmund & Siegel 2008). Diatoms need mixed water for resuspension, whereas flagellates take advantage of stabilized waters (Harrison et al. 1986). Since 1999 the photoautotrophic ciliate *Mesodinium rubrum* has been a dominant species during spring (Wasmund & Siegel 2008). This may also be due to mild winters because an acceleration of ciliate growth and higher biomass maxima at elevated temperatures were observed in response to winter warming in mesocosm studies (Aberle et al. 2007).

The abundance and spawning stock biomass of Baltic cod decreased dramatically in the 1980´s (Fig. 4) caused by a combination of recruitment failure and increasing fishing pressure (Köster et al. 2001b, Köster et al. 2003b). The changed inflow regime apparently lead to less favourable spawning conditions for the adults and survival of the eggs (Hammer et al. 2008). The collapse of

Baltic cod caused a release of predation pressure and a drastic increase of sprat abundance (Möllmann et al. 2003, Köster et al. 2003b, Möllmann et al. 2008, Casini et al. 2011). A positive correlation between sprat recruitment and the average water column temperature in spring has been observed which may be due to the reduced adverse effects of winter cooling on egg and larval development (Mackenzie & Köster 2004, Baumann et al. 2006). Furthermore, sprat larvae prey mainly on *Acartia* spp. (Voss et al. 2003, Dickmann et al. 2007) and they are most likely favoured by the increased standing stock of this copepod. The recruitment of herring has declined concurrently with the decreasing growth and condition of the Baltic herring stocks, suggesting that the low condition have adverse effects on egg survival (Hammer et al. 2008). Eutrophication of coastal areas, resulting in degradation of algal communities, has furthermore caused the disappearance of herring spawning beds (Hammer et al. 2008).

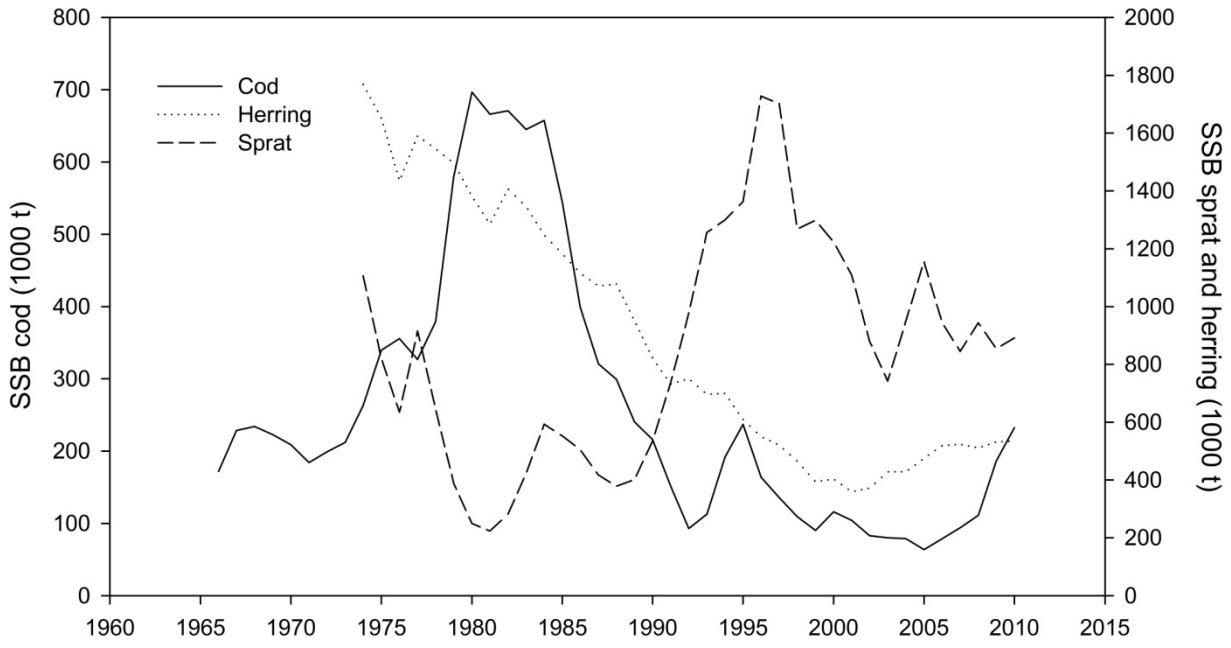


Fig. 4. Time series of spawning stock biomass (SSB) Baltic cod (full line, subdivision 25-32), herring (dotted line, subdivision 25-29, 32) and sprat (broken line, subdivision 22-32). High SSB of Baltic cod around 6-700.000 t in 1979-1985 was followed by a decrease and in the period 1990-2010 SSB varied form 64.000-237.000 t. Data from ICES Advice 2011, Book 8.

FISH REPRODUCTION IN BALTIC SEA

Herring

Herring migrate between feeding areas and spawning grounds every year. In the central Baltic Sea the main feeding grounds are around Bornholm and in the Gdansk Basin and the spawning grounds are at the coastline around the feeding grounds (Aro 1989). Herring is a total spawner which means that all eggs are spawned during one spawning event (Murua & Saborido-Rey 2003) and eggs are deposited on algal covered sea beds and in shallow littoral waters (Aneer 1989, Rajasilta et al. 1993). The coastal spring spawning herring (March-May) dominates, but open sea spring spawning herring (April-June) and coastal autumn spawning herring (August-November) are also present (Aro 1989). Individual maturation cycles of herring vary in the Baltic Sea and timing of spawning is primarily determined by the feeding conditions prior to spawning (Rajasilta 1992a).

Sprat

The main spawning grounds of sprat in the central Baltic Sea are the Bornholm Basin, the Gdansk Deep and the Gotland Basin (Aro 1989, Köster et al. 2003a). Baltic sprat is a serial batch spawner with pelagic eggs (Wahl & Alheit 1988a, Alheit 1988b) and the bulk of eggs are laid above the 80-100 m depth zone (Ojaveer & Kalejs 2010). Sprat has a protracted spawning season from February to August (Ojaveer & Kalejs 2010) with peak spawning in May-June in the Bornholm Basin (Kraus & Köster 2001, Kraus & Köster 2004). Sprat batch fecundity is positively related to fish length and weight and mean winter temperature but fish condition is not related to batch fecundity (Haslob et al. 2011). Development and survival of sprat eggs are depending on ambient water temperature (Petereit et al. 2008).

Cod

Baltic cod show adaptations to the brackish water conditions in the Baltic Sea. The salinity gradient also affects intraspecific biodiversity in cod due to local genetic populations adapted to spatial differences in environmental conditions (Ojaveer et al. 2010). Two distinct stocks of cod exist in the Baltic Sea; a western stock that extends to the southernmost part of the Kattegat and to the west of Bornholm (longitude 14°30'E) and an Eastern Baltic cod stock that extends from Bornholm to about 63°N (Bagge et al. 1994). One adaption to the brackish water is an increase in egg size and higher water content which results from increased water uptake during final oocyte maturation due

to higher intracellular contents of FAA, Cl^- and $NH4^+$ (Thorsen et al. 1996). Egg diameter of Baltic cod is between 1.4 and 1.8mm (Vallin & Nissling 2000) compared to 1.1 - 1.5mm of Atlantic cod (Kjesbu 1989, Trippel 1998). As a consequence Baltic cod eggs are more buoyant than those of other cod stocks and they attain neutral buoyancy within and below the halocline in the deep basins (Nissling & Vallin 1996, Nissling & Westin 1997).

The hydrographic conditions in this depth range fluctuate depending on the water exchange with the North Sea. Viable hatch requires a minimum oxygen level of 2 ml^{-1} (Wieland et al. 1994) and the minimum salinity that allows cod eggs to float is 11‰ (Bagge et al. 1994). Hence, the volume suitable for cod reproduction (reproductive volume) is the water volume with salinity not less than 11‰ and oxygen content not less than 2 ml/l, (Mackenzie et al. 2000). This condition is not always met in periods with rare inflows of high saline and oxygenated water. The three main spawning areas for the Eastern Baltic cod stock are the Bornholm Basin, the Gotland Basin and The Gdansk Basin (Fig. 5) and a secondary spawning site in the Slupsk Furrow (Bagge et al. 1994).

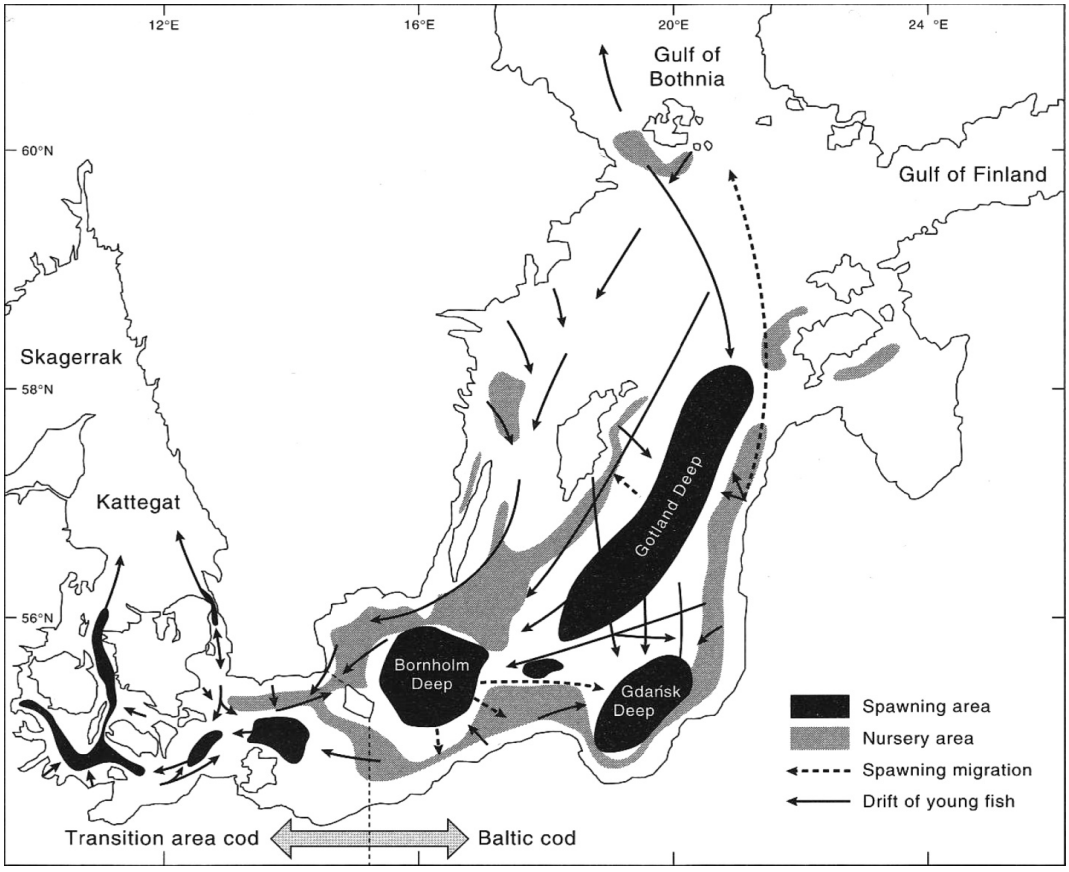


Fig. 5. Map of the Baltic Sea showing the stock boundaries of the western and the eastern stock (dotted line) and spawning area of Baltic cod (black areas), nursery grounds (grey areas), spawning migration (dotted arrows) and juvenile drift (black arrows). Historically the three basins Bornholm Deep, Gdansk Deep and Gotland Deep comprised the main spawning areas of the eastern stock but today only Bornholm Deep has condition enable egg and larval survival. From Bagge, O. et al 1994.

However since the beginning of the 1980s cod egg abundance has been very low in Gdansk and Gotland Basins (Baranova 1995, Karasiova & Voss 2004, Karasiova 2011). Cod exhibit determinate fecundity and a multiple batch spawning strategy where selected oocytes successively undergo final maturation during the process of batch development (Tomkiewicz et al. 2003). The cod spawning can be extensive. Kjesbu et al. (1989) found in an experimental study that captive North Sea cod spawned up to 17 – 19 batches over a period of 50 – 60 days. Cod are in general very fecund and females can shed up to $4.8*10^6$ eggs or 1.6 l egg kg^{-1} bodyweight during a spawning season (Kjesbu 1989). However, 99.9% of the eggs do not survive in nature (Wieland et al. 2000a).

DIETARY LIPIDS AND FISH REPRODUCTION

Origin of polyunsaturated fatty acids in fish

In the marine ecosystem, polyunsaturated fatty acids (PUFAs) originate from the primary producers, phytoplankton, and are transmitted through the food web. Despite large variations in individual fatty acids within the different taxonomic classes common features of marine algae species can still be recognized (Ackman et al. 1968). One example is that diatoms in general have high levels of 16:1n-7 and dinoflagellates in general have high levels of 16:0 and the 16:1n-7/16:0 proved to be a valid food web tracer of diatom/dinoflagellate ratio in larval North Sea cod (St John & Lund 1996).

Seasonal patterns of phytoplankton are pronounced in temperate ecosystems and temporal in the mean concentration of individual FA in general peaks during the spring bloom (Kattner et al. 1983). In diatoms PUFA increased when in active growth under nutrient rich conditions, and declined when nutrients were exhausted simulating the situation at the end of a diatom bloom (Diekmann et al. 2009). The majority of small zooplankton species from temperate regions are omnivorous and their feeding behavior is tightly coupled to food availability and hence the seasonal changes in FAC generally follow the temporal dynamics in phytoplankton dominance (Kattner & Krause 1989a, Cotonnec et al. 2001). Copepods have the ability to modify FAC of phytoplankton diet due to de novo biosynthesis of long chain MUFA (Sargent & Henderson 1986, Graeve et al. 2005) and some species are cable of storing lipid (Lee et al. 2006). However, in species such as *Acartia tonsa* with limited storage capacity no differences is found in FAC between females, their eggs and prey (Acheampong et al. 2011).

The role of dietary lipid in parent fish

Lipids, and specifically fatty acids, are the preferred source of metabolic energy for growth, swimming and reproduction (Sargent et al. 2002, Tocher 2003). Links between capelin availability and cod diet, growth, condition, and reproductive potential have been observed in Newfoundland (Rose & O'Driscoll 2002). Potential fecundity is found to be positively correlated with the availability of prey during vitellogenesis in Arcto-Norwegian cod (Kjesbu et al. 1998) and starvation significantly decreased fecundity in farmed Atlantic cod (Karlsen et al. 1995). Liver is the primary storage site of lipid energy in cod (Kjesbu et al. 1991a) and energy deposition is closely related to dietary lipid content (Karlsen et al. 2006). A positive association between recruitment and liver weights of spawners and a highly significant linear relationship between total egg production and total lipid energy was observed in the Barents Sea cod stock (Marshall et al. 1999). These studies strongly indicate that recruitment is constrained by the amount of lipid energy stored in the liver. Furthermore, fecundity and total egg dry weight was higher in female cod in good condition compared to females of poor condition (Lambert & Dutil 2000). Lipids are incorporated into oocytes yolk from dietary sources or from reserves that are stored prior to vitellogenesis and subsequently mobilized and transferred to the ovary (Wiegand 1996). Vitellogenin is a high molecular weight lipoglycophosphoprotein synthesized by maternal hepatocytes after induction by estradiol (Mommsen & Korsgaard 2008).

Fish reproduction is not only influenced by the quantity of dietary lipids but also the lipid quality i.e. fatty acid composition (FAC). Long chain poly-unsaturated fatty acids (PUFA) have been identified as major dietary factors that determine successful reproduction.

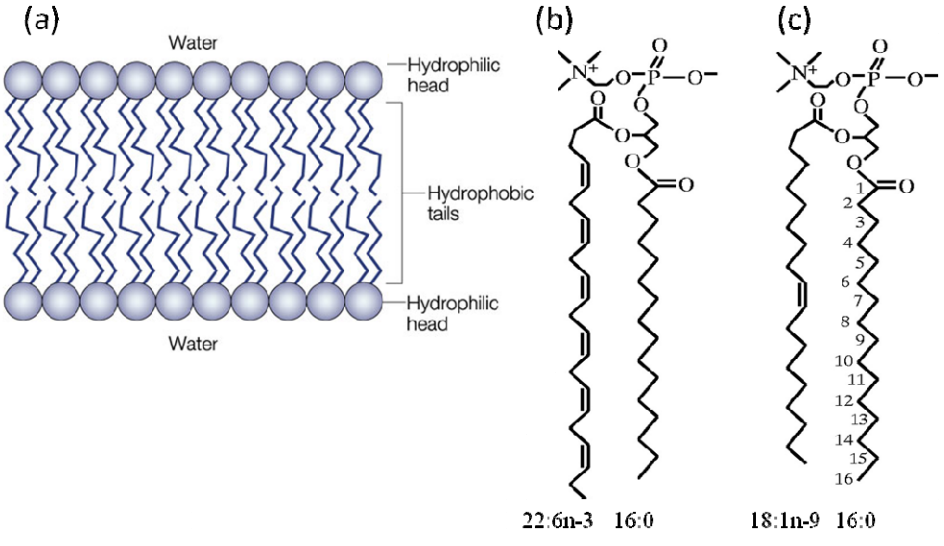


Fig. 6. Diagrammatic representation of a biological membrane bilayer (a) and the molecular structure of two representative phospholipids: a polyunsaturated (b) and a monounsaturated phosphatidylcholine (c). (a) from Pietzsch. J. 2005 and (b, c) modified from Hulbert A.J., J Exp Biol 2003.

Especially the fatty

acids docosahexaenoic acid (22:6n-3 = DHA), eicosapentaenoic acid (20:5n-3 = EPA) and arachidonic acid (20:4n-6 = ARA) are important for maturation and egg quality (Sargent et al. 1999b, Tocher 2003). The proportion of PUFA is higher in phospholipids (PL) than in triacylglycerols (TAG) in marine fish species (Tocher & Sargent 1984a, Sargent et al. 2002) due to the general role of PUFA in PL of membrane bilayers to help maintain the structural and functional integrity of cells (Fig. 6).

EPA and ARA furthermore have a more specific role as precursors of eicosanoids e.g. prostaglandins (Bell et al. 1986, Sargent et al. 1995, Tocher 2003), which have a wide range of physiological actions including oocyte maturation and ovulation in fish (Stacey & Goetz 1982, Mustafa & Srivastava 1989a, Goetz & Garczynski 1997, Sorbera et al. 2001, Lister & Van der Kraak 2008, Planas J.V. & Swanson 2008). The sex hormone 17β-estradiol (E2) is another important player in fish reproduction by inducing hepatic Vtg synthesis (Babin et al. 2007) and regulating oocyte maturation and ovulation (Planas J.V. & Swanson 2008). Eicosanoids produced from ARA (i.e. 2-series prostaglandins, 4-series leukotrienes) are generally more active than those produced from EPA (i.e. 3-series prostaglandins, 5-series leukotrienes); however EPA competitively interferes with eicosanoid production from ARA and competes for the same membrane receptors (Sargent et al. 1999a, Sargent et al. 1999b, Sargent et al. 2002). Thus eicosanoid action in marine fish is determined by the ratio of EPA/ARA in cellular membranes this in turn being determined by the dietary intake of PUFA (Sargent et al. 1999b, Tocher 2003). Lipid in TAG is on the other hand dominated by saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) which are primarily metabolized to produce energy for the various physiological processes (Sargent et al. 2002).

The role of PUFA for fecundity and egg quality

Stored oil must support not only the immediate energy requirements of the parent fish but also synthesis of new cellular lipid for reproduction and the future requirements of the progeny. Egg quality is a major factor determining the degree of hatching success and the subsequent survival rate of fish larvae (Kjørsvik et al. 1990) and much effort has been put into research of enhancing broodstock feed. Compositions of egg lipid are quite different from those of the body oils consistent with selective catabolism of certain fatty acids and/or selective transfer of other fatty acids to the eggs. The proportion of PL and PUFA is high in eggs and larvae of marine fish (Tocher & Sargent

1984a, Fraser et al. 1988a, Rainuzzo et al. 1992). Eggs from marine fish usually have a DHA/EPA ratio of around 2:1 and an (*n*-3)/(*n*-6) PUFA ratio between 5:1 and 10:1 (Sargent 1995).

Increased dietary n-3 PUFA was correlated with egg quality in several fish species (Fernández-Palacios et al. 1995, Navas et al. 1997, Furuita et al. 2000, Watanabe & Vassallo-Agius 2003, Zakeri et al. 2011). DHA is especially high in vertebrate neural tissue i.e. eyes and brain (Tocher & Harvie 1988, Bell & Dick 1991, Parrish 2009) and dietary DHA was found to be important in terms of survival and eye development in halibut larvae (Shields et al. 1999) and visual performance in juvenile herring (Bell et al. 1995). Fecundity decreased with dietary n-3 deficiency in sea bass (Cerda et al. 1995) and yellowfin sea bream (Zakeri et al. 2011) but not in cod (Lie et al. 1993).

Proportions of ARA are generally higher in eggs of wild fish than in farmed fish and egg quality is often found to be higher in wild compared to farmed broodstock (Pickova et al. 1999, Salze et al. 2005, Pickova et al. 2007). ARA supplementation has been shown to have a profound effect on spawning performance in several species either directly or by changing the ratios of ARA to other fatty acids (Fernández-Palacios et al. 2011). Percentage of viable eggs was improved in sea bass (*Dicentrarchus labrax*) when fed trash fish with high ARA content compared to dry pellets with four times lower ARA level (Bell et al. 1997). An enhanced reproductive performance has also been reported in Atlantic halibut (*Hippoglossus hippoglossus*) in terms of higher fertilization rates, blastomere morphology scores and hatching rates with supplemented ARA (Mazorra et al. 2003). Furthermore, in Japanese flounder (*Paralichthys olivaceus*) supplemental ARA improved total egg production, percentage of buoyant eggs, hatching success, larval survival and normality of larvae was higher when the proportion of dietary ARA was increased from 0.6 to 3.6% of total fatty acids (Furuita et al. 2003).

Studies on cod, however, have shown inconsistent results on the effects of different ARA levels on egg quality. Fecundity in cod (mean number per batch of fertilized eggs per kg female) has been improved with short-term ARA supplementation but there was no correlation with supplementation period (Sawanboonchun 2009). In a large scale study on the effect of ARA supplementation (0.5, 1, 2 or 4% of total fatty acids) realized fecundity was high with 1% long-term ARA supplementation but low with 4% (Norberg et al. 2008). In some studies on cod, low level of dietary ARA and high EPA/ARA ratio was correlated with poor egg quality (Pickova et al. 1999, Salze et al. 2005, Pickova et al. 2007) and low larval hatching success (Pickova et al. 1997), while in other studies it was not (Penney et al. 2006, Sawanboonchun 2009).

Biosynthesis of fatty acids

The saturated fatty acids 16:0 and 18:0 can be biosynthesized de novo by all known organisms, including fish, by the conventional pathway catalyzed by the cytosolic fatty acid synthetase (Sargent et al. 1989). Likewise, all organisms are capable of desaturating 18:0 by the microsomal fatty acid Δ9 desaturase, to yield 18:1n-9 (Fig. 7). But all vertebrates lack the Δ12 and Δ15 desaturase and so cannot form 18:2n-6 and 18:3n-3, respectively, from 18:1n-9 (Bell & Tocher 2009). However 18:2n-6 and 18:3n-3, can with varying efficiencies be further desaturated and elongated in vertebrates to form C20/22 PUFA and the extent varies with species and is associated with their capacity for fatty acyl desaturation and elongation (Tocher 2003).

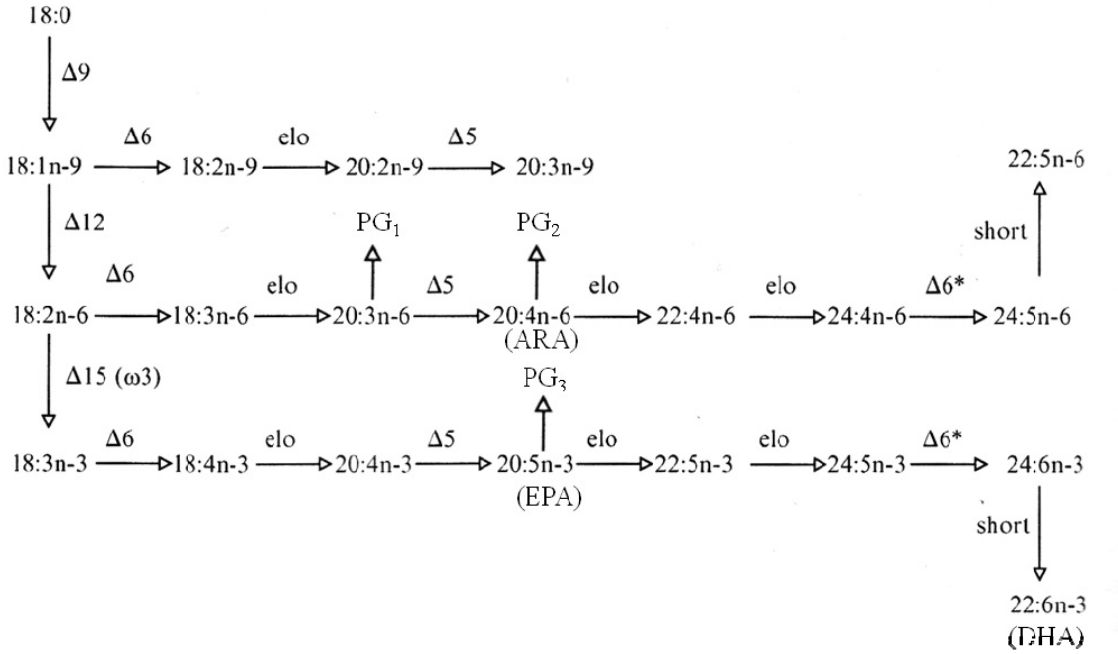


Fig. 7. Pathways of biosynthesis of C20 and C22 highly unsaturated fatty acids from n-3, n-6 and n-9 C18 precursors. Δ5, Δ6, Δ6*, Δ9, Δ12, Δ15 (ω3) = fatty acyl desaturases; elo = fatty acyl elongases, short = chain shortening, PG = Prostaglandin, ARA = arachidonic acid, EPA = eicosapentaenoic acid and DHA = docosahexaenoic acid. Modified from Bell and Tocher, 2009.

The conversion of 18:3n-3 to EPA and DHA, by Δ5 and Δ6 desaturase and fatty acid elongases, is well established for many freshwater species of fish (Sargent et al. 1995) but occurs poorly, if at all, in those species of marine fish studied so far (Kanazawa et al. 1979, Yamada et al. 1980, Sargent et al. 1995, Sargent et al. 2002, Bell & Tocher 2009). The fresh water species Eurasian perch has the ability to biosynthesis DHA and EPA from 18:3n-3 but not ARA from 18:2n-6 (Henrotte et al. 2011a). Functional characterisation of cod desaturase cDNAs by heterlogous expression in the yeast *Saccharomyces cerevisiae*, which lacks the ability to synthesis PUFA, shows enzyme activity of Δ6 but no activity of Δ5 and Δ4 (Tocher et al. 2006) and very little or no elongase activity (Agaba et al.

2005). However the expression of Δ6 and PUFA biosynthesis activity measured in cod hepatocytes and enterocytes is very low, irrespective of diet (Tocher et al. 2006). As a consequence levels of DHA, EPA and ARA in cod are determined by the dietary intake and highly significant relationships have been found between feed oil and liver oil contents of 18:2n-6, 18:3n-3, EPA and DHA (Jobling & Leknes 2010).

Incorporation and mobilisation of dietary fatty acids

Dietary lipids are emulsified by bilesalts and hydrolysed by pancreatic lipases in the gut to form FFA, glycerols (from TAG) and lysophosholipids (from PL) which are absorbed into intestinal epithelial cells where they are re-esterfied into TAG and PL (Sheridan 1988, Sheridan 1994, Tocher 2003). The lipids are transported in the blood and delivered to lipid storage organs by lipoproteins and primarily stored as TAG in most species of fish. Dietary lipids are primarily stored in livers in cod from where they can be mobilized and transferred to other tissues (Wiegand 1996).

The metabolism of lipids and FA in fish is strongly linked to physiological and behavioral traits such as size, age, sex, state of maturity and spawning as well as biotic and abiotic factors such as food abundance, water temperature etc. (Dalsgaard et al. 2003). Starvation has often evolved as part of the reproductive cycle of fish. In Atlantic cod feeding activity ceased in females on average 36 days before the onset of spawning and feeding was suppressed by both sexes during the first three-quarters of females on average 42 days spawning period (Fordham & Trippel 1999). Starvation is accompanied by an increased mobilization of energy stores and mobilized FA are transported to the liver where they are oxidized via microsomal beta-oxidation, to provide energy (Henderson & Sargent 1985, Sargent et al. 1989). TAG is mobilized either simultaneously or after carbohydrates but usually before proteins and always before PL.

Modifications of dietary fatty acids

Modification of dietary FA prior to incorporation occurs in some fish but as mentioned above the desaturation and elongation activity in marine fish is very limited. Modifications of FAC in marine fish compared to its prey can, however, occur due to differentiated digestibility and selective retention and or catabolism of specific FA. Digestibility of different fatty acid increases with unsaturation (Sigurgisladottir et al. 1992, Koven et al. 1994b, Olsen et al. 2004) and hence the proportion of PUFA may be higher in a predator than in its prey. Selective catabolism of short chain SFA has been observed in sandeel where a slower uptake of ^{13}C labelled 16:0 than 18:3n-3

indicated a high metabolic turnover of SFA and very limited metabolism of PUFA (Dalsgaard & St John 2004). Similar a lower incorporation of 16:0 compared to 18:1n-9 was observed in rainbow trout storage lipid (NL) but not in PL and incorporation varied among tissues (Weber et al. 2003). In Atlantic capelin and herring a selective catabolism of MUFA for egg production was observed (Henderson et al. 1984, Henderson & Almatar 1989). Large amount of 20:1n-9 and 22:1n-11 was stored in TAG of the parent fish but only present in roe only very small amounts indicating a selective catabolism of these MUFA.

ANTIOXIDANTS

Marine fish are in general rich in PUFA. However, due to their polyunsaturated nature PUFA are highly susceptible to attack by free radicals followed by reaction with oxygen which results in peroxidation. The ease of peroxidation is proportional to the number of double bonds present (Sargent et al. 2002). Damage to PUFA in membrane phospholipids due to peroxidation can have serious consequences for cell membrane structure and function, with potential pathological effects on cells and tissues (Sargent et al. 2002). A highly efficient antioxidant protection is therefore essential. An antioxidant is a molecule which has the ability to remove free radicals from a system either by reacting with them to produce other innocuous compounds or disrupting the oxidation reactions (Britton, 1995).

Tocopherol (vitamin E) and carotenoids are both lipophilic antioxidants (Higuera-Ciapara et al. 2006) with a protective antioxidative role in fish (Syvaoja et al. 1985a, Britton 1995, Bell et al. 2000). Tocopherols and are not synthesized by animals and must be obtained in the diet, ultimately from higher plant and algal sources (Hess 1993). a-tocopherol (Fig. 8a) is identified as the major naturally occurring tocopherol in the lipids of marine fish (Ackman and Cormier 1978; Parazo et al. 1998) and astaxanthin (Fig. 8b) is the most abundant carotenoid in cod (Miki et al. 1982a, Grung et al. 1993a). A few algal species can produce astaxanthin, but in aquatic systems it is mainly synthesized by crustaceans from carotenoid precursors, especially b-carotene and zeaxanthin, provided by algae (Matsuno 2001). Astaxanthin is transported to the eggs with lipids in vitellogenin (Tyler & Sumpter 1996). In cod the uptake of astaxanthin into eggs from the broodstock diet was highly efficient (Sawanboonchun et al. 2008). Astaxanthin may have important functions related to reproduction: acceleration of sexual maturity, increasing fertilization and egg survival, and a better embryo development (Putnam 1992). However, the reports on the effect of carotenoid concentration and egg quality has been contradictory (Izquierdo et al. 2001). Nonetheless, in more recent studies it

was shown that a supplement of dietary astaxanthin increased egg quality in yellowtail (Watanabe & Vassallo-Agius 2003) and resulted in 47% more fertilized eggs per batch per kg female compared to the control groups in cod (Sawanboonchun et al. 2008).

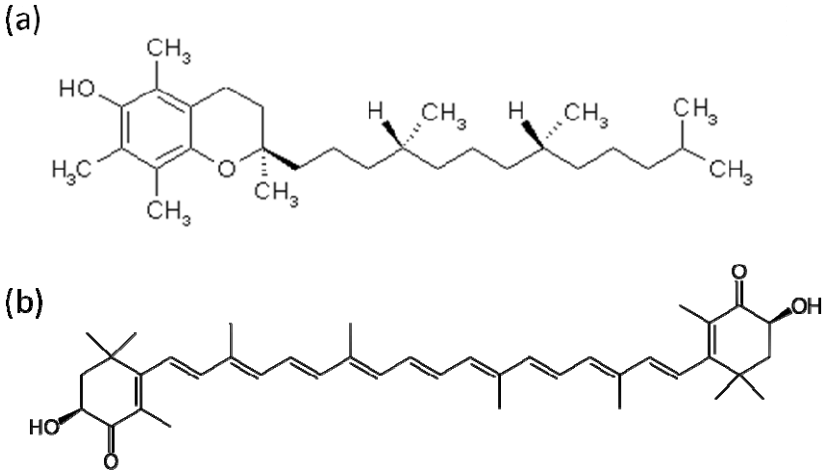


Fig. 8 Molecular formula of alpha-tocopherol (a) and astaxanthin (b). From www.wikipedia.org

THESIS RATIONALE

The rationale for the thesis was the hypothesis that recent changes in the food web of Baltic cod have decreased clupeid condition and resulted in a reduction in quantity and quality of lipid in clupeid prey. This may have affected the reproduction of Baltic cod, their main predator by prolonging the maturation period and reducing egg and larval quality.

Delay in spawning time of Baltic cod

Western Baltic cod stock have peak spawning in February to April (Bleil & Oeberst 1997). A remarkable change in the peak spawning time of cod in the central Baltic Sea (ICES SD 25-32) was observed in the 1990s and peak egg abundance changed from April-June to end of July (Wieland et al. 2000b, Kraus et al. 2002, Karasiova 2006, Karasiova et al. 2008) and has been observed as late as in end of august (Voss et al. 2011). Peak in cod egg abundance in July and August has also been observed in 1903 to 1938 in the Bornholm Basin (Kändler 1949). However these observations are subject to uncertainty as they were not carried out systematically but it indicates that spawning time could have had several historical alterations.

The later spawning has consequences for several processes influencing survival of early life stages. Oxygen content below the halocline decreases from spring to summer and hence egg mortality due to oxygen deficiency is assumed to be higher in years with late spawning and furthermore life cycle of important prey may be out of phase with food requirements of late hatching larvae (MacKenzie et al. 1996, Støttrup et al. 2008a). Experimental results for Atlantic cod report a delay in spawning time by 8-10 days with a decrease in temperature of 1°C (Kjesbu 1994). Atlantic cod kept at low temperatures (5°C) showed a delay in spawning of one month compared to cod kept at ambient temperatures (Kjesbu et al. 2010).

Seasonal peak egg abundance in Baltic cod was found to be correlated with ambient water temperature during gonadal maturation (Wieland et al. 2000b). This was only true at low levels of cod spawning biomass (Wieland et al. 2000b) and in the period succeeding this study from 1995-2005 spawning stock biomass was low and temperature relatively stable (Anon 2007). However, date of peak egg abundance still varied considerably (Voss, R, pers. comm.). So the temperature alone cannot explain the shift in spawning time. Timing of spawning of female cod from 1947 to 1992 in three regions off Newfoundland, Canada varied significantly among years and the effect of spawning time depend on regional hydrography (Hutchings & Myers 1994). Big females of Atlantic

cod are observed to start and end spawning later than smaller females but also spawn for a longer period (Hutchings & Myers 1993). Other variables such as age structure of the cod spawning stock and sprat biomass did not seem to affect timing of spawning significantly (Wieland et al. 2000b).
There are indications that spawning time might be diet related in cod as the proportion of ripe cod in the Gotland Basin in January during 1972 to 1995 decreased with feeding condition and spawning time was delayed one month in moderate feeding conditions and in years with large stock size and poor feeding conditions spawning time was both delayed and prolonged (Baranova 1995). In Sea bass a diet related delay in spawning season has also been observed (Cerda et al. 1994). Females fed a reduced feed ration had a delayed spawning compared to fish on normal feed ration and had reduced 17β-estradiol (E2) plasma levels and presented vitellogenic oocytes in the ovary a month later. In a large scale study on the effect of ARA supplementation (0.5, 1, 2 or 4% of total fatty acids) high ARA levels (4%) have been found to increase 17β-estradiol (E2) plasma levels whereas low ARA levels (0.5%) delayed peak of vitellogenin in plasma one month (Norberg et al. 2008, Norberg et al. 2009). The spawning period was extended in fish fed diet with high level of ARA, however the peak of spawning was identical between groups (Rosenlund, G., pers. comm.).

Recruitment and egg and larvae quality of Baltic cod

Cod has experienced a period with low recruitment in recent decades. Numbers of recruits for a given spawning stock biomass was lower in the period from 1982 to 2008 than in the previous period from 1966 to 1981 (Fig. 9). The decline in recruitment during the 1980s was related to the climate-induced changes in the physical environment resulting in anoxic conditions in spawning and severe egg mortalities (Köster et al. 2005). The lack of recovery in the mid-1990s despite improved hydrographic conditions was related to poor larval survival. There are further indications that egg and larval quality of Baltic cod is suboptimal. A high mortality and occurrence of malformations in Baltic cod larvae has been reported (Grauman & Sukhorukova 1982, Petersen et al. 1997). In rearing experiments, hatching rate was significantly lower for eggs of Baltic cod than those of Skagerrak cod in some studies (Pickova & Larsson 1992, Larsson 1994, Pickova et al. 1997). Mortality and abnormalities was higher in Baltic cod eggs than for those of the Barents Sea cod (Åkerman et al. 1996) indicating that spawn from the Baltic is of lower quality (Norrgren et al. 1998). However in one study viable hatch did not differ significantly between the two stocks (Nissling et al. 1998). Low condition in terms of RNA/DNA ratios has been observed in 70% of

field caught cod larvae in the Baltic (Grønkjær et al. 1997) and Baltic cod larvae is found to have a considerable lower growth rate, based on RNA/DNA ratios, compared to larvae from Georges Bank in the Gulf of Maine (Huwer 2009, Huwer et al. 2011). In Pickova et al. (1997) hatching success was correlated with the proportion of ARA in egg PL and Baltic cod eggs had low proportion of ARA and high EPA/ARA ratios compared to Skagerrak cod eggs, indicating that poor egg quality may be related to lipid composition.

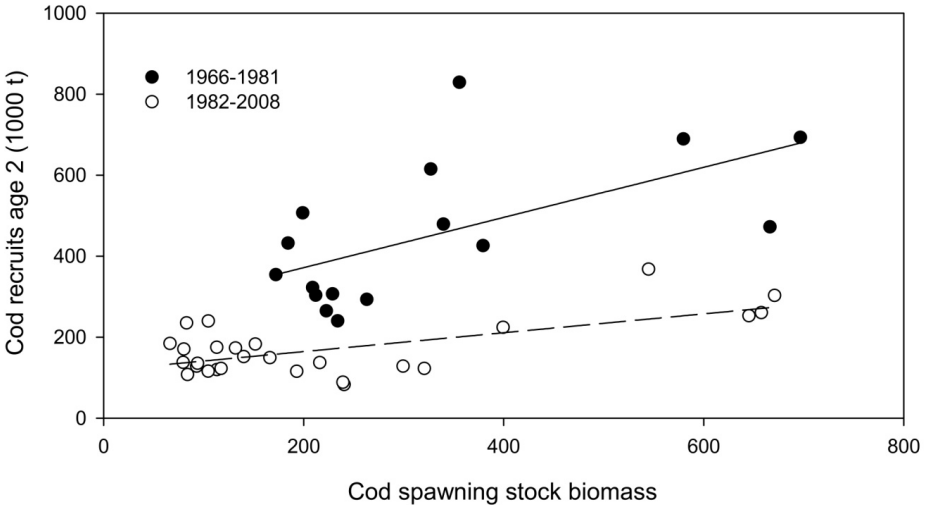


Fig. 9. Cod recruits at age 2 (backshifted to the recruitment year) in relation to SSB in the period from 1966-1981 (black circles) and the period 1982-2008 (white circles). Linear regression of the early period (full line): r^2, y = 0.62x + 247.7 and late period (broken line): $r^2 = 0.42$, y = 0.23x + 117.9. Data from ICES Advice 2011, Book 8.

Consequences of ecosystem alterations for diet of clupeids and cod

The most important food item for both sprat and herring in spring has been *Pseudocalanus* sp., but since the late 1970s the relative amount of this copepod in the diet of both sprat and herring decreased (Möllmann et al. 2003, Möllmann et al. 2004). In the 1990s *Temora longicornis* and *Acartia* spp. partly compensated for the decreased proportion of *Pseudocalanus* sp. in the diet of sprat and herring but the stomach fullness was still lower than in 1970s and 1980s (Möllmann et al. 2004). Condition has decreased in Baltic herring since the late 1980s and in Baltic sprat since the 1990s (Fig. 10) due to increased food competition caused by the changes in zooplankton composition concurrent with increased sprat abundance (Möllmann et al. 2005, Köster et al. 2005, Casini et al. 2006, Casini et al. 2010, Casini et al. 2011). Furthermore the proportion of sprat in relation to herring in diet of Baltic cod has most likely increased in the period with high sprat abundance.

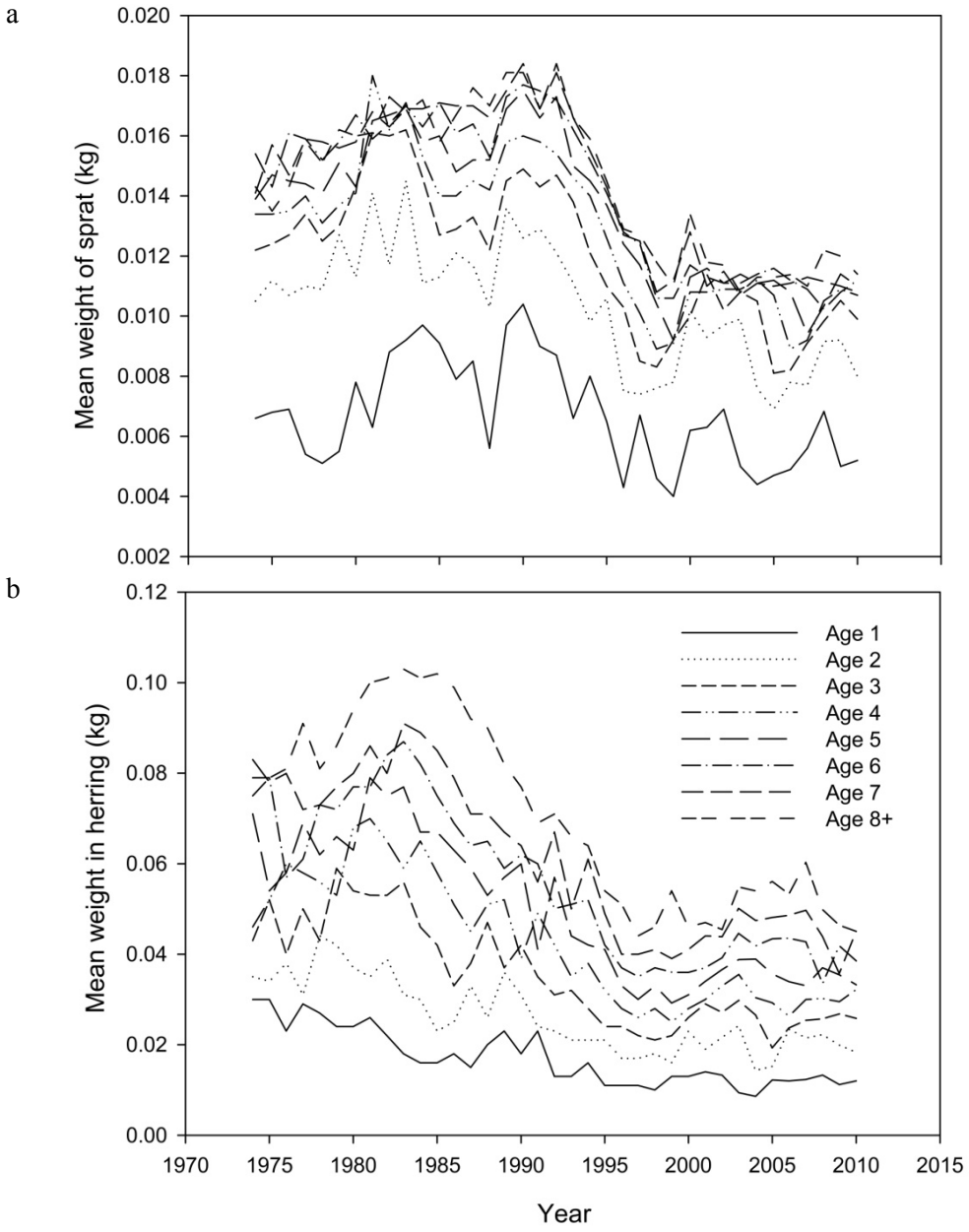


Fig.10. (a) Time series of mean weight at age 1-8+ of Baltic sprat (a) and herring (b). Mean weight of herring decreased in the 1980s and 1990s and in sprat mean weight at age has decreased in the 1990s. Data from ICES WGBFAS report 2011.

The widespread oxygen deficiency has resulted in habitat loss over vast areas and severely reduced macro benthic communities below the halocline in the Baltic Proper and the Gulf of Finland which has severely disruption of benthic food webs (Karlson et al. 2002, Zillen et al. 2008, Conley et al. 2009). Ecosystem models show that hypoxia-enhanced diversion of energy flows into microbial pathways to the detriment of higher trophic levels (Diaz & Rosenberg 2008) and the overall forecast is for hypoxia to worsen, with increased occurrence, frequency, intensity and duration (Diaz & Rosenberg 2011). Mysids, an alternative prey for herring, occurs in low abundance in the central

Baltic possibly due to inaccessibility of the oxygen deficient bottom layer (Barz & Hirche 2009a). The crustaceans *Saduria entomon* was an important prey in the 1980s where it contributed up to 30% of food consumption in the Central Baltic Sea (Zalachowski 1985a, Uzars 1994) and is still occasionally found in stomachs of Eastern Baltic cod (Uzars 1994, Neuenfeldt & Beyer 2003). A decline in abundance of *S. entomon* in the deep water has been observed (Cederwall & Elmgren 1980) as well as low abundance in hypoxic areas of in Gdansk (Janas et al. 2004) and during 1985-1990 the proportion of this species declined in cod diet by at least a factor 2 (Uzars 1994). The present abundance and distribution of S. *entomon* in the Baltic is not known but in 2007 it was listed as threatened and/or declining in the Southern Baltic Proper (HELCOM 2007).

The changes in phytoplankton composition has most likely changed FAC in the base of the food web as fatty acid composition (FAC) differs between phytoplankton groups (Ahlgren et al. 1992a, Viso & Marty 1993, Dijkman & Kromkamp 2006) and even between species from the same taxonomic group (Mansour et al. 1999). Very high DHA/ARA ratios has been observed in Baltic plankton and the authors suggested that imbalance in FAC may prevail in the basic food web (Ahlgren et al. 2005). Low levels of ARA in Baltic copepods (Peters 2006) supporting this theory. Low ARA level in dinoflagellates compared to diatoms was strongly indicated by a study where the calanoid copepod *Acartia tonsa* fed diatoms had 15 times higher ARA compared *A. tonsa* fed dinoflagellates (Støttrup et al. 1999). Likewise, ARA levels was higher in cod larvae feeding on *A. tonsa* fed diatoms than *A. tonsa* fed dinoflagellates (Grønkjær et al. 1995).

Low ARA levels in the base of the food web may cause low ARA levels in higher trophic levels. Consequences of low ARA levels remain uncertain. Previous feeding studies on the effects of different ARA levels on egg production and quality in cod have shown no or inconsistent results (Lie et al. 1993, Salze et al. 2005, Norberg et al. 2008, Sawanboonchun 2009). These studies were based on batch spawning from several females, in contrast to studies on individual females of sea bass (Bruce et al. 1999), Atlantic halibut (Mazorra et al. 2003) and Japanese flounder (Furuita et al. 2003) in which correlation between FAC and egg quality was found (Fig. 11).

Fig. 11. Table compiling previous studies on the effect of different levels of dietary ARA in cod and selected studies of other marine species. Size and number of broodstock, period of feeding and spawning, Batch (B) or individual (I) spawning, number of replicates and feed type and relative content of docosahexaenoic acid (DHA) eicosapentaenoic acid (EPA) and arachidonic acid (ARA), DHA/EPA and EPA/ARA ratios in feed are indicated. Crosses indicate measurements taken and L and H indicate significantly low and high values respectively. DP} indicates that fish were fed first diet a and then diet b. I* indicates that individual spawning was assumed due to homogeneity in egg development in analyzed batches.

Species	Size (yr or kg)	Fish (n)	Feeding period (months)	Spawning (B/I)	Spawning period (months)	Tank replicates	Feed type	DHA	EPA	ARA	DHA/EPA	EPA/ARA	Total egg production	Floating eggs production	Egg size	Blastomere symmetry	Fertilization rate	Fatty acid compostion	Lipid classes	Astaxanthin	Hormones	Hatching rate	Survival rate	Reference
Atlantic cod	2 yr	3200 (subsamp.)	>8	B	1-4	2	DP	10.8	7.2	0.5	1.5	14.4	x					x			x			Norberg, B. et al, 2008 + Rosenlund, G. pers. comm.
						2	DP	10.8	7.2	1	1.5	7.2	H					x			x			
						2	DP	10.8	7.2	2	1.5	3.6	x					x			x			
						2	DP	10.2	6.8	4	1.5	1.7	L					x			x			
Atlantic cod, W/F	4 kg	98	0	B	3-5	No	DP	11.1	6.6	0.65	1.7	10.7	x	x			x	x	x			x		Sawanboonchun, J., 2008
			1			No	DP+	10.4	6.3	3.2	1.7	2.0	x	x			x	x				x		
			2			No	DP+	10.4	6.3	3.2	1.7	2.0	x	x			x	x				x		
			3			No	DP+	10.4	6.3	3.2	1.7	2.0	x	x			x	x				x		
Atlantic cod, F		300	24	B	3-4	No	DP}	[a]7.1	7.5	0.3	1.0	25.0				L	L	x	x				L	Salze, G et al., 2006
Atlantic cod, W/F		355	2-8			No		[b]16.6	8.9	1.1	1.9	8.1				H	H	x	x				H	
Atlantic cod W		27	0			No	No									H	H	x	x					
Atlantic cod, F	3 yr	1140	24	B	1-4	No	DP	4.5	4.5	0.1	1	45.0	x		x		x	x						Lie, Ø. Et al., 1993
						No	DP	6.8	7.7	0.2	0.8	38.5	x		x		x	x						
						No	DP	10.8	13	0.8	0.8	16.3	x		x		x	x						
Atlantic halibut	8-	20	18	I	3-5	No	DP	10.8	8.3	1.8	1.3	4.6	x			H	H	x				H		Mazorra, C. et al., 2003
	19 kg					No	DP	11	8.6	0.4	1.3	21.5	x			L	L	x				L		
	2 yr	24	3+	I	3-5	2	DP	9.9	5.1	0.6	1.9	8.5		L								L	L	Furuita, H. et al., 2003
Japanese flounder							DP	9.7	5.1	3.6	1.9	1.4		H								H	H	
							DP	9.4	4.9	7.3	1.9	0.7		L								L	L	
European Sea bass	0.3-	40	6	I		No	DP	23.5	18.7	1.3	1.3	14.4		L				x	x			L		Bell, J.G. et al., 1997
	1.3 kg						Fish	23.2	7	4.8	3.3	1.5		H				x	x			H		
European Sea bass	2 yr	108- 180	>12	I*		3	DP	7.8	5.8	0.4	1.3	14.5	x		x			x				L		Bruce, M. et al., 1999
						3	DP	19.5	5.6	1.4	3.5	4.0	x		x			x				H		
						No	Fish	22.1	6.7	4.6	3.3	1.5						x						
Gilthead seabream		36	7	I		No	DP	4.3	8.8	0.78	0.5	11.3		H			H	x				x		Almansa, E. et al., 2001
							DP	0	tr	tr				L			L	x				x		

STUDY APPROACH

Field work

The studies in this PhD work combine data from existent field data (paper I and III) and new field data collected in 2008-2009 (paper I and II). Existent data was cod, herring and sprat collected in the Baltic Sea and cod collected in the North Sea in 2002-2004. New field data was cod, herring and sprat collected during trawl surveys with research vessels Dana in November 2008 and March 2009, with German Research vessels in May and August 2009 and with local fisher boats in February and July 2009. Size range of sprat and herring was chosen according to sizes of sprat and herring found in cod stomach analyses 1981-1993 (Stefan Neuenfeldt, pers. comm.). Total length, TL, whole body weight, BW, and for cod furthermore eviscerated body weight, EBW, gonad weight GW and liver weight LW was measured and gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated.

From left: the German Research vessel ALKOR, maturity determination of Baltic herring and a histological section of a female cod in late ripening stage (MIV). Photographs Maria Røjbek

Determination of gonadal maturity stage

Gonadal maturity of sprat and herring were determined from macroscopic characters in sprat according to the manual of Baltic International Trawl Surveys (Anon 2001) with subdivisions according to (ICES 2011b) and in herring according to Bucholtz et al. (2008).

Maturity stage of cod gonads was determined by using histological methods. The preserved ovarian tissue was embedded in paraffin using standard procedures, sectioned at 7 µm and stained using hematoxylin and eosin. Sections were analyzed using light microscopy and maturity stages were distinguished on basis of morphological characteristics of the larger and more advanced oocytes (Tomkiewicz et al. 2003). In the early ripening stage (MIII) a passage from endogenous to exogenous vitellogenesis occurs which coincides with the beginning of yolk production in the

oocytes. In relation to lipid composition this is a very important and dynamic maturity stage. MIII was therefore further divided into three sub-stages.

Experimental work

The study combines knowledge from aquaculture and population ecology by comparing the result of a broodstock diet with a FAC similar to Baltic prey with diets with more optimal FAC according to existing knowledge from aquaculture. In contrast to previous studies this study focuses on the effect of fatty acid composition in single female cod. The experiment was carried out at St. Andrews Biological Station, New Brunswick, Canada in collaboration with Edward A. Trippel, Research Scientist at Fisheries and Oceans Canada.

Farmed cod broodstock were fed diets 6.5 month prior to spawning with either low or high ARA level and/or low and high EPA level resulting in a low, intermediate or high EPA/ARA ratio (Fig. 12). During the spawning period 53 cod were divided into 8 replicate tanks (A=3, B=2 and C=3) and total egg production (floating and sunken) of was measured in each tank. All fish were stripped three times: at the beginning, peak and end of spawning and 34 crosses of individual females and males investigated. Egg lipid composition was analysed and egg quality in terms of fertilization success, egg diameter and dry weight, and larval quality in terms of hatching success and survival to 8 days post hatch was measured (Fig. 13).

Fatty acid	Diet A	Diet B	Diet C
ARA	2.5	2.5	0.8
EPA	7.5	11.0	11.0
EPA/ARA	3.0	4.4	13.8

Fig. 12. Composition (% of total fatty acids) of arachidonic acid (ARA), eicosapentaenoic acid (EPA) and the EPA/ARA ratios in the three diets tested.

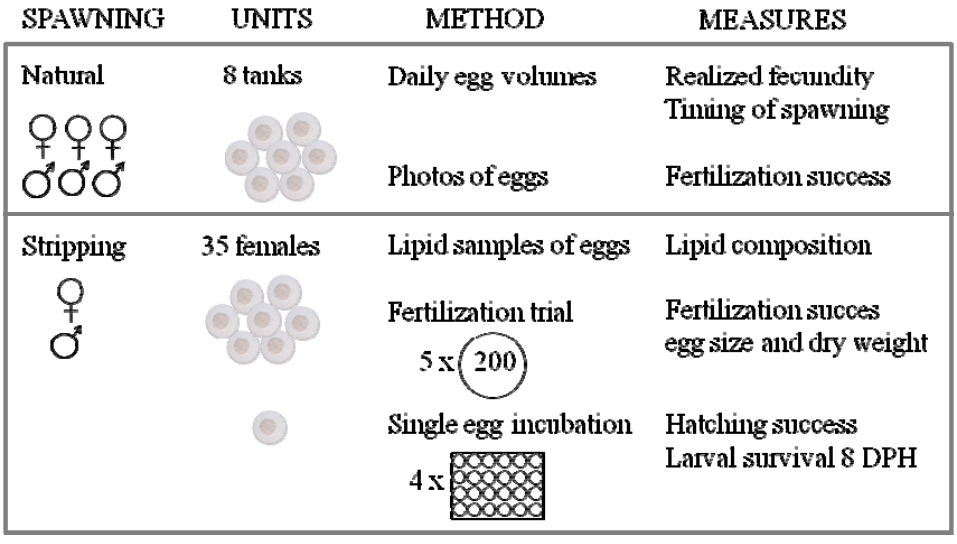
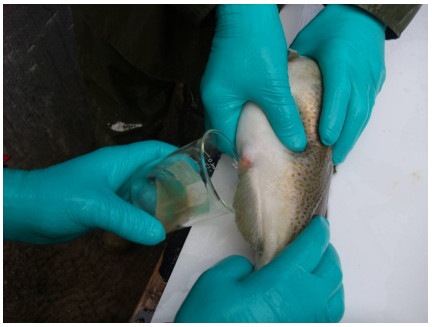
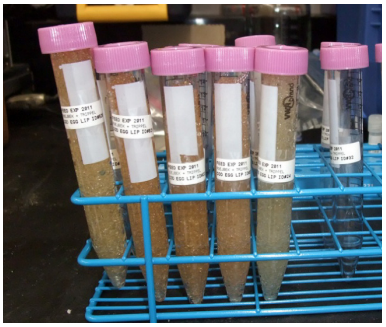


Fig. 13. Experimental design of feeding trial with Atlantic broodstock cod. Methods and measurements of egg production and egg and larval quality.

From left: St. Andrews Biological Station, New Brunswick, Canada, egg collection by stripping of broodstock fish and samples of cod eggs for lipid analysis. Air photo from: http://www.tbs-sct.gc.ca/dfrp-rbif/pn-nb/photo/56247-eng.aspx and the other photos by Maria Røjbek.

Analytical work

The collected sprat and herring were divided in groups of different size, sex and maturity from each cruise and whole fish of pooled samples were homogenized before analyses of lipid content and FAC in total lipid. Lipid content and FAC in PL and NL in ovaries and livers of individual cod was analysed for cod of different season and different maturity stage and for ovaries furthermore lipid classes. Finally lipid content and FAC of total lipid in diet ingredients, batches of experimental feed and eggs from the experimental work was analysed. Lipids were extracted with a homogeneous mixture of chloroform, methanol and water following the method of Bligh & Dyer (1959) and lipid content was determined by gravimetry after evaporation of chloroform. Lipid extracts were used for the preparation of fatty acid methyl esters (FAME) which were analysed with gas chromatography. Each fatty acid was quantified by calculating its peak area relative to the total peak area (%) or relative to the internal standard (mg g^{-1}). Lipid classes were determined using thin-layer chromatography with flame ionization detection (TLC-FID) with Iatroscan and peak areas were quantified using calibration curves obtained from scans of standards. Tocopherol and astaxanthin was measured in cod ovary samples and in whole sprat and herring and *Saduria entomon*. Tocopherol was analyzed with HPLC and the concentration calculated from the peak area relative to the internal standard and equivalent sample size (µg g^{-1}). Astaxanthin was measured spectrophotometrically at 485 nm and concentration was calculated by using the extinction coefficient $E_{1\%}$ = 2460 and equivalent sample size (µg g^{-1}). All lipid analyses were carried out in laboratories of National Food Institute, DTU Food.

Fatty acids as trophic markers in marine ecosystems

Fatty acid trophic markers (FATM) are incorporated into consumers in a conservative manner, thereby providing information on predator-prey relationships. FATM provide information on the

dietary intake over a longer period than traditional gut content analyses (Dalsgaard et al. 2003). In fish lipids FA composition is a blend of endogenous and exogenous sources, determined by de novo biosynthesis of short-chain SFA and MUFA and uptake, direct incorporation and modification of dietary FA and fatty alcohols (Dalsgaard et al. 2003). Hence as trophic levels increase, the ability to use unique fatty acids alone to trace feeding to a specific food type is reduced (Iverson 2009). In contrast, evaluating differences in the full array of fatty acids and looking at the fatty acid composition in predator and prey is a promising way to examine at trophic interactions at higher trophic levels and enables one to identify a fatty acid "signature" indicating dietary intake (Iverson 2009). When Atlantic cod switched to diets consisting of a single prey, i.e. low-fat squid or high-fat mackerel, the whole body fatty acid signature of these cod clearly reflected the fatty acids of their diets after only three weeks (Kirsch et al. 1998). In cod, dietary lipids are primarily stored in the livers (Lambert & Dutil 1997), and as most marine predators cod consume their prey whole. The approach in this thesis work was therefore to trace trophic pathways by comparing FAC in TAG of storage lipid with FAC of total lipid of whole prey as recommended by Iverson (2009).

Statistical analyses

Due to the high number of fatty acids and other variables in the data statistical analysis was mainly obtained by multivariate analysis. Multivariate analysis is a tool to find patterns and relationships between several variables simultaneously and it accounts for one variables effect on other variables. Principal Component Analysis has a highly graphical approach that allows examination of hidden structure of large data sets and to visually identify the factors which influence the results. The main objective of a PCA is to reduce the dimensionality of a set of data and give the best possible two-dimensional representation (Jolliffe 2002). Multi-way ANOVA was calculated using R version 2.12. Multi-way ANOVA can provide an overall statistical test on data with several correlated dependent variables, instead of performing multiple individual tests and allows test for interaction between variables. Multi-way ANOVA was followed by pair-wise comparisons test to pin-point specific differences between means. Statistical differences in fertilization, hatching success and larval survival between diets and time were calculated with logit-linear binomial models with random effects using AD-model builder (Fournier et al. 2011). The random variation due to different females, males, crosses (female and male combinations) and replicate handling were included.

MAIN RESULTS AND DISCUSSION

Lipid composition of Baltic clupeids and Saduria entomon

There was a significant seasonal variation in lipid content and EFA (ANOVA, $p < 0.01$) of sprat in the size range of 120-130 mm sprat and herring in the size range of 205-210 mm (Fig. 14). The mean content of lipid and ARA in sprat (10.3%, 0.5mg g^{-1} ww) were significantly higher ($p < 0.05$) than in herring (6.7%, 0.3mg g^{-1} ww). The highest lipid levels were found in November and this is consistent with the end of the annual zooplankton production cycle succeeded by a decline in lipid content during winter when zooplankton abundance is low. In spite of favorable feeding conditions in spring and summer, lipid content continued to decrease in sprat and herring. Seasonal variation in zooplankton lipid composition is linked to variation in composition of phytoplankton and protists (Linko et al. 1985a, Peters et al. 2006, Peters et al. 2007). In herring, the average lipid content changed during the reproductive cycle, with significantly higher lipid content in herring in early maturation in spring compared to spent fish in summer. Season and hence the availability and composition of zooplankton is the main cause of the variation in lipid levels and composition in sprat (ANOVA: $F = 79$, $p < 0.001$) and herring ($F = 78$, $p < 0.001$) and maturity has an additional effect on lipid content in herring ($F = 4$, $p < 0.01$).

Lipid content and FAC differed significantly between sprat, herring and *Saduria entomon* (Paper I, Fig. 15). Sprat samples had in general high lipid content and proportion of the MUFA, 18:1n-9, compared to herring which in contrast had high proportion of the major PUFA, DHA. Hence, altered abundance of sprat and herring available for cod may impact lipid content and FAC in cod. Herring has been observed to switch from feeding mainly on adult stages of *Pseudocalanus* sp. to feed mainly on young stages of *Temora longicornis* with increasing competition from sprat (Möllmann & Köster 2002). Results in the present study suggest that herring in contrast to sprat, indeed feeds more on *Temora longicornis* than *Pseudocalanus* sp. indicated by a high DHA/18:1n-9 ratio, with DHA being very abundant in *T. longicornis* compared to *Pseudocalanus* sp., which on the other hand are very rich in 18:1n-9 (Peters 2006, Peters et al. 2006). FAC of *S. entomon* differed from FAC of clupeid particular in having high proportions of ARA and EPA which reflects FAC of its main prey; the amphipod *Monoporeia affinis*, which feed on settled phytoplankton and detrital

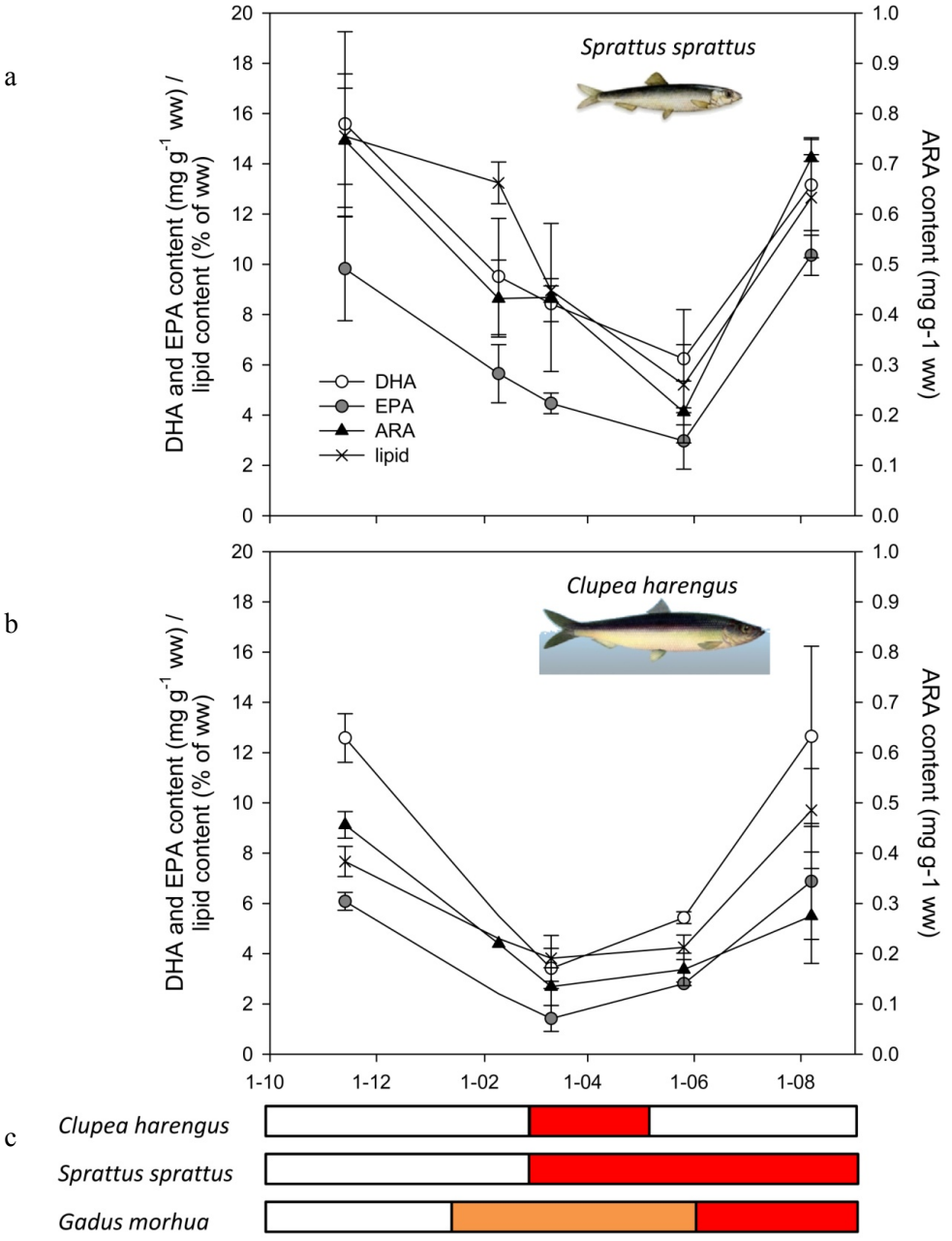


Fig. 14. Seasonal content of lipid and absolute content of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA) in wet weight of whole 120-130 mm sprat (a) and 205-210 mm herring (b) from November 2008, February, March, May and August 2009. Lowest values were observed in March and May in both species. Illustrated length classes of sprat include the most frequent encountered by cod. Values are means per length class ± SD, $n_{(sprat)}$ =13, $n_{(herring)}$ = 11. (c) Horizontal bars indicate the spawning period of herring, sprat and cod based on litterature; red bar is peak spawning and orange is the vitellogenic period in cod.

organic matter (Lahdes et al. 2010). Furthermore, *S. entomon* had in average 8 times more astaxanthin than sprat and herring in the present study and so had many other Baltic crustaceans (Czeczuga 1976a). High proportions of ARA, EPA and astaxanthin, in *S. entomon* render it a

valuable constituent in the diet of Baltic cod. The difference in FAC of sprat, herring and *S. entomon* proved that FAC is a valuable tool for distinguish between differences in diet of these species, in spite of a dietary overlap between sprat and herring (Möllmann et al. 2004). The pigment astaxanthin furthermore proved an valuable indicator of crustacean signatures (Oechsler-Christensen et al. 2012). In addition phytoplankton pigments is an alternative method used to trace trophic interactions (Head & Harris 1994) which has most recently proved successfully in an experimental study on food selection in Baltic copepods (Oechsler-Christensen et al. 2012). FAC in combination with phytoplankton pigments may be useful to distinguish phytoplankton types dominating the ecosystem.

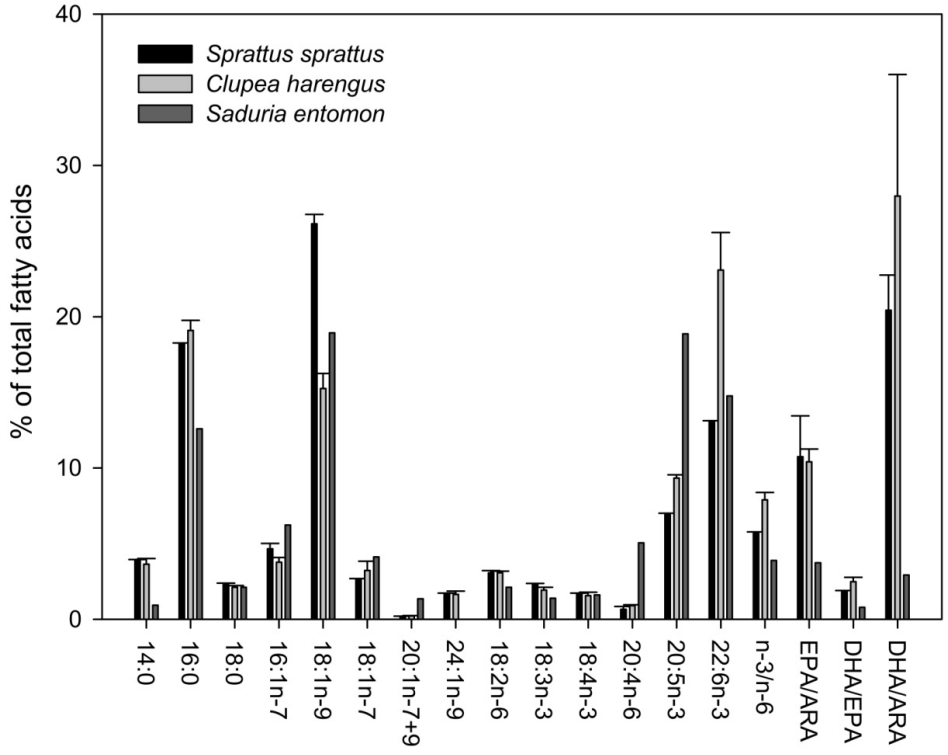


Fig. 15. The fatty acid composition of 120-130 mm sprat (*sprattus sprattus,* black bars), 205-210 mm herring (*clupea harengus*, grey bars) from the Baltic Sea in March 2009 and various sizes of *Saduria entomon* (dark grey bars) in March 2010. Values are mean ± SD and only fatty acids with more than 1 % in one of the species are shown, n = 2, 3 and 1 samples (pooled of 29, 25, ~100 specimens) of sprat, herring and *S. entomon* respectively. The main difference between sprat and herring is in the high proportion of 18:1n-9 in sprat and high proportion of 22:6n-3 (DHA) in herring. FAC of *S. entomon* differs from clupeids, particular with low proportion of 14:0, 16:0 and high values of 20:4n-6 (ARA) and 20:5n-3 (EPA).

Lipid dynamics in Baltic cod

A substantial amount of lipids was invested in reproduction by cod analyzed in the present study. Lipid content increased in ovaries during ovarian maturation and a notable decrease in lipid content

of ovary and liver during spawning was observed, most likely due to metabolic investment in oogenesis (Paper II). Lipid transferred from liver to ovary increased the amount of lipid in ovary relative to liver 10 times during gonadal development both due to the increase in size and lipid content of ovary. However, the food intake seemed to sustain high levels of liver energy because cod had ample lipid energy and liver lipid did not decrease until late in the reproductive cycle (Paper II) and lipid content was significantly higher in Baltic cod than in North Sea cod (Paper III). Proportions of DHA increased while ARA decreased in Baltic cod ovarian PL, TAG and liver PL with progressive ovarian development (Paper II and III) and GSI (Fig. 16), but remained constant at a low level in liver TAG. The trend of EPA was less clear. However a selective retention of both DHA and ARA in ovaries during ovarian maturation was evident (Paper II). Despite mobilization of ARA from liver, the level was not sufficient to keep up with the requirement in ovaries and ARA decreased in late maturation and during spawning (Fig. 17). The decrease in ARA results in an increased EPA/ARA ratio in late maturation which can have a negative effect on the activity of eicosanoids, as discussed further below.

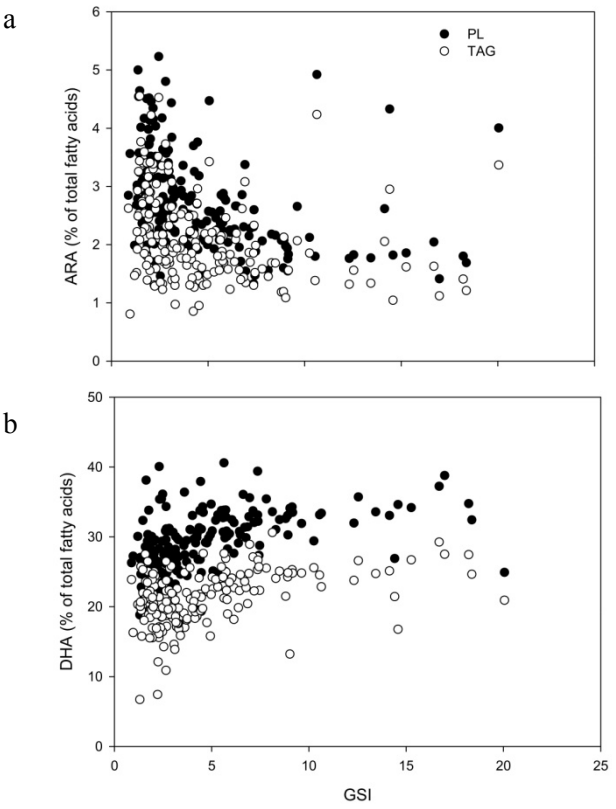


Fig. 16. (a) The content of arachidonic acid (ARA) and (b) docosahexaenoic acid (DHA) in phospholipids (PL) and triacylglycerols (TAG) in ovaries of cod (*Gadus morhua*) in maturity stage II+IX, III and IV from the Baltic Sea in relation to gonadosomatic index (GSI).

The 18:1n-9/DHA ratio was almost twice as high in sprat than in herring and hence this ratio can be used as a fatty acid trophic marker in clupeid predators, indicating the ratio of sprat and herring in their diet (Paper I). The 18:1n-9/DHA ratio in cod liver compared to proportions in sprat and herring (Fig. 18) indicates that some cod fed mainly on sprat and others mainly on herring but the majority has a mixed diet consisting of both clupeid species (Paper II). The antioxidants, *α*-tocopherol and astaxanthin, were accumulated in cod ovaries compared to prey and decreased in late maturation and spawning due to antioxidant protection activity (Paper II). An accumulation of α-tocopherol in ovaries is consistent with transport from muscle to ovary during vitellogenesis as indicated in a study of salmon (Lie et al. 1994a). Average concentration of α-

tocopherol in cod ovaries was 12 - 89 times greater than in the Baltic herring and sprat respectively and hence an accumulation in ovary compared to dietary concentration was evident. Astaxanthin levels in cod ovaries were higher than in whole sprat and herring indicating a selective retention in ovaries. This result is consistent with an accumulation of astaxanthin in ovaries compared to other tissues found in a recent study on Baltic cod (Nie et al. 2011).

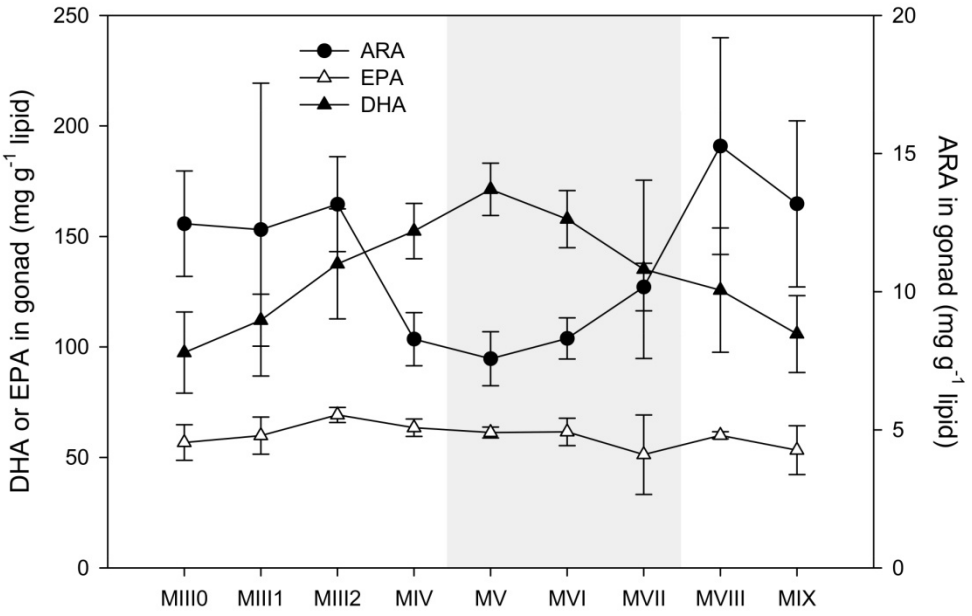


Fig. 17. Estimated content of docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) in total lipids (sum of phospholipids (PL) and triacylglycerols (TAG)) of cod ovaries in and maturity stages MIV – MIX. Gonadal maturity stages MIII=early ripening (maturity sub stages $MIII^{0-2}$), MIV=late ripening, MV=initiation of spawning, MVI=main spawning, VII=cessation of spawning, VIII=spent and MIX=resting. The spawning stages are indicated with the grey box. Values are mean ± SD, n = 45.

Trophic interactions and ecosystem changes

Lipid content in Baltic herring (Paper I) was very low compared to whole spring spawning herring from Norway (Slotte 1999) and herring fillet from Scottish waters (Henderson & Almatar 1989). This low lipid content is most likely due to a combination of reduced amount of preferred zooplankton and changes in species composition of zooplankton associated with the decrease in salinity and increased temperature (Möllmann et al. 2008). Furthermore, mysids, an alternative prey for herring, occur in low abundance in the central Baltic possibly due to inaccessibility of the oxygen deficient bottom layer (Barz & Hirche 2009a).

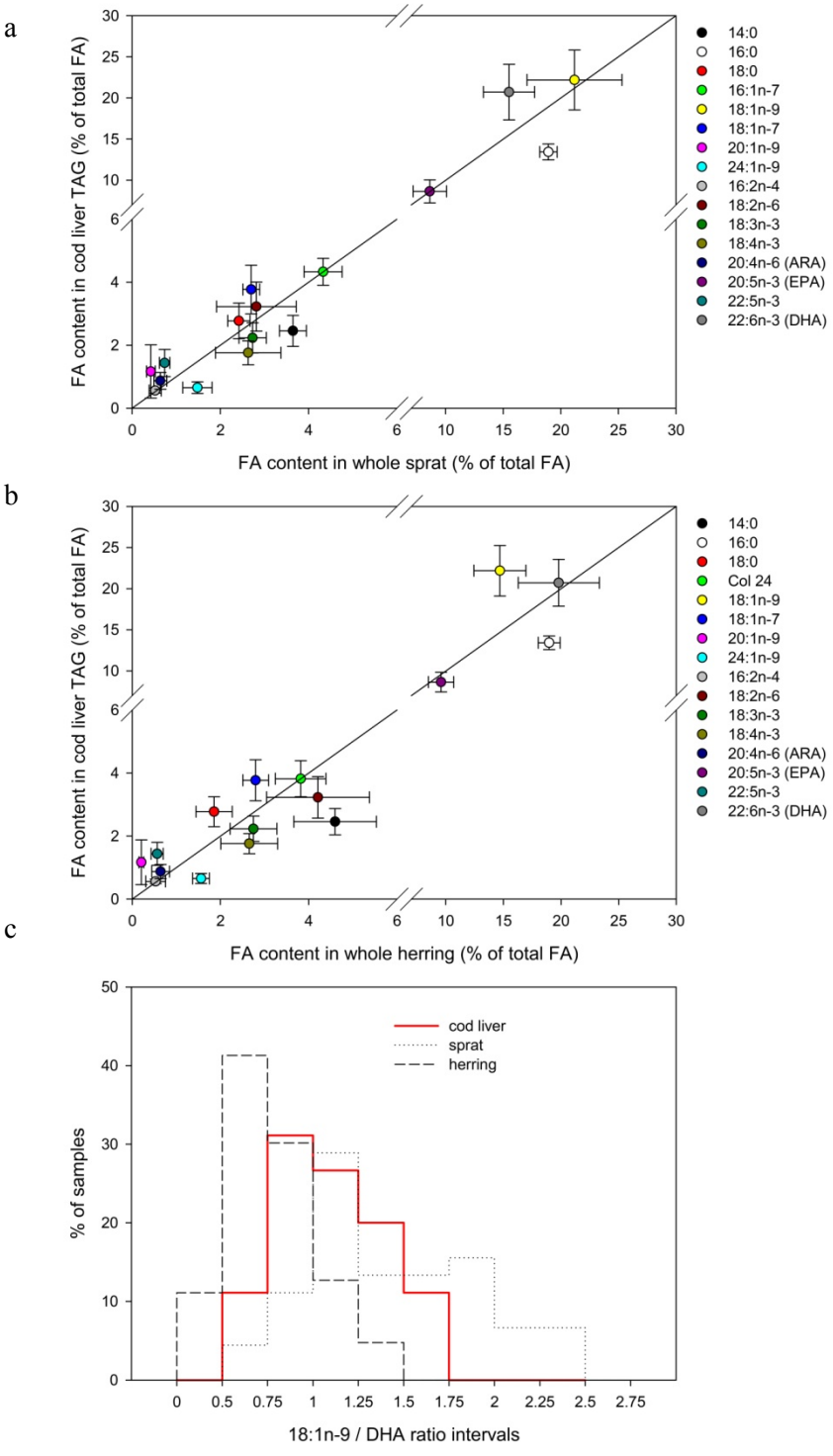


Fig. 18. The ratio between average FA content ± SD of cod liver TAG and FA content ± SD of total lipid in whole herring (a) and sprat (b) and the frequency of different 18:1n-9/DHA ratio intervals in cod, sprat and herring. The line in (a) and (b) illustrates a ratio = 1, cod: n = 45, herring: n = 62, sprat: n = 45.

Fatty acids biomarkers indicate that the alterations in the Baltic Sea ecosystem may have resulted in a reduction of lipid quantity in herring and of ARA level in both sprat and herring (Paper I). EPA/DHA and 16:1n-7/16:0 ratios are valuable fatty acid trophic markers (FATM) for discriminating between diatoms and dinoflagellates (Parrish et al. 2000, Dalsgaard et al. 2003). EPA/DHA ratios ≤ 0.8 and 16:1n-7/16:0 ratios ≤ 0.3 in sprat and herring (Paper I) strongly indicate that the central Baltic ecosystem was dominated by dinoflagellates and other flagellates in the investigated periods. The levels of EPA/DHA of sprat and herring were reflected in cod liver (Paper II) but 16:1n-7/16:0 ratios were higher in cod (≤ 0.7) most likely due to selective catabolism of 16:0. A domination of dinoflagel-lates most likely results in low ARA levels in zooplankton because ARA level in Baltic copepods fed dinoflagellates was found to be 15 times lower compared to copepods

fed dinoflagellates (Støttrup et al. 1999). This is supported by a mesocosm study where ARA levels were below detection limit in phytoplankton and copepods from the Baltic Sea compared to plankton from the Norwegian Sea (Ahlgren et al. 2005). Furthermore, significantly lower ARA levels were found in Baltic cod ovaries and livers than in North Sea cod, (Paper III, Fig. 19).

Effect of EFA on cod spawning pattern

Low content of ARA and high ratio of EPA/ARA coincide with the timing of cod ovarian development in the central Baltic Sea (Paper II). ARA proportion was significantly lower and EPA/ARA ratio higher in Baltic cod than in cod from the North Sea, that do not have a delayed spawning (Paper III). High EPA/ARA ratio may affect eicosanoid activity, because eicosanoids produced from ARA are more biologically active than those produced from EPA (Sargent et al. 2002). A low eicosanoid activity may impair prostaglandin production important for ovulation. Optimal EPA/AA ratios in eicosanoid production is likely to be species specific (Sargent et al. 2002) and are at present not known for cod. But in gold fish it has been shown that only ARA was able to induce the production the hormone produced by vitellogenic follicles undergoing final meiotic maturation, as well as the production of PGE2 and PGF2a by the follicles, whereas DHA and EPA did not have this ability (Henrotte et al. 2011b). ARA limitation in the Baltic Sea food

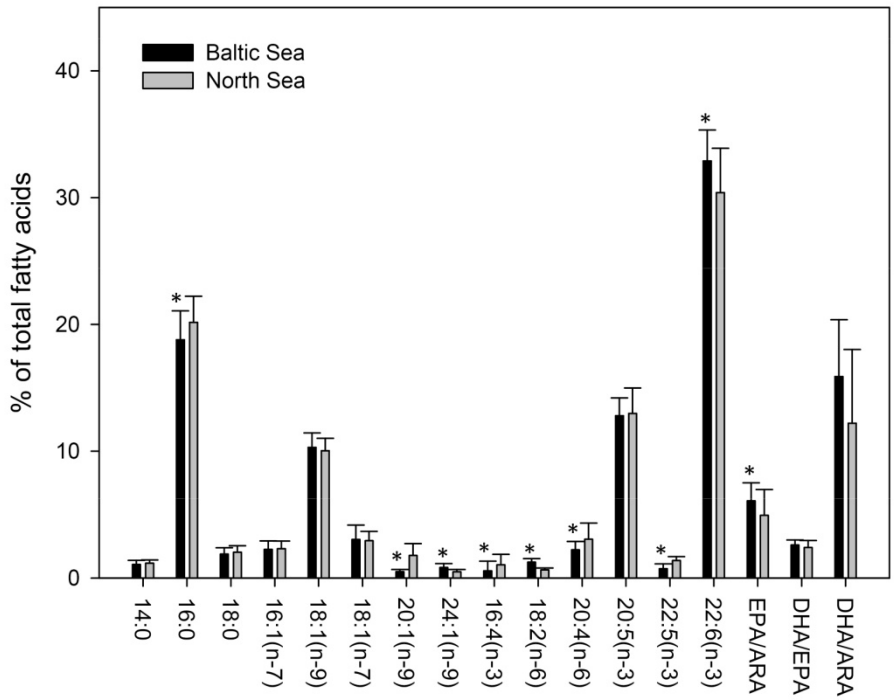


Fig. 19. The fatty acid composition of ovarian phospholipids (PL) of cod (*Gadus morhua*) in maturity stage MIV from the Baltic Sea (black bars) and the North Sea (grey bars). Values are mean ± SD and only fatty acids with more than 0.5 % in one of the origins are shown, Baltic Sea, n = 49, North Sea, n = 25. The main differences were between proportions of 16:0, 20:1n-9, 20:4n-6 (ARA), 20:5n-3 (EPA) and 22:6n-3 (DHA). Asterisks indicate significant differences between origins ($p < 0.05$). Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA).

web therefore has the potential to prolong cod maturation period and affect timing of spawning (Paper III). However, feeding cod with diets with different ARA levels and EPA/ARA ratios did not seem to influence the timing of spawning in farmed Atlantic cod (Paper IV). Females in all tanks had started spawning in mid-February and the main spawning period was from end of February to end of March. By end of March 84 - 94% of total eggs were spawned except for B1 which only had spawned 76% at this point and eggs were observed until the 9th of May (Fig. 20).

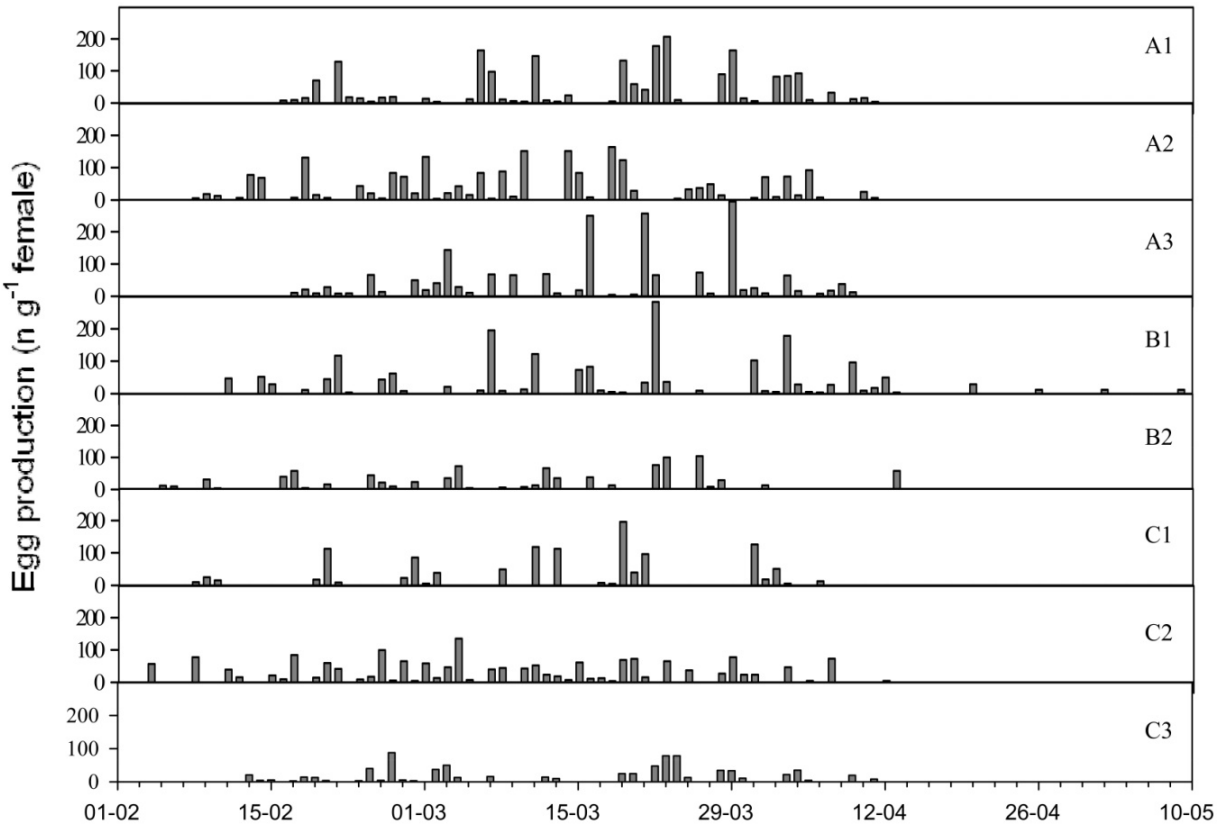


Fig. 20. Total egg production in replicate tanks of cod fed either diet A, B and C. The duration of spawning in tanks was between 55 and 70 days and started between 3rd and 16th of February and ended between 11th and 13th of April except for tank B2 where egg were observed in a period of 88 days until the 9th of May. No obvious trend in spawning time with diet was evident.

As the spawning period was extended in only one out of five tanks fed high ARA levels this study does not support the findings of Norberg. et al. (2009) who reported an extended spawning period with high level of ARA (Rosenlund, G., pers. comm.). Measuring spawning period in tanks with several females implies a risk of obscuring differences due to outliers however individual assessment of spawning was not possible with the available facilities. If the assumption is correct, that the underlying process is similar in Atlantic and Baltic cod stock, low ARA level in the Baltic Sea does not seem to be the cause of the delayed spawning. Time series of lipid composition in cod,

sprat and herring is needed to further investigate timing of spawning to test if low prey quality (EFA content) is correlated with delayed spawning.

Effects of dietary fatty acids on realized fecundity

Uptake of ARA into cod eggs from broodstock diet was highly efficient (Paper IV). This is in agreement with a study of Norberg et al (2009) who also found a strong correlation between dietary and ovarian FAC after minimum 8 months of feeding. Cod fed diet with high ARA level and low EPA level had significantly highest realized egg production with 472 eggs g^{-1} female compared to cod fed diet B and C which produced around 242 and 235 eggs g^{-1}. This is in contrast to the study of Norberg et al. (2009) where realized fecundity was higher at EPA/ARA around 7 compared to a ratio around 2 (Grethe Rosenlund, pers. comm.). Due to the differences in ingredients between diets and it remains unclear if this was solely due to lower EPA/ARA ratio.

The experiment was carried out on first time spawning cod so extrapolating the results to wild stock is not straightforward, because first time spawners are observed to have significantly lower realized egg production than second time spawners (Trippel 1998). In comparison, relative fecundity observed in wild Baltic cod in the period from 1987 to 2000 and 2002 Baltic cod varied between 500 and 900 eggs per g female (Kraus et al. 2002, Kraus et al. 2012). The median length of these Baltic was between 45 and 58 cm for the different years and so the main proportion of fish is most likely repeat spawners as the size of maturity is less than 40 cm in Baltic cod (Cardinale & Modin 1999).

Effects of dietary fatty acids on egg and larval quality

Eggs from fish fed diet with high ARA level (2.5% of total fatty acids) had higher fertilization success and survival to 8 days post hatch (DPH) than fish fed low levels (0.8%) of ARA (Fig. 21a). The odds ratios for fertilization success indicated that high levels of ARA and EPA are equally important for fertilization success and both fatty acids are also important in terms of larval survival but here ARA is of higher importance than EPA (Paper IV). The additional enhancement of fertilization success and larval survival with EPA was not significant and did not compensate for the reduced egg production (Fig. 21b).

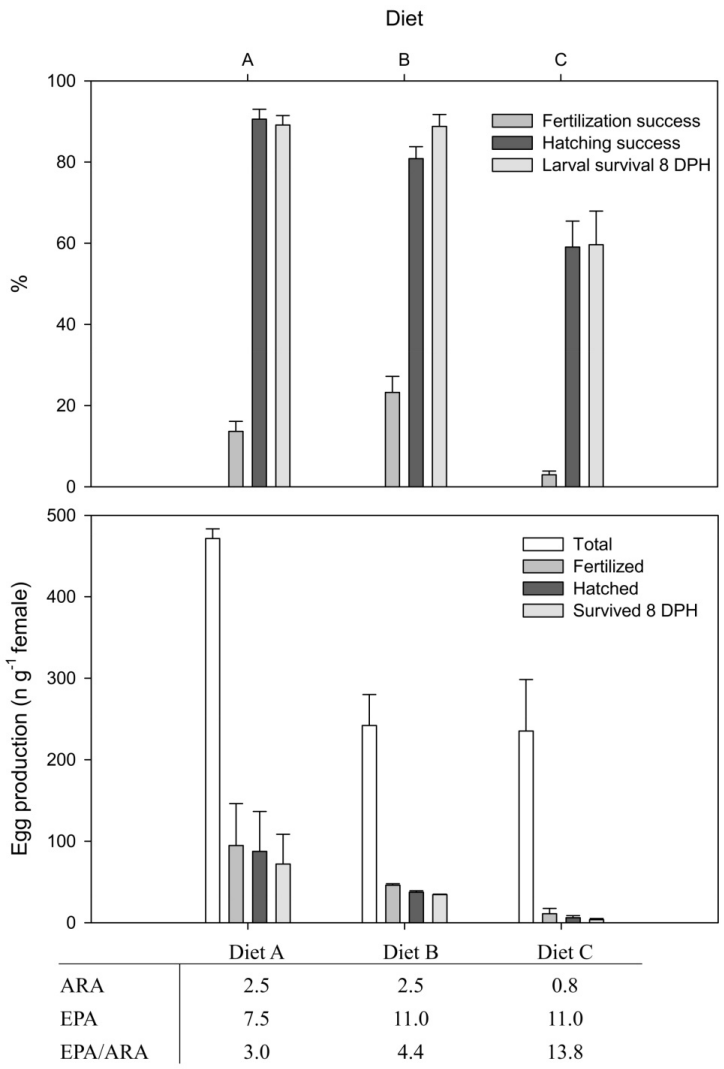


Fig. 21. (a) Fertilization success (% of total eggs), hatching success (% of fertilized eggs) and larval survival to 8 days post hatch (DPH, % of hatched egg) and (b) measured total egg production and estimated production of fertilized, hatched and larvae survived to 8 DPH in replicate tanks of cod in cod fed either diet A, B and C. Values are mean ± standard error, (a) n = 31 crosses and (b) n = 8 tanks. Composition (% of total fatty acids) of arachidonic acid (ARA), eicosapentaenoic acid (EPA) and the EPA/ARA ratios in the three diets are indicated below.

Paper IV is the first to show a positive effect of dietary ARA and EPA on fertilization success and larval survival in Atlantic cod. ARA seems to have a positive effect on hatching success in mid spawning only. A low and very variable fertilization success observed in the present study has previously been reported in first-time spawners of young cod of wild origin from the northwest Atlantic (Trippel 1998). Farmed cod broodstock has previously been reported to exhibit extremely variable fertilization success (Kjesbu 1989) and lower than in wild stock (Salze et al. 2005) indicating that diet is of prime importance and composition of extruded diet used commercially was not optimal. The number of hatched larvae g^{-1} female (first-spawners) was in the study of Trippel (1998) on average 19 which was higher than the 4 g^{-1} female (viable larvae 8 days post hatch) observed in diet C in the present study but lower than 71 eggs g^{-1} in diet A and B, indicating that diet A and B may be at least comparable and maybe even better than natural diet.

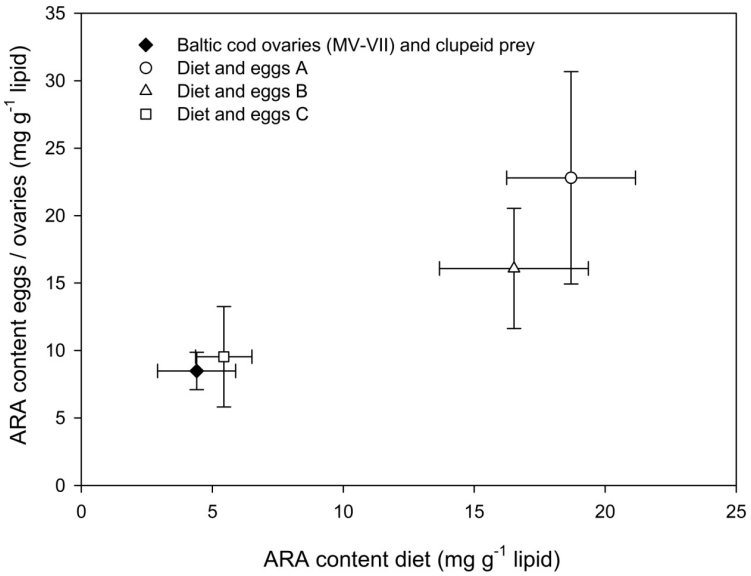


Fig. 22. Absolute content of arachidonic acid (ARA) in total lipids of diet and eggs from the feeding experiment (white symbols) and in sprat + herring and ovaries in spawning condition (MV-VII) from the Baltic Sea (black symbol, total lipid in ovaries was estimated as the sum of phospholipids (PL) and triacylglycerols (TAG)). Values are mean ± SD, n diet samples; diet A: n = 6, diet B: n = 8, diet C: n = 6 and sprat + herring: n = 45 + 63 and n egg samples; diet A: n = 11, diet B: n = 9, diet C: n = 11 and ovaries of Baltic cod: n = 45.

Absolute ARA content in Baltic sprat and herring was similar to diet C and ARA content in Baltic cod ovaries similar to ARA in eggs fed diet C (Fig. 22). The results from this study strongly indicate that the low ARA levels in Baltic cod ovaries, reflecting ARA levels in prey, result in low fertilization success and survival of eggs and larvae. This is important as the period between the late egg and the early larval stage is critical for cod recruitment (Köster et al. 2003a). Nutritional value of prey in terms of lipid and EFA content may therefore affect recruitment dynamics in Baltic cod, in addition to size, structure, and distribution of the spawning stock (Köster et al. 2001b) and environmental variability and species interaction (Köster et al. 2001a).

Low levels of EFA in Baltic cod females and their eggs could explain the low hatching success (Pickova & Larsson 1992, Larsson 1994, Pickova et al. 1997) and high mortality in Baltic cod larvae reported (Grauman & Sukhorukova 1982, Åkerman et al. 1996, Petersen et al. 1997). This is supported by the finding of Pickova et al. (1997) where hatching success was correlated with the proportion of ARA in egg PL and Baltic cod eggs had low proportion of ARA and high EPA/ARA ratios compared to Skagerrak cod eggs. Furthermore, EFA limitations in Baltic cod larvae could explain the lower growth rate reported as compared to larvae from Georges Bank, Gulf of Maine (Huwer 2009, Huwer et al. 2011), even though this may also be related to low food availability in the deep water layer (Grønkjær et al. 1997).

CONCLUSION AND PERSPECTIVES

This PhD provides novel information about seasonal lipid dynamics in Baltic sprat and herring which is important due to their central position in the Baltic ecosystem, representing the link between lower trophic levels and top predators. Information on lipid content in Baltic clupeids can improve the understanding of the present low condition and used to estimate the energy available for spawning. Low levels of lipid and EFA in both species in spring and low lipid content in herring in general may have adverse effects on clupeid reproduction and reduce the lipid quantity and quality available to their main predator cod. Low abundance of the copepod *Pseudocalanus* sp. and mysids may have caused the reduction in lipid content observed in herring. Fatty acids trophic markers indicated that dinoflagellates dominated during the years examined which may have resulted in a reduction of ARA level in both sprat and herring compared to periods with domination of diatoms (Paper I).

Lipid content and fatty acid composition (FAC) differs significantly between sprat, herring and *S. entomon* (Paper I). Sprat has in general high lipid content and proportion of the monounsaturated fatty acid, oleic acid, compared to herring, which in contrast, has high proportion of the polyunsaturated fatty acid, docosahexaenoic acid (DHA). This suggests that sprat feeds more on the copepods *Pseudocalanus* sp. than herring which mainly feeds on *Temora longicornis*. Hence, altered ratios of sprat and herring abundance available for cod may impact lipid content and FAC in cod. High proportions of ARA, EPA and the antioxidant, astaxanthin, in *S. entomon*, compared to clupeids, render it a valuable constituent in the diet of Baltic cod and investigations of its availability and presence in cod diet would be of great value.

The results contribute to a better understanding of the lipid requirements and fatty acid mobilization during maturation in cod. Selective retention of ARA in ovaries during ovarian maturation is evident (Paper II) but despite mobilization of ARA from liver, ARA level decreases in ovaries in late maturation and during spawning. The antioxidants, α-tocopherol and astaxanthin, were accumulated in cod ovaries and decreased in late maturation and spawning due to antioxidant protection activity (Paper II). No limitation in lipid energy was evident but a deficiency in the essential fatty acid ARA was indicated (Paper II). ARA level, important for eicosanoid activity

(Sargent et al. 2002), was lower in ovaries of Baltic Sea cod than in North Sea cod with no delay in spawning time (Paper III) indicating that this fatty acid may be important for spawning time. However the spawning period was not influenced by different ARA levels and EPA/ARA ratios in farmed Atlantic cod (Paper IV). More studies on the exact role of ARA and other EFA in cod reproduction are warranted before clear conclusions about the requirements for ARA in cod can be made.

Uptake of DHA, EPA and ARA into cod eggs from broodstock diet is highly efficient (Paper IV). Diet with low EPA/ARA ratio has significantly higher realized fecundity and eggs from fish fed a diet with high ARA level has higher fertilization success and survival to 8 days post hatch compared to fish fed low level of ARA. The combined results strongly indicate that the low ARA levels in cod ovaries, reflecting prey, results in low fertilization success and larval survival of Baltic cod eggs.

The improved knowledge of the effect of dietary EFA on realized fecundity and egg and larval quality in cod is of great importance for estimating recruitment, since the relatively higher mortality of larvae in the Baltic cod stock constitutes a major bottleneck for this population (Köster et al. 2003a). Knowledge on dietary EFA effects on realized fecundity and egg and larval quality is also important to cod farming because egg quality in cultured cod is one of the limiting factors for successful mass production of fish fry (Kjørsvik et al. 1990, Penney et al. 2006).

EFA proved a useful tool as trophic markers in the Baltic Sea ecosystem however, more studies on seasonal and annual variation of FAC in phyto- and zooplankton and clupeids are recommended. In the future EFA may also be useful as ecosystem state indicators in ecosystem based management, because they link effects of climatic changes on the base of the food web with changes in higher trophic levels.

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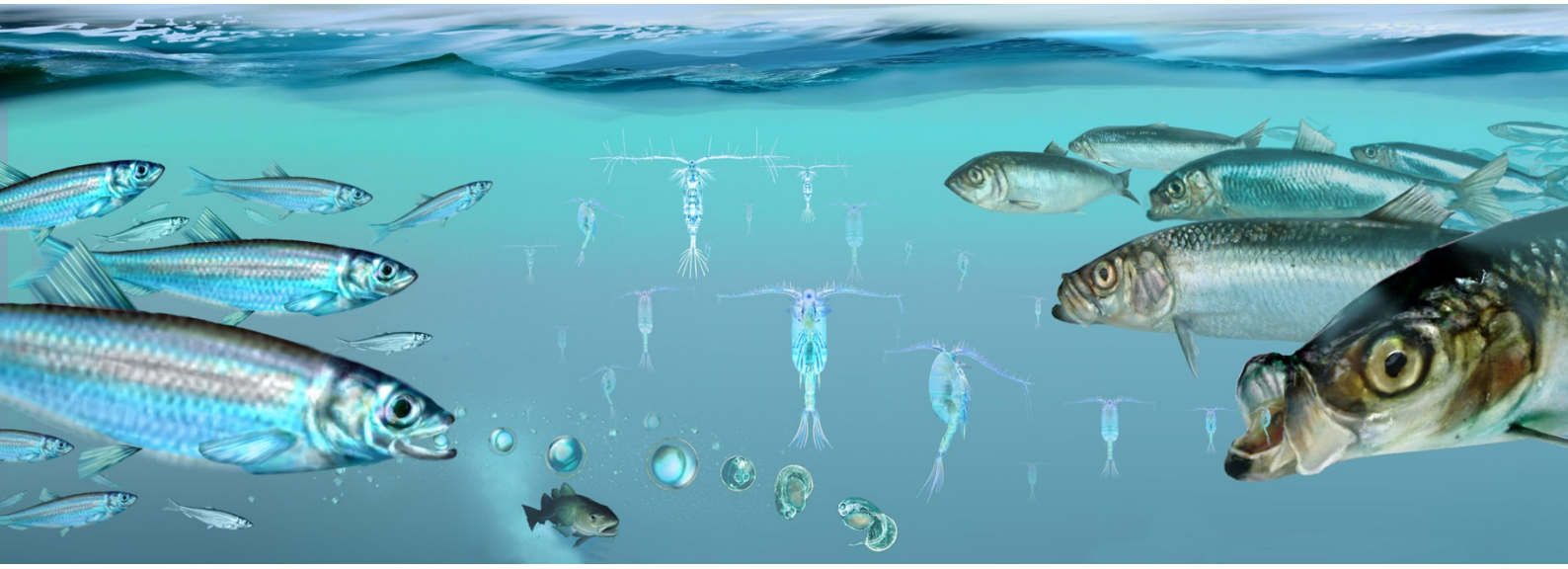
Paper I



Lipid dynamics of herring (*Clupea harengus* L.) and sprat (*Sprattus sprattus* L.) as major prey species in the Baltic Sea

by Maria C. Røjbek, Jonna Tomkiewicz, Charlotte Jacobsen & Josianne G. Støttrup

Illustration is part of image by Glynn Gorick for the ICES/GLOBEC project: Cod and Climate Change

Lipid dynamics of herring (C*lupea harengus* L.) and sprat (S*prattus sprattus* L.) as major prey species in the Baltic Sea

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RUNNING TITLE: Lipid dynamics in Baltic clupeids

KEY WORDS**:** Clupeids · essential fatty acids · fatty acid composition · *Gadus morhua* · prey quality · reproduction · *Saduria entomon* · trophic interactions

ABSTRACT

This study investigates seasonal changes in lipid content and composition of sprat (*Sprattus sprattus*) and herring (*Clupea harengus*) being important components in the Baltic Sea food web and main prey for top-predators, such as cod (*Gadus morhua*). Lipid dynamics of sprat and herring sampled in the Central Baltic Sea during three periods in 2002/2003, 2003/2004 and 2008/2009 following the annual reproductive cycle was studied according to fish length, sex and maturity. The isopod *Saduria entomon*, an alternative prey of Baltic cod, was additionally sampled for comparison of lipid composition and nutritional value to clupeids. Lipid composition differed significantly between sprat and herring. Sprat samples had an average lipid content of 9% of wet weight and had a high proportion of monounsaturated fatty acid (MUFA) compared to herring which in contrast had an average lipid content of 6% and a high proportion of polyunsaturated fatty acids (PUFA). The average lipid content increased with fish length in sprat while the lipid content changed during the reproductive cycle in herring, with significantly higher lipid content in herring in early maturation compared to spent fish. In both species, lipid content and absolute contents (mg g^{-1} wet weight) of essential fatty acids (EFA) varied seasonally with high levels in November at the end of the annual zooplankton production cycle, succeeded by a decline during winter when zooplankton abundance and feeding activity is low. Low lipid and EFA content in spring and summer of both sprat and herring coincide with the maturation period of cod and may impact cod nutrition and reproductive success. High levels of EFA in *S. entomon* render it a valuable constituent in the diet of Baltic cod.

INTRODUCTION

Sprat (*Sprattus sprattus*) and herring (*Clupea harengus*) occupy a central position in the Baltic ecosystem, constitute the bulk of fish biomass (Parmanne et al. 1994), consume large amounts of zooplankton (Möllmann & Köster 2002) and serve as prey for higher trophic levels including Baltic cod (*Gadus morhua*). Small herring and all sizes of sprat are strictly zooplantivorous (Möllmann et al. 2004, Casini et al. 2004) and their PUFA content is obtained from their main prey, the calanoid copepods; *Pseudocalanus* sp., *Temora longicornis* and *Acartia* spp. Sprat and herring are primary prey of cod (Bagge et al. 1994, Uzars 1994), salmon (Mikkonen et al. 2011) and seals (Lundström et al. 2010) in the Central Baltic Sea. Baltic cod additionally prey on benthic species: the crustaceans *Saduria entomon*, *Pontoporeia* and mysids and the polychaetes *Harmothoe* and other invertebrates (Uzars 1994). Cod stomach analysis showed that the crustacean *Saduria entomon* was a frequent prey of cod in the Central Baltic Sea in the early 1980s (Zalachowski 1985b), while its occurrence in cod diet declined in the late 1980s (Uzars 1994) concurrent with an increasing proportion of clupeids in their diet (Uzars et al. 2000). In the summer 1999, sprat and herring generally constituted 80-95% of total mass in stomachs of cod over 30 cm (Neuenfeldt & Beyer 2003).

In recent decades, the pelagic Baltic Sea ecosystem experienced shifting regimes due to atmospheric and anthropogenic pressure (Köster et al. 2003b, Alheit et al. 2005, Möllmann et al. 2008, Casini et al. 2008, Möllmann et al. 2009). Spawning stock biomass of cod decreased from early 1980's to early 1990's, whereas sprat increased dramatically from the end of the 1980's to mid 1990's (ICES 2011a). Herring showed a declining trend throughout the 1970's-1990's, with a reversal of the trend in the most recent decade, while cod recovery started later in mid 2000's. Sprat in contrast shows a declining trend with present spawning stock biomass values corresponding to the early 1990's (ICES 2011a). The phytoplankton composition varied between 1979 and 2005 due to an increase in temperature and a decrease in salinity and inorganic nitrogen concentrations (Wasmund & Uhlig 2003, Wasmund et al. 2011). The reduced salinity also caused a substantial decrease in the abundance of *Pseudocalanus* sp. in the late 1980's, while the abundance of *Temora longicornis* and *Acartia* spp. increased due to increased sea temperature during the same period (Möllmann et al. 2005, Möllmann et al. 2008). The most important food item for both sprat and herring in spring has been *Pseudocalanus* sp., but since the late 1970s the relative amount of this

copepod in the diet of both sprat and herring decreased (Möllmann et al. 2003, Möllmann et al. 2004). This change in zooplankton composition concurrent with increased sprat abundance resulted in reduced condition in terms of g at the grand mean length of herring since the late 1980s and sprat since the 1990s (Möllmann et al. 2005, Casini et al. 2010, Casini et al. 2011). This was due to hydro-climatic forces (Casini et al. 2010) and a decrease in *Pseudocalanus* sp. abundance (Möllmann et al. 2005) in the former period and due to inter and intra-specific density-dependence mediated by the size and distribution of the sprat population in the latter period (Casini et al. 2011).

Information on seasonal lipid dynamics in sprat is documented for autumn to spring 1967-1968 at the British Isles (Hardy & Mackie 1969) and to our knowledge information on lipid content in the subspecies Baltic sprat (*Sprattus sprattus balticus*) is not available. Baltic sprat is a serial batch spawner and income-breeding species (Alheit 1988a, Wahl & Alheit 1988b) with a prolonged spawning season lasting from February to August in the Baltic Proper (Ojaveer & Kalejs 2010). Seasonal variations in lipid content in fillets of North Sea herring (*Clupea harengus*) is documented for autumn to spring in spring-spawning fish from Norway 1994-1998 (Slotte 1999, Hamre et al. 2003) and for all seasons from the British Isles in spring-spawning fish 1985 (Henderson & Almatar 1989) and in autumn-spawning fish 1999-2001 (Aidos et al. 2002). Sesonal lipid dynamics in the small-sized subspecies Baltic herring (*Clupea harengus membras*) is documented only for the Northern part of the Baltic Sea in spring to autumn 1976 (Linko et al. 1985b) and 1996-1998 (Aro et al. 2000) where the majority of herring spawn between May and July. In contrast, there are very few studies on seasonal variation in lipid content of whole herring (Slotte 1999). Seasonal changes in lipid composition of herring is related to changes in lipid composition of plankton (Linko et al. 1985b) and reproductive stage (Henderson & Almatar 1989). Spring spawning herring (March-May) dominates in the Baltic Sea (Aro 1989) and timing of spawning is primarily determined by the feeding conditions prior to spawning (Rajasilta 1992b). In contrast to sprat, herring follow a capital-breeding strategy (Jager et al. 2008). Herring develop its gonads over a long period, spawn during a short period and have long intervals between reproductive events compared to sprat. The difference in reproductive pattern may influence the lipid dynamics of these clupeid species in different ways.

Marine fishes have a dietary requirement for PUFA as they are unable to synthesize them in adequate levels, if at all, in those species studied so far (Sargent et al. 2002, Bell & Tocher 2009). Lipids, and specifically fatty acids, are the preferred source of metabolic energy for growth,

swimming and reproduction in marine fish (Sargent et al. 2002, Tocher 2003). In particular long chain polyunsaturated fatty acids (PUFA) have been identified as major dietary factors essential to successful reproduction. Especially the fatty acids docosahexaneoic acid (22:6n-3 = DHA), eicosapentaenoic acid (20:5n-3 = EPA) and arachidonic acid (20:4n-6 = ARA) are important, the two latter being precursors to eicosanoids including prostaglandins. Long chain PUFA originate from primary producers, primarily phytoplankton, and are transferred up through zooplankton to higher trophic levels. The content of PUFA may also have changed in Baltic clupeids due to changes in the phytoplankton composition because FAC differs between phytoplankton groups (Ahlgren et al. 1992b, Viso & Marty 1993, Dijkman & Kromkamp 2006) and even between species from the same taxonomic group (Mansour et al. 1999).

The rationale for this study was the hypothesis that these alterations in the Baltic Sea ecosystem have resulted in a reduction in lipid quantity and quality in sprat and herring, affecting clupeid condition and reproduction as well the nutritional quality for Baltic top-predators, such as cod. Main objectives were to determine the content of lipid and EFA in sprat and herring as major prey species of cod and to investigate the variation in lipid and EFA content linked to size, season, sex and gonadal maturation of sprat and herring. A comparison between the lipid content and composition of the crustacean S*aduria entomon* was included for comparison with an alternative prey being at times important for cod as prey species.

MATERIALS AND METHODS

Sample collection and lipid sampling procedure

Sprat and herring and were sampled on trawl surveys and fisheries covering the Bornholm Basin (ICES Sub-division 25) of the Central Baltic Sea during three periods in 2002-2003, 2003-2004 and in 2008-2009, following the reproductive cycle from autumn to summer (Fig. 1, Table 1a and b).

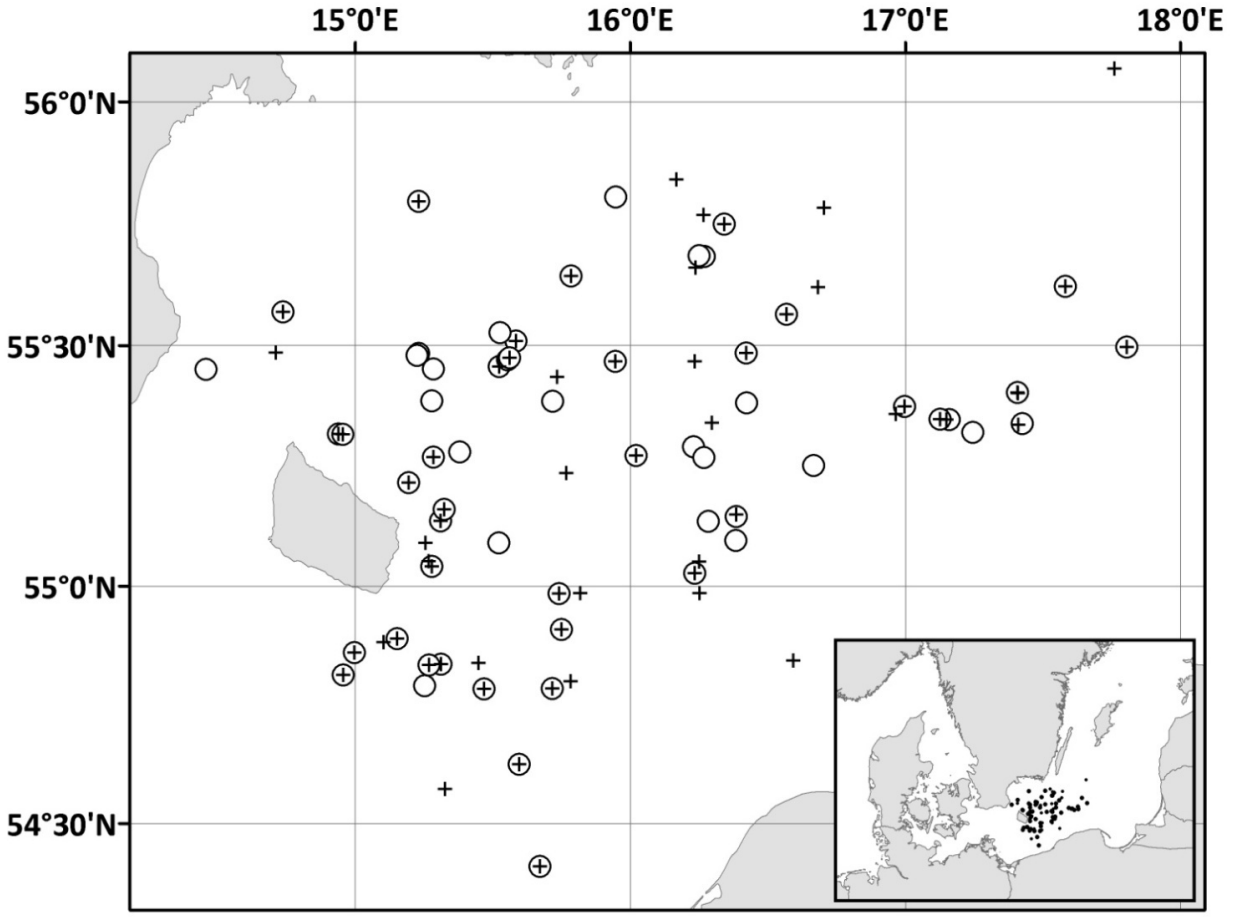


Fig. 1. Sampling location in the Bornholm Basin of the Central Baltic Sea i.e. ICES Sub-division 25, grid lines and codes indicate squares (ICES Statistical Rectangles). In total *Sprattus sprattus* were sampled at 60 trawl stations (circles) and *Clupea harengus* (pluses) at 62 trawl stations.

In total, samples from 12 research cruises and one from fisheries (Feb. 2009) were included. For each species fish were sampled at random from catches using a length based sampling design. Total length of each was measured to the nearest lower ½-cm and specimens were grouped according to length class. Isopods *S. entomon* of variables sizes were sampled in the Bornholm Basin in March 2009 and November 2010 from one and three stations, respectively.

Table 1. Number of stations, fish and lipid samples of *Sprattus sprattus* (a) and *Clupea harengus* (b) sampled by length class during three study periods. Sampling date refers to the median when sampling covered several days.

a

Sampling time Period	Date	Length class (mm)	Stations (no.)	Fish (no.)	Lipid samples
2002-2003	10. Nov	80-99	3	26	1
		100-149	2	18	1
	21. Jan	80-99	1	8	1
		100-149	3	33	1
	15. Mar	80-99	2	20	1
		100-149	2	20	1
	7. Jul	80-99	4	46	1
		100-149	5	53	1
2003-2004	12. Nov	80-99	3	30	2
		100-129	3	14	2
		130-149	3	16	2
	28. Jan	80-99	5	138	4
		100-129	5	78	4
		130-149	5	35	4
	18. Mar	80-99	6	63	5
		100-129	6	27	5
		130-149	6	29	5
	6. Jun	80-99	2	66	2
		100-129	3	93	3
		130-149	3	63	3
		Total no.	72	876	49

Sampling time Period	Date	Length class (mm)	Stations (no.)	Fish (no.)	Lipid samples
2008-2009	13. Nov	80-89	5	17	1
		90-99	4	14	1
		100-109	7	11	2
		110-119	9	19	2
		120-129	9	19	2
	9. Feb	80-89	3	34	3
		90-99	2	30	2
		100-109	2	30	2
		110-119	3	34	3
		120-129	3	44	3
	11. Mar	80-89	18	30	3
		90-99	13	34	2
		100-109	9	20	2
		110-119	13	28	2
		120-129	11	29	2
	26. May	90-99	2	20	1
		100-109	4	43	2
		110-119	4	35	2
		120-129	7	52	4
	7. Aug	110-119	6	27	2
		120-129	12	53	2
		Total no.	146	623	45

b

Sampling time Period	Date	Length class (mm)	Stations (no.)	Fish (no.)	Lipid samples
2002-2003	10. Nov	100-149	2	18	1
	21. Jan	100-149	3	19	1
		150-199	3	16	1
	15. Mar	100-149	2	20	1
		150-199	2	20	1
	7. Jul	100-149	2	6	1
		150-199	5	52	1
2003-2004	12. Nov	100-124	2	8	2
		125-149	2	12	2
		150-174	3	13	3
		175-199	3	29	3
		200-225	1	19	1
		225-229	1	3	1
	28. Jan	100-124	3	19	1
		125-149	3	32	1
		150-174	4	27	2
		175-199	4	41	2
		200-225	4	28	2
		225-229	3	5	2
	18. Mar	80-99	3	3	2
		100-124	6	29	5
		125-149	6	27	5
		150-174	6	18	5
		175-199	6	35	5
		200-225	1	5	1
		Total no.	80	504	52

Sampling time Period	Date	Length class (mm)	Stations (no.)	Fish (no.)	Lipid samples
2008-2009	13. Nov	130-139	3	10	1
		145-154	7	15	1
		160-169	6	10	1
		175-184	7	11	2
		190-199	7	10	2
		205-214	5	13	2
		220-229	8	13	2
	11. Mar	115-124	3	7	1
		130-139	5	17	1
		145-154	6	19	2
		160-169	10	18	4
		175-184	12	18	3
		190-199	10	20	2
		205-214	15	25	3
		220-229	11	18	3
	26. May	145-154	4	17	4
		160-169	3	16	3
		175-184	13	34	4
		190-199	10	57	3
		205-214	6	45	2
		220-229	5	27	2
	7. Aug	145-154	1	5	1
		160-169	9	36	2
		175-184	12	58	2
		190-199	11	48	2
		205-214	17	48	4
		220-229	9	26	4
		Total no.	215	641	63

Sampled sprat, herring and isopods were stored at -40°C for later lipid analysis. In the laboratory, sprat and herring were thawed, and whole body weight of individual fish was recorded (g). Fish were sexed and the gonadal maturity determined from macroscopic characters. Herring maturity

Table 2. Clasification of the 8-level maturity stages of herring and 6-level scale of sprat.

	Herring	Sprat
Immature	I - II	I
Ripening	III - V	II
Spawning	VI	III_{a+b} - IV
Regeneration	VII	V
Abnormal	VIII	VI

was classified according to an 8-level scale (Bucholtz et al. 2008) and sprat with successive batches, maturity was staged according to a 6-level scale (ICES 2011b) with stage III subdivided into III_a re-ripening and III_b active spawning with running gametes (Table 2). In both species, smaller specimens that could not be staged macroscopically were assigned to stage I.

The sampling strategy during 2002-2004 focused variation among species and length classes as well as between locations i.e. squares (ICES Statistical Rectangles, Fig. 1) within Sub-division 25. Sex and maturity was assessed to identify the progression of the reproductive cycle starting in autumn with spawning in spring-summer (Table 3a), and sampled fish were combined in length classes by month in the lipid analysis. Sampling in 2008-2009 focused on resolving the influence of sex and maturity stages on the seasonal variation in lipid content and composition within species and between length classes. Sampled sprat and herring were separated according to sampling month, length class, sex and maturity. Sprat maturity stage MII and MIIIa were combined as they are both characterized by vitellogenic eggs for females and morphology is similar also for males, while stage IIIb characterized by hydrated eggs was kept separate (Table 3b). For herring maturity stage MIV and MV were similarly grouped in the lipid analysis as both stages are characterized by a progressed vitellogenesis and for males progressed development. Specimens *S. entomon* were separated according to sampling month and station and pooled irrespective of size for lipid analysis.

Analyses of lipid content and fatty acid methyl esters

Fish and isopods were cut into small pieces and frozen with liquid nitrogen and blended. Lipids were extracted from 10 g blended fish with a homogeneous mixture of chloroform, methanol and water (2:2:1.8) following the method of Bligh & Dyer (1959). Lipid content was determined by gravimetry after evaporation of chloroform and is expressed as percentage of thawed wet weight (ww) of fish sample throughout the paper. Lipid extracts were used for the preparation of fatty acid methyl esters using AOCS Official Method Ce 2-66 (1998). C23:0 methyl ester was used as an internal standard.

Table 3. Distribution of sampled *Sprattus sprattus* and *Clupea harengus* on sex and maturity class per month for the three periods 2002-2003 and 2003-2004 (a) and 2008-2009 (b). During 2002-2004 fish were subsequently pooled by length class and ICES squares for lipid analysis. During 2008-2009 the sexed and staged fish were pooled according to fish length class, sex and maturity. Numbers in parenthesis refer to the number of pooled lipid samples analysed. Juvenile specimens where sex was not distinguished formed a separate group. Maturity stages for sprat (a): MI = immature, MII = maturing, IIIa = rematuring, MIIIb = active spawning, MIV = spent and MV = abnormal. Maturity stages for herring (b): MI = immature, MII = early ripening, MIII = mid ripening, MIV + MV = late ripening, MVI = spawning, MVII = spent and MVIII = abnormal

a

Sampling time		No. of female sprat					No. of male sprat					No sex	Total no.
Period	Month	I	II	III_{a+b}	IV	V	I	II	III_{a+b}	IV	V	I	I - VI
2002-	Nov	11	6	0	0	2	3	1	0	0	0	21	44 (2)
2003	Jan	7	16	0	0	0	5	13	0	0	0	0	41 (2)
	Mar	5	6	4	0	0	10	10	0	0	0	5	40 (2)
	Jul	9	18	16	6	1	10	33	2	4	0	0	99 (2)
2003-	Nov	6	18	0	0	0	6	11	0	0	0	19	60 (6)
2004	Jan	21	54	24	0	0	3	106	41	1	0	1	251 (12)
	Mar	27	24	15	3	0	13	19	13	0	0	5	119 (15)
	Jun	40	3	50	2	0	35	12	62	4	0	14	222 (8)
Total no.		126	145	109	11	3	85	205	118	9	0	65	876 (49)

Sampling time		No. of female herring					No. of male herring					No sex	Total no.
Period	Month	I	II	III	IV+V	VII	I	II	III	IV+V	VII	I	I - VIII
2002-	Nov	0	0	0	0	0	0	0	0	0	0	18	18 (1)
2003	Jan	7	3	1	0	0	4	0	1	0	0	19	35 (2)
	Mar	12	1	5	2	0	1	0	3	3	0	13	40 (2)
	Jul	22	2	0	0	2	24	3	0	0	4	1	58 (2)
2003-	Nov	13	27	5	0	0	5	7	5	7	0	15	84 (12)
2004	Jan	38	27	10	1	0	15	1	0	18	0	42	152 (10)
	Mar	26	16	8	0	0	14	6	1	9	0	37	117 (23)
Total no.		118	76	29	3	2	63	17	10	37	4	145	504 (52)

b

Sampling time		No. of female sprat					No. of male sprat					No sex	Total no.
Period	Month	I	$II+III_a$	III_b	IV	V	I	$II+III_a$	III_b	IV	V	I	I - VI
2008-	Nov	6(1)	16(2)	0	0	0	0	27(3)	0	0	0	31(2)	80 (8)
2009	Feb	0	75(5)	14(1)	4(1)	0	0	75(5)	0	0	0	4(1)	172 (13)
	Mar	0	49(5)	0	0	0	0	75(5)	0	0	0	17(1)	141 (11)
	May	0	30(3)	20(1)	0	0	0	97(4)	3(1)	0	0	0	150 (9)
	Aug	0	40(2)	0	0	0	0	40(2)	0	0	0	0	80 (4)
Total no.		6	210	34	4	0	0	314	3	0	0	52	623 (45)

Sampling time		No. of female herring					No. of male herring					No sex	Total no.
Period	Month	I	II	III	IV+V	VII	I	II	III	IV+V	VII	I	I - VIII
2008-	Nov	0	38(5)	8(1)	0	0	0	11(3)	0	0	0	25(2)	82 (11)
2009	Mar	7(2)	0	8(2)	45(5)	0	4(1)	0	0	28(4)	3(1)	47(4)	142 (19)
	May	0	5(1)	0	12(3)	80(6)	4(1)	8(2)	0	0	87(5)	0	196 (18)
	Aug	0	109(6)	6(2)	3(1)	0	0	97(5)	0	6(1)	0	0	221 (15)
Total no.		7	152	22	60	80	8	116	0	34	90	72	641 (63)

Fatty acid methyl esters (FAME) were analysed on a HP 5890A gas chromatograph (Hewlett-Packard, Palo Aalto, CA) equipped with an Omegawax 320 (30 m x 3.2 mm x 0.25 μm) column from Supelco (Bellefonte, PA) using AOCS method Ce 1b-89 (1998). The oven temperature programme was 15°Cmin^{-1} to 160°C, hold 2 min, 3°Cmin^{-1} to 200°C, hold 1 min and 3°Cmin^{-1} to 220°C, hold 17 min. A split ratio of 1:25 was used. Fatty acids were identified by comparison of retention times with a mixture of standards containing all the fatty acids identified in this study. Each fatty acid was quantified by calculating its peak area relative to the total peak area or relative to the internal standard. These values are referred to as fatty acid proportion of total lipid (%) or absolute content per gram fish (mg g^{-1}) throughout the paper.

Statistical analyses

Multivariate principal component analysis (PCA) was performed using the Unscrambler® v9.1 (CAMO, Oslo, Norway) software. Fatty acid variables were column mean centred and normalised to unit column standard deviation (SD) before calculation of the models and models were validated by full cross-validation. A PCA was performed on all samples comparing the overall lipid composition of the two species including information on 27-37 identified FAs, sum and ratios of FAs, lipid content, sampling month and period (years from autumn to summer). For the data from 2008 - 2009 separate PCAs were performed for herring and sprat including FAs, sum and ratios of FAs, lipid content, sampling month, length class, sex and maturity stage. Statistical differences in lipid and fatty acid content in sprat and herring data from 2003 - 2004 and 2008 - 2009 were calculated with R version 2.12. The data were checked for normal distribution with a residual analysis.

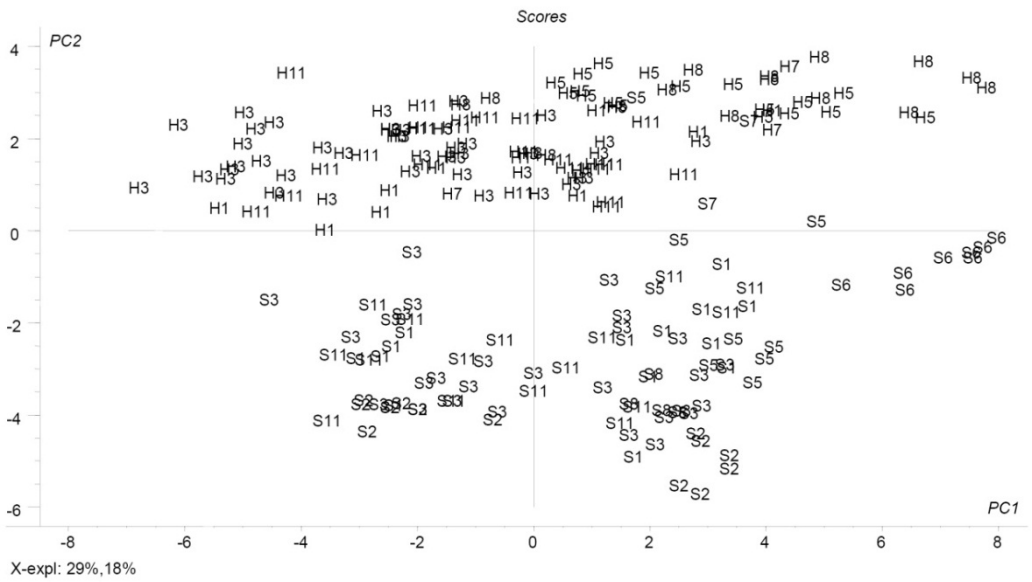
Multi-way analysis of variance (ANOVA) was used to analyse the influence of sampling month, length class, and location of sample stations (squares) in data from the period; November 2003 to June 2004 and differences between sampling month, length class, sex, and maturity stage in sprat and herring samples from November 2008 to August 2009. Analysed samples where sex was not determined were excluded from the ANOVA. The ANOVA was supported by multiple comparison tests. A significance level (p) of 0.05 was applied in all tests. Due to the low number of samples only first order terms were included in the ANOVA and data from the period; November 2002 to July 2003 were excluded. Due to the different size classes and sampling design between the three study periods no attempt was carried out to make statistical comparison between years.

RESULTS:

Comparison between sprat, herring and *Saduria entomon*

In total 1499 sprat and 1145 herring were included in this study, which were pooled into 94 sprat samples and 115 herring samples for lipid analysis (Table 1 and 3). The sampling was distributed relatively evenly over different months within periods. Size intervals were refined during the study to enhance coverage of pronounced peaks and lows in the size distributions.

a



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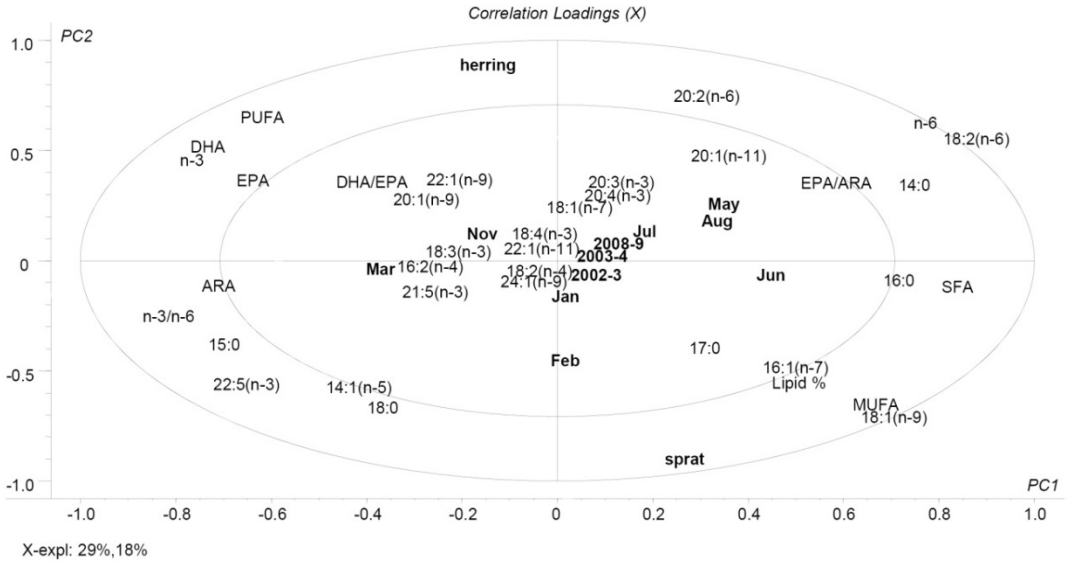


Fig. 2. PCA scores plot (a) and loadings plot (b) of variation in lipid content (% of ww), FA content of total lipid and summed fatty acid groups (% of total fatty acids) in all pooled lipid samples between *Sprattus sprattus* (S) and *Clupea harengus* (H). Related numbers indicate sampling month. Sprat: n = 94 and herring: n = 115. SFA = Saturated fatty acids, MUFA = Monounsaturated fatty acids, PUFA = Polyunsaturated fatty acids, DHA = docosahexaneoic acid (22:6n-3), EPA = eicosapentaenoic acid (20:5n-3) and ARA = arachidonic acid (20:4n-6).

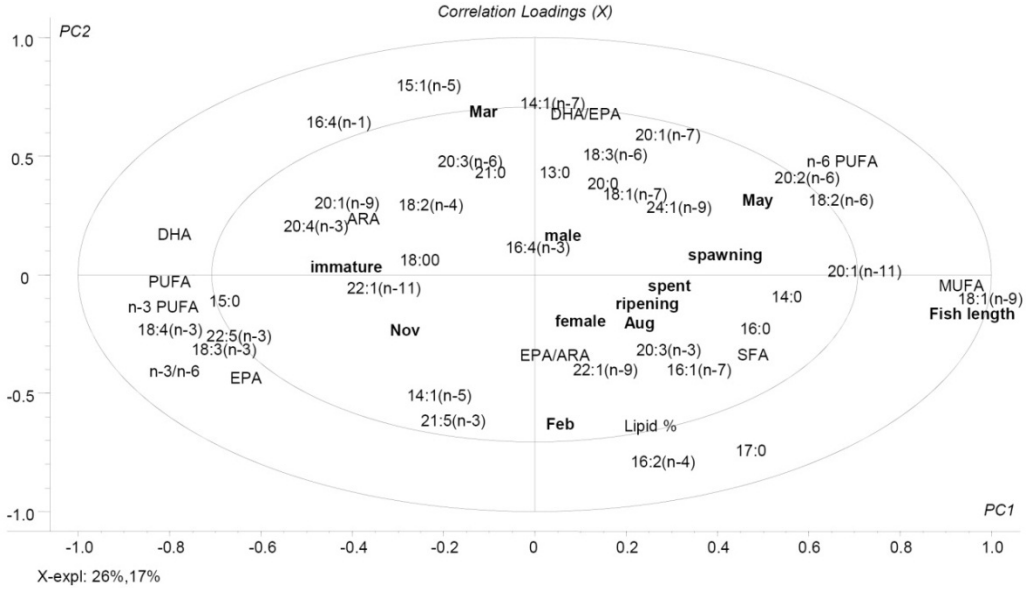
The lipid composition differed significantly between sprat and herring samples (Fig. 2). The PCA analyses of all herring and sprat samples explained 47% (PC1 + PC2) of the variation in samples. The difference in FAC is reflected in the PCA scores plot where herring and sprat samples are placed in two entirely separate groups, except for two samples of sprat located amongst herring samples (Fig. 2a). Differences between species were mainly expressed by PC2; with herring samples located in 1st and 2nd quadrant and sprat samples dominating the 3rd and 4th quadrant (Fig. 2b). The seasonal variation was expressed by PC1 with most samples from May to August located in the 1st quadrant for herring and 4th quadrant for sprat. Differences between periods (years from autumn to summer) were of minor importance compared to differences between species and seasonal variation, since periods are all placed close to the center of the loadings plot (Fig. 2b). However the lack of difference between periods could not be tested with ANOVA due to the different sampling design. Herring samples were characterized by high proportions of total PUFA mainly due to DHA and EPA while lipid content and proportions of MUFA was low. In contrast sprat samples had high lipid content and proportions of total MUFA mainly due to high levels of 16:1n-7 and 18:1n-9, but low PUFA including DHA and EPA. Proportions of SFA and n-6 PUFA varied between seasons (Fig. 2b).

Table 4 shows average FAC of the length groups most frequently encountered by predators, 120 mm for sprat and 205 mm for herring in different months from November 2008 and to August 2009 and include information about *Saduria entomon* sampled in March 2009 and November 2010. The most abundant fatty acids were 16:0, palmitoleic acid 16:1n-7, oleic acid 18:1n-9, EPA and DHA in all three species but the proportions differed between species and season. The ratio between 18:1n-9 and DHA was on average 1.6 in *S. entomon*, 1.4 in sprat and 0.8 in herring whereas the 16:1n-7/16:0 ratio was on average 0.9 in *S. entomon* and 0.2 in both clupeid species. Furthermore *S. entomon* had low proportion of SFA but high proportion of PUFA particularly EPA and ARA and the proportion of ARA varied between 2.2 and 5.0% in *S. entomon* in November and March compared to only 0.7-0.9% in sprat and herring.

Table 4. FAC of total lipid (% of total fatty acids) in pooled samples of whole 120-129 mm *Sprattus sprattus* and 205-209 mm *Clupea harengus* sampled in November 2008, and March and May 2009 as well as *Saduria entomon* sampled in March 2009 and November 2010. Females and males were grouped and values are means ± SD of all samples across sex and maturity stage. Only fatty acids with a proportion higher than 1% in at least one sampling are included, which account for around 88-90% of the total fatty acids.

	Sprattus sprattus					*Clupea harengus*				*Saduria entomon*	
	Nov	Feb	Mar	May	Aug	Nov	Mar	May	Aug	Nov	Mar
Fatty acid	(n = 2)	(n = 3)	(n = 2)	(n = 4)	(n = 2)	(n = 2)	(n = 3)	(n = 2)	(n = 4)	(n = 3)	(n = 1)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean	Mean
SFA											
14:0	3.7 ± 0.1	3.7 ± 0.2	3.9 ± 0.1	3.7 ± 0.2	3.8 ± 0.1	5.0 ± 0.5	3.6 ± 0.4	5.5 ± 0.1	5.5 ± 0.4	3.3	0.9
16:0	18.9 ± 0.2	19.0 ± 0.2	18.3 ± 0.0	18.9 ± 0.8	19.1 ± 0.1	19.1 ± 0.1	19.1 ± 0.7	20.4 ± 0.0	19.3 ± 0.4	13.4	12.6
18:0	2.2 ± 0.1	2.4 ± 0.3	2.3 ± 0.1	2.4 ± 0.3	2.5 ± 0.2	1.6 ± 0.1	2.1 ± 0.1	1.7 ± 0.1	1.3 ± 0.0	2.1	2.1
MUFA											
16:1n-7	4.6 ± 0.2	4.8 ± 0.4	4.7 ± 0.3	4.1 ± 0.6	4.6 ± 0.2	4.6 ± 0.2	3.8 ± 0.3	3.9 ± 0.0	4.1 ± 0.4	13.4	6.2
18:1n-9	21.3 ± 2.2	26.2 ± 1.0	26.1 ± 0.6	24.7 ± 1.2	22.5 ± 0.3	14.2 ± 0.4	15.2 ± 1.0	17.4 ± 0.1	15.9 ± 1.9	17.9	18.9
18:1n-7	2.6 ± 0.1	2.8 ± 0.1	2.6 ± 0.0	2.7 ± 0.1	2.6 ± 0.0	2.4 ± 0.0	3.2 ± 0.6	2.8 ± 0.0	2.7 ± 0.2	3.5	4.1
20:1n-7+9	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	3.0	1.3
20:1n-11	0.4 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.1	1.1	0.7
24:1n-9	0.7 ± 0.9	1.7 ± 0.1	1.7 ± 0.0	1.8 ± 0.2	1.1 ± 0.1	1.6 ± 0.0	1.6 ± 0.2	1.4 ± 0.0	1.4 ± 0.0	0.0	0.0
PUFA											
18:2n-6	2.5 ± 0.0	2.8 ± 0.2	3.1 ± 0.2	4.2 ± 0.2	2.6 ± 0.4	3.8 ± 0.1	3.1 ± 0.1	5.6 ± 0.1	5.3 ± 0.3	2.3	2.1
18:3n-3	2.7 ± 0.1	2.5 ± 0.2	2.4 ± 0.0	2.4 ± 0.2	2.7 ± 0.1	3.0 ± 0.2	1.9 ± 0.2	2.8 ± 0.0	3.3 ± 0.3	1.0	1.4
18:4n-3	2.5 ± 0.1	1.9 ± 0.1	1.7 ± 0.0	1.8 ± 0.1	2.3 ± 0.2	2.5 ± 0.0	1.6 ± 0.2	2.9 ± 0.1	3.3 ± 0.3	1.9	1.6
20:4n-6	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.2	0.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.9 ± 0.1	0.5 ± 0.1	0.4 ± 0.0	2.2	5.1
20:5n-3	9.5 ± 0.6	7.5 ± 0.1	6.9 ± 0.1	6.7 ± 0.2	10.4 ± 0.6	9.8 ± 0.2	9.3 ± 0.2	8.1 ± 0.2	9.1 ± 0.8	14.4	18.9
22:6n-3	14.9 ± 0.6	12.6 ± 0.5	13.0 ± 0.1	14.3 ± 1.6	13.1 ± 0.0	20.2 ± 0.8	23.1 ± 2.5	15.7 ± 0.7	16.8 ± 0.9	10.2	14.8
Total SFA	26.1 ± 0.1	26.4 ± 0.5	25.8 ± 0.1	26.2 ± 1.0	26.5 ± 0.0	26.7 ± 0.3	25.5 ± 0.8	28.4 ± 0.2	27.0 ± 0.6	19.6	16.4
Total MUFA	30.6 ± 0.9	37.1 ± 0.8	36.9 ± 0.1	34.9 ± 1.5	31.9 ± 0.7	24.8 ± 0.3	25.8 ± 1.1	27.0 ± 0.3	25.6 ± 1.5	39.3	30.6
Total PUFA	35.9 ± 1.7	30.7 ± 0.8	30.7 ± 0.1	32.4 ± 1.8	34.3 ± 0.1	42.9 ± 1.0	43.3 ± 1.9	38.4 ± 0.8	41.2 ± 1.7	35.5	46.1
n-3/n-6	8.4 ± 0.2	6.6 ± 0.2	5.8 ± 0.0	5.0 ± 0.1	7.5 ± 0.7	6.8 ± 0.3	7.9 ± 0.5	4.6 ± 0.2	5.3 ± 0.3	4.6	3.9
EPA/ARA	13.1 ± 0.4	13.1 ± 1.6	10.7 ± 2.7	14.2 ± 1.2	14.6 ± 1.0	13.3 ± 0.0	10.4 ± 0.8	16.9 ± 2.9	25.1 ± 1.8	6.5	3.7
DHA/EPA	1.6 ± 0.0	1.7 ± 0.1	1.9 ± 0.0	2.1 ± 0.2	1.3 ± 0.1	2.1 ± 0.0	2.5 ± 0.3	1.9 ± 0.0	1.9 ± 0.1	0.7	0.8
18:1n-9/DHA	1.4 ± 0.2	2.1 ± 0.2	2.0 ± 0.1	1.7 ± 0.3	1.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.1	1.1 ± 0.1	1.0 ± 0.2	1.8	1.3
16:1n-7/16:0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	1.0	0.5

a



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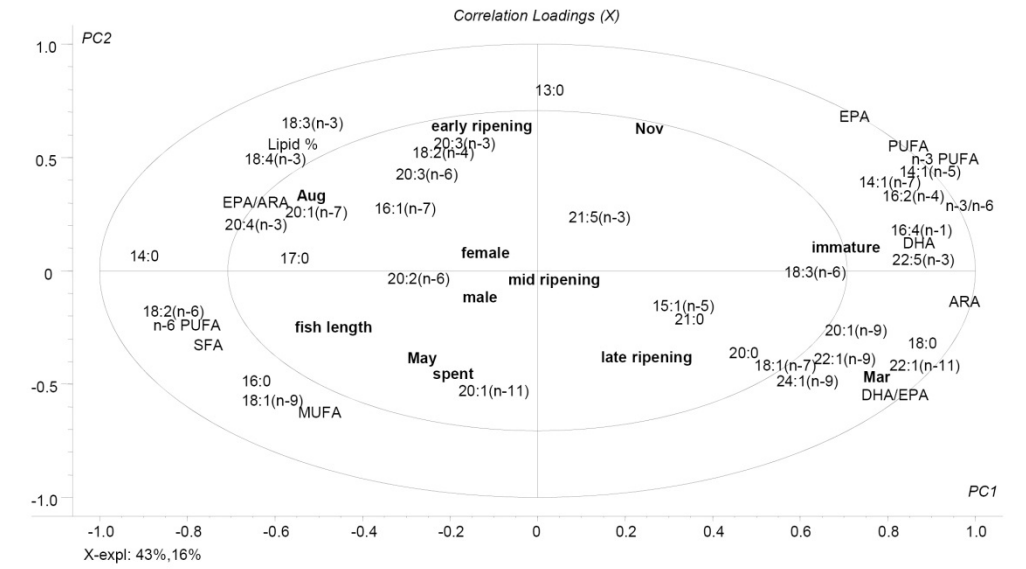

Fig. 3. PCA loadings plot illustrating the relationship between lipid content (% of ww), FA content of total lipid and summed fatty acid groups (% of total fatty acids) in pooled samples of *Sprattus sprattus* (a) and *Clupea harengus* (b) collected in November 2008, February, March, May and August 2009. n = 45 (a) and n = 63 (b). See Fig. 2 for abbreviations.

Variation in FAC of sprat and herring

The PCA loadings plot of the 45 sprat samples from 2008-2009 in Figure 3a shows that season, size, and maturity explained 43% of the variation between samples, while females and males differed only little. High proportions of n-3 PUFA, DHA, EPA, ARA, 18:3(n-3) and 18:4(n-3) were observed in November relative to larger ripening and spawning sprat in May where high

proportions of 18:2(n-6), 20:2(n-6) were found. Big sprat had high proportions of MUFA especially 18:1(n-9). Herring showed substantial seasonal variation in lipid content and FAC (Fig. 3b). PCA loadings plot of the 63 herring samples from 2008-2009 showed that season, size and maturity explained 59% of the variation between samples whereas variation with sex was negligible (Fig.

Table 5. Results of ANOVA analysis of variation in fish body weight, lipid contents, proportions and absolute values of six important fatty acids in pooled samples of *Sprattus sprattus* and *Clupea harengus* in relation to month of sampling, length class and area of sampling i.e. ICES squares in 2003 – 2004 samples (a) and month, length class, sex and maturity stage in 2008–2009 (b). Fish without sex determination were excluded from the ANOVA. (a) n = 41 (sprat) and n = 45 (herring) and (b) total body weight: n = 623 (sprat) and n = 641 (herring), lipid content and fatty acids and n = 41 (sprat) and n = 57 (herring).

a

	Sprattus sprattus						*Clupea harengus*					
Fatty acid	Month		Size		Square		Month		Size		Square	
Degree of freedom	3		2		7		3		3		7	
	F	p	F	p	F	p	F	p	F	p	F	p
Lipid (% of ww)	19.9	***	7.6	**	0.2		30.1	***	8.2	***	2.4	*
FA (% of total FA)												
16:0	10.5	***	0.2		1.1		13.6	***	2.5	*	2.1	
18:1n-9	3.1	*	82.6	***	0.5		3.4	*	16.6	***	3.0	*
18:2n-6	107	***	25.4	***	1		25.4	***	23.3	***	7.7	***
20:4n-6	26.3	***	0.2		1.2		7.7	***	1.2		4.4	**
20:5n-3	6.3	**	27	***	3.1	*	3.6	*	6.4	***	1.9	
22:6n-3	28	***	33.6	***	0.1		11.5	***	8.1	***	1.3	
FA (mg g-1 ww)												
16:0	14.4	***	4.6	*	2.2		34.9	***	6.8	***	2.1	
18:1n-9	9.3	***	12.2	***	2.3		29.5	***	12.8	***	2.3	
18:2n-6	23.9	***	6.7	**	1.5		28.8	***	13.1	***	3.3	*
20:4n-6	13.1	***	9.4	***	5.7	***	30.5	***	6.8	***	2.3	
20:5n-3	23	***	2.6		3.3	*	32.8	***	4.8	**	1.6	
22:6n-3	17.1	***	2.5		3.1	*	26.0	***	4.8	**	3.2	

b

	Sprattus sprattus								*Clupea harengus*							
	Month		Size		Sex		Maturity		Month		Size		Sex		Maturity	
Degree of freedom	4		4		1		3		3		5		1		4	
	F	p	F	p	F	p	F	p	F	p	F	p	F	p	F	p
Total weight (g ww)	47.9	***	784	***	10.4	**	1.2		56.6	***	1032	***	15.7	***	40.3	***
Lipid (% of ww)	79.3	***	18.2	***	0.6		0.8		78	***	2.4		0.3		4.2	**
FA (% of total FA)																
16:0	4.8	**	1.3		1.01		1.9		13.4	***	8.2	***	0.5		0.4	
18:1n-9	7	***	65.5	***	0.11		1.8		6.1	**	17	***	5.1	*	1.9	
18:2n-6	31.4	***	0.7		0.1		0.3		94.4	***	16.5	***	0.5		0.2	
20:4n-6	17.4	***	3.3	*	0.0		0.3		46.3	***	4.6	**	0.8		0.4	
20:5n-3	46.8	***	37.4	***	0.8		1.7		11.4	***	15.9	***	3.2		1	
22:6n-3	4.7	**	27.9	***	0.6		0.9		41.2	***	18.3	***	0.2		0.3	
FA (mg g-1 ww)																
16:0	33	***	7.2	***	5	*	0.8		84.6	***	4.2	**	4.3	*	3.5	*
18:1n-9	18.6	***	16.1	***	2.6		0.3		75.6	***	7.2	***	1.5		3.3	*
18:2n-6	12.4	***	5.8	**	6.3	*	0.8		57.1	***	6	***	3.4		3.4	*
20:4n-6	63.2	***	6.9	***	2.9		1.6		76.5	***	1.6		0.4		0.7	
20:5n-3	88.7	***	3.3	*	5.7	*	1.3		103	***	1.3		6.7	*	4.1	**
22:6n-3	40.6	***	3.0	*	4.2	*	1.2		95.4	***	0.8		4.4	*	2.4	

*p<0.05, **p<0.01, ***p<0.001

3b). As in sprat, high proportions of n-3 PUFA and ARA were observed in herring in November and in immature fish relative to spawning sprat in May where high proportions of MUFA were observed. Herring length was correlated with increase in proportions of SFA and MUFA and decrease in proportion of PUFA.

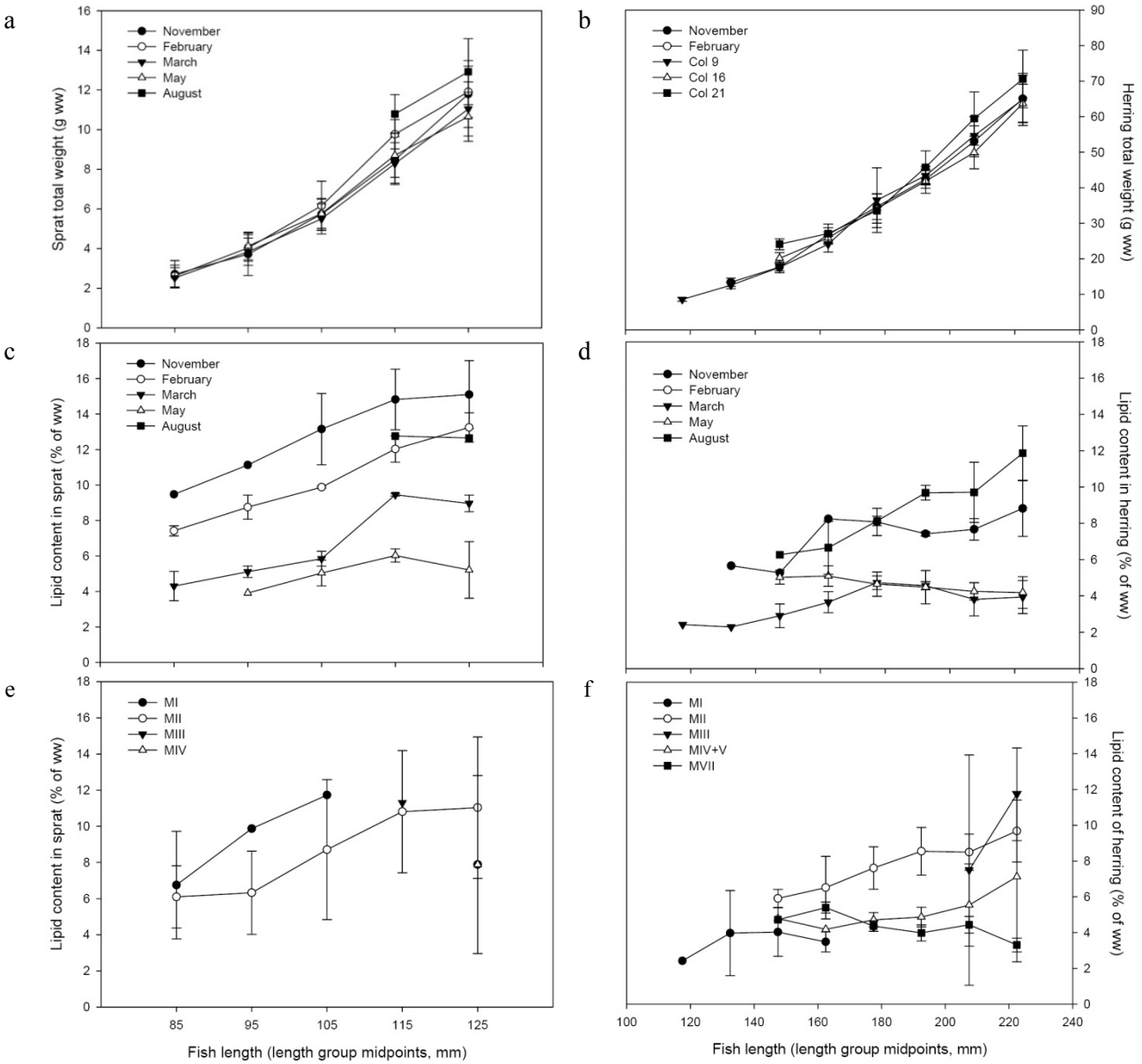

Fig. 4. Average total body weight (g ww) of *Sprattus sprattus* (a) and *Clupea harengus* (b) and lipid content (% of ww) in pooled lipid samples of *Sprattus sprattus* (c and e) and *Clupea harengus* (d and f) from 2008 – 2009 in relation to fish length class (midpoint, mm) in months (c, d) and maturity stages (e, f). Females and males were grouped and values are means ± SD, n = 623 (a), n = 641 (b), n = 45 (c and e) and n = 63 (d and f).

The multi-way ANOVA on lipid samples from 2003 – 2004 showed a significant variation in lipid content and most of the important fatty acids between months and length classes in lipid samples, while the location of the station sampled in the Bornholm Basin (ICES squares) in general had no or little influence on lipid content and FAC (Table 5a). In data from 2008 - 2009 location was therefore not considered, and the analysis focused on lipid content and FAC in relation to sex and maturity besides seasonal influence (month) and length class for both species (Table 5b). The ANOVA showed that average bodyweight varied significantly with month, size and sex for both

species and for herring, weight furthermore varied with maturity. Lipid content and most of the important fatty acids also varied significantly with month and length class for both species in 2008-2009. No significant differences in lipid content and FAC were found between male and female for neither sprat nor herring except for the proportion of 18:1n-9 and a few minor FAs in herring; males and females were accordingly grouped in Fig. 4 and Table 4. Variation with maturity was only significant for total weight, lipid content and absolute values of important fatty acids in herring. The analysis of influence of maturity was hampered by the inability to distinguish sex in small specimens and in sprat the limited variation in stages among sampled specimens (Table 3).

Seasonal and size related differences in total weight and lipid content

In Fig. 4 the average body weight of sprat and herring and content of lipid in the pooled lipid samples are shown for each species by length group in the different seasons in 2008-2009. As the smallest length class was not represented in all months and sex often not determined, these samples were excluded from the ANOVA analysis for both species but included in Figure 4. Average total weight of sprat varied between 2.5-13 g ww for sprat measuring 80 to 130 mm (Fig. 4a) and in herring the total weight varied between 8 and 71 g ww for fish between 115 and 225 mm (Fig. 4b). The lipid level varied between 3.6 and 17.5% of ww in samples of sprat (Fig. 4c) and between 2.1 and 13.6% in samples of herring (Fig. 4 d). Pair wise comparisons showed that all months except August and November were significantly different in sprat ($p \leq 0.03$) and that lipid content in size 80 – 100 mm was significantly lower than 110 and 120 mm sprat ($p \leq 0.02$). Pair wise comparisons of herring samples showed that all months except March and May differed significantly ($p \leq 0.03$) and size 160 mm was significantly lower than 220 mm herring ($p = 0.05$).

Variation in lipid content in relation to maturity stages

The majority of sprat sampled was in maturity stage II and III with the onset of the spawning period in February-March and proceeding into August (Table 3). Lipid content and absolute levels of fatty acids did not vary with maturation in sprat (Table 5b) most likely due to the low number of samples in maturity stage I, III and IV. Samples of successive maturity stages from immature to spent were represented for herring (MVI) except the spawning stage though not in equal numbers. No spawning herring were caught in the samples as herring migrate to coastal spawning areas (Aro 1989). Herring with ripening gonads were primarily sampled in January and March and spent herring in May 2009 (Table 3). The majority of herring are spring spawners, which mature during

November to April, leave the basin to spawn around May and return as spent, while a low proportion are autumn spawners. Lipid content in samples from 2008 – 2009 in relation to maturity in different length groups is shown in Fig. 4e for sprat and in Fig 4f for herring. Lipid content varied significantly between maturity stages in herring (Table 5b) however in pair wise comparison only mid ripening (MIII) and spent fish (MVII) were significantly different ($p = 0.05$). Absolute levels of 16:0, 18:1n-9, 18:2n-6 and 20:5n-3 (EPA) varied also significantly with maturity stage in herring.

Variation in absolute PUFA content

The average lipid content and the absolute content of the essential PUFAs; DHA, EPA and ARA in selected comparable length classes of sprat and herring for the three periods are shown in Fig. 5. Lipid and EFA content varied seasonally with highest levels in November and decreased in general during winter and spring in both species (ANOVA, $p < 0.01$). Pair wise analysis of 2008-2009 data showed that lipid and EFA content in sprat decreased significantly from November to March and

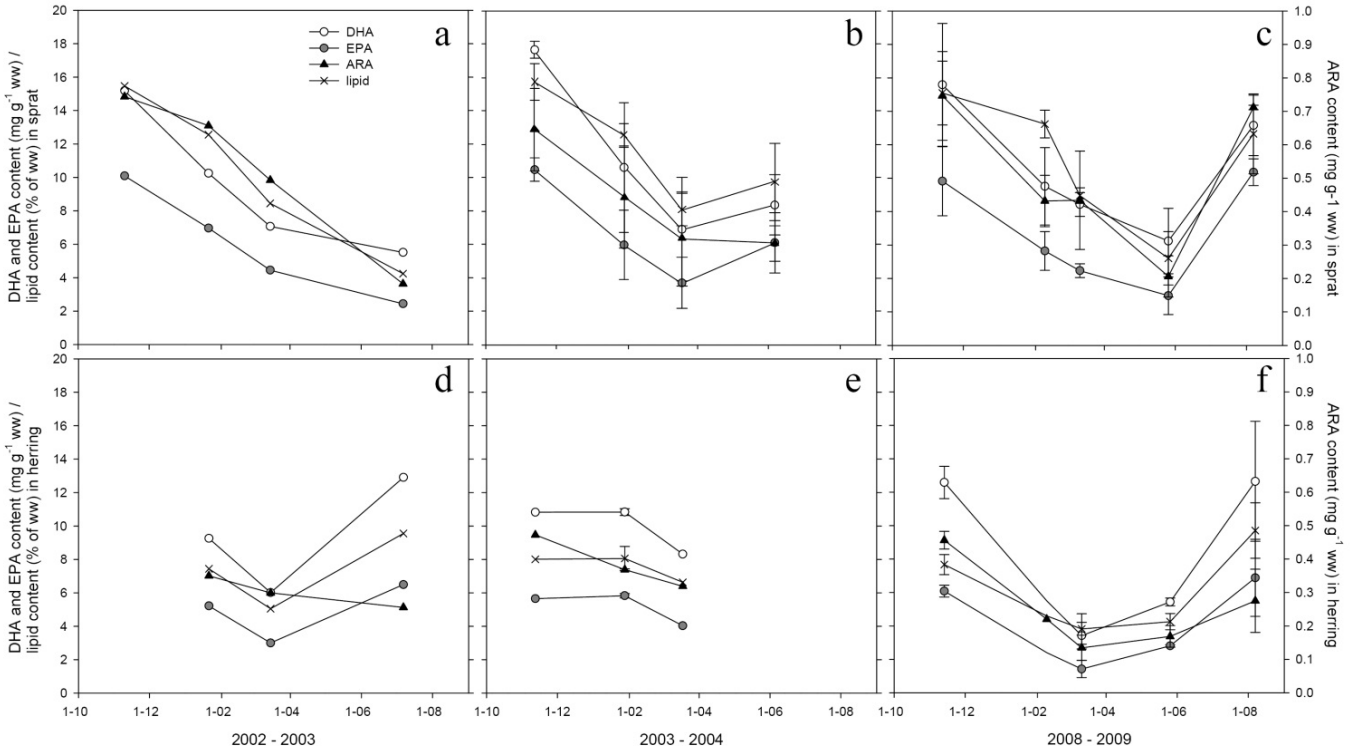

Fig. 5. Seasonal content of lipid (% of ww, bars) and absolute content of DHA, EPA and ARA (mg g^{-1} ww, line plots) in pooled lipid samples of *Sprattus sprattus* (a, b, c) and *Clupea harengus* (d, e, f) including comparable length classes sampled in 2002-2003 (a), 2003-2004 (b) and 2008-2009 (c). Illustrated length classes of sprat include 100-150mm (a), 100-130mm (b) and 120-130 (c) and for herring 150-200mm (a), 200-220 (b) and 205-210 (c). Values are means per length class ± SD, n = 4 (a), n = 14 (b), n = 13 (c), n = 3 (d), n = 4 (e) and n = 11 (f).

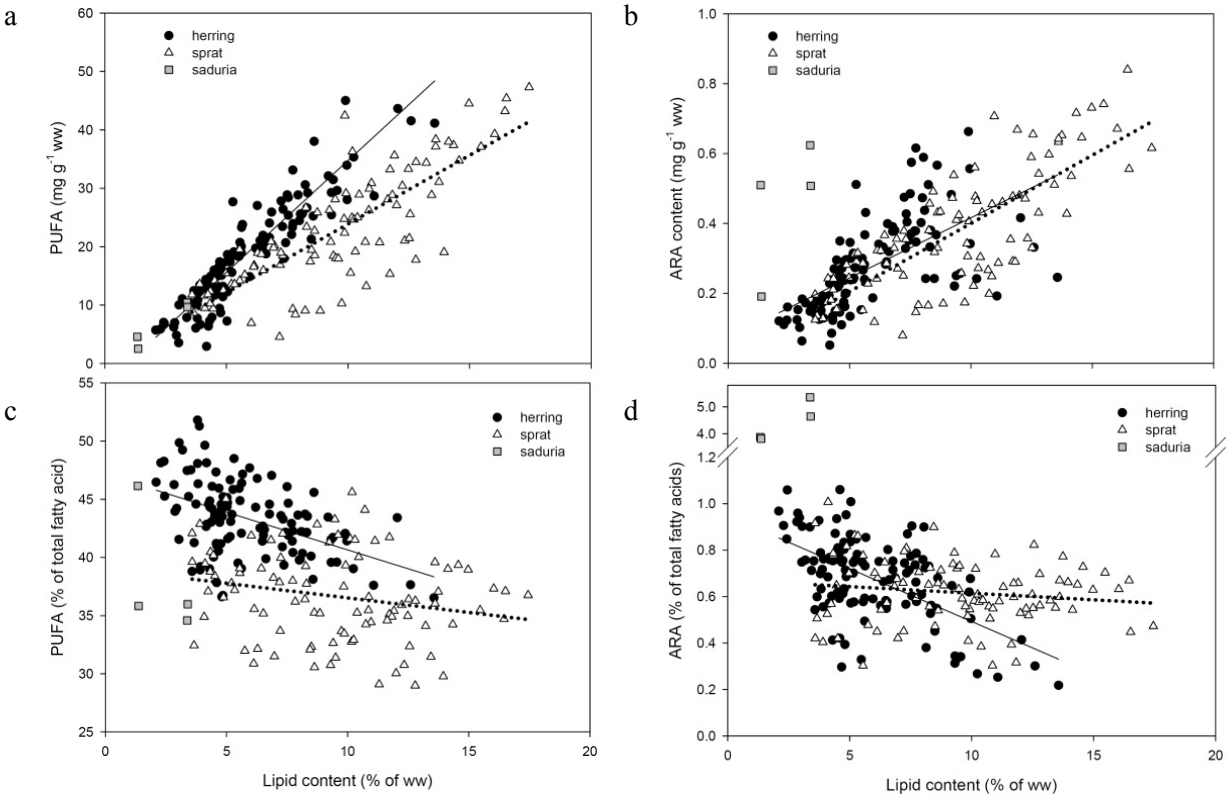

Fig. 6. (a) Content of PUFA (mg g^{-1} ww), (b) proportion of PUFA (% of total fatty acids), (c) ARA content (mg g^{-1} ww) and (d) ARA proportion (% of total fatty acids) in pooled samples of *Sprattus sprattus* (white triangles), *Clupea harengus* (black circles) and *Saduria entomon* (grey squares) in relation to lipid content (% of ww). Solid lines are regression lines for herring, dotted lines are regression lines for sprat. Herring: n = 115, $p < 0.001$ all plots, (a) $r^2 = 0.84$, (b) $r^2 = 0.22$, (c) $r^2 = 0.38$, (d) $r^2 = 0.33$. Sprat: n = 94, (a) $p < 0.001$, $r^2 = 0.66$, (b) $p = 0.03$, $r^2 = 0.05$, (c) $p < 0.001$, $r^2 = 0.60$, (d) $p < 0.001$, $r^2 = 0.02$ and *Saduria entomon*: n = 4.

May ($p < 0.05$) except for non significant decrease in DHA content from November to March and content of lipid and EFA increased again from May to August ($p < 0.05$). In herring lipid content and EFA was significantly lower in March than in November and August ($p < 0.05$) except for a non significant increase in ARA content from March to August. Some fatty acids varied in absolute content between sex and maturity stages, including DHA and EPA in both species (Table 5b). Absolute values of DHA was on average 1.0 mg g^{-1} higher in female than in male sprat ($p = 0.05$) and herring ($p = 0.04$) and values of EPA was on average 0.7 mg g^{-1} higher in female than in male sprat ($p = 0.02$) and herring ($p = 0.01$). Absolute content of ARA did not differ between sex and maturity stages.

Average lipid content was highest in sprat (9.1% of ww), intermediate in herring (6.0% of ww) and lowest in *S. entomon* (2.4% of ww). PUFA content (mg g^{-1} ww) was correlated with lipid content (%) (Fig. 6a, $p < 0.001$, $r^2 = 0.66$ for sprat and 0.92 for herring) but some sprat samples in spring and summer had very low level of ARA despite a high lipid level. The proportion of PUFA (Fig.

6b) was significantly negatively correlated with lipid content in herring ($p < 0.001$, $r^2 = 0.22$) while there was no correlation in sprat ($p = 0.03$, $r^2 = 0.05$). ARA content (mg g^{-1} ww) was positively correlated with lipid content (%) for both species (Fig. 6c, $p < 0.001$, $r^2 = 0.60$ for sprat and 0.38 for herring) while the proportion of ARA (Fig. 6d) was negatively correlated with lipid content in herring ($p < 0.001$, 0.33 for herring) but not in sprat ($r^2 = 0.02$). Despite low lipid levels *S. entomon* had a relatively high ARA level of 0.51 mg g^{-1} in March 2009 compared to sprat that varied between 0.25 and 0.43 mg g^{-1} and herring between 0.11 and 0.16 mg g^{-1} ARA.

DISCUSSION:

Differences in FAC between sprat, herring and Saduria entomon

In spite of a 50% dietary overlap between large sprat and small herring (Möllmann et al. 2004) a significant difference in FAC between sprat and herring was found. Herring has been observed to switch from feeding mainly on adult stages of *Pseudocalanus* sp. to feed mainly on young stages of *Temora longicornis* with increasing competition from sprat (Möllmann & Köster 2002). Results in the present study suggest that herring in contrast to sprat, indeed feeds more on *Temora longicornis* than *Pseudocalanus* sp. indicated by a high DHA/18:1n-9 ratio, with DHA being very abundant in *T. longicornis* compared to *Pseudocalanus* sp., which on the other hand are very rich in 18:1n-9 (Peters 2006, Peters et al. 2006). The difference between sprat and herring lipid composition can furthermore be related to the vertical distribution of both clupeid and zooplankton prey. *T. longicornis* and *Acartia* spp. inhabit the upper 30 m of the water column of Bornholm basin while older copepodid stages and adults of *Pseudocalanus* sp. inhabit the halocline and deep water layer (Hansen et al. 2006). In spring both sprat and herring prey mainly on copepods but in addition, sprat feeds on cladocerans in summer and autumn (Möllmann et al. 2004) and larger herring favour mysids, amphipods and polychaetes especially in autumn and winter (Casini et al. 2004). The different FAC in sprat and herring confirms significant differences in diet in both clupeids.

High proportions of PUFA especially DHA and very low proportions of 20:1 and 22:1 MUFA was found in sprat and herring in this study compared to sprat from Britain (Hardy & Mackie 1969) and herring from Scotland (Henderson & Almatar 1989) and Nova Scotia (Budge et al. 2002). High proportion of DHA was found in seston from the Baltic (Peters et al. 2006) compared to seston from the Dutch Wadden Sea (Koski et al. 2010). DHA in Baltic seston is coherent with high biomass of

dinoflagellates (Peters et al. 2006). High levels of 22:1(n-11) and 20:1(n-9) in North Sea herring is most likely due to the calanoid copepods rich in these fatty acids and abundant in the North Sea (Sargent & Falk-Petersen 1988, Kattner & Krause 1989b), which do not exist in the Baltic Sea. The lack of these MUFA will inherently increase the proportion of PUFA in Baltic clupeids. Levels of PUFA were high in Baltic clupeids compared to other ecosystems mainly due to differences in species and lipid composition of phyto- and zooplankton.

Despite low lipid levels, *Saduria entomon* had higher ARA content than sprat or herring in March. The low lipid content in *S. entomon* is consistent with studies of other crustaceans (Iverson et al. 2002). *S. entomon* is a benthic crustacean and in the Northern Baltic its diet has been found to consist mainly of deposit-feeding amphipods *Monoporeia affinis* (Haahtela 1990a, Englund et al. 2008). FAC of *M. affinis*, which feed on settled phytoplankton and detrital organic matter (Lahdes et al. 2010), is quite similar to FAC of *S. entomon*. The proportion of ARA was 2.2-3.7% in PL of *M. affinis* which is higher than in phytoplankton of the same area (Kaitaranta et al. 1986). The results from the present study indicate that *S. entomon* is potentially an important source of ARA in cod diet especially in periods with low lipid levels in clupeids. For assessing the importance of this prey species, however, it would be necessary to investigate the seasonal and spatial variability in abundance and lipid composition of this species as well as its presence in cod diet composition.

Seasonal variation in lipid content in sprat and herring

A seasonal variation in lipid content of sprat and herring, as observed in the present study,has previously been reported for whole Norwegian spring spawning herring (Slotte 1999) and in fillets of Baltic herring (Linko et al. 1985b, Aro et al. 2000). The high lipid levels found in the present study in November are consistent with the end of the annual zooplankton production cycle succeeded by a decline during winter when zooplankton abundance is low. Zooplankton biomass is highest in spring and summer in the south eastern Baltic Sea (Aleksandrov et al. 2009) and it is also in this period sprat and herring exhibit highest feeding activity (Möllmann et al. 2004). In spite of favorable feeding conditions in spring and summer, lipid content continued to decrease in sprat and herring. This could be due to seasonal variation in lipid content in zooplankton as low lipid content was observed in April in zooplankton from the Bornholm Deep in 1974 (Renk et al. 1985). Seasonal variation in zooplankton lipid composition is linked to variation in composition of phytoplankton and protists as well as changes in life stages, and reproductive cycles of zooplankton (Linko et al. 1985b, Peters et al. 2006, Peters et al. 2007). The low lipid content in herring in

summer can furthermore be due to energy spent on reproduction in spring. This is similar to a study of Pacific herring, where very low amounts of flesh lipids in spawning fish were observed in March (2.1% of ww) compared to non-spawning fish in September (10.8% of ww) (Huynh et al. 2007). The results from the present study suggest that season and hence the availability and composition of zooplankton is the main cause of the variation in lipid levels and composition in sprat and herring.
In addition to seasonal differences in zooplankton, variation in lipid composition of clupeids can be due to selective metabolism and retention of fatty acids in clupeids particularly during reproduction. Low ARA and total PUFA content was correlated with low lipid levels in both sprat and herring but the proportion of PUFA and ARA was highest in herring with lowest lipid content indicating that herring preferably used MUFA as an energy source, conserving PUFA important for reproduction. This was in agreement with the study of Pacific herring where spawning fish in March had very low amounts of flesh lipid but significantly higher proportions of DHA and ARA in organ tissues (roe, milt and liver) compared to non-spawning fish in September (Huynh et al. 2007). The increasing lipid level with size in sprat is likely a result of metabolic rate decreasing with body size because the variability in diet composition is low between different size classes (<10cm, 10-12cm and >12cm) of sprat (Möllmann et al. 2004). Lipid content did not increase linearly with size in herring in the present study, which is most likely due to interaction between size and maturity because maturity was often more progressed in larger herring. From a predator viewpoint, larger sprat are therefore nutritionally a better prey than herring in the period from November to March, while both clupeids are lean and low in absolute levels of EFA in May.

Effects of ecosystem changes on lipid storage of Baltic clupeids

Lipid content of whole herring in this study was comparable with levels found in fillet of Baltic herring caught in 1996-1998 (Aro et al. 2000) but much lower than medium sized Baltic herring fillet in June to October in 1976 before the regime shift in the Baltic sea (10.5 to 15.5% of wet weight, using a dry to wet weight conversion ratio of 5) (Linko et al. 1985b). Lipid content in Baltic herring was furthermore very low compared to whole spring spawning herring from Norway (Slotte 1999) and herring fillet from Scottish waters (Henderson & Almatar 1989) where the lipid content was between 18 and 26% from June to November. This low lipid content is most likely due to a combination of reduced amount of preferred zooplankton and changes in species composition of zooplankton associated with the decrease in salinity and increased temperature (Möllmann et al. 2008). Mysids, an alternative prey for herring, occur in low abundance in the central Baltic possibly

due to inaccessibility of the oxygen deficient bottom layer (Barz & Hirche 2009b). Lipid content was in general higher in sprat than in herring supporting the hypothesis that sprat outcompetes herring (Möllmann & Köster 2002) and indicating that low abundance of *Pseudocalanus* sp. and mysids influences lipid content in herring negatively. The low lipid content observed in herring is likely to affect its reproduction because, survival and hatching success of eggs were positively linked with the condition of females in Baltic herring from the Archipelago Sea (Laine & Rajasilta 1999), However, dedicated studies on the linkage between nutritional condition and recruitment are presently missing. Low lipid content in herring and sprat may affect population dynamics of cod, their main predator, because a highly significant linear relationship between total lipid energy and total egg production has been observed in cod from the Barents Sea (Marshall et al. 1999).

Effects of ecosystem changes on EFA levels in clupeids

The recent ecosystem changes may have resulted in changed levels of PUFA in the base of the Baltic cod food web. EPA/DHA and 16:1n-7/16:0 ratios were found to be valuable fatty acid trophic markers (FATM) for discriminating between diatoms and dinoflagellates or other flagellates (Parrish et al. 2000, Dalsgaard et al. 2003). EPA/DHA ratios around 0.5 and 16:1n-7/16:0 ratios around 0.2 found in both sprat and herring in this study, both in samples from 2002 - 2004 and 2008 - 2009, strongly indicate that the central Baltic ecosystem was dominated by flagellates in these periods. A domination of dinoflagellate not only decreases EPA/DHA ratio but also decreases ARA in zooplankton and clupeids compared to diatoms because Baltic copepods fed diatoms have 15 times higher ARA compared to copepods fed dinoflagellates (Støttrup et al. 1999). This is supported by a mesocosm study where ARA levels were below detection limit in phytoplankton and copepods from the Baltic Sea compared to plankton from the Norwegian Sea (Ahlgren et al. 2005). A shift towards an increased dominance of dinoflagellates was actually observed in the 1990s in spring and summer in the Baltic proper (Wasmund & Uhlig 2003) although strong spring blooms of diatoms were observed in the period from 2000 to 2005 (Wasmund et al. 2011). This apparent contradiction could be explained by a preferential ingestion of flagellates or dinoflagellates by copepods in spring irrespective of a rather constant portion of total biomass of dinoflagellates, as observed in *Pseudocalanus* sp. in the Baltic Sea during all seasons examined (Peters et al. 2006). In contrast FATM of *Temora longicornis* from the North Sea suggests that this species is a non-selective feeder (Peters et al. 2007). A shift in zooplankton composition during the regime shift has most likely changed FAC in zooplankton available for clupeids as FAC varies among the species

Pseudocalanus sp., *T. longicornis* and *Acartia* sp. (Peters 2006, Peters et al. 2006, Peters et al. 2007).

CONCLUSIONS:

Lipid content and FAC differed significantly between sprat and herring and varied with season and size in both species. In herring lipid content varied furthermore between maturity stages. The results indicate that the alterations in the Baltic Sea ecosystem have resulted in a reduction of lipid quantity in herring and of ARA level in both sprat and herring. Low levels of lipid and EFA in both species in spring and low lipid content in herring in general may have adverse effects on clupeid reproduction and reduce the lipid quantity and quality available to higher trophic levels, such as its main predator cod. The crustacean *Saduria entomon* may provide an important source of PUFA in the Baltic cod diet and its availability can potentially impact EFA levels in cod.

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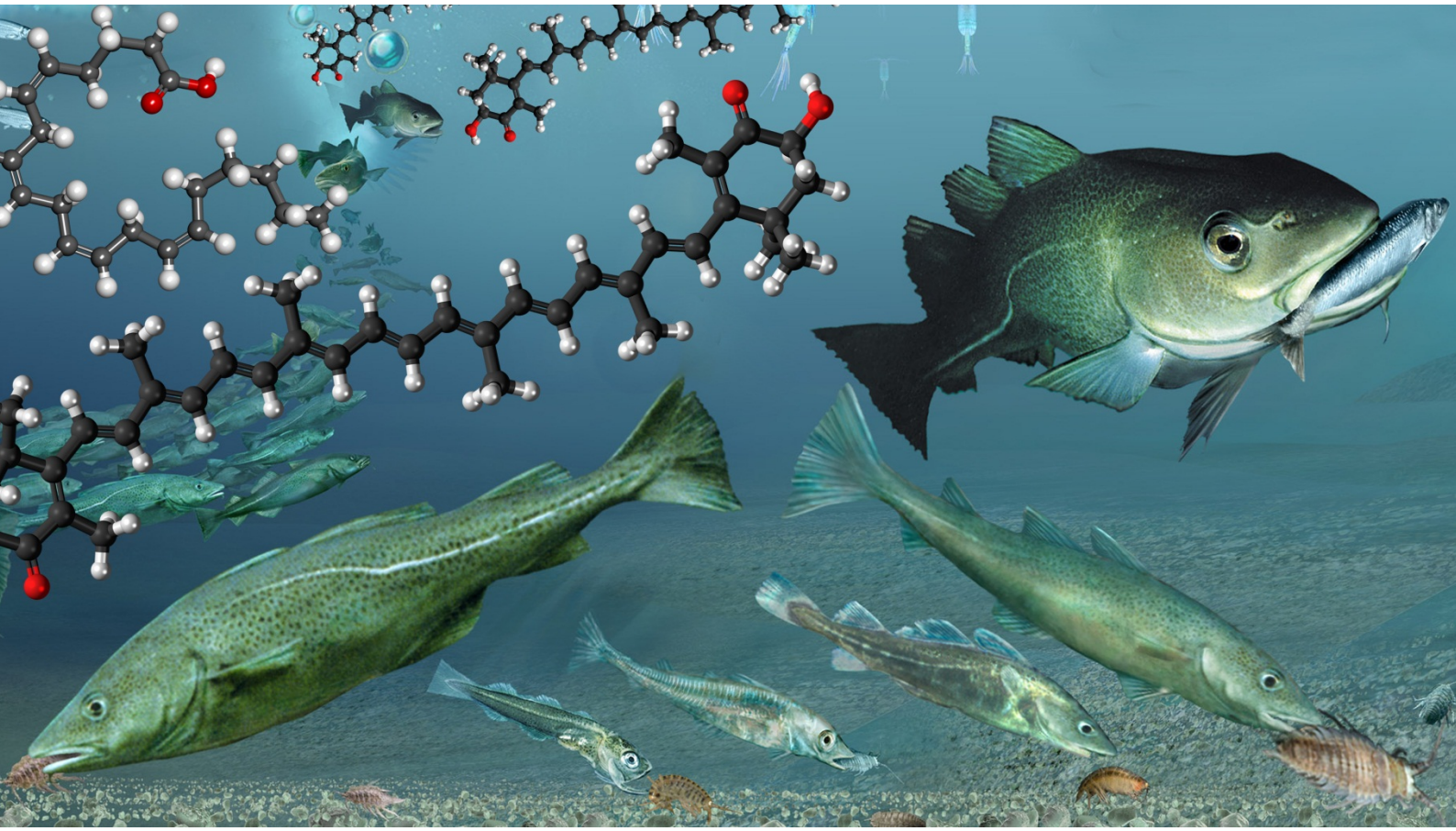
Paper II



Linking lipid dynamics with reproductive cycle in Baltic cod (*Gadus morhua* L.)

by Maria C. Røjbek, Charlotte Jacobsen, Jonna Tomkiewicz & Josianne G. Støttrup

Illustration modified from image by Glynn Gorick for the ICES/GLOBEC project: Cod and Climate Change

Linking lipid dynamics with reproductive cycle in Baltic cod (*Gadus morhua* L.)

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RUNNING TITLE: Lipid dynamics and cod reproduction

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ABSTRACT

This study describes lipid composition and antioxidant protection of Baltic cod (*Gadus morhua*) during the reproductive cycle and investigates whether cod fatty acid composition (FAC) reflected its dominant prey and whether levels of fatty acids important for reproductive performance were low. Reasons for a shift in peak spawning time of Baltic cod from spring/early summer to midsummer prevailing since the early 1990s remain unresolved and may partly be diet related. This study demonstrated that a substantial amount of lipid was invested in cod ovarian development and that lipid composition varied substantially with the reproductive cycle. A selective retention of the essential fatty acids; docosahexaenoic acid (DHA) and arachidonic acid (ARA) in ovaries during maturation was evident, but despite mobilization from liver, ARA level appeared insufficient to keep up with the requirement in ovaries during late maturation and spawning. Astaxanthin and α-tocopherol accumulated in cod ovaries and decreased in late maturing and spawning fish most likely due to their antioxidant protection activity. FAC of cod liver reflected its clupeid prey and 18:1n-9/DHA indicated the ratio of sprat (*Sprattus sprattus*) and herring (*Clupea harengus*) and 16:1n-7, and astaxanthin the presence of the isopod *Saduria entomon* in cod diet. It is likely that food web alterations in the Baltic ecosystem related to environmental and hydrographic changes caused a decrease in ARA availability. Low content of ARA coincides with cod ovarian development in the central Baltic Sea and may be associated with the delay in spawning and affect egg and larval survival; however, this needs further verification in experimental studies.

INTRODUCTION

A remarkable shift in the peak spawning time of cod in the central Baltic Sea (ICES SD 25-32) was observed in the 1990s and peak egg abundance changed from April-June to end of July (Wieland et al. 2000b, Kraus et al. 2002, Karasiova et al. 2008). The later spawning has consequences for several processes influencing survival of early life stages (MacKenzie et al. 1996, Støttrup et al. 2008b). Identification of mechanisms causing variations in spawning time is of great importance to understanding of the recruitment dynamics of this stock. Ambient water temperature and fishery-induced changes of age composition can affect timing of spawning (Wright & Trippel 2009) but these factors cannot fully explain the delayed spawning in the Baltic Sea. Periods with low feeding condition have been correlated with delayed spawning in Baltic cod (Baranova 1995, Karasiova et al. 2008) indicating that delayed spawning may also be diet related. Low energetic status, particularly lipid levels, can reduce the levels of hormones involved in gonadal development (Cerda et al. 1994, Matsuyama et al. 1994) and a recent study indicated that the composition of lipids which differs among cod stocks may influence maturation and spawning time in Baltic cod (Tomkiewicz et al. 2009).

Lipids, and particularly fatty acids, are major dietary factors determining successful reproduction and serve not only as the preferred source of metabolic energy of the parent fish, but are also crucial for the future requirements of the progeny (Sargent et al. 2002, Tocher 2003). Lipids are incorporated into oocyte yolk from dietary sources or from reserves that are stored prior to vitellogenesis and subsequently mobilized and transferred to the ovary (Wiegand 1996). Not only the quantity, but also the quality of dietary lipids is important and long chain polyunsaturated fatty acids (PUFA) have been identified as a major dietary factor determining successful reproduction. Especially the PUFA docosahexaenoic acid (22:6n-3 = DHA), eicosapentaenoic acid (20:5n-3 = EPA) and arachidonic acid (20:4n-6 = ARA) are important for egg and larval quality. DHA and EPA have a structural role in membrane phospholipids (PL) and EPA and ARA are precursors for eicosanoids including prostaglandins (Sargent et al. 1999b, Tocher 2003), which have a role in oocyte maturation and ovulation in fish (Mustafa & Srivastava 1989b, Goetz & Garczynski 1997, Sorbera et al. 2001, Lister & Van der Kraak 2008).

However, due to their polyunsaturated nature PUFA are highly susceptible to attack by free radicals followed by a reaction with oxygen, which results in lipid peroxidation. The ease of lipid peroxidation is proportional to the number of double bonds present (Sargent et al. 2002). Peroxidation can have serious consequences for cell membrane structure and function and a highly efficient antioxidant protection is essential. α-tocopherol and carotenoids, in cod particularly astaxanthin (Miki et al. 1982b, Grung et al. 1993b) both have a protective antioxidative role in fish quenching harmful excited oxygen and free radicals (Syvaoja et al. 1985b, Miki 1991).

The majority of marine fishes do not possess the ability to synthesize either PUFA or α-tocopherol and carotenoids themselves (Sargent et al. 2002). The extent to which fish can convert C18 to C20/22 varies with species and is associated with their capacity for fatty acyl desaturation and elongation (Tocher 2003). Δ6 desaturase activities and PUFA biosynthesis are very low in cod hepatocytes and enterocytes irrespective of diet (Tocher et al. 2006). As a consequence the biosynthesis of PUFA is very low in cod and PUFA levels are therefore determined by the dietary intake of PUFA. Hence, cod depend on PUFA and tocopherols that are synthesized in phytoplankton or plants and transferred up through the food web, while astaxanthin is synthesized by crustaceans from carotenoid precursors, especially β-carotene and zeaxanthin, provided by algae (Matsuno 2001).

Phytoplankton composition varied in the Baltic Sea between 1979 and 2005 due to an increase in temperature and decrease in salinity and inorganic nitrogen concentrations (Wasmund & Uhlig 2003, Wasmund et al. 2011). Strong spring blooms of Diatomophyceae occurred in the 1980s and again from 2000, whereas those of Dinophyceae occurred in the 1990s. It is most likely that these changes have altered fatty acid composition (FAC) in the base of the food web since FAC differs between phytoplankton groups (Ahlgren et al. 1992b, Viso & Marty 1993, Dijkman & Kromkamp 2006). Elevated levels of n-3/n-6 fatty acids in phytoplankton and very high DHA/ARA ratios observed in copepods from the Baltic Sea compared to the Norwegian Sea in a mesocosm enrichment study from 2001 (Ahlgren et al. 2005) could indicate such changes taking place within the plankton community. The Baltic copepods are the main prey of Baltic sprat and herring (Möllmann et al. 2004), which again are the most important prey items for adult cod in the Eastern Baltic Sea (Bagge et al. 1994, Neuenfeldt & Beyer 2003). The benthic crustacean Saduria entomon is furthermore sporadically found in cod stomachs. Condition has decreased in Baltic herring since

the late 1980s and in Baltic sprat since the 1990s due to increased food competition caused by the changes in zooplankton composition concurrent with increased sprat abundance (Möllmann et al. 2005, Köster et al. 2005, Casini et al. 2006, Casini et al. 2010, Casini et al. 2011). The amount of mesenteric fat declined significantly in herring from 1985 to 1991 (Flinkman et al. 1998) and hence it is most likely that the decreased condition reduced the nutritional quality of sprat and herring as prey for cod. (Flinkman et al. 1998).

Differences in PUFA were found in gonad and liver of late maturing cod from the Baltic Sea compared to the North Sea suggesting that deficiencies in PUFA might be the underlying cause for the delay in spawning time of cod (Tomkiewicz et al. 2009) because no delay in spawning is observed in North Sea cod. To investigate if PUFA requirements are met for cod reproduction, measurements of absolute levels are needed. Cod exhibit determinate fecundity and a multiple batch spawning strategy where selected oocytes successively undergo final maturation during the process of batch development (Tomkiewicz et al. 2003). The cod spawning can be extensive. Kjesbu et al. (1989) found in an experimental study that captive North Sea cod spawned up to 17 – 19 batches over a period of 50 – 60 days. Stores of lipids and fatty acids are gradually emptied during the reproductive process potentially impacting egg quality over the spawning.

The aim of the present study was to investigate variation in lipid content as well as proportions and absolute values of PUFA of female Baltic cod gonads and livers during a complete reproductive cycle. In gonads we furthermore investigated levels of α-tocopherol and astaxanthin to measure antioxidant protection. Liver is the primary storage site of lipid energy in cod (Kjesbu et al. 1991b) and we examined whether the lipid composition of cod liver reflected its major prey sprat and herring and if it was possible to detect fatty acid "finger prints" of the isopod *Saduria entomon*.

MATERIALS AND METHODS

Sample collection

Female cod were sampled onboard trawl surveys and fisher boats in the central Baltic Sea in February, March, May, July and August 2009 (Fig. 1). The aim was to sample 5-10 females per length group per sampling event in the length classes 30-39, 40-49, 50-59 and >60cm to distribute samples over the most common size groups of reproducing females and including a minimum of

three female cod of each prevailing maturity stage per sampling. For each female, total length, TL, was measured to the nearest lower integer (cm), whole body weight, BW, and eviscerated body weight, EBW (gonad, liver, stomach and viscera removed), was measured to the nearest gram. Gonad weight GW and liver weight LW were assessed to the nearest 0.1 g. Gonadosomatic Index (GSI) and Hepatosomatic Index (HSI) were calculated as: GSI = GW × 100 / EBW and HSI = LW × 100 / EBW. Gonadal maturity stages of cod (MIII=early ripening, MIV = late ripening, MV = initiation of spawning, MVI = main spawning, VII = cessation of spawning, VIII = spent and MIX = resting) were judged from macroscopic characters according to Tomkiewicz et al. (2003) during sampling. A sub-sample of the ovarian tissue was preserved in formalin buffered with NaH_2PO_4-H_2O and Na_2HPO_4-$2H_2O$ for histological validation.

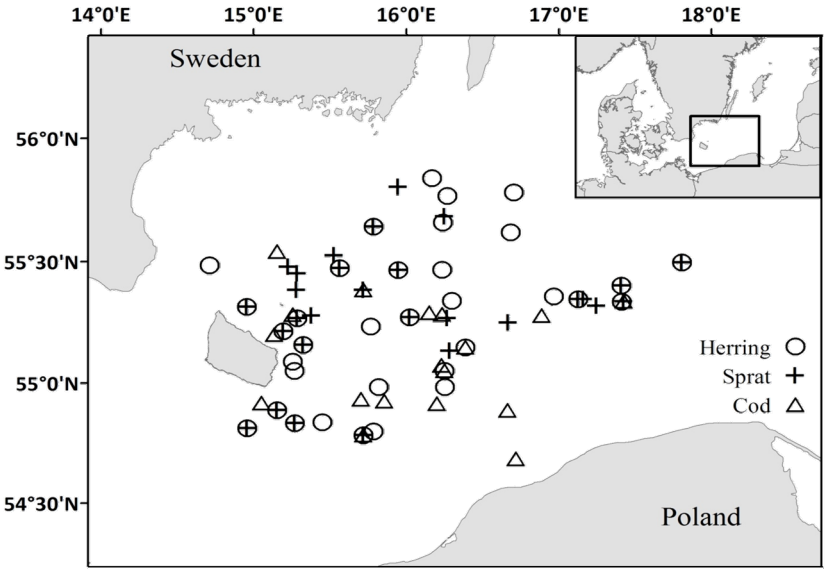


Fig. 1. Map of study area. Samples were collected in ICES subdivision 25 at or near the Bornholm basin in the Baltic Sea. Symbols represent stations from the collection of the three species in November 2008 to August 2009.

The remaining ovarian tissue and liver were frozen and stored at -40˚C for analysis of lipids, astaxanthin and tocopherol in the laboratory. Muscle dry weight was determined on board the research vessels or in the laboratory. 10 g of fillet was dried in an oven in aluminium trays at 105ºC and weighed (± 0.001 g) after 18, 48 and 72 hours. Sprat (*Sprattus sprattus*) and herring (*Clupea harengus*) were sampled in November 2008, February, March, May and August 2009 and the isopod *Saduria entomon* was sampled and in March 2009 and November 2010. Total length of sprat and herring was measured to the nearest lower ½-cm and specimens were grouped according to length class. Sampled sprat, herring and isopods were stored at -40°C for later lipid analysis.

Histology

To verify the maturity stage of female cod, ovarian development was evaluated using histological sections. The preserved ovarian tissue was embedded in paraffin using standard procedures, sectioned at 7 μm and stained using hematoxylin and eosin. Sections were analyzed using light microscopy and maturity stages were distinguished on basis of morphological characteristics of the larger and more advanced oocytes (Tomkiewicz et al. 2003). The early ripening stage (MIII) is a very dynamic maturity stage and lipid composition may differ in different sub-stages. MIII was therefore further divided into three sub-stages according to the characteristics of the most advanced oocytes: oocytes with cortical alveoli (CA) only = $MIII^0$; peripheral yolk granules among CA = $MIII^1$; and yolk granules fill most but not the entire cytoplasm = $MIII^2$ (Tomkiewicz et al. 2003).

Sub sampling of females for lipid analysis

Only adult females ≥40cm were chosen for lipid analysis. The histological classification, regrouped the samples and the aimed minimum of three females of each maturity stage (or sub-stage of MIII) was not achieved in all sampling months and smaller sample sizes for lipid analysis were accepted in these cases. In case of more than three females within maturity stages and sampling event, samples were randomly chosen by using Research Randomizer (http://www.randomizer.org/).

For each sampling month, sprat and herring mixed from several stations, were separated into 10 mm length classes and pooled (5-58 fish per sample) for lipid analysis. Five classes of sprat (80-130 mm) and eight classes of herring (130-230 mm) were considered, representing the dominant size range found in cod stomachs (Stefan Neuenfeldt, pers. comm.). Specimens of *S. entomon* in various sizes were pooled according to sampling month and station for lipid analysis.

Lipid extraction and determination of lipid content

Sprat, herring and isopods were cut into small pieces, frozen using liquid nitrogen and blended. Lipids were extracted from 5-10 g of the blended samples. For cod the samples of ovary and liver as well as ten samples of muscle were analysed on an individual basis. The extraction was done with a homogeneous mixture of chloroform, methanol and water (2:2:1.8) following the method of Bligh & Dyer (1959). The method was modified to use a smaller volume of solvents, but the original ratio between chloroform, methanol and water was maintained. Lipid extracts were used for the subsequent lipid class fractionation and determination of FAC as well as for determination of lipid content. Lipid content was determined by gravimetry after evaporation of chloroform and is expressed as percentage of wet weight of ovary and liver respectively throughout the paper.

Lipid class separation

Lipids from ovary and liver extracts were separated in phospholipids (PL) and neutral lipids (NL) by chromatography on a solid phase consisting of aminopropyl modified silica. Solvents with increasing polarity were used to separate lipid classes. A lipid extract corresponding to 10-100 mg lipid was used for the lipid class separation. Solvents from the lipid extraction were evaporated and the extract was resolubilized in 0.5 ml chloroform and transferred to a Sep-Pak column (Waters Corporation, Milford, Massachusetts). NLs were eluated using 4 ml chloroform/2-pronanol (2:1), and PLs were eluated with 6 ml methanol. Eluates were evaporated to almost dryness (NL) or to 1-2 ml (PL) under nitrogen. The only lipid class of NL present in cod that contains fatty acids is triacylglycerols (TAG) and this term is used throughout this paper.

Preparation of fatty acid methyl esters (FAME) and analysis of FAC

Eluates of ovary or liver extracts from the lipid class separation and extracts of total lipid from sprat herring and isopods were used for the preparation of FAME using AOCS Official Method Ce 2-66 (Anon 1998b). FAME were analysed on a HP 5890A gas chromatograph (Hewlett-Packard, Palo Aalto, CA) equipped with an Omegawax 320 (30 m x 3.2 mm x 0.25 μm) column from Supelco (Bellefonte, PA) using AOCS Official Method Ce 1b-89 (Anon 1998a). Oven temperature programme was 15°Cmin-1 to 160°C, hold 2 min, 3°Cmin-1 to 200°C, hold 1 min and 3°Cmin-1 to 220°C, hold 17 min. A split ratio of 1:25 was used and C23:0 methyl ester was used as internal standard. The proportion of fatty acids was quantified by calculating its peak area relative to the total peak area (% of total FA) and absolute content of fatty acids was calculated relative to the internal standard and equivalent sample size (mg g-1 ww). Absolute total content of fatty acids in ovarian and liver lipid (mg g-1 lipid) was estimated as the sum of absolute content of identified fatty acids (mg g-1 ww) in PL and TAG divided by the percentage of lipid content in ovary and liver respectively, assuming that the sum of unidentified fatty acids were more or less constant between samples.

Lipid class determination

Lipid classes were determined using thin-layer chromatography with flame ionization detection (TLC-FID) with Iatroscan MK5. Lipid extracts from gonad and liver were either concentrated or diluted to ensure a concentration ranging from 10-20 mg ml^{-1}. Triplicates of 1 μl samples of lipid extracts were spotted on silica gel coated Chromarods® (SIII) using an Autospotter (Model SES

3202). Chromarods® were developed in the following solvent system at 20°C: n-heptane/diethyl ether/formic acid (70:10:0.02, vol/vol/vol) until solvent reach the 90% mark (around 20 min). Solvent was then dried of by heating the chromarods at 110°C for 5 min. and scanned in the Iatroscan. This single development separates the NL classes: TAG, free fatty acids and cholesterol and total PL. Data were analyzed using SES ChromStar software and peak areas were quantified using calibration curves obtained from scans of standards (trilinolein, lynoleic acid, cholesterol and L-alpha-phosphatidylcholin, Sigma-Aldrich, Germany).

Tocopherol and astaxanthin

Tocopherol was measured in cod ovary samples and in whole sprat and herring using AOCS Official Method Ce 8-89 (Anon 1997). Samples of 2 g lipid extract was evaporated under oxygen-free nitrogen, re-dissolved in 1 ml heptanes and mixed for 30 sec. Separation of tocopherols was performed by HPLC (Agilent 1100) equipped with a 3 μm Silica column (150 mm * 4.6 mm) from Water Spherisorb. Concentration of tocopherol was calculated from the peak area relative to the internal standard (α, β, γ, δ standards, Calbiochem 613424) and equivalent sample size (μg g^{-1}). Astaxanthin was measured spectrophotometrically (UV mini 1240) at 485 nm in lipid extract from cod ovary samples and in whole sprat, herring and *Saduria entomon* and concentration was calculated by using the extinction coefficient $E_{1\%}$ = 2460 and equivalent sample size (μg g^{-1}).

Statistical analyses

Multivariate data analysis was performed using the Unscrambler® v9.1 (CAMO, Oslo, Norway) software. Principal Component Analyses (PCA) were performed and variables were including relative peak areas of 30 identified fatty acids, groups and ratios of fatty acids, HSI, GSI, lipid content, season and maturity stage. Variables were column mean centred and normalised to unit column standard deviation (SD) before calculation of the models and models were validated by full cross-validation. Statistical differences in oil and fatty acid content were calculated with R version 2.12. The data were checked for normal distribution and tested with one-way analysis of variance (ANOVA). A comparison between model 1: ANOVA including only maturity stage and model 2: ANOVA including both maturity stages and season was tested with Bonferroni correction. If no significant differences were found between the models the simpler model 1 was used to test differences between maturity stages, followed by a multiple comparison test. If significant differences were found model 2 was used. A significance level (p) of 0.05 was applied in all tests.

RESULTS

Sample characteristics

In total, 144 female cod were sampled. After histological validation, 51 % of the samples were in developing condition (MIII and MIV), 20% in spawning stages (MV – MVII), 24 % in spent or resting stages (MVIII and MIX) and 5% immature (MII). Females in spawning stages were obtained during May, July and August representing 50%, 93% and 15% of the females sampled within each sampling event, indicating the timing of peak spawning and duration of the spawning season. A subsample of 45 cod in maturity sub stages $MIII^{0-2}$ and maturity stages MIV – MIX were chosen for lipid analysis (Table 1). Total length of the female cod for lipid analysis varied between 40 - 92 cm with an average length of 53.1 cm and weight of 1.6 kg and with no significant differences in length and weight among sampled females between maturity stages or seasons (Table 1). The size range of sampled cod reflected the composition of spawners in the stock where the highest proportion is below 2 kg (ages 3-6) in the sample area ICES Sub-division 25 (ICES 2010). Water content in muscle (Table 1) was significantly higher in fish at the end of spawning (86% in MVII) than at early maturation (81% in $MIII^{1}$). Due to windy weather in May it was not possible to use the fine balance on board and the lower accuracy of the balance used (± 1 g) resulted in a high variation in muscle water content of maturation stage MIV, MV and MVII. Lipid content of fillet was on average 0.66%. In total 623 sprat and 641 herring were included in this study. They were pooled into 45 sprat and 62 herring samples and *Saduria entomon* were pooled into 4 samples.

Table 1. *Gadus morhua*. Female cod sampled for analysis of lipid content and fatty acid composition (FAC). Number of specimens per maturity stage (MIII-MIX) after histological validation and total length (TL), body weight (BW) and water content in muscles samples. Values are mean, n = 45.

Maturity	Feb	Mar	May	Jul	Aug	Tot	TL (cm)	BW (kg)	Water (%)
$MIII^{0}$	3	3	0	0	0	6	55.2 ± 10.4	1.6 ± 0.7	82.0 ± 1.0
$MIII^{1}$	4	3	0	0	0	7	61.0 ± 18.8	2.6 ± 2.2	81.2 ± 0.7
$MIII^{2}$	0	3	0	0	0	3	48.3 ± 2.5	1.4 ± 0.8	81.0 ± 0.5
MIV	1	3	2	1	2	9	50.3 ± 6.8	1.4 ± 0.4	82.9 ± 3.1
MV	0	0	2	3	0	5	45.8 ± 4.5	1.2 ± 0.3	82.6 ± 2.0
MVI	0	0	0	3	0	3	50.7 ± 7.4	1.6 ± 1.0	84.8 ± 0.9
MVII	0	0	3	0	0	3	48.3 ± 5.1	1.2 ± 0.3	86.1 ± 3.7
MVIII	0	0	0	0	2	2	54.5 ± 7.8	1.6 ± 0.6	82.0 ± 0.6
MIX	4	0	0	0	3	7	57.0 ± 6.5	1.7 ± 0.5	82.2 ± 0.6

Multivariate analysis

PCA analysis of stored TAG in liver shows that FAC in liver vary with maturation and principal component (PC) 1 explains 22% and PC2 explains additional 15% of the variation in samples (Fig. 2). The scores plot shows that samples of spawning female cod (MV and MVI) are grouped together in the lower right corner, except for one outlier of MVI (Fig. 2a). Likewise samples of cod at the end of spawning (MVII) and resting (MIX) are relatively similar; however some samples of the two groups are overlapping. FAC of maturing (MIII and MIV) and resting fish (MVIII) varied highly, however MIII samples were mainly located in the left side of the plot.

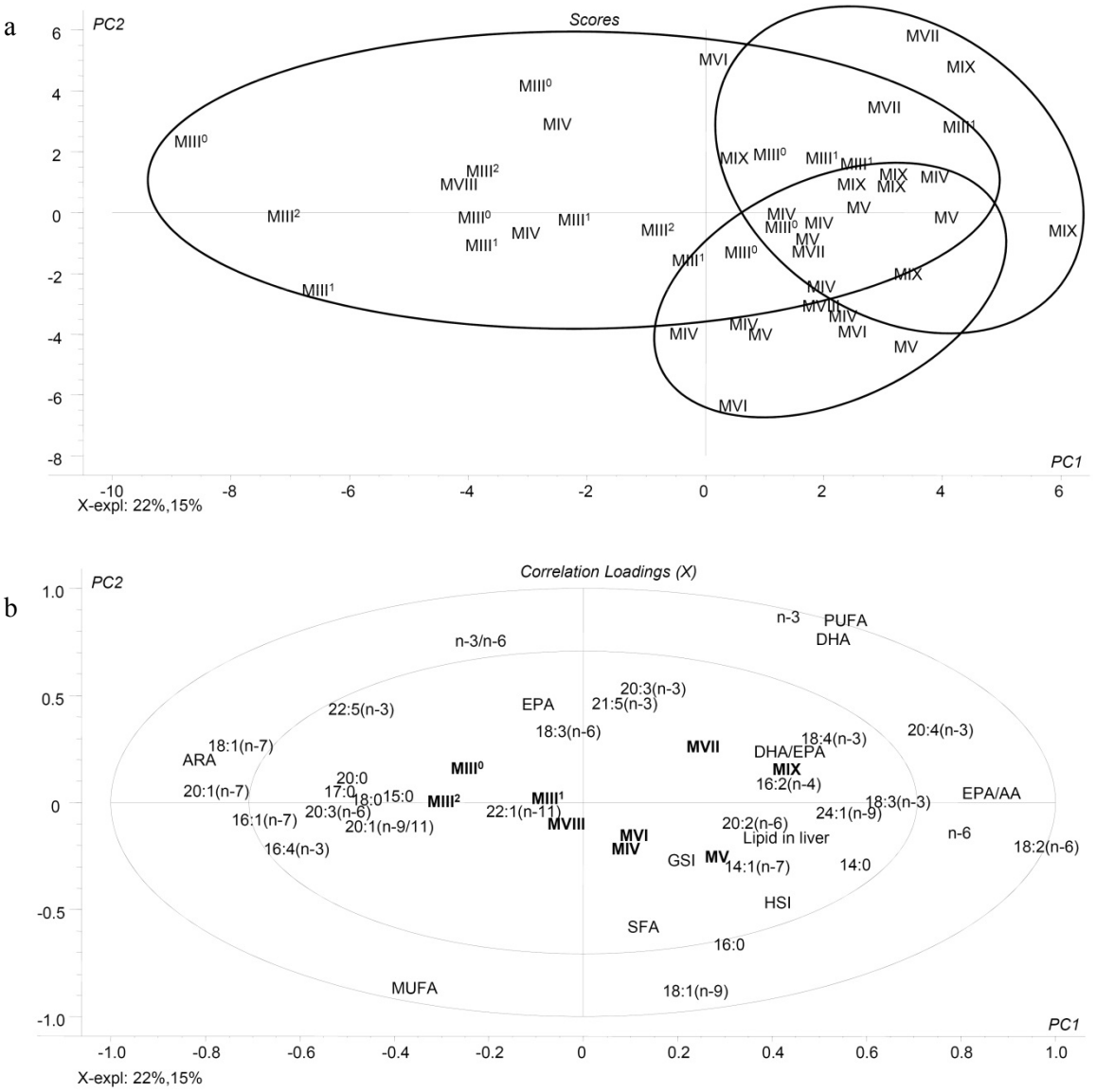


Fig. 2. *Gadus morhua*. Principal component analysis (PCA) scores plot (a) and loadings plot (b) of liver triacylglycerol (TAG) including the variables: fatty acid composition (FAC), lipid content in liver, hepatosomatic index (HSI), gonadosomatic index (GSI), maturity sub stages $MIII^{0-2}$ and maturity stages MIV – MIX, n = 45. SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, DHA = docosahexaenoic acid, EPA = eicosapentaenoic acid and ARA = arachidonic acid.

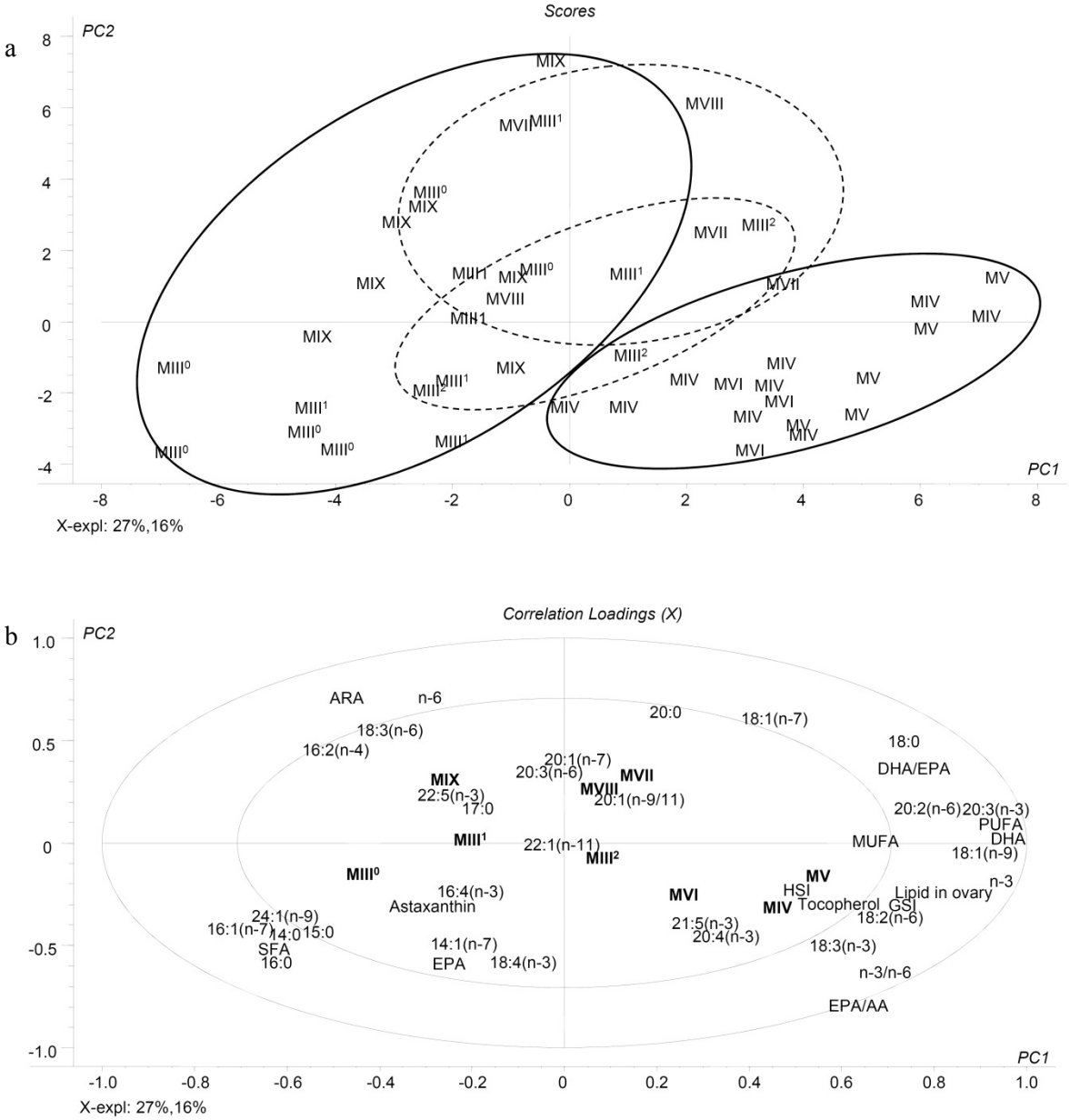


Fig. 3. *Gadus morhua*. Principal component analysis (PCA) scores plot (a) and loadings plot (b) of ovarian phospholipid (PL) including the variables: fatty acid composition (FAC), lipid content in ovary, hepatosomatic index (HSI), gonadosomatic index (GSI), astaxanthin, α-tocopherol, maturity sub stages $MIII^{0-2}$ and maturity stages MIV – MIX, n = 45. SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, DHA = docosahexaenoic acid, EPA = eicosapentaenoic acid and ARA = arachidonic acid.

The corresponding loadings plot (Fig. 2b) shows that liver TAG of cod MIII was characterized by high ARA, 18:1(n-7) and 20:1(n-7) and that DHA, total n-3 PUFA and EPA/ARA was highest in liver of MVII and MIX cod while MUFA was low.

PCA analysis of ovary PL shows that FAC and other sample characteristics varied with maturation and indicates a cyclic dynamics with gonadal maturation (Fig. 3a) and PC1 explains 27% and PC2 explains additional 16% of the variation in samples. FAC of fish in early maturation ($MIII^0$ and

$MIII^1$) was similar to the composition of resting fish (MIX) in 2nd and 3rd quadrant while fish in late maturation (MIV) is situated close to the spawning cod (MV and MVI) in 1st and 4th quadrant except for one outlier. Fish in $MIII^2$ and end of spawning and spent (MVII and MVIII) are in transition zones (dotted lines). The corresponding loadings plot (Fig. 3b) shows that ovary PL of cod in early maturation, particular $MIII^0$, were characterized by high levels of 16:1n-7, 24:1n-9, 14:0, 15:0, 16:0 and total SFA and cod in resting stage (MIX) were characterized by high levels of ARA, 18:3n-6, total n-6 PUFA and 16:2n-4. Ovary PL in late maturation and spawning were characterized by high GSI, lipid content and high levels of DHA, n-3 PUFA, PUFA, 18:1n-9, total MUFA and tocopherol.

Lipid dynamics and gonadal maturation

FAC in TAG and PL of liver and ovary in the different maturation stages is shown in Table 2. The ANOVA-model including both maturity stage (and sub stages of MIII) and season was not statistically different from the ANOVA-model with maturity stage alone for any variable. The only effect of season on FAC was therefore indirectly through maturity stage and ANOVA model 1 was therefore applied to Table 2. More fatty acids differed significantly between maturation stages in PL of ovary and liver than in TAG of both organs where only a few fatty acids differed significantly. The sum of SFA, dominated by 16:0, did not vary with maturation in any lipid fraction but were in general higher in PL than in TAG in both ovary and liver. Sum of MUFA, dominated by 18:1(n-9), only varied with maturation in ovary PL and were much higher in TAG than PL of both ovary and liver. Sum of PUFA varied significantly with maturation in PL and TAG of ovaries, but not in livers and were higher in PL than TAG of both ovary and liver.

Proportion of DHA increased with gonadal maturation and decreased again after spawning in ovary PL and TAG and liver PL, but did not vary with maturation stage in liver TAG. The proportion of ARA decreased significantly from early to late maturation, and/or spawning, in all lipid fractions and increased significantly again after spawning in ovary PL and tended also to increase in ovary TAG. EPA on the other hand did not vary significantly with maturity stage in any lipid fraction. The decrease in ARA resulted in an increase in EPA/ARA ratios in late maturation in ovary PL and TAG and liver PL.

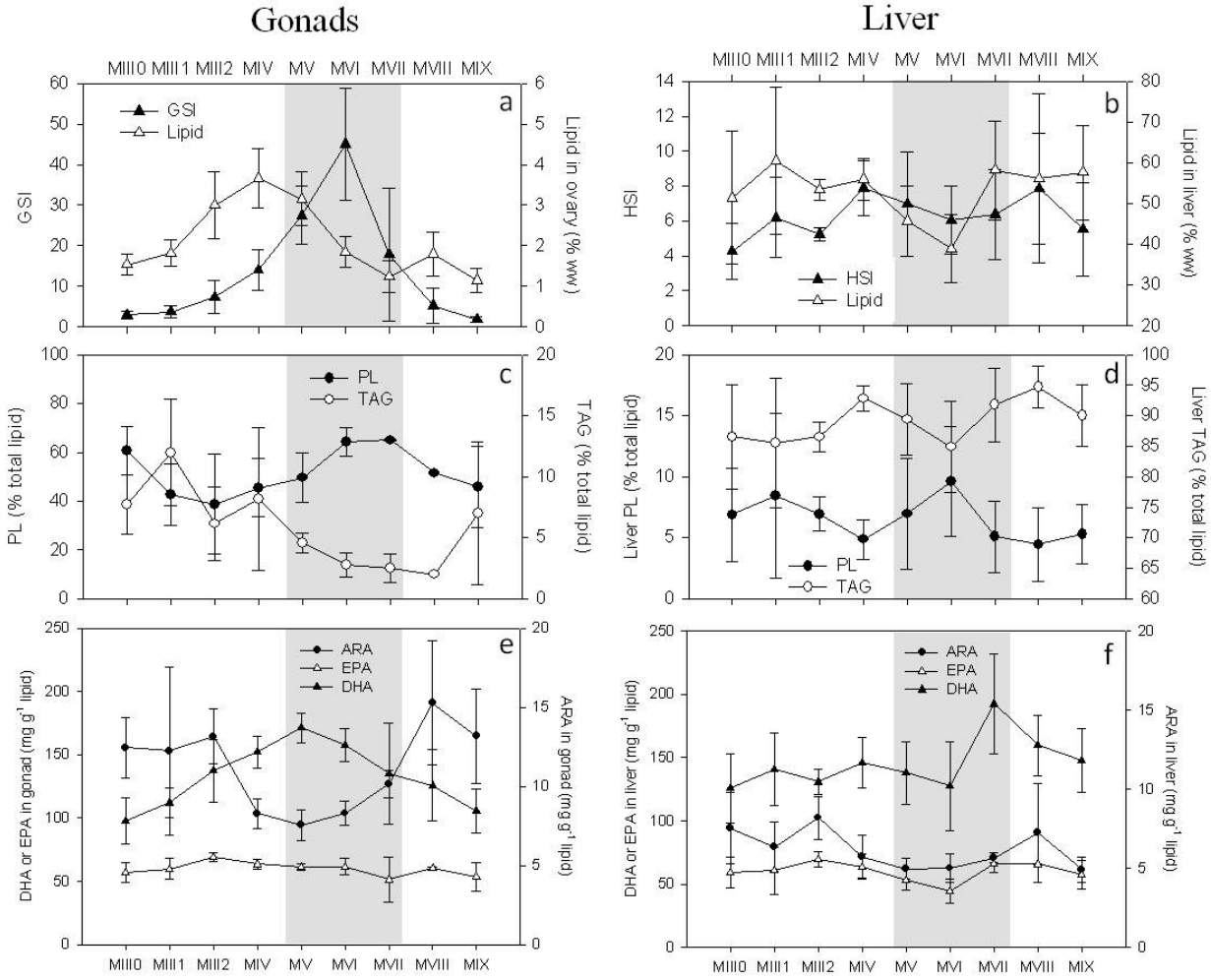
Tabel 2a. *Gadus morhua.* Fatty acid composition (% of total fatty acids) and fatty acid ratios of phospholipids (PL) and triacylglycerols (TAG) in ovaries from Baltic cod in maturity sub stages MIII$^{0-2}$ and maturity stages MIV - IX. Values are means ± SD.

Fatty acid	MIII[0] (n = 6)	MIII[1] (n = 7)	MIII[2] (n = 3)	MIV (n = 9)	MV (n = 5)	MVI (n = 3)	MVII (n = 3)	MVIII (n = 2)	MIX (n = 7)
Phospholipids									
14:0	1.5 ± 0.4	1.2 ± 0.4	1 ± 0.4	1 ± 0.3	0.9 ± 0.4	1.1 ± 0.1	0.9 ± 0.1	0.9 ± 0.5	1.6 ± 0.4
16:0	22.4 ± 2.5	21.2 ± 2.6	20 ± 3.2	20.1 ± 2.4	18.9 ± 2.2	21.1 ± 0.6	19.9 ± 2.0	20.1 ± 3.2	21.1 ± 1.9
18:0	1.1 ± 0.3 b	1.2 ± 0.3 ab	1.6 ± 0.4 bc	1.9 ± 0.6 bc	2.3 ± 0.7 c	1.6 ± 0.2 bc	2.5 ± 0.3 c	2.4 ± 0.7 ac	1.9 ± 0.4 bc
SFA	26 ± 2.9	24.4 ± 2.9	23.4 ± 3.4	23.6 ± 2.2	22.7 ± 2.0	24.5 ± 0.3	23.9 ± 2.1	23.9 ± 3.2	25.3 ± 2.0
16:1(n 7)	3.7 ± 1.2 a	3.1 ± 0.5 ab	2.5 ± 0.7 ac	2 ± 0.6 bc	1.7 ± 0.5 c	2 ± 0.3 bc	1.3 ± 0.0 c	1.5 ± 0.2 bc	2.2 ± 1.0 bc
18:1(n 9)	8 ± 0.9 a	7.9 ± 0.7 a	9.7 ± 1.7 ab	11.2 ± 1.8 bc	12.5 ± 0.7 b	11.7 ± 1.8 bc	12.6 ± 2.0 b	11.2 ± 0.4 ab	9.2 ± 1.4 ac
18:1(n 7)	2.1 ± 0.5	2.4 ± 0.8	2.7 ± 0.7	2.4 ± 0.3	2.6 ± 0.2	2.4 ± 0.7	2.8 ± 0.4	3.5 ± 2.1	2.3 ± 0.5
20:1(n 9)	0.5 ± 0.2	0.5 ± 0.2	0.4 ± 0.1	0.6 ± 0.7	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.3	0.5 ± 0.1
24:1(n 9)	1.2 ± 0.3 b	1.1 ± 0.4 ab	0.8 ± 0.3 bc	0.7 ± 0.2 ac	0.6 ± 0.1 c	0.6 ± 0.2 ac	0.4 ± 0.1 c	0.7 ± 0.4 bc	0.8 ± 0.2 bc
MUFA	15.8 ± 1.5 ab	15.3 ± 1.1 a	16.4 ± 1.4 ab	17.2 ± 1.2 ab	17.9 ± 0.5 b	17.2 ± 1.3 ab	17.8 ± 1.5 ab	17.6 ± 1.5 ab	15.2 ± 1.3 a
16:2(n 4)	1.4 ± 0.3 bd	1.5 ± 0.3 ab	1 ± 0.2 acd	0.9 ± 0.2 cd	0.7 ± 0.1 c	0.9 ± 0.1 acd	1.5 ± 0.6 bd	1.4 ± 0.7 bc	1.9 ± 0.3 b
18:2(n 6)	0.8 ± 0.2 b	0.8 ± 0.2 ab	0.9 ± 0.1 bc	1.3 ± 0.2 ac	1.5 ± 0.4 c	1.5 ± 0.3 c	1.1 ± 0.2 bc	1.2 ± 0.5 bc	1.1 ± 0.4 bc
18:3(n 3)	0.4 ± 0.0 ab	0.4 ± 0.0 a	0.5 ± 0.1 ab	0.5 ± 0.1 ab	0.5 ± 0.1 ab	0.6 ± 0.1 b	0.4 ± 0.1 ab	0.4 ± 0.1 ab	0.4 ± 0.2 ab
18:4(n 3)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.2	0.3 ± 0.1
20:4(n 6)	3.2 ± 0.7 bc	2.8 ± 1.1 ab	2.9 ± 0.1 ab	1.8 ± 0.2 a	1.6 ± 0.2 a	1.8 ± 0.1 ac	2.9 ± 0.8 ab	4 ± 1.0 b	3.7 ± 1.0 b
20:5(n 3)	13.7 ± 2.3	12.7 ± 2.8	14.1 ± 2.1	13.3 ± 1.0	12.1 ± 0.7	13 ± 0.8	11.3 ± 2.1	13.1 ± 2.0	12.6 ± 1.0
22:5(n 3)	0.9 ± 0.2	1 ± 0.4	0.9 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.9 ± 0.2	0.7 ± 0.1
22:6(n 3)	26 ± 4.7 a	29.9 ± 4.3 ab	30.9 ± 4.0 ab	33.5 ± 2.4 b	35.6 ± 2.0 b	33.2 ± 0.6 ab	31.7 ± 1.8 ab	28.5 ± 3.9 ab	28.1 ± 2.0 a
PUFA	48.2 ± 3.6 a	50.8 ± 2.0 ab	53 ± 1.9 ab	53.7 ± 1.9 b	54.7 ± 1.5 b	53.6 ± 1.8 b	51.2 ± 2.6 ab	51.4 ± 1.5 ab	50.6 ± 2.4 ab
n-6	4.7 ± 0.7 bc	4.3 ± 1.0 ab	4.4 ± 0.4 bc	3.7 ± 0.2 b	3.8 ± 0.3 b	3.9 ± 0.2 ab	4.8 ± 0.9 bc	6.1 ± 0.4 ac	5.9 ± 1.3 c
n-3	42.1 ± 3.5 b	45 ± 1.9 abc	47.6 ± 1.7 cd	49.1 ± 1.9 d	50.1 ± 1.5 d	48.9 ± 1.7 ad	44.9 ± 4.0 bd	43.9 ± 1.8 bd	42.8 ± 1.8 bc
DHA/EPA	2.0 ± 0.6	2.5 ± 0.9	2.3 ± 0.6	2.5 ± 0.3	3.0 ± 0.3	2.6 ± 0.1	2.9 ± 0.4	2.2 ± 0.6	2.3 ± 0.3
EPA/AA	4.4 ± 1.3 b	5 ± 1.8 ab	4.9 ± 0.9 bc	7.4 ± 0.6 c	7.7 ± 1.0 c	7.3 ± 0.8 ac	4.2 ± 1.7 ab	3.4 ± 1.4 b	3.6 ± 1.0 b
Triacylglycerols									
14:0	1.9 ± 0.3	1.8 ± 0.3	1.6 ± 0.4	1.6 ± 0.3	1.6 ± 0.3	1.7 ± 0.6	1.3 ± 0.1	1.6 ± 0.9	2.1 ± 0.6
16:0	16 ± 3.4	18.5 ± 2.7	15.2 ± 3.6	17.1 ± 2.4	18.6 ± 1.6	15.2 ± 2.0	20.6 ± 0.8	17.7 ± 6.0	19 ± 4.2
18:0	1.1 ± 0.3 ac	1 ± 0.3 a	1.5 ± 0.3 ab	1.5 ± 0.2 ab	1.4 ± 0.2 ab	1.9 ± 0.4 bc	1.6 ± 0.2 ab	2.1 ± 0.6 b	1.6 ± 0.4 ab
SFA	20.1 ± 3.0	22.1 ± 2.4	19.2 ± 2.9	20.9 ± 2.4	22.4 ± 1.8	19.5 ± 1.3	24.2 ± 0.8	22 ± 4.5	23.3 ± 3.3
16:1(n 7)	6.2 ± 1.0 a	6.1 ± 0.7 a	5.4 ± 2.0 ab	3.9 ± 1.1 bc	2.9 ± 0.3 c	4 ± 1.2 ac	2.2 ± 0.1 c	3 ± 0.7 bc	3.4 ± 1.1 bc
18:1(n 9)	16.5 ± 4.2	15.2 ± 4.4	18 ± 3.4	17.3 ± 2.8	15.6 ± 1.2	19.6 ± 6.0	15.2 ± 1.4	15.9 ± 5.0	14.8 ± 2.4
18:1(n 7)	3.1 ± 0.8	2.6 ± 0.8	3.5 ± 1.3	2.4 ± 0.7	2.1 ± 0.3	3.2 ± 0.8	2 ± 0.3	3 ± 0.1	2.1 ± 0.4
20:1(n 9)	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.6	0.3 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.6 ± 0.3	0.5 ± 0.2
24:1(n 9)	2.3 ± 0.4 ad	2.6 ± 0.7 a	1.6 ± 0.6 ab	1.2 ± 0.3 bc	0.7 ± 0.4 b	0.9 ± 0.4 bc	0.6 ± 0.3 b	0.9 ± 0.6 bcd	2.1 ± 0.8 ac
MUFA	29.4 ± 5.7	27.5 ± 5.6	29.6 ± 6.9	25.9 ± 3.8	21.9 ± 1.1	28.8 ± 7.6	20.7 ± 1.2	24 ± 6.8	23.6 ± 3.5
16:2(n 4)	0.8 ± 0.1	0.9 ± 0.2	0.7 ± 0.3	0.7 ± 0.2	0.8 ± 0.1	0.6 ± 0.1	1 ± 0.2	0.8 ± 0.1	0.9 ± 0.3
18:2(n 6)	1.2 ± 0.4	1.2 ± 0.3	1.4 ± 0.2	1.8 ± 0.6	2 ± 0.5	2.6 ± 0.9	1.5 ± 0.2	2.1 ± 1.5	2.1 ± 1.1
18:3(n 3)	0.8 ± 0.2	0.7 ± 0.2	1 ± 0.2	1.1 ± 0.3	1.1 ± 0.2	1.5 ± 0.4	0.8 ± 0.0	1.2 ± 0.9	1.2 ± 0.7
18:4(n 3)	0.6 ± 0.2	0.5 ± 0.2	0.7 ± 0.1	0.7 ± 0.3	0.7 ± 0.2	1.1 ± 0.5	0.5 ± 0.1	0.9 ± 0.8	1.1 ± 0.7
20:4(n 6)	2.6 ± 0.7 a	2.4 ± 1.0 ab	2.1 ± 0.5 ab	1.5 ± 0.2 b	1.3 ± 0.2 b	1.2 ± 0.3 ab	2 ± 0.4 ab	2.4 ± 1.2 ab	2.4 ± 0.7 ab
20:5(n 3)	11.8 ± 2.5	12.3 ± 1.3	11.7 ± 2.0	11.6 ± 0.9	11.2 ± 0.5	10.1 ± 1.9	11.5 ± 0.7	12.1 ± 0.9	13 ± 1.2
22:5(n 3)	1.1 ± 0.3	1.2 ± 0.4	1.1 ± 0.3	0.8 ± 0.3	0.8 ± 0.1	1.1 ± 0.3	0.8 ± 0.1	1 ± 0.2	0.9 ± 0.1
22:6(n 3)	19.9 ± 2.2 a	20.1 ± 2.5 a	22.1 ± 4.5 abc	26.8 ± 2.8 cd	30.6 ± 1.1 d	26 ± 6.8 ad	29.5 ± 2.1 bd	25.3 ± 5.1 ad	22.6 ± 2.1 ac
PUFA	40.8 ± 4.0 b	41.1 ± 3.3 ab	43.1 ± 6.0 bc	47 ± 2.5 ac	50.2 ± 1.4 c	46.2 ± 7.8 bc	49.5 ± 1.7 c	47.9 ± 3.5 bc	46.1 ± 2.4 bc
n-6	4.3 ± 0.5 ab	4.1 ± 0.8 a	4.1 ± 0.3 ab	3.9 ± 0.5 a	3.9 ± 0.5 a	4.4 ± 0.7 ab	4.2 ± 0.5 ab	5.2 ± 0.5 ab	5.2 ± 0.8 b
n-3	35.7 ± 3.7 a	36.1 ± 3.1 a	38.3 ± 5.5 ab	42.4 ± 2.6 b	45.6 ± 1.2 b	41.2 ± 8.3 ab	44.3 ± 2.4 b	41.9 ± 4.1 ab	40 ± 2.0 ab
DHA/EPA	1.7 ± 0.4 a	1.6 ± 0.1 a	1.9 ± 0.1 ab	2.3 ± 0.3 bc	2.7 ± 0.2 c	2.6 ± 0.4 c	2.6 ± 0.1 c	2.1 ± 0.3 ac	1.7 ± 0.2 a
EPA/AA	4.7 ± 0.7 a	5.7 ± 1.8 a	5.6 ± 0.4 abc	7.9 ± 0.6 bd	8.6 ± 1.1 d	8.5 ± 1.2 cd	5.9 ± 1.6 ad	5.5 ± 2.3 ad	5.7 ± 1.4 ac

Tabel 2b. *Gadus morhua.* Fatty acid composition (% of total fatty acids) and fatty acid ratios of phospholipids (PL) and triacylglycerols (TAG) in livers from Baltic cod in maturity sub stages MIII$^{0-2}$ and maturity stages MIV – IX. Values are means ± SD.

Fatty acid	MIII^{0}	MIII^{1}	MIII^{2}	MIV	MV	MVI	MVII	MVIII	MIX
Phospholipids									
14:0	1.6 ± 0.3 bc	2.1 ± 0.5 ab	1.5 ± 0.0 bc	1.8 ± 0.4 bc	1.3 ± 0.2 c	1.1 ± 0.1 c	1.3 ± 0.3 ac	1.4 ± 0.5 bc	2.4 ± 0.6 b
16:0	20 ± 0.6 ab	19.4 ± 1.1 ab	19.7 ± 0.4 ab	20.3 ± 1.0 ab	21 ± 0.8 b	21.5 ± 1.6 b	21.3 ± 1.1 b	18.1 ± 0.4 a	19.8 ± 1.5 ab
18:0	5.6 ± 0.9 ab	6.1 ± 1.6 a	4.6 ± 0.5 ab	4.6 ± 1.1 ab	3.5 ± 0.6 b	3.9 ± 1.5 ab	3.6 ± 0.6 ab	4.4 ± 2.0 ab	5.1 ± 1.6 ab
SFA	28.3 ± 1.1	29 ± 3.2	26.7 ± 0.4	27.4 ± 1.7	26.5 ± 0.8	27.2 ± 0.7	26.9 ± 0.8	24.6 ± 3.0	28.5 ± 2.4
16:1(n 7)	1.3 ± 0.2 ab	1.5 ± 0.7 ab	1.2 ± 0.1 ab	1.2 ± 0.4 ab	0.9 ± 0.2 a	0.9 ± 0.1 ab	1.4 ± 0.4 ab	1.2 ± 0.2 ab	1.9 ± 1.0 b
18:1(n 9)	10 ± 0.9	10 ± 1.1	10.7 ± 1.0	11.1 ± 1.5	11.9 ± 1.0	10.7 ± 1.9	10.9 ± 1.8	9.9 ± 1.3	9.7 ± 2.9
18:1(n 7)	2.5 ± 0.6 ab	2.5 ± 0.8 ab	2.8 ± 0.4 ab	2.5 ± 0.5 ab	2.4 ± 0.1 ab	2.5 ± 0.3 ab	2.6 ± 0.1 ab	3.6 ± 1.2 a	2 ± 0.3 b
20:1(n 9)	0.4 ± 0.1	0.4 ± 0.2	0.3 ± 0.1	0.5 ± 0.6	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.2	0.4 ± 0.1
24:1(n 9)	0.3 ± 0.2 bc	0.3 ± 0.1 ab	0.2 ± 0.1 ac	0.1 ± 0.1 c	0.2 ± 0.0 ac	0.2 ± 0.0 ac	0.3 ± 0.1 bc	0.3 ± 0.1 bc	0.4 ± 0.1 b
MUFA	14.9 ± 1.4	15.3 ± 1.9	15.3 ± 0.8	15.6 ± 1.6	15.8 ± 1.2	14.6 ± 1.6	15.5 ± 1.7	15.5 ± 0.4	14.6 ± 4.3
16:2(n4)	0.7 ± 0.2 a	0.5 ± 0.1 ab	0.5 ± 0.1 ab	0.4 ± 0.1 b	0.4 ± 0.1 b	0.4 ± 0.1 bc	0.6 ± 0.1 ac	0.5 ± 0.1 ab	0.6 ± 0.1 ac
18:2(n 6)	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.2	1.4 ± 0.3	1.4 ± 0.3	1.4 ± 0.2	1.2 ± 0.1	1.4 ± 0.6	1.7 ± 0.6
18:3(n 3)	0.5 ± 0.2 ab	0.3 ± 0.3 a	0.4 ± 0.3 ab	0.7 ± 0.2 ab	0.6 ± 0.1 ab	0.6 ± 0.1 ab	0.5 ± 0.1 ab	0.6 ± 0.1 ab	0.9 ± 0.6 b
18:4(n 3)	0.4 ± 0.1 ab	0.5 ± 0.1 ab	0.3 ± 0.1 b	0.3 ± 0.2 b	0.3 ± 0.1 b	0.2 ± 0.1 b	0.3 ± 0.1 b	0.3 ± 0.1 ab	0.7 ± 0.3 a
20:4(n 6)	2.6 ± 0.6 b	2.4 ± 0.7 ab	1.9 ± 0.2 bc	1.3 ± 0.2 c	1.5 ± 0.2 ac	1.8 ± 0.4 bc	1.7 ± 0.3 bc	2.5 ± 1.4 bc	1.9 ± 0.4 bc
20:5(n 3)	14.3 ± 0.7 ab	13.7 ± 1.8 ab	13.2 ± 2.1 ab	12.1 ± 0.7 ab	11.4 ± 0.5 a	11.7 ± 1.0 ab	13.3 ± 3.3 ab	15.9 ± 3.2 ab	14.9 ± 2.6 b
22:5(n 3)	0.7 ± 0.2	0.8 ± 0.5	0.7 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.5 ± 0.2	0.6 ± 0.3	0.5 ± 0.2
22:6(n 3)	30.4 ± 1.5 ab	29.7 ± 2.9 ab	34.1 ± 1.2 bc	35 ± 2.1 c	36.8 ± 1.1 c	36.9 ± 1.7 c	34.2 ± 2.0 bc	32.3 ± 1.4 ac	28.3 ± 3.3 a
PUFA	52.2 ± 1.2	50.7 ± 2.1	53.9 ± 1.5	53.3 ± 1.5	54.3 ± 0.5	54.8 ± 2.4	53.8 ± 2.6	55.9 ± 2.9	51.4 ± 4.4
n-6	4.4 ± 0.5 ac	4.4 ± 0.7 a	3.6 ± 0.4 ab	3.2 ± 0.3 bc	3.4 ± 0.4 b	3.7 ± 0.4 ab	3.5 ± 0.4 ab	4.5 ± 0.8 ac	4.4 ± 0.5 a
n-3	47.2 ± 1.2 ab	45.8 ± 2.2 a	49.8 ± 1.4 ab	49.7 ± 1.5 b	50.5 ± 0.7 b	50.6 ± 1.9 ab	49.6 ± 2.2 ab	50.8 ± 2.2 ab	46.4 ± 4.1 ab
DHA/EPA	2.13 ± 0.2 a	2.20 ± 0.4 a	2.63 ± 0.5 ab	2.90 ± 0.3 b	3.22 ± 0.2 ab	3.19 ± 0.4 ab	2.69 ± 0.8 ab	2.08 ± 0.5 ab	1.95 ± 0.4 ab
EPA/AA	5.8 ± 1.4 a	6.2 ± 1.8 a	7.1 ± 0.6 ab	9.1 ± 1.1 b	7.9 ± 0.8 ab	6.6 ± 1.8 ab	7.8 ± 1.5 ab	7.1 ± 2.8 ab	7.9 ± 1.7 ab
Triacylglycerols									
14:0	2.3 ± 0.3	2.4 ± 0.5	2 ± 0.5	2.4 ± 0.6	2.8 ± 0.4	2.3 ± 0.7	2.4 ± 0.1	2.2 ± 0.2	2.9 ± 0.4
16:0	13.2 ± 0.9	13.4 ± 0.9	13 ± 0.4	13.2 ± 0.9	14 ± 1.2	13.9 ± 1.9	12.7 ± 1.1	13 ± 0.4	13.9 ± 0.8
18:0	3.1 ± 0.4	3.2 ± 0.6	3.2 ± 0.7	2.8 ± 0.5	2.5 ± 0.4	2.9 ± 0.6	2.2 ± 0.5	2.9 ± 0.6	2.3 ± 0.3
SFA	19.6 ± 0.9	19.7 ± 1.5	19.3 ± 0.8	19.2 ± 1.0	20 ± 1.9	19.8 ± 2.0	18 ± 1.4	18.8 ± 1.2	19.8 ± 1.1
16:1(n 7)	5.3 ± 0.9	5.3 ± 1.5	5.5 ± 0.3	4.7 ± 0.6	4.5 ± 0.2	4.9 ± 0.4	4.3 ± 0.2	5.2 ± 1.3	4.5 ± 0.3
18:1(n 9)	20.6 ± 3.5	21.6 ± 2.7	21.9 ± 2.3	23.7 ± 4.0	24.1 ± 2.9	23.3 ± 6.8	20.8 ± 5.0	23.1 ± 5.1	20.8 ± 3.0
18:1(n 7)	4.3 ± 1.0	3.9 ± 0.9	4.4 ± 0.8	3.5 ± 0.7	3.3 ± 0.2	4.1 ± 0.6	3.3 ± 0.4	4.5 ± 1.0	3.3 ± 0.3
20:1(n 9)	1.3 ± 0.7	1.3 ± 0.8	1.2 ± 0.2	1.4 ± 1.6	0.9 ± 0.1	1 ± 0.2	0.7 ± 0.0	1.4 ± 0.9	0.9 ± 0.2
24:1(n 9)	0.6 ± 0.2	0.6 ± 0.3	0.4 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.2
MUFA	33.2 ± 2.9	34 ± 4.8	34.6 ± 1.2	35.3 ± 2.8	34.2 ± 2.3	34.9 ± 6.5	30.3 ± 4.5	36.2 ± 1.1	31.1 ± 2.7
16:2(n4)	0.5 ± 0.1 b	0.6 ± 0.0 ab	0.5 ± 0.1 b	0.5 ± 0.1 b	0.6 ± 0.1 ab	0.7 ± 0.1 a	0.6 ± 0.1 a	0.6 ± 0.0 ab	0.6 ± 0.1 ab
18:2(n 6)	2.7 ± 0.8	2.8 ± 0.6	2.5 ± 0.7	3.3 ± 0.7	3.8 ± 0.7	3.5 ± 0.8	3.4 ± 0.3	3.3 ± 1.0	3.7 ± 0.8
18:3(n 3)	2.2 ± 0.5	2.2 ± 0.6	2.1 ± 0.3	2 ± 0.8	2.5 ± 0.1	2.3 ± 0.2	2.3 ± 0.1	2.2 ± 0.5	2.4 ± 0.4
18:4(n 3)	1.7 ± 0.5	1.7 ± 0.5	1.6 ± 0.3	1.9 ± 0.4	1.8 ± 0.3	1.8 ± 0.4	1.6 ± 0.0	1.4 ± 0.3	1.8 ± 0.3
20:4(n 6)	1.2 ± 0.4 a	0.9 ± 0.2 ab	1.2 ± 0.2 ab	0.8 ± 0.2 b	0.7 ± 0.1 b	0.8 ± 0.1 ab	0.7 ± 0.0 ab	0.8 ± 0.3 ab	0.7 ± 0.2 b
20:5(n 3)	9.6 ± 1.8	8.6 ± 2.0	9.9 ± 0.8	8.7 ± 1.2	7.9 ± 0.6	7.1 ± 1.4	8.7 ± 0.5	7.8 ± 0.3	8.7 ± 1.2
22:5(n 3)	1.5 ± 0.5	1.5 ± 0.7	1.6 ± 0.3	1.2 ± 0.2	1.3 ± 0.2	1.5 ± 0.6	1.7 ± 0.5	1.8 ± 0.8	1.3 ± 0.3
22:6(n 3)	20.1 ± 2.9	20.5 ± 3.0	18.4 ± 0.7	19.7 ± 2.2	20.1 ± 2.3	20.4 ± 6.1	25.4 ± 5.4	19.2 ± 2.6	22.7 ± 3.5
PUFA	42.4 ± 3.2	41.6 ± 5.4	40.7 ± 1.4	40.9 ± 3.1	41.4 ± 3.2	40.8 ± 8.0	47.2 ± 5.7	40 ± 1.6	44.8 ± 2.9
n-6	4.8 ± 0.5	4.6 ± 0.4	4.6 ± 0.6	4.9 ± 0.5	5.4 ± 0.8	5.2 ± 0.6	4.9 ± 0.3	5 ± 0.7	5.3 ± 0.8
n-3	37.1 ± 3.0	36.4 ± 5.1	35.6 ± 0.8	35.5 ± 2.9	35.5 ± 3.2	35 ± 8.2	41.6 ± 5.4	34.4 ± 2.3	38.9 ± 3.0
DHA/EPA	2.18 ± 0.6	2.47 ± 0.5	1.87 ± 0.2	2.29 ± 0.3	2.54 ± 0.3	2.88 ± 0.6	2.96 ± 0.8	2.46 ± 0.4	2.71 ± 0.7
EPA/AA	8.4 ± 1.8	10.2 ± 4.0	8.7 ± 1.9	11.5 ± 2.1	11 ± 1.6	9.2 ± 1.9	11.8 ± 0.7	10.1 ± 4.3	12.2 ± 3.2

GSI varied significantly with maturity stage from an average of 2.8 in MIII^0 to a maximum of 45 in stage MVI and followed by a decrease to 1.8 in MIX (Fig. 4a). Lipid content in ovaries peaked with 4% in stage MIV. Average HSI and lipid content in liver (Fig. 4b) did not vary significantly considering all maturation stages, but attained lowest levels in MVI. The amount of lipid in ovaries relative to lipid in liver ((GW*lipid content)/(LW*lipid content)) increased significantly with maturation from 2.5 in average in MIII^0 to 21 in MV and decreased to 1 in MIX. The average proportion of PL in gonads increased to 88% in MVI while the proportion of TAG decreased to 3% (Fig. 4c). Average proportions of cholesterol varied between 7 and 40% and free fatty acids (FFA) between 1 and 8% (not shown).



* 4 samples were excluded in Fig. 4c due to technical problems

Fig. 4. *Gadus morhua*. Variation in female cod (n = 45) in maturity sub stages MIII$^{0\text{-}2}$ and maturity stages MIV – MIX in: gonadosomatic index (GSI) and ovarian lipid content (a), hepatosomatic index (HSI) and liver lipid content (b), proportion of phospholipids (PL) and triacylglycerols (TAG) in ovary* (c) and liver (d) estimated content of docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) in total lipids (sum of phospholipids (PL) and triacylglycerols (TAG)) of cod (n = 45) in ovarian lipid (e) and liver lipid (f). Grey boxes represent the maturity stages with spawning fish.

In livers, the average proportion of TAG was 89% and PL 7% with no variation with maturation (Fig. 4d) and cholesterol and FFA varied between 0 - 4% (not shown). Average DHA content in ovary lipid increased to 171 mg g^{-1} lipid in MV and decreased again in MIX (Fig. 4e). In contrast ARA decreased to around 8 mg g^{-1} lipid in MIV and during spawning and then increased again while EPA was more or less constant. In liver lipid absolute content of DHA was relatively stable until an increase in MVII while ARA decreased already in MIV and was only around 5 mg g^{-1} lipid during spawning while EPA was relatively constant (Fig. 4f). ARA in ovary lipid was positively correlated (slope = 1.0) with ARA in liver (Fig. 5, $r^2 = 0.25$, $p<0.01$) while no correlation was found for DHA ($r^2 = 0.01$, $p<0.01$) and EPA ($r^2 = 0.00$, $p<0.01$) (not shown).

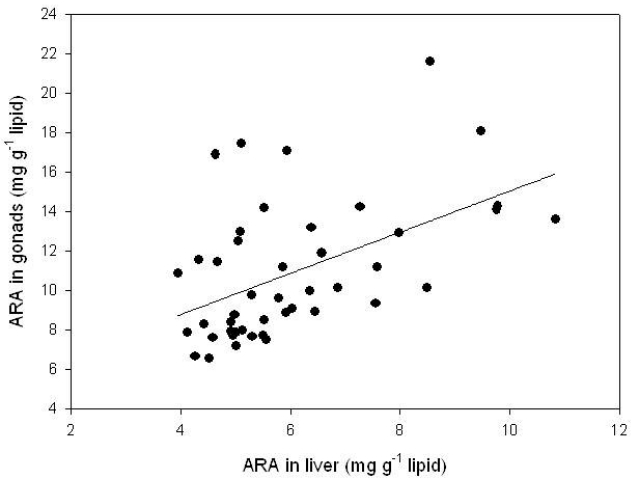


Fig. 5. *Gadus morhua*. Estimated contents of arachidonic acid (ARA) of total lipids (sum of phospholipids (PL) and triacylglycerols (TAG)) in ovary as a function of estimated contents in liver, n = 45.

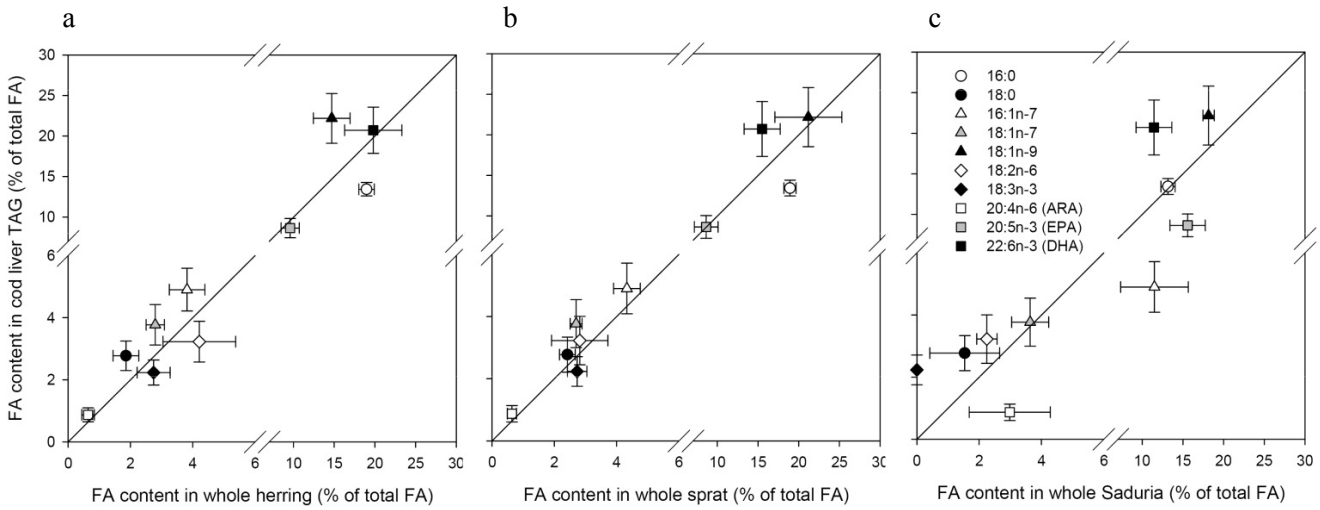


Fig. 6. The relationship between average content of important FA ± SD of cod liver TAG and FA content ± SD of total lipid in whole herring (a), sprat (b) and *Saduria entomon* (c). Values are the grand mean of all seasons and the line illustrates line of equality, cod: n = 45, herring: n = 62, sprat: n = 45 and *S. entomon*: n = 4.

Predator-prey relationships

FA content of cod liver TAG and FA content of total lipid in herring, sprat and *Saduria Entomon* was compared to identify predator-prey relationships (Fig. 6). The proportion of 18:0, 16:1n-7, 18:1n-7, and ARA were higher in cod liver than in sprat and herring while 16:0 and 18:3n-3 were lower. The average proportion of 18:1n-9 in cod was similar to the content in sprat while level of DHA was similar to that of herring. Level of 18:2n-6 in cod was higher than in sprat, but lower than in herring. The proportion of 16:0 and 18:1n-7 was identical in cod liver and *S. entomon* while 16:1n-7, EPA and ARA were found in higher proportions in *S. Entomon*.

Antioxidants in cod and its prey

The only tocopherol homologue detected in ovaries was α-tocopherol. The concentration of α-tocopherol in gonads at different maturity stages (Fig. 7a). Average concentration of α-tocopherol varied between 18.5 and 76.5 µg g^{-1} with highest levels in late maturing cod. Average concentration of astaxanthin varied between 1.2 and 10.3 µg g^{-1} (Fig. 7b), but only $MIII^0$ and MIX differed significantly. The outlier in $MIII^0$ had an astaxanthin level of 27.6 µg g^{-1} and this ovary had a clear orange colour. The level of tocopherol was closely correlated with the level of lipid in ovaries ($r^2 = 0.73$, $p < 0.01$)

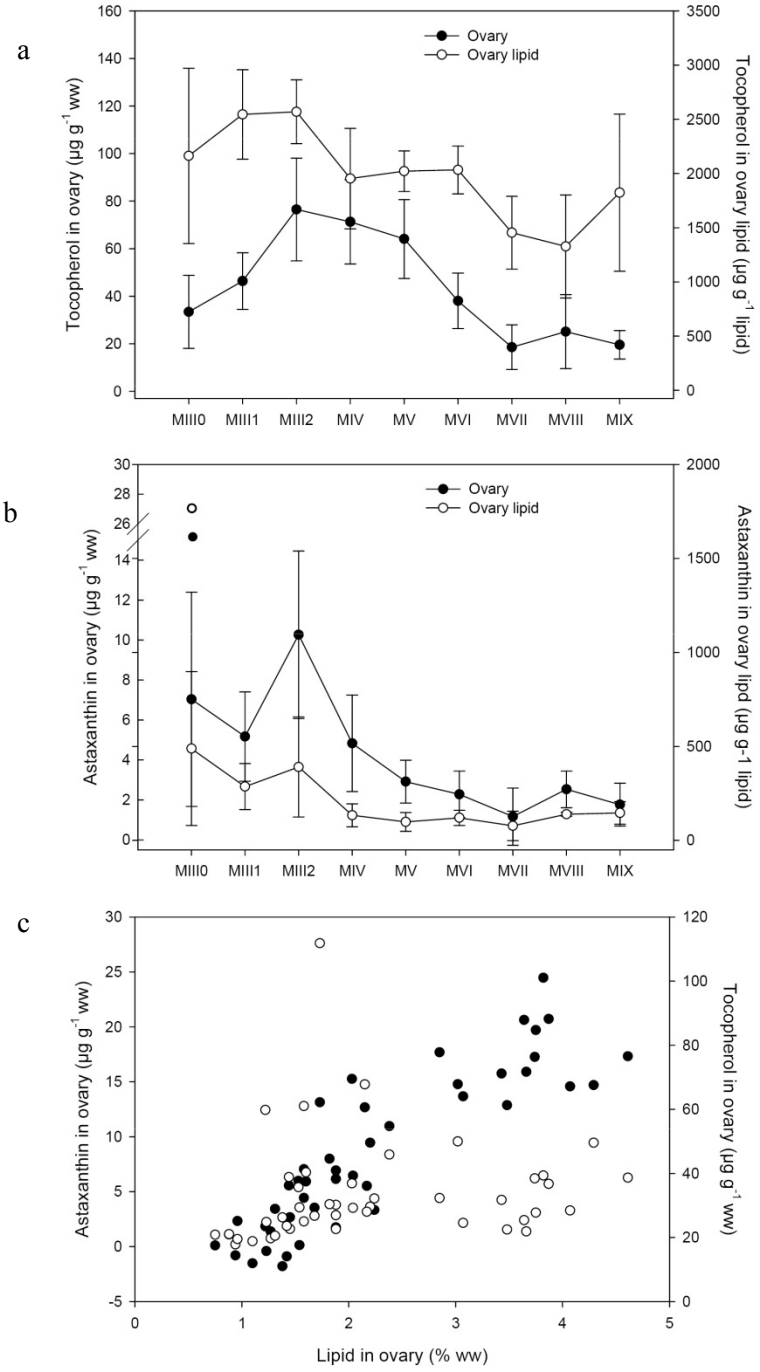


Fig. 7. *Gadus morhua*. Content of α-tocopherol (a) and astaxanthin (b) in ovaries of cod in maturity sub stages $MIII^{0-2}$ and maturity stages MIV – MIX and as a function of lipid content in ovaries (c), n = 45.

while astaxanthin showed no relation to lipid level (r^2 = 0.004, p = 0.67) (Fig. 7c). Average astaxanthin concentration was 1.2 ± 0.6 μg g^{-1} in sprat (n = 45), 1.3 ± 0.7 μg g^{-1} in herring (n = 64) and 10.1 ± 5.4 μg g^{-1} in *Saduria entomon* (4.2 - 14.7 μg g^{-1}, n = 3) and average α-tocopherol concentration was 1.7 ± 3.4 μg g^{-1} in sprat and 0.9 ± 2.1 μg g^{-1} in herring. No significant correlation between lipid level and astaxanthin and α-tocopherol concentration was found in sprat and herring.

DISCUSSION

Investment of lipid energy in reproduction

A substantial amount of lipids was invested in reproduction by cod analyzed in the present study. Lipid transferred from liver to ovary increased the amount of lipid in ovary relative to liver 10 times during gonadal development both due to the increase in size and lipid content of ovary. However, the food intake seemed to sustain high levels of liver energy because cod had ample lipid energy and liver lipid did not decrease until late in the reproductive cycle. This is later than usually reported for wild cod: in Baltic cod, a 45% decrease in liver lipid was detected from early (MIII) to late (MIV) maturation (Shatunovskiy 1971) and a pronounced decrease in lipid was found before spawning from winter to spring in Atlantic cod from Balsfjorden in Norway (Eliassen & Vahl 1982) and Nova Scotia in Canada (Jangaard et al. 1967b). The high lipid content in livers at the end of spawning (MVII) in the present study was not expected. The histological evaluation reclassified females of this maturity stage sampled in July and August, where this stage is normally most abundant and only females from May remained for lipid analysis. Females in late spawning are not common for the Eastern Baltic in May and it cannot be excluded that these fish originated from the Western Baltic cod stock that have peak spawning in February to April (Bagge et al. 1994, Bleil & Oeberst 1997).

Average GSI and HSI was similar to earlier reports for this stock (Tomkiewicz et al. 2003). HSI tended to decrease from the spent (MVIII) to resting stage (MIX) in the present study which could indicate that those fish had "skipped" spawning because this has previously been correlated with low HSI compared to a high HSI in resting fish which had spawned (Tomkiewicz et al. 2003). It is also noteworthy that there are some discrepancies between the trend in HSI and lipid content, most likely due to variation in water content in liver, which has previously been reported for Norwegian cod (Eliassen & Vahl 1982). The amount of lipid energy stored in the liver is crucial for recruitment as a highly significant linear relationship between total egg production and total lipid energy was

observed in the Barents Sea cod stock (Marshall et al. 1999). The increased proportion of PL during spawning in both ovary and liver was most likely caused by a selective retention of PL and a selective catabolism of neutral lipids especially TAG for reproduction particularly egg production. Similarly PL was observed to increase with GSI in Baltic herring and farmed trout (Kaitaranta & Ackman 1981). The variation in proportion of PL during a complete reproductive cycle has not earlier been described in cod, but an increase from MIII to MIV in absolute content of PL and from MIV to MV in relative content was reported for Baltic cod but PL did not exceed 39% total lipid in ovary (Shatunovskiy 1971) compared to 61% in the present study. The proportion of PLs in ovary was reported to be 72-76% of total lipid for cod (Kaitaranta & Ackman 1981, Tocher & Sargent 1984b) and was also reflected in over 70% PL in egg and larvae of cod (Fraser et al. 1988b).

Selective incorporation of PUFA in ovaries

This study demonstrated a selective incorporation of PUFA in ovaries during ovarian maturation. The PCA plots of ovary PL visualized the gradual shift from high levels of SFA in early maturation to high lipid content and high levels of n-3 PUFA and low levels of n-6 PUFA in spawning fish and a shift back towards immature again after spawning. The PCA plots also confirmed that PUFA contributed significantly to the differences observed among spawning and non-spawning cod. Proportion of PUFA was highest in PLs compared to TAG, which is consistent with an earlier study of ovaries of cod and other marine fish species (Tocher & Sargent 1984b) and is due to the structural role of PLs in cell membranes (Sargent et al. 2002). The increased absolute content of DHA in ovary lipid in late maturation and spawning indicated a selective retention of DHA during ovarian development. The ratio of absolute content of both DHA and EPA in ovary and liver lipid was close to one during maturation, but increased in MV and MVI, which indicated a selective transfer of these PUFA from liver to gonad during spawning. The increased DHA/EPA ratio in both ovary and liver during late maturation and spawning indicated furthermore a selective catabolism of EPA relative to DHA in fatty acid oxidative processes producing energy for oogenesis as suggested by Sargent et al. (2002). The increase in absolute content of EPA and particular DHA in liver lipid in females at the end of spawning (MVII) could be attributed to inclusion of Western stock cod in the samples, as explained above.

The role of ARA in maturation processes

Low content of ARA and high ratio of EPA/ARA coincided with cod ovarian development. ARA decreased in ovarian lipid in late maturation and spawning, in contrast to an increase in DHA. However, a high absolute content of ARA in ovary lipid compared to liver lipid together with the strong correlation between absolute ARA levels in lipid of gonad and liver provided strong evidence for selective incorporation of this fatty acid into ovaries. The mechanisms underlying lipid deposition in maturing oocytes is not clearly understood, but the link between low levels of ARA and delayed maturation may be during oocyte maturation with the lack of sufficient essential fatty acids for transfer to individual oocytes slowing down the process. However, this needs further verification in experimental studies in vivo and in vitro.

The decrease in ARA resulted in an increased EPA/ARA ratio in late maturation which could have a negative effect on the activity of eicosanoids. Eicosanoids produced from ARA are more biologically active than those produced from EPA and thus eicosanoid action is determined by the ratio of ARA to EPA in cellular membranes (Sargent et al. 2002). Mean ARA level of 1.5-1.8 in PL and TAG of ovaries in late maturation (MIV) in the present study were lower than the 2.1-3.0 observed in ovaries of cod from the North Sea cod from 2004, and EPA/ARA ratio of 7-8 in Baltic cod was higher than the 5-6 in North Sea cod, that does not have a delayed spawning (Tomkiewicz et al. unpublished data). However, mean ARA and EPA/ARA in cod from the North Sea around Scotland in 1984 was similar to Baltic cod (Tocher & Sargent 1984b). ARA levels of 2.7 in total lipids of cod from Nova Scotia (Jangaard et al. 1967b) that primarily spawns in winter was also higher than in the Baltic, however EPA/ARA was 8 similar to Baltic cod. However, the two latter studies were based on very few specimens and the study of Jangaard et al. (1967) reported a high individual variability. Hence, due to these contradictions and the low number reference data the effect of ARA and EPA/ARA on ovarian maturation and spawning time remains unsolved.

A DHA/EPA/ARA ratio around 15/8/1(2) has been recommended for larval nutrition (Sargent et al. 1999a). The composition of ovaries in the present study did not deviate substantially from this ratio. However, the low ARA level in cod ovaries in late maturation and spawning could have negative effects on egg quality as similar ARA levels in eggs has been reported to be correlated with poor quality in cod (Pickova et al. 1997, Salze et al. 2005). In rearing experiments, hatching rate was significantly lower for eggs of Baltic cod than those of Skagerrak cod (Pickova & Larsson 1992, Larsson 1994, Pickova et al. 1997), indicating poor egg and larval quality of Baltic cod. Poor larval survival was observed in the mid-1990s and was related to the lack of recovery of the Baltic cod stock despite improved hydrographic conditions in this period (Köster et al. 2005). Hence, the low

ARA level observed in cod may have affected egg and larval viability in the Baltic Sea. However, this needs further verification in experimental studies.

Other factors influencing spawning time

Low temperatures were shown to delay spawning in cod. Experimental results for Atlantic cod report a delay in spawning time by 8-10 days with a decrease in temperature of 1°C (Kjesbu 1994) and cod kept at low temperatures (4°C below ambient) showed a delay in spawning of one month compared to cod kept at ambient temperatures (Kjesbu et al. 2010). Seasonal peak egg abundance in Baltic cod was also negatively associated with ambient water temperature in January to April in the period 1986 to 1996 but only at low levels of cod spawning biomass (Wieland et al. 2000b). In the succeeding period 1996-2005 spawning stock biomass was low and temperature relatively stable (ICES 2007). However, date of peak egg abundance still varied considerably (Rüdiger Voss, pers. comm.). Time series of temperature in the central Baltic Sea showed an increasing trend and annual temperature minimum in intermediate winter water increased 1.5°C since 1988 (Meier et al. 2006). Therefore temperature did not fully explain the shift in spawning time. In contrast, high temperatures was suggested to delay spawning time in halibut, *Hippoglossus hippoglossus,* another, coldwater species (Brown et al. 2006). However, according to the study by Wieland et al., (2000) this seems unlikely to be the case in Baltic cod.

Changes in age composition were suggested to influence the timing of spawning because many fish species spawn progressively earlier within a season and may produce more egg batches over a longer period as they get older (Wright & Trippel 2009). This phenomenon was observed in cod (Tomkiewicz & Köster 1999, Marteinsdottir & Björnsson 1999) but the timing of peak spawning of Baltic cod females did not differ substantially relative to size (Tomkiewicz & Köster 1999, Tomkiewicz & Kraus 2005). Hence it was concluded that changes in age composition were unlikely to be a major cause of the delayed spawning.

Predator-prey relationships

The approach of tracing trophic pathways by comparing TAG of storage lipid and total lipid of whole prey followed the recommendation by Iverson (2009). In captive cod it was shown that FA of whole cod reflected their diet after 3 weeks or maybe less, as no samples were taken earlier (Kirsch et al. 1998). In cod, dietary lipids are primarily stored in the livers (Lambert & Dutil 1997), and like most marine predators, cod consume their prey whole. Thus, liver samples most likely reflect

dietary content earlier than muscle samples. The present results indicated a selective catabolism of 16:0 and selective incorporation of DHA, ARA, 18:1n-9 and 18:1n-7 in cod liver TAG. This is consistent with SFA being predominantly mobilized for metabolic purposes, whereas 18:1 (Weber et al. 2003) and PUFA is mainly retained (Sargent et al. 2002, Dalsgaard & St John 2004). Low SFA can furthermore be due to low assimilation because the digestibility of fatty acids decreases with saturation (Koven et al. 1994a, Olsen et al. 2004). The average proportion of 18:1n-9 and DHA in cod liver compared to proportions in sprat and herring indicated a mixed diet consisting of these two clupeids. The ratio between 18:1n-9 and DHA was almost twice as high in sprat than in herring and could function as a biomarker indicating the ratio of sprat and herring in cod diet.

Difference in FAC between sprat and herring was most likely due to differences in prey selectivity. In spring both sprat and herring prey mainly on copepods but in addition, sprat feeds on cladocerans in summer and autumn (Möllmann et al. 2004) and larger herring favour mysids, amphipods and polychaetes especially in autumn and winter (Casini et al. 2004). Herring was observed to switch from feeding mainly on adult stages of *Pseudocalanus* sp. to feed mainly on young stages of *Temora longicornis* with increasing competition from sprat (Möllmann & Köster 2002). DHA was very abundant in *T. longicornis* compared to *Pseudocalanus* sp., which on the other hand were very rich in 18:1n-9 (Peters 2006, Peters et al. 2006) and could explain the difference in 18:1n-9/DHA ratio between sprat and herring.

The level of 16:1n-7 could indicate a trace of the crustacean *Saduria entomon* in cod diet. Dietary 16:1n-7 is a good lipid biomarker because it seems not to be altered by marine animals. The content of this FA does not enter the internal biosynthetic pathway in copepods as it may only be elongated to longer-chain (n-7) isomers (Sargent & Henderson 1986), which are generally not detected in large amounts in calanoid copepods (Sargent & Falk-Petersen 1988). This is probably the same in fish as indicated by a study where cod larvae fed monocultures of either diatom or dinoflagellates reflected the 16:1n-7/16:0 ratio of the phytoplankton after 13 days (St John & Lund 1996). We would therefore expect this fatty acid to be similar in cod liver and prey. However, the average proportion of 16:1n-7 was slightly higher in cod than its main prey, sprat and herring, which indicated a small proportion of *S. entomon* in the diet of Baltic cod. *S. entomon* was an important prey in the 1980s where it contributed up to 30% of food consumption in the Central Baltic Sea (Zalachowski 1985a, Uzars 1994) but it declined by at least a factor 2 during 1985-1990 (Uzars 1994). The hypoxic zone in the Baltic Sea has increased in area about fourfold since 1960 (Zillen et

al. 2008) and abundance of *S. entomon* was low in hypoxic areas (Janas et al. 2004). A low proportion of *S. entomon* in cod diet is most likely to affect the ARA level in cod.

Antioxidant dynamics

The present study indicated an accumulation of α-tocopherol (vitamin E) during early ovarian development. Tocopherol levels in cod ovaries with maturation have not earlier been published but the concentration in this study was on average 46 times higher than in cod muscle from the Northern part of the Baltic Sea (Syvaoja et al. 1985b). An accumulation of α-tocopherol in ovaries is consistent with transport from muscle to ovary during vitellogenesis as indicated in a study of salmon (Lie et al. 1994b). Average concentration of α-tocopherol in cod ovaries was 12 - 89 times greater than the Baltic herring and sprat respectively and hence an accumulation in ovary compared to dietary concentration was evident. However, in a feeding trial with Atlantic cod, vitamin E concentration in muscle was not correlated with dietary vitamin E level, but was instead negatively correlated with dietary lipid content (Hemre et al. 2004). Higher dietary vitamin E concentrations significantly decreased muscle concentrations of thiobarbituric acid reactive substances (TBARS) which are an index of lipid peroxidation and oxidative stress. We suggest that tocopherol was actively transported to the ovaries during vitellogenesis and the decrease in concentration from late maturation ($MIII^2$) to end of spawning (MVII) was caused by the antioxidant protection activity due to the increased levels of DHA.

Astaxanthin levels in cod ovaries were higher than in whole sprat and herring indicating a selective retention in ovaries. This result is consistent with an accumulation of astaxanthin in ovaries compared to other tissues found in a recent study on Baltic cod (Nie et al. 2011). Astaxanthin levels were much higher in stomachs of sprat and herring compared to muscle indicating that a direct transfer of crustacean astaxanthin from the clupeid stomach to piscivores is important (Nie et al. 2011). Astaxanthin levels in clupeids are therefore influenced by diet and transfer of astaxanthin to higher trophic levels is consequently low in periods of starvation. However, the measured astaxanthin concentration in whole sprat and herring in the present study was approximately 10 times higher than calculated from the weighted concentration of gonad, liver, muscle and stomach by Nie et al. (2011). This could indicate that a substantial proportion of astaxanthin is found in other body parts of clupeid such as skin, brain, kidneys and plasma as reported for cod and salmon (Bell et al. 2000, Ytrestoyl & Bjerkeng 2007). *Saduria entomon* had on average 8 times more astaxanthin than sprat and herring in the present study and so had many other Baltic crustaceans (Czeczuga

1976b). Crustaceans can thus be considered an important diet component and consequently a decreased intake of crustaceans would affect astaxanthin levels. The concentration of astaxanthin was on average 17 times lower than α-tocopherol but the defense mechanisms of astaxanthin were observed to be 100 times greater than that of *α*-tocopherol (Miki 1991). Low astaxanthin level during late maturation and spawning may cause an inadequate antioxidant protection during maturation which is critical due to high levels of PUFA.

CONCLUDING REMARKS

This study demonstrated an increase in relative lipid content in ovaries during ovarian development and a notable decrease during maturation. FAC of ovary and liver varied with maturity stage in Baltic cod and a selective retention of DHA and ARA in ovaries during ovarian maturation was evident. Despite mobilization of ARA from liver, the level was not sufficient to keep up with the requirement in ovaries and ARA decreased in late maturation and during spawning. Low content of ARA and high ratio of EPA/ARA coincided with cod ovarian development in the central Baltic Sea. The antioxidants *α*-tocopherol and astaxanthin were accumulated in cod ovaries compared to prey and decreased in late maturation and spawning due to antioxidant protection activity. The novel information on linkage between reproduction and lipid dynamics in Baltic cod provided in this manuscript is important because it enhances the understanding of lipid requirement in cod. However experimental investigations of the detailed role of ARA in gonadal maturation, ovulation and for egg and larval quality in cod are needed to fully understand the underlying physiological processes involved.

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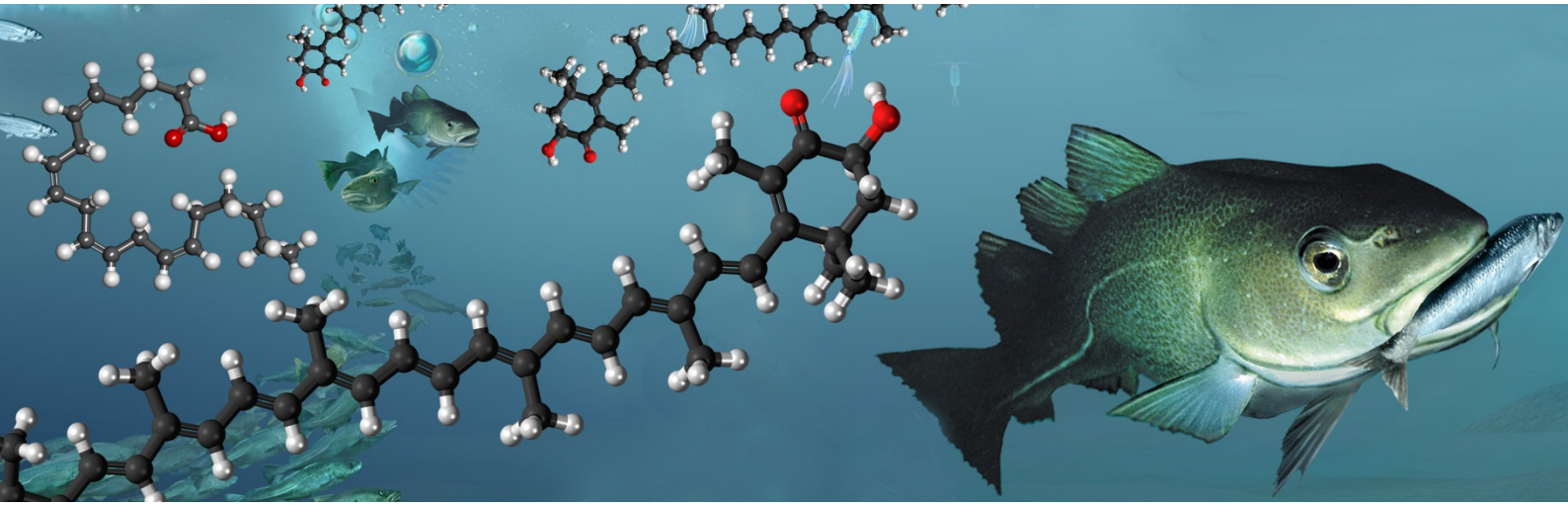
Paper III



Lipid energy reserves and fatty acids in reproduction of Baltic and Atlantic cod (*Gadus morhua* L): Imprint of trophic regime

by Jonna Tomkiewicz, Maria C. Røjbek, Josianne G. Støttrup, Charlotte Jacobsen & Friedrich W. Köster

Illustration modified from image by Glynn Gorick for the ICES/GLOBEC project: Cod and Climate Change

Lipid energy reserves and fatty acids in reproduction of Baltic and Atlantic cod (*Gadus morhua* L): Imprint of trophic regime

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ABSTRACT

The present study focused on female Baltic cod (*Gadus morhua*) lipid energy reserves and fatty acid (FA) dynamics during ovarian development from immature until prespawning. A comparison between prespawning Baltic and North Sea cod was included to elucidate potential limitation in lipid energy and essential fatty acids (EFA) potentially impacting on Baltic cod reproduction. We analysed lipid content and FAC in phospholipids (PL) and triacylglycerides (TAG) in ovaries and liver of individual female cod. Ovarian lipid content increased as development progressed in Baltic cod and was similar in ovaries of prespawning cod from both areas. By contrast, liver lipid content and hepatosomatic index were significantly higher in Baltic cod than in North Sea cod. FAC including EFAs important to reproduction in cod ovarian and liver PL and TAG reflected ecosystem characteristics and differed among areas. In particular, we found proportions of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) to be significantly higher in Baltic cod ovaries and livers than in North Sea cod, while arachidonic acid (ARA) levels were significantly lower. Proportions of ARA significantly decreased while DHA significantly increased in ovarian PL, TAG and liver PL as development progressed in Baltic cod, while both remained constant at a low level in liver TAG. Ample lipid energy in Baltic cod livers and high ratios of DHA/ARA and EPA/ARA indicate ARA limitations in the Baltic food web potentially affecting cod reproduction. Suboptimal egg lipid composition may affect reproduction success and potentially prolong the maturation period contributing to a delay in spawning time of Baltic cod observed since early 1990ies. The present study confirms studies reporting an imbalance in fatty acid composition at the base of the food web of the Baltic Sea by addressing effects on the upper trophic level.

INTRODUCTION

Reproduction of cod (*Gadus morhua*) in the Baltic Sea is highly dependent on the hydrograhic conditions affecting survival of eggs and larvae (Köster et al. 2003a). Cod in the central Baltic Sea declined in the late 1980's from historic high levels to low levels in the 1990's due to intense fisheries and reproduction failure (Köster et al. 2005) followed by a delay in spawning time (Wieland et al. 2000, Kraus et al. 2002). Hydrographic stagnation and eutrophication of the estuarine waters during the 1980's and 1990's altered the planktonic food web and caused a decline in macrobenthos (Alheit et al. 2005, Karlson et al. 2007, Österblom et al. 2007). A new ecological regime dominated by high sprat (*Sprattus sprattus*) abundance led to increased planktivore food competition and a declining nutritional condition of both sprat and herring (*Clupea harengus*) (Möllmann et al. 2005, Casini et al. 2010). Especially in stagnation periods, these clupeids are the primary prey of cod (Uzars 1994) and their abundance and nutritional condition impact cod energy intake and diet composition including fatty acids (FA) (Neuenfeldt & Beyer 2006, Røjbek et al. 2012).

Lipid storage and fatty acid dynamics are particularly important aspects of fish physiology and population dynamics because they have a large influence on growth, reproduction and survival. Lipids are obtained directly from the diet or synthesised *de novo* by the fish, however some fatty acids considered essential must be obtained through the diet (Sargent et al. 1999, Sargent et al. 2002). The liver plays a critical role in various aspects of lipid metabolism such as the uptake, oxidation and conversion of fatty acids and the supply of long chain polyunsaturated fatty acids (PUFA) to other tissues (Tocher 2003). Fish store reserve lipid in the liver or muscle and during maturation dietary nutrients and lipid reserves are mobilized and used in ovaries for egg development. Analyses of lipid content and FA profiles of body, liver and ovaries from fish under natural or controlled feeding conditions can yield valuable information, which may elucidate their nutritional status and FA requirements.

Fish need different classes of PUFA in right proportions for normal development, behaviour and reproduction (Henderson et al. 1984, Sargent et al. 1999, Sargent et al. 2002, Tocher 2003). PUFA include essential fatty acids (EFA) that help maintain structural and functional integrity of cells and regulate vital physiological processes in all life stages (Bell et al. 1997, Henderson et al. 1997, Sargent et al. 1999, Izquierdo et al. 2001, Tocher 2003, Masuda, 2003, Glencross 2009). In particular, the incorporation of PUFA into fish eggs during ovarian development is crucial to

embryonic and larval development (Wiegand 1996). In general, fish eggs contain high proportions of EFAs as the larvae depend on FA in yolk during early development. EFA important for fish reproduction and early life stages include in particular docosahexaenoic acid (22:6n-3, DHA), eicosapentaenoic acid (20:5n-3, EPA) and arachidonic acid (20:4n-6, ARA), (Bell et al. 1997, Sargent et al. 1999, Bell & Sargent, 2003 Tocher 2003, Masuda 2003).

Freshwater fish produce EPA and DHA by chain elongation and desaturation of α-linolenic acid (18:3n-3 = ALA) (Kanazawa et al. 1979, Yamada et al. 1980) and similarly ARA can be produced from linoleic acid (18:2n-6 = LA) (Cowey et al. 1976). By contrast, most marine fish species have low or no capacity to synthesize DHA, EPA and ARA *de novo* (Sargent et al. 1989, Bell & Tocher 2009) and thus have a dietary requirement for these PUFA (Lie et al. 1992, Sargent et al. 1999, Sargent et al. 2002). PUFA in the marine environment originate from primary producers mainly phytoplankton and macroalgae (Bergé & Barnathan 2005, Sargent et al.1995) and vary considerably both within and between phytoplankton taxa (Dalsgaard et al. 2003). These FAs are often transmitted conservatively through the food web and cause substantial spatial and temporal variation in food web lipid dynamics. The contents and composition of PUFAs in marine fish thus relates to the composition of their prey and specific PUFAs are retained by predators at different trophic levels (Kainz et al. 2004). Hence, FAC and lipid dynamics may reveal trophic relations in food webs through energy transfer and FA acting as biomarkers (Arts et al. 2001, Ahlgren et al. 2005, Parrish 2009).

The Baltic ecosystem changes that occurred during the late 1980's and early 1990' included a pronounced decline in diatom biomass and an increase in flagellates and dinoflagellates, associated with increasing temperatures, decreasing salinities and inorganic nitrogen concentrations (Wasmund & Uhlig 2003; Wasmund et al. 2011). These change had an impact on the production of PUFAs in the system, especially ARA (Ahlgren et al. 2005). Observed changes in the mesozooplankton community may partly be caused by this switch from diatom to dinoflagellate dominance and partly by hydrographic changes, i.e. increasing temperatures and declining salinities favouring and penalizing specific copepod species (Alheit et al. 2005, Möllmann et al. 2008). The lipid rich copepod *Pseudocalanus acuspes* declined in abundance, showing strong negative anomalies throughout the 1990's and most of the 2000's, while lipid poor copepods such *Temora longicornis* increased in standing stocks (Peters 2006). The fatty acid composition of these copepods is affected by the dominance of dinoflagellates and characterized by extremely low level of ARA independent of the overall lipid content (Ahlgren et al. 2005, Peters 2006). These changes in the mesozooplakton

community enforced by increasing intra- and interspecific competition in sprat and herring stocks had a negative impact on the nutritional condition of these clupeids (Möllmann et al. 2005, Casini et al. 2010). The imbalance of fatty acids at the base of the Baltic Sea food web and changed trophic dynamics may influence the growth and reproduction success of the Baltic cod that remained at a low level until recently.

Acknowledging the substantial changes in the food web structure of the central Baltic Sea and the potential limitation of certain EFA for reproduction, our study focused on lipid and FA dynamics of female cod during maturation in the Baltic Sea. The study included a comparison between prespawning Baltic and North Sea cod to elucidate potential limitation in lipid energy and EFAs during maturation that may impact cod reproductive performance including a shift in spawning time. The North Sea ecosystem has not experienced similar ecosystem changes and the cod has a fundamentally different and more diverse diet (Kikkert 1993), with likely more balanced fatty acid composition and thus serves a suitable reference.

In the present study, we posed the hypothesis that foodweb changes affect lipid energy reserves, FAC and ovary EFA accumulation in Baltic cod potentially prolonging the maturation period and delaying spawning time. We investigated lipid content and FAC in phospholipids (PL) and triacylglycerides (TAG) in ovaries and liver of individual female cod including three maturity stages immature, maturing and prespawning for Baltic cod as well as the prespawning stage for North Sea cod. The study describes i) the accumulation of total lipid in ovary and liver, followed by a detailed analysis of ii) changes in ovarian FAC in Baltic cod during maturation, and differences in FAC between Baltic and North Sea cod in the prespawning stage, iii) changes in FAC of livers during Baltic cod maturation as well as differences among stocks and lastly iv) relationships between DHA, EPA and ARA during maturation in relation to accumulated ovary and liver lipid reserves. We discuss lipid dynamics and FAC, utilisation of lipid reserves and accumulation of dietary fatty acids in ovaries and eggs considering the reproductive potential and shift in spawning time of Baltic cod.

MATERIALS AND METHODS

Field sample collection

Cod were sampled onboard seven trawl surveys in the central Baltic Sea (B) (15-18°E, 54-56°N) and the North Sea (N) (Scotia -3-2°E, 58-61°N and Dana -1-8°E, 54-58°N) during 2003 and 2004 (Fig. 1, Table 1). The Baltic Sea sampling extended to from November - January to include the

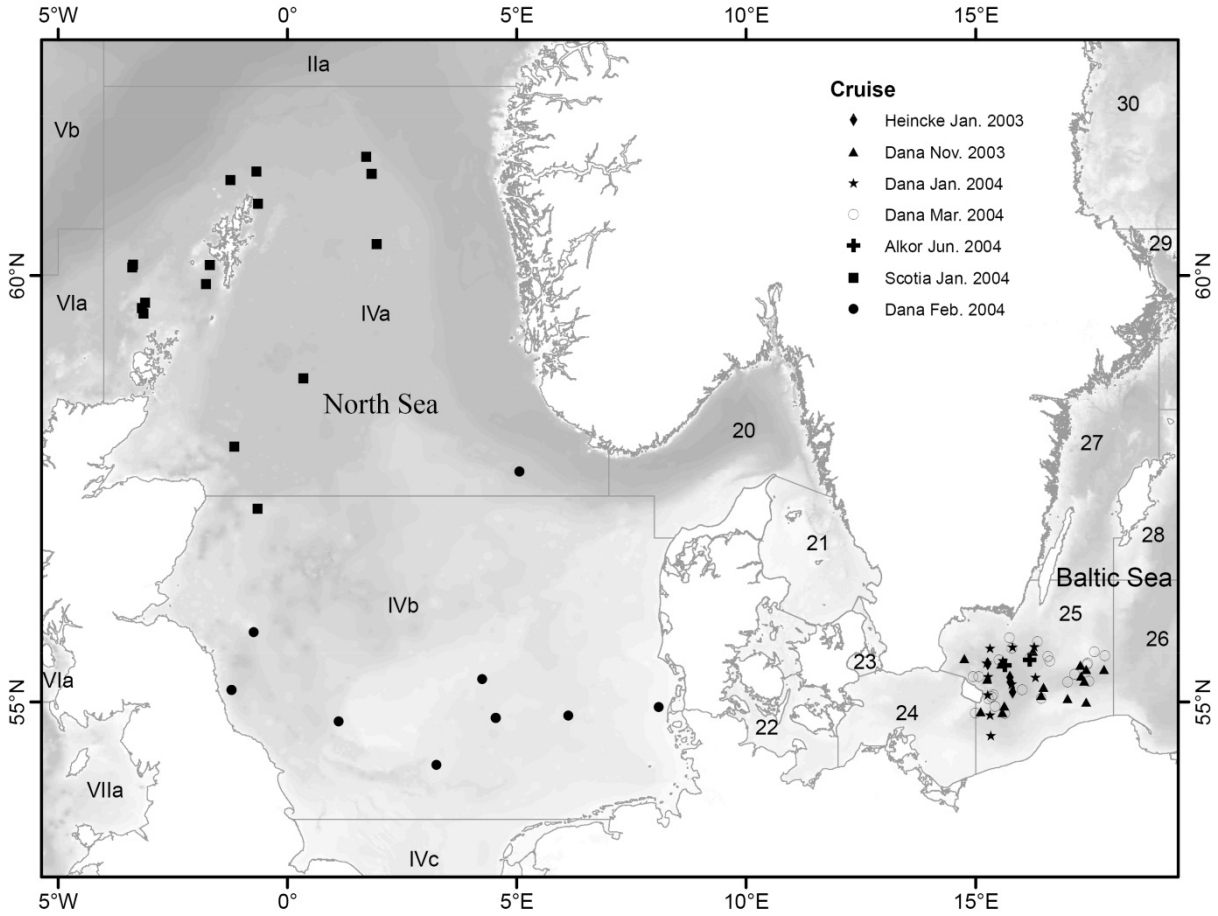

Fig. 1. Map of study area. Symbols represent sample stations from seven trawl surveys. In the North Sea Roman numerals and lowercase letters indicate ICES area divisions and subdivisions. In the Baltic Sea Arabic numerals identify ICES sub-divisions.

entire ripening period, whereas North Sea sampling took place in January - February to obtain ripe specimens. Onboard research vessels sex and gonadal maturity of cod was graded from macroscopic characters according to Tomkiewicz et al. (2003). Sampling of Baltic cod (B) focused on maturity stages II, III, IV and IX and for North Sea cod (N) on stage IV and aimed at min. 10 specimens per 10 cm length group starting at 30 cm. For each female, total length (TL) was measured to the nearest lower integer (cm), whole body weight (BW), and eviscerated body weight (EBW i.e. gonad, liver, stomach and viscera removed) was measured (g). Ovary weight (GW) and liver weight (LW) were recorded (0.1 g). A sub-sample of the ovarian tissue was preserved in formalin buffered with NaH_2PO_4-H_2O and Na_2HPO_4-$2H_2O$ for histological validation of maturity. The remaining ovarian tissue and livers were frozen and stored at minimum -40˚C for analysis of lipid content and fatty acid composition FAC.

Table 1. *Gadus morhua.* Female cod sampled for analysis of lipid content and fatty acid composition (FAC): Cruise information, number of specimens per maturity stage (MII-MIX) after histological validation, total numbers, total length (TL) and body weight (BW).

Research Vessel	Survey area	Year	Month	No. of specimens						Length (cm) Mean ± SD	Weight (kg) Mean ± SD
				MII	MIII	MIV	MV-VIII	MIX	Total		
Heincke	Baltic Sea	2003	Jan.	0	22	0	0	0	22	41.27 ± 07.33	0.69 ± 0.39
Dana	Baltic Sea	2003	Nov.	2	25	2	3	21	53	49.92 ± 08.57	1.28 ± 0.82
Dana	Baltic Sea	2004	Jan.	0	21	2	0	2	25	48.43 ± 13.38	1.38 ± 1.45
Dana	Baltic Sea	2004	Mar.	0	44	36	1	0	81	51.85 ± 12.60	1.62 ± 1.13
Alkor	Baltic Sea	2004	Jun.	0	2	9	1	0	12	40.00 ± 06.39	0.70 ± 0.35
Scotia	North Sea	2004	Jan.	0	2	17	3	0	22	67.79 ± 20.68	3.88 ± 2.99
Dana	North Sea	2004	Feb.	6	1	8	1	0	16	51.13 ± 19.96	1.42 ± 1.43
			Total	8	117	74	9	23	231	49.78 ± 11.65	1.41 ± 1.12

Histology

The preserved ovarian tissue was embedded in paraffin using standard procedures, sectioned at 7 µm and stained using hematoxylin and eosin. Ovarian development was evaluated histologically according to the morphological characteristics of the larger and more advanced oocytes (Tomkiewicz et al. 2003). Ovaries characterized by oocytes with circumnuclear ring (CNR) i.e. immature cod in preparation (MII) or resting stage (MIX) were grouped in the analyses as these stages show no signs of reproductive development. The early maturation stage, MIII, characterized by oocytes with cortical alveoli (CA) and/or presence of yolk granules i.e. vitellogenic oocytes (VTG) was subdivided according the appearance of progressively more advanced characteristics: oocytes with CA only = $MIII^0$; peripheral yolk granules among CA (VTG1) = $MIII^1$; and yolk granules filling most but not the entire cytoplasm (VTG2) = $MIII^2$. The late ripening stage, MIV, was characterized by considerably enlarged oocytes with yolk granules filling the entire cytoplasm. Stages MV-VII comprised stages where spawning had started evidenced by the presence of hydrated eggs or post ovulatory follicles.

Lipid extraction and determination of lipid content

Lipids in ovaries and livers were extracted from 5 g samples with a homogeneous mixture of chloroform, methanol and water (2:2:1.8) following the method of Bligh & Dyer (1959). The method was modified to use a smaller volume of solvents, but the original ratio between chloroform, methanol and water was maintained. Lipid extracts were used for the subsequent lipid class fractionation and determination of FAC as well as for determination of lipid content. Lipid

content was determined by gravimetry after evaporation of chloroform and is expressed as percentage of wet weight of ovary and liver respectively throughout the study.

Lipid class separation and preparation of fatty acid methyl esters

Ovary and liver lipids extracts were separated in phospholipids (PL) and the triacylglyceride (TAG) fraction of neutral lipids (NL) by chromatography on a solid phase consisting of aminopropyl modified silica. Solvents with increasing polarity were used to separate lipid classes. A lipid extract corresponding to 10-100 mg lipid was used for the lipid class separation. Solvents from the lipid extraction were evaporated and the extract was resolubilized in 0.5 ml chloroform and transferred to a Sep-Pak column (Waters Corporation, Milford, Massachusetts)**.** TAGs were eluated using 4 ml chloroform/2-pronanol (2:1), and PLs were eluated with 6 ml methanol. Eluates were evaporated to almost dryness (TAG) or to 1-2 ml (PL) under nitrogen.

Eluates from lipid class separation of gonad or liver extracts were used for the preparation of FAME using AOCS Official Method Ce 2-66 (1992b). C23:0 methyl ester was used as internal standard. FAME were analysed on a HP 5890A gas chromatograph (Hewlett-Packard, Palo Aalto, CA) equipped with an Omegawax 320 (30 m x 3.2 mm x 0.25 μm) column from Supelco (Bellefonte, PA) using AOCS Official Method Ce 1b-89 (1992a). The oven temperature programme was: 3 $^{\circ}$C min^{-1} to 200^{o}C, hold 1 min, 3 $^{\circ}$C min^{-1} to 220 $^{\circ}$C, hold 17 min and 15 ^{o}C min^{-1} to 160 $^{\circ}$C, hold 2 min. A split ratio of 1:25 was used. FAs were identified by comparison of retention times with a mixture of standards containing all the FAs identified in this study. Each FA was quantified by calculating its peak area relative to the total peak area and is expressed as percentage of total FA throughout the paper.

Sample size

The samples comprised ovaries and livers from 231 female cod sampled in 2003-2004 (Table 1). The size range of cod caught in the Baltic Sea was similar to cod in the southern North Sea (N^{b}), while cod caught in the northern North Sea (N^{a}) tended to be larger. The histological validation of maturity stage was used to establish uniform categories for the analyses. The analysis related to Baltic cod maturation comprised 188 specimens in undeveloped stages MII+MIX: immature/resting, MIII: early maturation, and MIV; late maturation/prespawning. The spatial analysis comparing prespawning female cod of different origin included 49 MIV specimens from the Baltic Sea and 25 MIV specimens from the North Sea. The resulting data for analyses of liver

and ovary lipid and FAC included in total 213 female cod. The remaining 18 specimens reclassified to adjacent stages by the histological validation were excluded from this analysis.

Analysis

The following maturation and conditions indices were calculated: Gonadosomatic Index (GSI) = GW × 100 / EBW, hepatosomatic index (HSI) = LW × 100 / EBW, Lipid Hepatosomatic Index (LHSI) and Lipid Gonadosomatic index (LGSI) computed by the formulas LHSI = ANSL / EBW x 100 and LGSI = ABSG / EBW x 100, where ABSL and ABSG are the absolute lipid content in the liver and ovary, respectively, computed by multiplying the respective lipid contents (in % wet weight) by total liver and ovary wet weights (Lloret et al. 2008). Proportions of each FA in PL and TAG in ovary and liver, total ovarian or lipid content, GSI and HSI, and length were calculated as means ± SD.

Statistical analyses were performed using the SPSS package (version 15.0 for Windows). Data were checked for normal distribution with a Shapiro-Wilks test and for homogeneity of variances with a Levene test. Differences between prespawning cod from the Baltic Sea and North Sea (MIV) were tested with T-test when data were normally distributed and the non-parametric Kolmogorov-Smirnov Z-test when the normality test failed. Differences between the maturity stages MII+MIX, MIII and MIV in Baltic cod were analysed using one-way analysis of variance followed by Tukey's multiple comparison test. When normal distribution or homogeneity of variances was not achieved, data were subjected to the Kruskall-Wallis non-parametric test followed by Dunnett T3 test for non-parametric multiple comparison. Spearman Rank Correlation Coefficient r_s was calculated to determine relationships between lipid content, levels of specific FAs and GSI / HSI. A significance level (p) of 0.05 was applied in all tests.

Multivariate data analysis was performed using the Unscrambler® v9.1 (CAMO, Oslo, Norway) software. Data were organised as a matrix with liver and ovary samples (TAG + PL) as rows and variables as columns including relative peak areas of 33 identified FAs, groups and ratios of FAs, lipid content, GSI, HSI, total length, maturity stage and cod origin Baltic (B) or North Sea (N). The latter was sub-divided into a northern area, N^a (ICES Sub-div. IVa) and southern part, N^b (ICES Sub-division (IV b) (Fig. 1). Models were validated by full cross-validation. Fatty acid variables were column mean centred and normalised to unit column standard deviation (SD) before calculation of the models. Weighting with 1/SD before calculation prevents the FAs found in large quantities from dominating the model ignoring FAs found in smaller quantities. Analyses were

carried out by using principal component analysis (PCA). Analyses of PL and TAG in livers and ovaries from 1) Baltic cod in maturity stages MII+MIX, $MIII^0$, $MIII^1$, $MIII^2$ and MIV and 2) cod in maturity stage MIV from the Baltic Sea and North Sea were compared.

RESULTS

Lipid accumulation in ovary and liver

Total ovarian lipid content in the sampled cod increased significantly with GSI (r_s = 0.77, $p < 0.001$, Fig. 2a) from immature and resting in maturity stage MI+MIX through early maturation MIII to prespawning MIV. Lipid contents ranged from about 1 to 4 % with a substantial scatter of observed values for maturity stage MIV including both Baltic and North Sea cod. The liver lipid content was positively correlated with HSI (r_s = 0.47, $P < 0.001$, Fig. 2b) and ranged from about 10 to 80 %. The relationship tended to be curvilinear approaching an upper threshold at app. 80 %. Mean GSI differed significantly among maturity stages ($p < 0.001$) in Baltic cod (Fig. 3a) and the corresponding lipid content in ovaries was significantly higher in MIV cod than in MII+MIX and MIII ($p < 0.001$). Mean GSI of North Sea females in stage MIV tended to be higher than in Baltic cod females, but neither GSI ($p = 0.33$) nor ovarian lipid content ($p = 0.66$) differed significantly among stocks. Mean HSI of Baltic cod in Stage MIV was significantly higher than in MII+MIX ($p < 0.01$) and MIII ($p = 0.03$) while the liver lipid content did not differ significantly among stages (Fig. 3b). Mean HSI and lipid content were significantly higher in prespawning MIV Baltic cod than in North Sea cod ($p = 0.02$ and $p < 0.01$

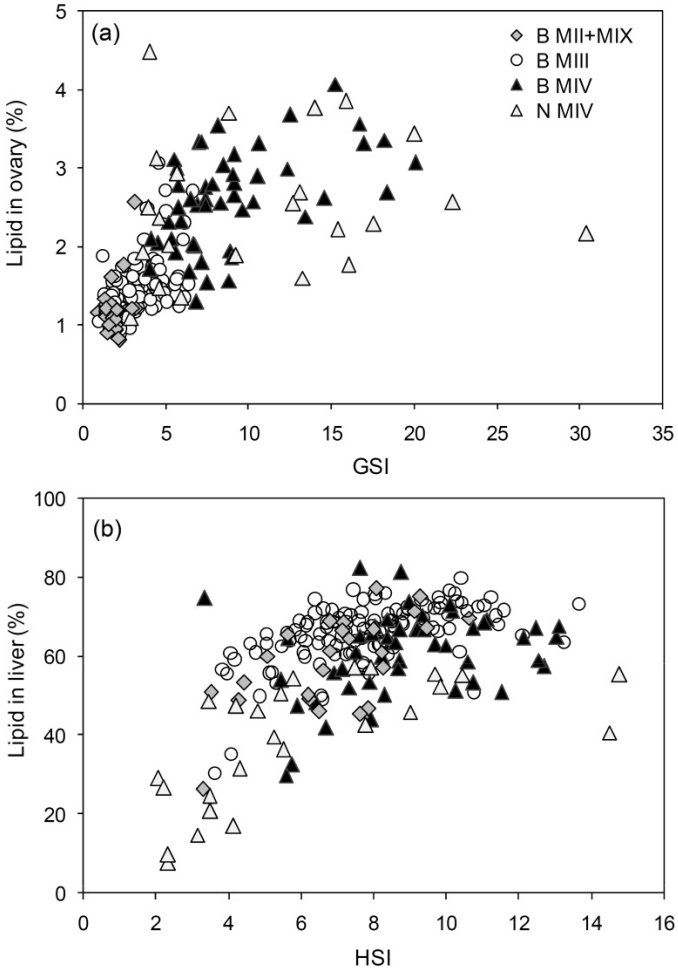

Fig. 2. Gadus morhua. (a) Percent lipid of individual ovaries in relation to gonadosomatic index (GSI) and percent lipid in liver in relation to hepatosomatic index (HSI) of Baltic Sea (B) and North Sea (N) cod ovaries in progressive maturity stages MII+MIX, MIII and MIV. See list of abbreviations.

respectively). The difference in mean lipid content was substantial i.e. 61 % in MIV Baltic cod livers compared to 39 % in MIV North Sea cod. The differences in GSI and HSI among areas resulted in a gonad/liver ratio in the prespawning stage MIV being up to twice as high in the North Sea as in the Baltic Sea.

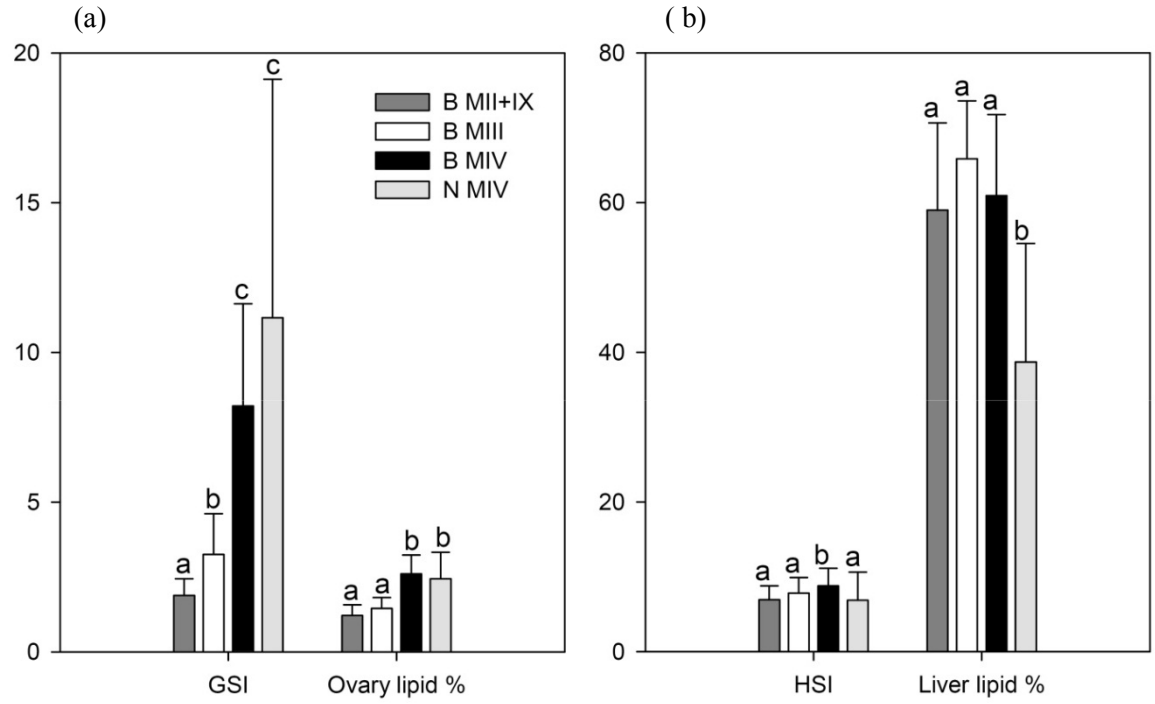

Fig. 3. *Gadus morhua*. (a) Gonadosomatic index (GSI) and percent lipid and (b) hepatosomatic index (HSI) and percent liver lipid in cod from the Baltic Sea (B; n = 49) in relation to maturity stage, MII+MIX, MIII and MIV and in cod from the North Sea (N; n = 25) in maturity stage MIV. Values are means ± SD. Letters define significant difference ($p < 0.05$).

Changes in ovarian FAC during maturation

The relative content of most FAs in phospholipids (PL) and triacylglycerols (TAG) in Baltic cod ovaries changed with progressive ovarian development (Table 2). The proportion of total SFA decreased from stage MII+MIX to MIV, while the proportion of total PUFA in ovarian PL increased significantly. In particular, the proportions of DHA, EPA, ARA and other PUFA e.g. linoleic acid (LA, 18:2 (n-6)) and α-linolenic acid (ALA, 18:3(n-3)) changed. Proportions of DHA increased significantly from stage MII+MIX to MIV, EPA showed a more moderate but also significant increase with progressive maturation. In contrast, the relative content of ARA significantly decreased from MII+MIX to MIV. Low ARA in combination with high DHA and EPA resulted in a pronounced increase in the DHA/ARA ratio from 7 to 16 and in EPA/ARA from 3 to 6, while the DHA/EPA ratio remained fairly constant.

Tabel 2. *Gadus morhua.* Fatty acid composition (% of total fattya acids) in ovaries from Baltic cod in maturity stages MII + IX, MIII and MIV and from North Sea cod in stage MIV separated into phospholipids (PL) and triacylglycerols (TAG) Values are means ± SD.

Fatty acid	Baltic Sea MII+IX	Baltic Sea MIII	Baltic Sea MIV	North Sea MIV
Phospolipids	(*n*=25)	(*n*=114)	(*n*=49)	(*n*=25)
SFA				
14:0	1.41 ± 0.25 ^{a}	1.31 ± 0.35 ^{a}	1.07 ± 0.32 ^{b}	1.16 ± 0.26 ^{z}
16:0	23.65 ± 2.04 ^{a}	21.79 ± 2.62 ^{b}	18.78 ± 2.28 ^{cx}	20.16 ± 2.06 ^{z}
18:0	2.54 ± 0.40 ^{a}	1.65 ± 0.50 ^{a}	1.89 ± 0.50 ^{c}	2.04 ± 0.51
MUFA				
16:1(n-7)	2.50 ± 0.48 ^{a}	3.09 ± 0.85 ^{b}	2.25 ± 0.67 ^{a}	2.31 ± 0.61
18:1(n-9)	9.94 ± 1.14 ^{a}	8.84 ± 1.32 ^{b}	10.29 ± 1.14 ^{a}	10.03 ± 0.99
18:1(n-7)	2.73 ± 0.53 ^{a}	2.33 ± 0.60 ^{b}	3.05 ± 1.13 ^{a}	2.94 ± 0.74
20:1(n-9)	0.57 ± 0.19 ^{a}	0.47 ± 0.15 ^{b}	0.51 ± 0.16 ^{abx}	1.78 ± 0.93 ^{z}
20:1(n-11)	0.40 ± 0.21 ^{a}	0.18 ± 0.16 ^{b}	0.18 ± 0.15 ^{bx}	0.11 ± 0.03 ^{z}
22:1(n-11)	0.00 ± 0.00 ^{a}	0.05 ± 0.02 ^{b}	0.04 ± 0.02 ^{cx}	0.31 ± 0.19 ^{z}
24:1(n-9)	1.14 ± 0.34 ^{a}	1.18 ± 0.39 ^{a}	0.83 ± 0.31 ^{bx}	0.49 ± 0.17 ^{z}
PUFA				
16:4(n-3)	1.10 ± 1.15 ^{ab}	1.12 ± 1.38 ^{a}	0.56 ± 0.76 ^{bx}	1.03 ± 0.83 ^{z}
18:2(n-6)	0.86 ± 0.24 ^{a}	1.00 ± 0.29 ^{a}	1.25 ± 0.28 ^{bx}	0.64 ± 0.15 ^{z}
18:3(n-3)	0.26 ± 0.10 ^{a}	0.38 ± 0.10 ^{b}	0.45 ± 0.11 ^{cx}	0.21 ± 0.08 ^{z}
18:4(n-3)	0.24 ± 0.09 ^{a}	0.30 ± 0.09 ^{b}	0.26 ± 0.08 ^{b}	0.27 ± 0.15
20:4(n-6) (ARA)	3.67 ± 0.80 ^{a}	2.76 ± 0.67 ^{b}	2.23 ± 0.65 ^{cx}	3.05 ± 1.27 ^{z}
20:5(n-3) (EPA)	11.77 ± 1.68 ^{a}	12.30 ± 2.16 ^{ab}	12.79 ± 1.41 ^{b}	12.97 ± 2.00
22:5(n-3)	1.11 ± 0.57 ^{a}	0.87 ± 0.27 ^{ab}	0.73 ± 0.38 ^{bx}	1.38 ± 0.30 ^{z}
22:6(n-3) (DHA)	24.82 ± 3.08 ^{a}	28.79 ± 3.73 ^{b}	32.89 ± 2.43 ^{cx}	30.39 ± 3.50 ^{z}
Total SFA	28.11 ± 2.26 ^{a}	25.34 ± 2.86 ^{b}	22.15 ± 2.30 ^{cx}	24.03 ± 2.10 ^{z}
Total MUFA	17.36 ± 1.23 ^{a}	16.35 ± 1.81 ^{b}	17.33 ± 1.59 ^{ax}	18.07 ± 0.89 ^{z}
Total PUFA	46.48 ± 3.51 ^{a}	50.07 ± 3.77 ^{b}	53.71 ± 2.41 ^{c}	52.66 ± 2.16
n-3 PUFA	39.50 ± 3.79 ^{a}	44.00 ± 3.78 ^{b}	48.20 ± 2.66 ^{cx}	46.86 ± 2.84 ^{z}
n-6 PUFA	4.86 ± 0.76 ^{a}	4.04 ± 0.62 ^{b}	3.84 ± 0.59 ^{bx}	4.00 ± 1.15 ^{z}
n-3/n-6	8.36 ± 1.78 ^{a}	11.16 ± 2.03 ^{b}	12.89 ± 2.56 ^{cx}	12.76 ± 3.99 ^{z}
EPA/ARA	3.36 ± 0.91 ^{a}	4.68 ± 1.21 ^{b}	6.09 ± 1.41 ^{cx}	4.95 ± 2.02 ^{z}
DHA/EPA	2.14 ± 0.33 ^{a}	2.46 ± 0.80 ^{b}	2.61 ± 0.39 ^{b}	2.41 ± 0.54
DHA/ARA	7.19 ± 2.29	11.14 ± 3.27	15.88 ± 4.49	12.19 ± 5.82
Triacylglycerols	(*n*=25)	(*n*=114)	(*n*=49)	(*n*=25)
SFA				
14:0	1.85 ± 0.37 ^{a}	2.14 ± 0.90 ^{b}	1.83 ± 0.32 ^{a}	1.96 ± 0.51
16:0	12.75 ± 1.41 ^{a}	15.22 ± 3.42 ^{b}	16.81 ± 2.46 ^{cx}	14.03 ± 2.27 ^{z}
18:0	3.19 ± 0.89 ^{a}	1.96 ± 1.24 ^{b}	1.45 ± 0.22 ^{cx}	1.72 ± 0.30 ^{z}
MUFA				
16:1(n-7)	3.66 ± 1.13 ^{a}	5.10 ± 1.28 ^{b}	4.53 ± 0.88 ^{c}	4.68 ± 1.35
18:1(n-9)	15.25 ± 3.18 ^{a}	17.56 ± 3.70 ^{b}	16.88 ± 3.77 ^{ab}	15.89 ± 2.05
18:1(n-7)	3.88 ± 1.48 ^{a}	3.07 ± 0.60 ^{b}	2.84 ± 0.62 ^{bx}	3.68 ± 1.04 ^{z}
20:1(n-9)	1.22 ± 0.72 ^{a}	0.53 ± 0.23 ^{b}	0.42 ± 0.10 ^{cx}	2.61 ± 1.23 ^{z}
20:1(n-11)	1.97 ± 1.71 ^{a}	0.40 ± 0.52 ^{b}	0.28 ± 0.28 ^{b}	0.19 ± 0.07
22:1(n-11)	0.25 ± 0.25	0.18 ± 0.18	0.11 ± 0.12 ^{x}	2.31 ± 1.30 ^{z}
24:1(n-9)	1.85 ± 0.82 ^{ab}	2.19 ± 0.72 ^{a}	1.62 ± 0.54 ^{b}	1.78 ± 0.66
PUFA				
16:4(n-3)	0.74 ± 0.71 ^{a}	0.22 ± 0.30 ^{b}	0.09 ± 0.13 ^{cx}	0.02 ± 0.03 ^{z}
18:2(n-6)	1.89 ± 0.57	1.86 ± 0.55	1.88 ± 0.61	1.64 ± 0.74
18:3(n-3)	1.07 ± 0.44	1.11 ± 0.47	1.01 ± 0.37 ^{x}	0.48 ± 0.23 ^{z}
18:4(n-3)	1.00 ± 0.46 ^{ab}	1.04 ± 0.43 ^{a}	0.88 ± 0.32 ^{bx}	0.81 ± 0.54 ^{z}
20:4(n-6) (ARA)	2.76 ± 0.79 ^{a}	2.10 ± 0.69 ^{b}	1.80 ± 0.62 ^{c}	2.13 ± 0.77
20:5(n-3) (EPA)	12.05 ± 1.75	11.97 ± 2.26	12.42 ± 1.68 ^{x}	10.91 ± 2.12 ^{z}
22:5(n-3)	1.13 ± 0.36 ^{a}	1.03 ± 0.28 ^{a}	0.87 ± 0.37 ^{bx}	1.76 ± 0.35 ^{z}
22:6(n-3) (DHA)	19.89 ± 3.04 ^{a}	20.45 ± 3.58 ^{a}	24.25 ± 3.08 ^{bx}	22.28 ± 3.43 ^{z}
Total SFA	18.21 ± 1.66 ^{a}	19.89 ± 4.81 ^{b}	20.58 ± 2.36 ^{bx}	18.39 ± 2.26 ^{z}
Total MUFA	28.50 ± 4.21 ^{ab}	29.57 ± 4.74 ^{a}	27.06 ± 4.81 ^{bx}	31.61 ± 3.63 ^{z}
Total PUFA	43.12 ± 4.39 ^{a}	41.92 ± 6.05 ^{a}	45.87 ± 3.19 ^{bx}	43.06 ± 2.84 ^{z}
n-3 PUFA	36.60 ± 4.17 ^{a}	36.40 ± 5.58 ^{a}	40.28 ± 3.45 ^{bx}	37.33 ± 3.41 ^{z}
n-6 PUFA	5.20 ± 0.84 ^{a}	4.32 ± 0.64 ^{b}	4.12 ± 0.56 ^{b}	4.09 ± 1.08
n-3/n-6	7.18 ± 1.29 ^{a}	8.52 ± 1.38 ^{b}	10.00 ± 1.84 ^{c}	9.79 ± 2.88
EPA/ARA	4.66 ± 1.23 ^{a}	6.08 ± 1.58 ^{b}	7.39 ± 1.64 ^{cx}	5.64 ± 1.72 ^{z}
DHA/EPA	1.67 ± 0.27 ^{a}	1.74 ± 0.29 ^{a}	1.98 ± 0.29 ^{bx}	2.11 ± 0.51 ^{z}
DHA/ARA	7.85 ± 2.70	10.61 ± 3.39	14.72 ± 4.24	12.27 ± 5.46

FAs with < 1.5 % in both ovary and liver are excluded but total values include all the 33 FAs analysed. Values with different superscripts across rows are statistically different; a, b and c represent test between maturity stages in the Baltic Sea while x and z represent test between origin in MIV cod, $p < 0.05$.

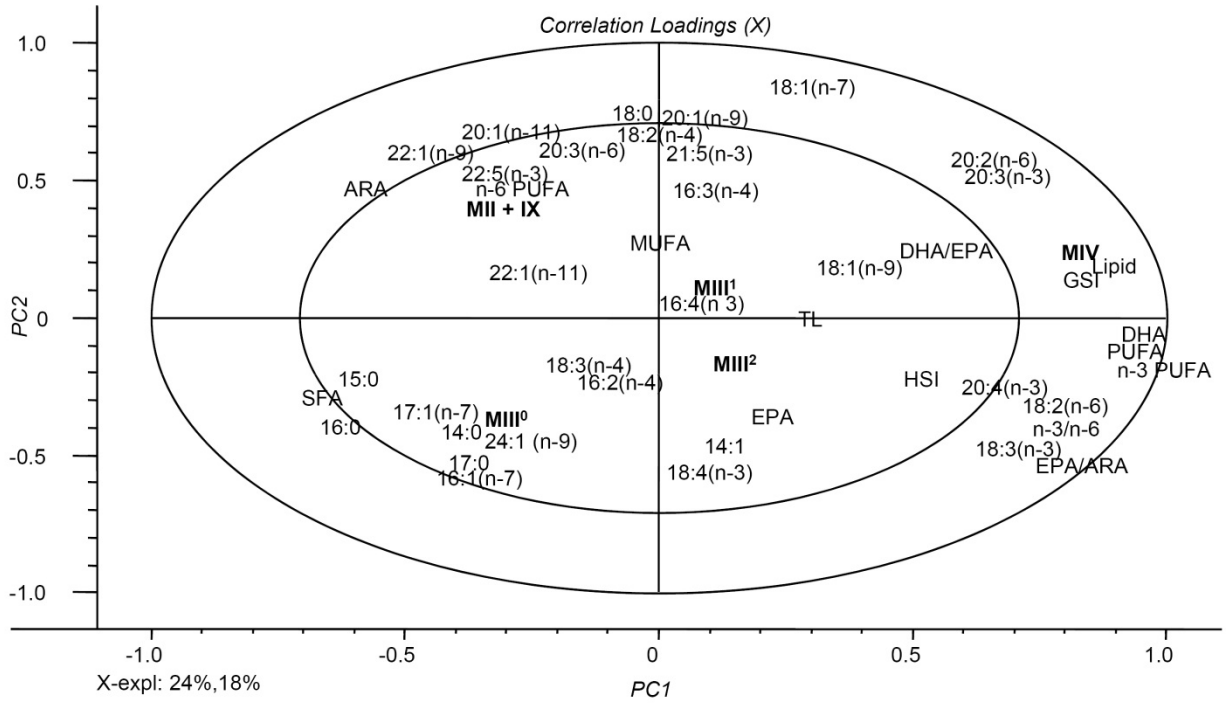

Fig. 4. *Gadus morhua*. Principal component analysis (PCA) loadings plot associating fatty acid composition (FAC) in ovarian phospholipids (PL) with maturity stage, ovarian lipid content, total length (TL), hepatosomatic index (HSI) and gonadosomatic index (GSI) of Baltic cod in progressive maturity stages, MII+MIX, MIII0-2 and MIV. See list of abbreviations.

The PCA loadings plot of FAC in ovarian PL of Baltic cod illustrates associations among FA, FA ratios, maturity stage, total ovarian lipid and morphometric data (TL, GSI, and HSI) (Fig.4). Ovarian development through successive maturity stages from MII+IX, $MIII^0$, $MIII^1$, $MIII^2$ to MIV was accompanied by significant changes in FAC with the Principal component (PC) 1 and 2 explaining 42 % of the observed variation. Stage MII+MIX samples were characterised by relatively high proportions of ARA, n-6 PUFA, and low HSI, DHA and n-3 PUFA proportions. Ovaries in early maturation $MIII^0$ prior to vitellogenesis were associated with high proportions of SFA and certain MUFA as well as low DHA/EPA, GSI and ovary lipid content. The stage $MIII^1$ and $MIII^2$ ovaries characterised by beginning oocyte yolk accumulation were located in the centre of the plot indicating intermediate values. Ovaries in MIV, prespawning, with large yolk filled oocytes were associated with high proportions of DHA, total PUFA and n-3 PUFA; high n-3/n-6, DHA/ARA, EPA/ARA and DHA/EPA ratios as well as high GSI, ovarian lipid and HSI. By contrast, stage MIV showed a negative correlation with ARA and SFA. ARA content showed a strong negative correlation with DHA and EPA, as well as a strong negative correlation with HSI, GSI and total lipid in ovaries.

In contrast to ovary PL, the proportion of total SFA in ovary TAG increased from MII+MIX to MIV (Table 2). PUFA was significantly higher in MIV than in MII+MIX, but the proportion of

DHA was lower than in PL and the increase less, EPA did not change significantly, while ARA was lower and decreased significantly from MII+MIX to MIV. Consequently, the DHA/ARA and EPA/ARA increased significantly from stage MII+MIX to MIV.

Difference in ovarian FAC among stocks

Ovarian FAC in the prespawning stage MIV differed depending on cod origin (Table 2). Total SFA and n-6 PUFA proportions in PL were higher in North Sea cod than in Baltic cod, while n-3 PUFA was lower. In particular, the proportion of ARA and docosapentaenoic acid (DPA, 22:5(n-3)) was low in Baltic cod ovaries compared to the North Sea samples, while proportions of DHA, 18:2n-6 and ALA were significantly higher in Baltic cod in both PL and TAG. Mean levels of EPA in ovarian PL did not differ between areas. Levels of DHA, EPA and ARA were lower in TAG than in PL. In both FAC in PL and TAG, proportions of e.g. 20:1(n-9) and 22:1(n-11) differed being significantly higher in North Sea cod ovaries than in Baltic cod, contrasting high proportions of e.g. LA and ALA in Baltic cod.

The PCA scores plot (Fig. 5a) illustrates the differences in ovarian FAC in PL between MIV Baltic and North Sea cod. The corresponding loadings plot in Figure 5b associates samples with FAs, lipid content, HSI, GSI and LT. PC1 and PC2 explained 40 % of the total variation in the data. In general, the samples from the northern (Na) and southern part (Nb) of the North Sea overlapped. The loaction of the parameter TL describing the length of the females near the ”North Sea” variable indicated that females from then North Sea were larger than Baltic cod (Fig 5a and b). Samples of Baltic cod ovaries were in general associated with high proportions of DHA, total PUFA, n-3 PUFA and ratios of n-3/n-6, EPA/ARA, DHA/EPA as well as HSI and negatively correlated with ARA and MUFA content. By contrast, North Sea cod samples were associated with high ARA, EPA, n-6 PUFA and SFA contents, but low DHA and HSI compared to the Baltic cod. Baltic cod samples possessed high proportions of LA, ALA, 18:3(n-4), while North Sea samples related to e.g. high proportions of 22:5(n-3), 21:5(n-3) and 20:3(n-6) as well as two specific long chain PUFA i.e. cetoleic acid, 22:1(n-11), and gadolic acid, 20:1(n-9). GSI was associated with lipid content, but negatively correlated with EPA proportions.

Changes in liver FAC during maturation

Table 3 show FAs in liver PL and TAG. In Baltic cod, proportion of SFA in liver PL remained fairly constant but lower than ovarian PL. The proportion of MUFA in liver PL remained constant at the same level as ovarian PL, which was substantially lower than in liver and ovarian TAG. The PUFA levels of DHA, n-3/n-6 and DHA/EPA in liver PL increased significantly with development,

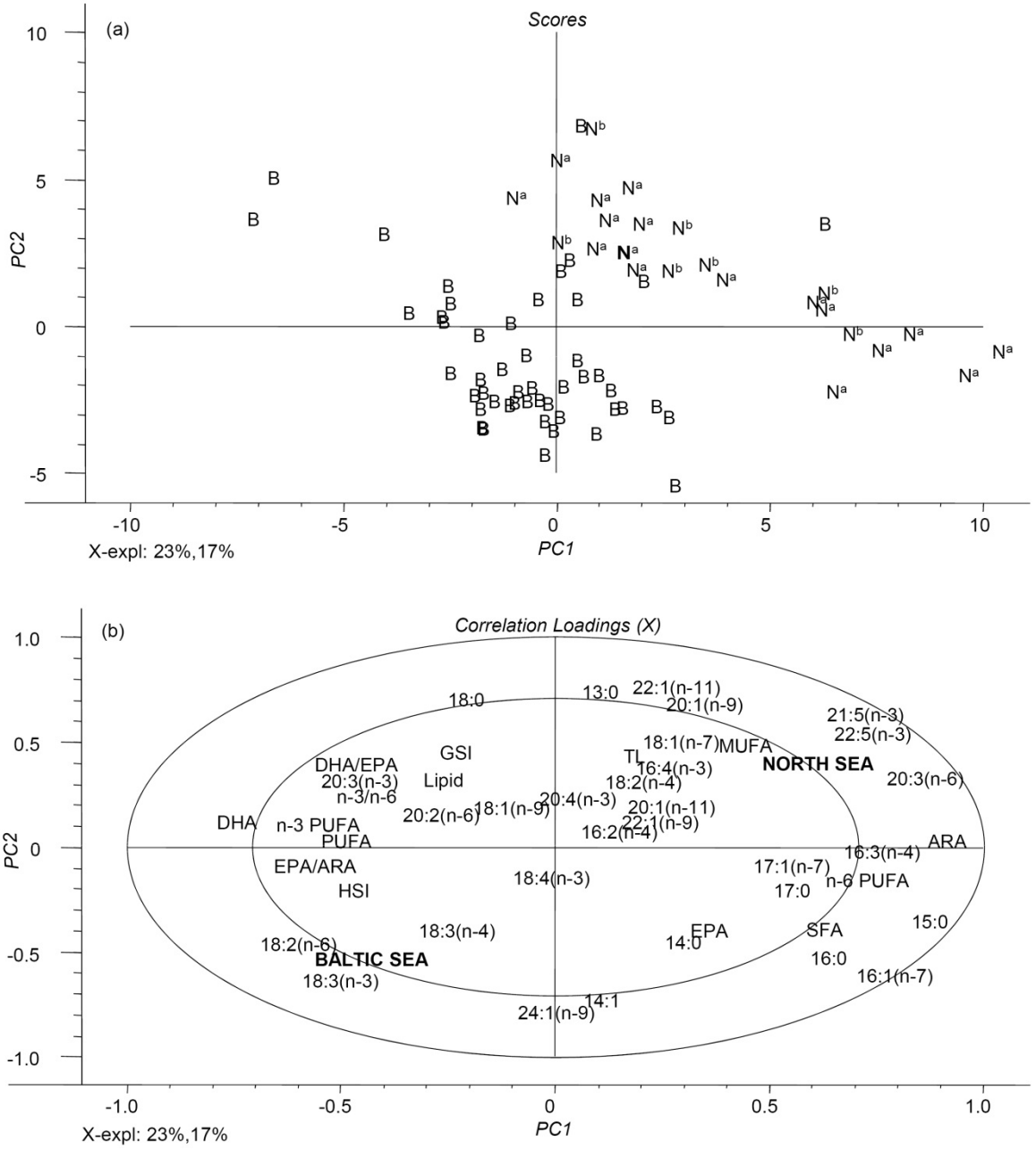

Fig. 5. *Gadus morhua*. (a) Principal component analysis (PCA) scores plot and (b) loadings plot of fatty acid composition (FAC) in ovarian phospholipids (PL), cod origin, ovarian lipid content, total length (TL), hepatosomatic index (HSI) and gonadosomatic index (GSI) from cod in maturity stage MIV. See list of abbreviations.

Tabel 3. *Gadus morhua*. Fatty acid composition (% of total fatty acids) in liver from female Baltic Sea cod in maturity stages MII + IX, MIII and MIV and North Sea cod in stage MIV separated into phospholipids (PL) and triacylglycerols (TAG). Values are means ± SD.

Fatty acid	Baltic Sea MII+IX	Baltic Sea MIII	Baltic Sea MIV	North Sea MIV
Phospolipids	(*n*=25)	(*n*=114)	(*n*=49)	(*n*=25)
SFA				
14:0	1.05 ± 0.29^{a}	1.28 ± 0.32^{b}	1.34 ± 0.30^{bx}	1.63 ± 0.44^{z}
16:0	16.72 ± 1.56	16.37 ± 1.67	16.61 ± 1.35^{x}	19.49 ± 2.97^{z}
18:0	4.19 ± 0.68^{a}	3.97 ± 1.03^{a}	3.48 ± 0.81^{b}	3.42 ± 1.20
MUFA				
16:1(n-7)	1.36 ± 0.42	1.47 ± 0.41	1.40 ± 0.37^{x}	1.74 ± 0.43^{z}
18:1(n-9)	11.30 ± 1.59	11.25 ± 1.31	11.59 ± 0.83	11.28 ± 1.15
18:1(n-7)	3.49 ± 1.10	2.99 ± 0.78	3.05 ± 0.86	3.75 ± 1.20
20:1(n-9)	0.51 ± 0.13^{a}	0.47 ± 0.13^{a}	0.40 ± 0.10^{bx}	2.15 ± 0.94^{z}
20:1(n-11)	0.60 ± 0.44^{a}	0.26 ± 0.25^{b}	0.18 ± 0.13^{c}	0.11 ± 0.04
22:1(n-11)	0.08 ± 0.00^{ab}	0.09 ± 0.06	0.04 ± 0.02^{x}	0.62 ± 0.38^{z}
24:1(n-9)	0.33 ± 0.12^{ab}	0.35 ± 0.11^{a}	0.25 ± 0.12^{bx}	0.21 ± 0.07^{z}
PUFA				
16:4(n-3)	0.21 ± 0.15	0.12 ± 0.08	0.12 ± 0.06^{x}	0.18 ± 0.14^{z}
18:2(n-6)	1.53 ± 0.40	1.55 ± 0.45	1.44 ± 0.35^{x}	0.81 ± 0.17^{z}
18:3(n-3)	0.65 ± 0.20^{a}	0.79 ± 0.23^{b}	0.72 ± 0.17^{ax}	0.31 ± 0.10^{z}
18:4(n-3)	0.43 ± 0.21^{ab}	0.54 ± 0.22^{a}	0.40 ± 0.11^{bx}	0.30 ± 0.13^{z}
20:4(n-6) (ARA)	2.32 ± 0.56^{a}	2.01 ± 0.60^{b}	1.52 ± 0.51^{cx}	2.09 ± 0.80^{z}
20:5(n-3) (EPA)	14.38 ± 1.91^{a}	13.47 ± 1.95^{a}	12.06 ± 1.20^{bx}	10.92 ± 2.10^{z}
22:5(n-3)	0.77 ± 0.31^{a}	0.61 ± 0.19^{b}	0.64 ± 0.36^{abx}	1.50 ± 0.47^{z}
22:6(n-3) (DHA)	32.60 ± 3.78^{a}	35.20 ± 3.30^{b}	38.40 ± 2.25^{cx}	32.23 ± 4.27^{z}
Total SFA	22.36 ± 1.77	22.27 ± 2.40	21.90 ± 1.85^{x}	25.50 ± 4.58^{z}
Total MUFA	17.60 ± 2.77	16.94 ± 1.93	16.95 ± 1.57^{x}	19.80 ± 1.53^{z}
Total PUFA	55.08 ± 3.21^{a}	56.24 ± 3.42^{a}	57.29 ± 1.94^{bx}	50.16 ± 5.84^{z}
n-3 PUFA	49.66 ± 3.46^{a}	51.26 ± 3.45^{a}	52.84 ± 2.14^{bx}	46.10 ± 5.73^{z}
n-6 PUFA	4.48 ± 0.51^{a}	4.08 ± 0.62^{b}	3.45 ± 0.48^{cx}	3.21 ± 0.82^{z}
n-3/n-6	11.28 ± 1.83^{a}	12.85 ± 2.09^{b}	15.64 ± 2.44^{c}	15.29 ± 4.27
EPA/ARA	6.50 ± 1.56^{a}	7.11 ± 1.68^{a}	8.51 ± 2.03^{bx}	5.90 ± 2.28^{z}
DHA/EPA	2.32 ± 0.51^{a}	2.67 ± 0.46^{b}	3.22 ± 0.41^{c}	3.02 ± 0.48
DHA/ARA	15.20 ± 5.19	19.11 ± 5.85	27.26 ± 6.80	17.72 ± 6.79
Triacylglycerols	(*n*=25)	(*n*=114)	(*n*=49)	(*n*=25)
SFA				
14:0	2.41 ± 0.42	2.63 ± 0.44	2.50 ± 0.43^{x}	3.48 ± 1.00$_{z}$
16:0	11.89 ± 1.22	12.18 ± 1.00	11.93 ± 0.98^{x}	11.15 ± 1.19$_{z}$
18:0	2.74 ± 0.52^{a}	2.63 ± 0.58^{a}	2.38 ± 0.56^{b}	2.59 ± 0.61
MUFA				
16:1(n-7)	5.35 ± 1.47	5.35 ± 1.39	5.66 ± 1.68	5.22 ± 1.25
18:1(n-9)	21.23 ± 3.19	21.87 ± 2.82	22.53 ± 2.15$_{x}$	14.33 ± 3.22$_{z}$
18:1(n-7)	4.75 ± 1.23$_{a}$	3.95 ± 0.89$_{b}$	4.08 ± 0.91$_{ab}$	4.31 ± 1.36
20:1(n-9)	1.39 ± 0.90$_{a}$	0.88 ± 0.34$_{b}$	0.89 ± 0.31$_{bx}$	6.95 ± 3.30$_{z}$
20:1(n-11)	1.10 ± 0.92$_{a}$	0.65 ± 0.74$_{ab}$	0.51 ± 0.37$_{b}$	0.39 ± 0.16
22:1(n-11)	0.22 ± 0.07	0.21 ± 0.10	0.17 ± 0.05$_{x}$	7.31 ± 4.47$_{z}$
24:1(n-9)	0.42 ± 0.18	0.43 ± 0.18	0.42 ± 0.16$_{x}$	0.66 ± 0.36$_{z}$
PUFA				
16:4(n-3)	0.10 ± 0.14	0.10 ± 0.09	0.10 ± 0.06$_{x}$	0.17 ± 0.10$_{z}$
18:2(n-6)	3.04 ± 0.99	3.15 ± 0.80	3.33 ± 0.84$_{x}$	1.44 ± 0.34$_{z}$
18:3(n-3)	1.97 ± 0.63	2.27 ± 0.69	2.26 ± 0.67$_{x}$	0.82 ± 0.34$_{z}$
18:4(n-3)	1.73 ± 0.64$_{a}$	2.52 ± 0.78$_{b}$	2.37 ± 0.59$_{bx}$	1.82 ± 0.99$_{z}$
20:4(n-6) (ARA)	0.88 ± 0.21	0.88 ± 0.27	0.84 ± 0.28	0.95 ± 0.52
20:5(n-3) (EPA)	9.35 ± 0.95$_{a}$	10.11 ± 1.26$_{b}$	9.72 ± 1.22$_{abx}$	7.61 ± 3.11$_{z}$
22:5(n-3)	1.33 ± 0.40$_{a}$	1.06 ± 0.35$_{b}$	1.14 ± 0.60$_{abx}$	2.16 ± 0.68$_{z}$
22:6(n-3) (DHA)	18.15 ± 3.24	18.29 ± 2.87	18.41 ± 2.21$_{x}$	17.00 ± 4.08$_{z}$
Total SFA	17.55 ± 1.65$_{ab}$	18.06 ± 1.29$_{a}$	17.36 ± 1.22$_{b}$	17.89 ± 1.54
Total MUFA	34.82 ± 3.20	33.67 ± 2.72	34.65 ± 2.82$_{x}$	39.73 ± 5.36$_{z}$
Total PUFA	39.90 ± 3.76	41.45 ± 3.17	41.57 ± 2.82$_{x}$	35.18 ± 5.05$_{z}$
n-3 PUFA	33.96 ± 3.40	35.56 ± 2.99	35.45 ± 2.65$_{x}$	31.13 ± 4.78$_{z}$
n-6 PUFA	4.66 ± 0.80	4.70 ± 0.68	4.84 ± 0.68$_{x}$	2.87 ± 0.59$_{z}$
n-3/n-6	7.44 ± 1.05	7.71 ± 1.25	7.45 ± 1.13$_{x}$	11.19 ± 2.32$_{z}$
EPA/ARA	11.03 ± 2.32	12.57 ± 6.55	12.43 ± 2.89$_{x}$	8.88 ± 3.32$_{z}$
DHA/EPA	1.97 ± 0.45	1.85 ± 0.47	1.93 ± 0.35$_{x}$	2.77 ± 1.86$_{z}$
DHA/ARA	22.12 ± 7.83	23.64 ± 11.92	24.20 ± 8.01	22.18 ± 10.51

FAs with < 1.5 % in both ovary and liver are excluded but total values include all the 33 FAs analysed. Values with different superscripts across rows are statistically different; a, b and c represent test between maturity stages in the Baltic Sea while x and z represent test between origin in MIV cod, $p < 0.05$.

while ARA, and total n-6 PUFA significantly decreased in liver PL. The levels of ARA in liver PL within maturity stages MII+MIX, MIII and MIV were lower than ovarian PL in the corresponding stages. In contrast, the level of DHA was higher in liver PL than in ovary PL ovary increasing from 33 to 38 % with development compared to 25 to 33 in ovary PL. The level of ARA, EPA and DHA was substantially lower in the liver TAG than in PL and remained at a fairly constant level.

The PCA loadings plot (Fig. 6) of FAC in liver PL of Baltic Sea cod illustrates the association among FAs and FA ratios, maturity stages, total liver lipid and morphometric data (TL, GSI and HSI). PC1 and PC2 explained 33 % of the variation in the data. The variation along the 1st axis is governed by a strong negative correlation between proportions of ARA and DHA, total PUFA and HSI. The association among stages and other variables was less pronounced than in ovaries. As in ovary FAC, MIV liver samples were associated with high proportions of DHA and PUFA, and DHA/EPA and EPA/ARA ratios, and negatively correlated with ARA and EPA. On the 2nd axis, liver FAC in the early developing stage $MIII^0$ was associated with low GSI and high EPA, while MIV was associated with high GSI and negatively correlated with EPA and ARA.

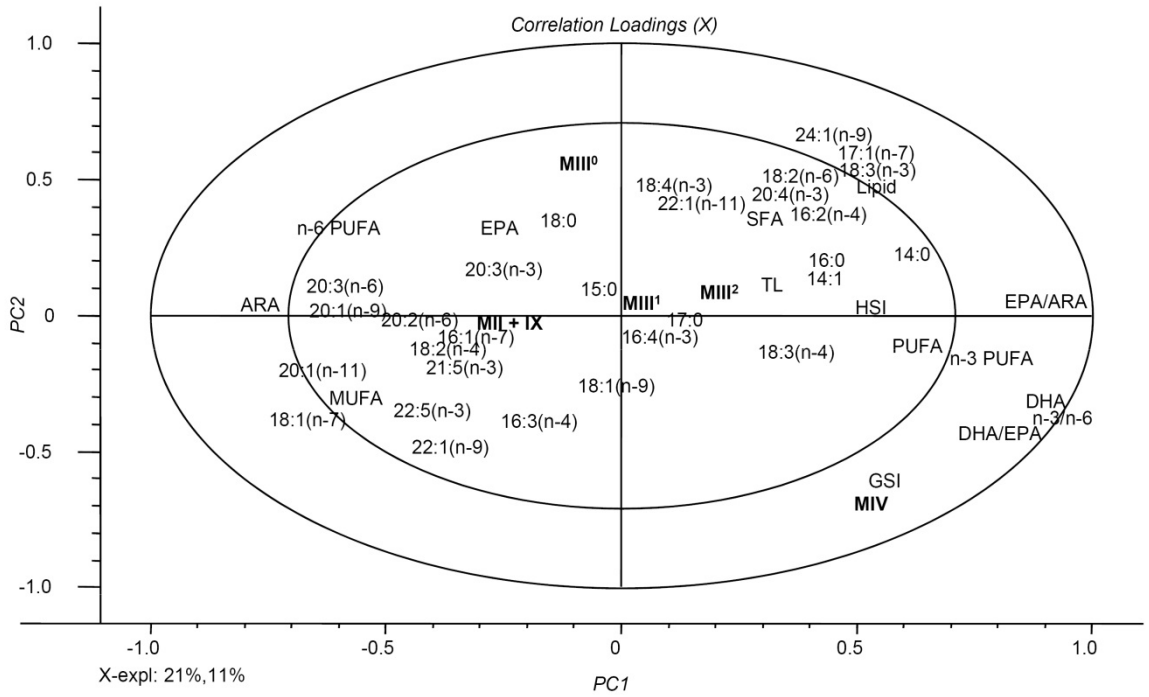

Fig. 6. *Gadus morhua*. Principal component analysis (PCA) loadings plot of fatty acid composition (FAC) in liver phospholipids (PL), maturity stage, liver lipid content, total length (TL), hepatosomatic index (HSI) and gonadosomatic index (GSI) of Baltic cod in progressive maturity stages. See list of abbreviations.

Difference in liver FAC among stocks

Liver FAC cod in the prespawning stage MIV differed significantly between Baltic and North Sea (Table 3). Proportions of ARA in liver PL as well as total SFA, MUFA, 20:1(n-9), 22:1(n:11 22:5(n-3) were higher in North Sea cod than in the Baltic cod. The level of total SFA was similar in liver TAG and much lower than in PL for both stocks. The proportion of MUFA in liver TAG was significantly higher in North Sea cod than in Baltic cod while total PUFA, n-3 PUFA, n-6 PUFA was lower. By contrast, EPA and DHA as well as LA, ALA, EPA/ARA, DHA/EPA and DHA/ARA in NL were higher in Baltic cod. The proportion of the long chain MUFA 20:1(n-9) in NL was almost 8 times higher and 22:1(n-11) was 42 times higher in North Sea cod than in Baltic Sea cod. The ARA content in NL was low and did not differ significantly between stocks.

The PCA scores plot (Fig. 7a) of FAC in liver PL of prespawning MIV cod showed that samples distributed according to area with Baltic samples aggregated on the right side and the more scattered North Sea samples towards left. The sub-divided North Sea samples showed that liver FAC in cod from the southern North Sea samples (N^b) were intermediate between northern North Sea samples (N^a) and the Baltic cod. PC1 and PC2 explained 47 % of the total variation in observations. The 1st principal axis of loadings plot of liver FAC showed a strong association of Baltic samples with high DHA, EPA, PUFA, n-3 PUFA and EPA/ARA ratios lipid content and HSI and a negative correlation with ARA. FAC in North Sea cod livers was associated with high ARA, MUFA, SFA (Fig 7b). The proportion of ARA was negatively correlated with HSI and EPA/ARA as well as n-6 with SFA, GSI and female size on the 2nd axis. The association of Baltic cod with high DHA and EPA and total lipid in liver PL was closer than in ovary PL and the negative correlation between samples from the two stocks stronger (Fig. 5b and 7b).

Dynamics of EFA during maturation

Figure 8 shows the variation in proportions of DHA and EPA in relation to proportion of ARA in ovary and liver PL and TAG for Baltic cod during maturation. A negative proportionality existed between DHA and ARA in ovary PL. During maturation the DHA increased while the proportion of ARA decreased (Fig 8a). DHA and ARA similarly showed negative proportionality in liver PL, but with proportions of DHA being higher and ARA lower (Fig. 8c). The proportion of DHA in relation to ARA was substantially lower in ovary TAG than in PL and observations more scattered (Fig 8b), while proportions of both DHA and ARA in liver TAG were strikingly low in comparison (Fig 8d). The proportion of EPA tended to be fairly constant independent of the ARA proportion in ovary PL (Fig. 8e). EPA and ARA showed a weak positive correlation in ovary TAG, liver PL and liver TAG

(Fig. 8f-h) with very low proportions of both ARA and EPA in liver TAG. The proportions of ARA were lowest in stage MIV cod and highest in MII+MIX except in liver TAG, where ARA was equally low in all stages. Three observations deviated in ARA level e.g. Figure 8d and h. These three females are identical with the three Baltic females to the left of the centre in Figure 7 in which liver FAC resembled southern North Sea cod.

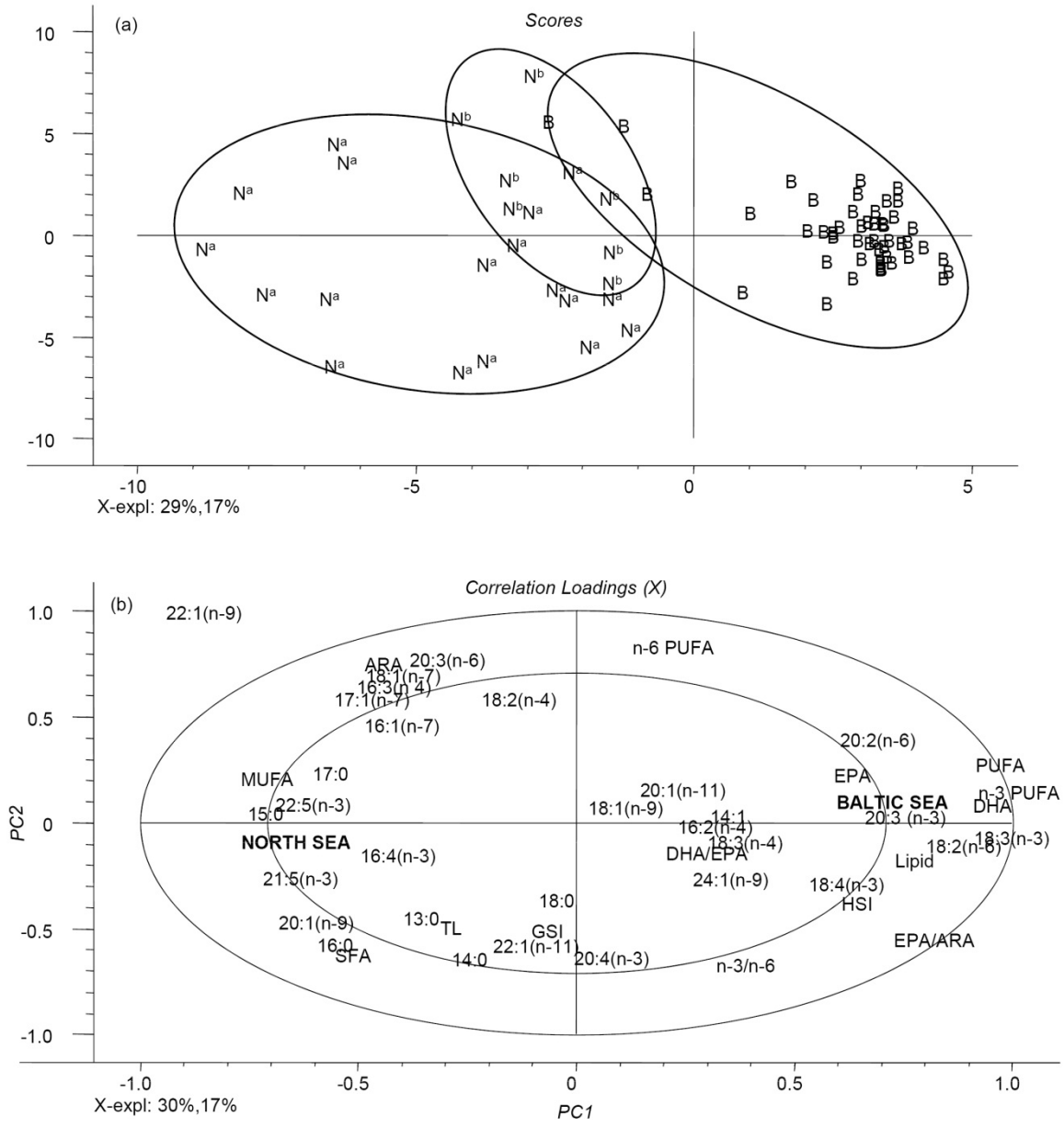

Fig. 7. *Gadus morhua*. (a) Principal component analysis (PCA) scores plot and (b) loadings plot of fatty acid composition (FAC) in ovarian phospholipids (PL), cod origin, ovary lipid content, total length (TL), hepatosomatic index (HSI) and gonadosomatic index (GSI) of cod in maturity stage MIV. See list of abbreviations.

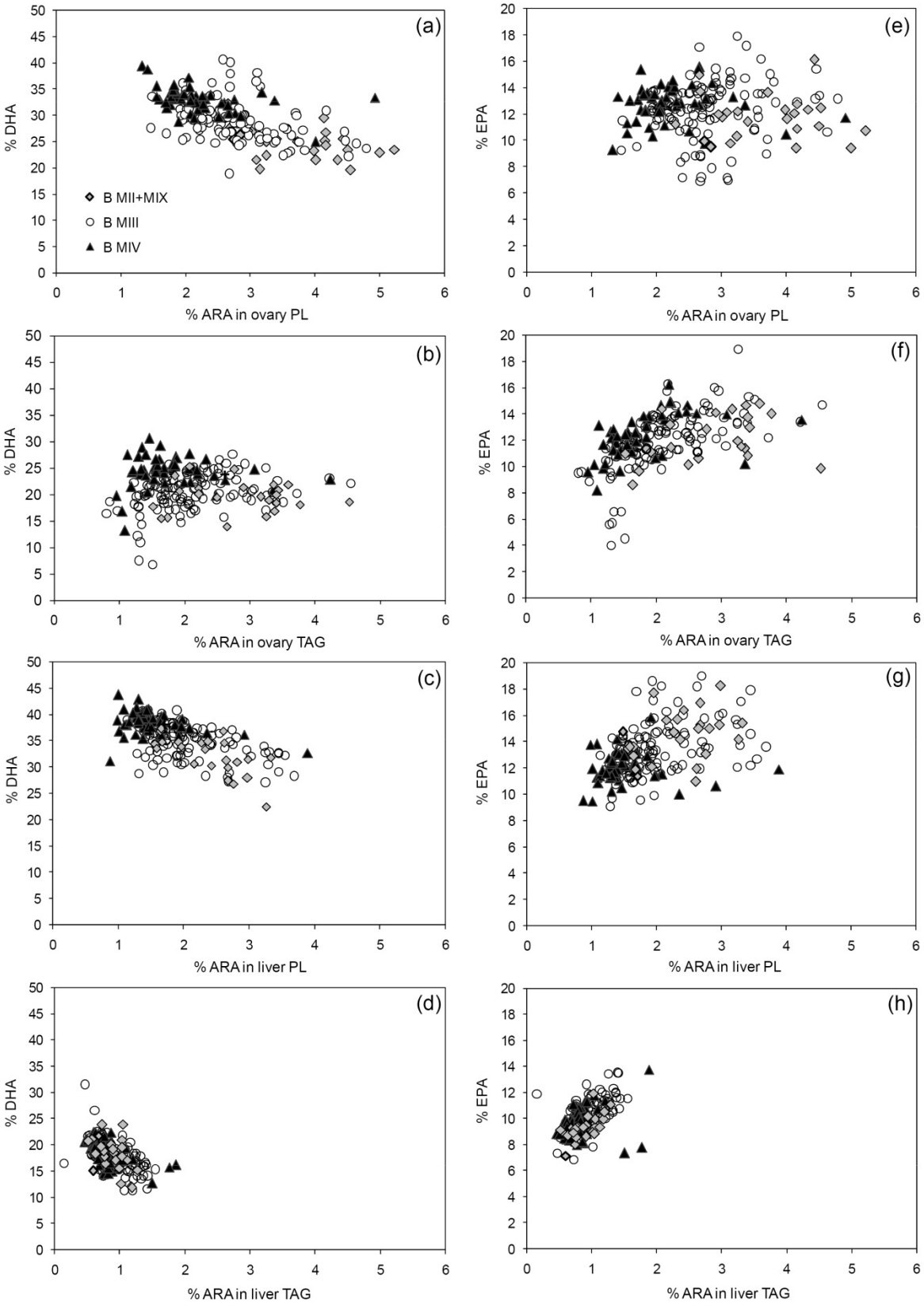

Fig. 8. *Gadus morhua*. Percent docosahexaenoic acid (DHA) (a-d) and eicosapentaenoic acid (EPA) (e-h) relative to arachidonic acid (ARA) in phospholipids (PL) and triacylglycerols (TAG) of ovaries and livers of Baltic cod in successive maturity stages MII+MIX, MIII and MIV.

EFA ratios in relation to ovary and liver lipid somatic index

Figure 9 shows DHA/ARA and EPA/ARA ratios in ovary and liver PL and TAG of cod in the Baltic and North Sea cod in prespawning stage MIV in relation to lipid gonadosomatic index, LGSI, and lipid hepatosomatic index LHSI i.e. the total lipid content of the ovary or liver standardised to be independent of female body size. The ratio of DHA/ARA in ovary PL (Fig. 9a) and TAG (Fig. 9b) tended to increase with increasing LGSI for North Sea MIV females while the ratio remained largely constant for Baltic cod. DHA/ARA and EPA/ARA in the Baltic cod were higher especially at low LGSI. A similar pattern was observed for the EPA/ARA ratio in ovary PL and TAG (Fig. 9 c and d). The DHA/ARA an EPA/ARA ratio also tended to increase with LHSI, except in liver TAG (Fig. 9 e-h). The ratios of DHA/ARA and EPA/ARA in relation to LHSI were in general higher in Baltic than in North Sea cod and some very low LHSI were encountered in the North Sea.

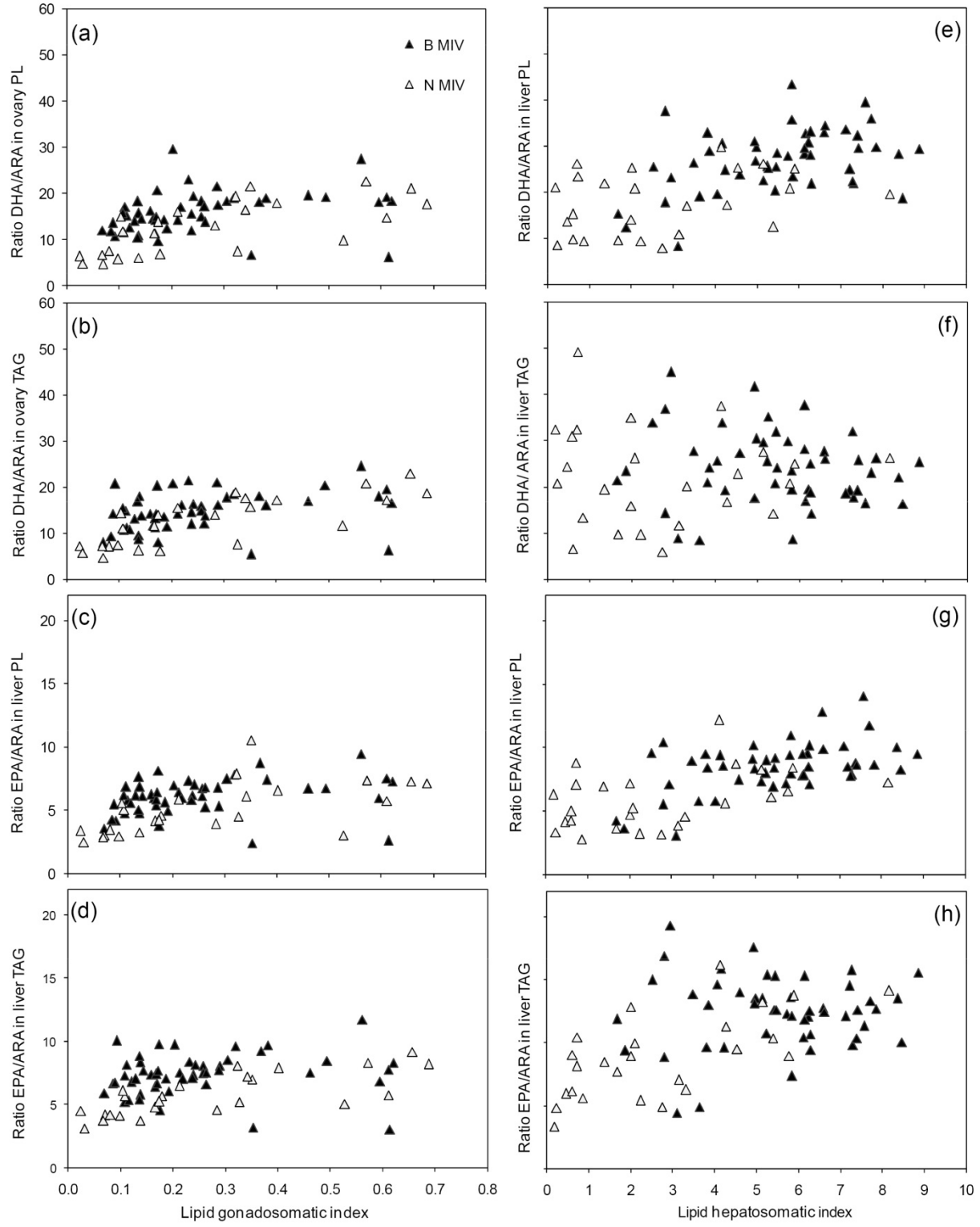

Fig. 9. *Gadus morhua*. Ratios of EFA in phospholipids (PL) and triacylglycerols (TAG) in relation to ovary and liver somatic indices, LIGS and LIHS in % of WW for Baltic (B) and North Sea (N) cod in maturity stage MIV. (a-b) DHA/ARA in ovary, (c-d) EPA/ARA in ovary, (e-f) DHA/ARA in liver and (g-h) EPA/ARA in liver. See list of abbreviations.

DISCUSSION

Baltic trophic regime and fatty acid signatures

Reproductive success of cod in the central Baltic Sea depends on the hydrographic conditions in the spawning areas affecting survival of eggs through reduced oxygen concentration in the dwelling depths and salinity driven availability of suitable food for larvae, i.e. juvenile stages of *Pseudocalanus acuspes* (e.g. Köster et al. 2005). Prolonged stagnation periods prevailing since the early 1980's caused a decline in cod recruitment, which in combination with high fishing intensity resulted in historic low stock levels from early 1990's to mid-2000's (ICES 2011). While processes affecting the survival of Baltic cod offspring have been elucidated (e.g. Köster et al. 2005, Huwer et al. 2011), the impact of parental characteristics, such as nutritional condition, on reproductive success is less clear, apart from a linkage of egg and larval survival to female size (Vallin et al. 1999).

Results from recent plankton mesocosm experiments comparing the Baltic with the Norwegian Sea suggest that an imbalance in fatty acid composition may prevail in the basic food web of the Baltic Sea also affecting higher trophic levels (Ahlgren et al. 2005). Extremely low ARA concentrations were reported in Baltic phytoplankton dominated by dinoflagellates (Ahlgren et al. 200%), and in the dominant mesozooplankton species in the central Baltic, i.e. *Temora longicornis*, *Pseudocalanus acuspes* and *Acartia longiremis* (Peters 2006). This signal of imbalanced DHA/ARA ratios can be traced also into the major zooplanktivorous fish species in the Baltic, sprat and herring (Røjbek et al. 2012), constituting a major part of the Baltic cod diet (Uzars 1994). In the present study, we found evidence that this imbalance is imprinted also in the FAC of Baltic cod, while North Sea cod show a different FAC signature with significantly higher proportions of ARA and lower DHA in both livers and ovaries. This difference can be explained by a fundamentally different primary production and more varied diet composition of cod in the North Sea (Kikkert, 1993).

An imbalanced EFA in the Baltic Sea may pose marine fish species with a physiological challenge as marine fish species have low or no capacity to synthesize DHA, EPA and ARA *de novo* (Sargent et al. 1989, Bell & Tocher 2009). Changes in the planktonic foodweb of the central Baltic during late 1980's and early 1990's (Alheit et al. 2005) may have amplified the problem of an imbalanced EFA for marine fish species in the Baltic. A pronounced decline in diatom biomass and an increase in dinoflagellates (Wasmund and Uhlig, 2003) impacted on the production of PUFAs in the system,

especially ARA (Ahlgren et al. 2005). Responsible hydrographic changes favoured additionally lipid poor copepods such *Temora longicornis* and penalized the lipid rich marine copepod *Pseudocalanus acuspes* (Alheit et al. 2005; Möllmann et al. 2005).

In contrast to cod, the sprat stock in the Baltic increased from lowest to highest level on record from late 1980's to mid-1990's, benefiting from reduced predation pressure by cod and hydrographic changes, e.g. warmer winter water temperatures, increasing reproductive capasity (Köster et al. 2003b). Increasing intra- and interspecific competition in sprat and herring stocks, however, enforced by the changes in the mesozooplankton community, affected the nutritional condition of both sprat and herring negatively (Möllmann et al. 2005; Casini et al. 2010). While the abundance of clupeids as primary prey of cod was high, their nutritional value and in particular ARA levels were low, especially in spring and early summer (Røjbek et al. 2012), corresponding to the period when cod relies almost entirely on clupeids as food resource. It has been demonstrated that clupeid prey availability is positively related to relative fecundity in Baltic cod and that relative fecundity increased in this stock from late 1980's/early 1990's to later in the 1990's (Kraus et al. 2002). The present study attempted to resolve the effect of high abundances of low quality food organisms on the lipid and FAC in Baltic cod reproduction compared to North Sea cod.

Liver lipid reserves and potential fecundity

Cod is a capital spawner and stores reserve lipids in the liver with the amount of energy reserves influencing and reproduction (e.g. Marshall et al. 1999, Lambert & Dutil 2000, Skjæraasen et al. 2010). Lambert & Dutil (1997) found that the relationships between energy content, water content, and simple measures such as HSI provide a fast and accurate assessment of available lipid energy reserves in cod. Lloret et al. (2008) used in a study of hake (*Merluccius merluccius*), LHSI as an enhanced size standardized index of the total available energy considering the non-linear nature of the relationship between % liver lipid and HSI. In the present study, we found the lipid content approached an asymptotic value of app. 80 % in liver tissue, while the liver size relative to the body continued to increase, which is similar to results of Skjæraasen et al. (2010).

The significantly higher liver lipid contents, HSI and LHSI in Baltic cod than in North Sea cod documented high levels of available energy in Baltic cod independent of the maturation stage. A study on captive cod similarly showed no net use of stored energy from the liver in the gonad development as long as the cod were offered excess feed (Hemre et al. 1995). These results indicate that the high prey abundance relative to cod provided ample energy in spite reduced condition of

clupeid prey, while the liver lipid content in North Sea cod was lower even at high HSI indicating that they invested more of their liver lipid reserves in egg production. High inter-individual variation in lipid reserves was observed, which is a common feature in wild fishes (Shulman & Love 1999), and may reflect differences in reproductive potential within and among populations.

Prey availability and available liver lipid energy has been used a proxies to predict fecundity in cod (Lambert & Dutil 2000, Kraus et al. 2002, Kjesbu et al. 1998, Skjæraasen et al. 2010) and population parameters such as egg production and recruitment success by fish stocks (e.g. Marshall et al. 1999). Skjæraasen et al. (2006) found lipid energy of cod around the start of vitellogenesis a useful proxy for potential fecundity of Northeast Arctic cod. Similarly, Kraus et al. (2002) found a significant relationship between potential relative fecundity of Baltic cod and the relative clupeid prey abundance during early maturation. In line with the higher liver lipid content and HSI in Baltic cod, also the average fecundity at length is higher in Baltic than in North Sea cod (Lambert et al 2005). Down regulation through atresia occurring during maturation may reduce fecundity and prevalence of atresia is related to cod condition i.e. Fulton K, HSI and liver energy (e.g. Skjæraasen et al. 2010). However, the relative intensity of atresia in maturing cod was low in Baltic cod in 2000 (Kraus et al. 2008) as would be expected from the high HSI and high lipid liver content observed in the present study.

Fatty acid during ovarian development

The liver plays an important role in the mobilization of hepatic lipids into the plasma as well as the production of the yolk protein, vitellogenin, and its incorporation into developing oocytes (e.g. Wiegand 1996). There was an obvious increase in the lipid contents of ovary during maturation of Baltic cod ovaries, which coincided with enhanced GSI. Ovarian lipid content reached similar levels in prespawning cod independent of origin, and GSI compares to previous studies of cod (e.g. Tomkiewicz et al. 2003, Skjæraasen et al. 2010). Few studies have focused on lipid content and FAC in wild cod ovaries. The mean lipid content in MIV ranged between 1.3 and 4.1 % which is similar to a study of ripening cod from Nova Scotia in 1964 where the ovary lipid content ranged from 1.0 to 3.2 % lipid (Jangaard et al. 1967a). The substantial scatter and differences in observed GSI and ovarian lipid content in prespawning cod may partly reflect differences in the degree of development within stage MIV. North Sea cod tended to be closer to spawning than Baltic cod as the histology revealed spawning specimens among the samples, which were however excluded from the FAC analysis.

FAC in maturity stages at a detailed level based on histological oocyte characteristics in order to examine changes in FAC during Baltic cod ovarian development (Kjesbu & Kryvi 1989, Tomkiewicz et al. 2003). The first sign of development is the appearance of cortical alveoli, stage $MIII^0$, preceded by progressive vitellogenic stages $MIII^1$, $MIII^2$ and MIV. During vitellogenesis, female fish require EFAs that are deposited in the eggs (Wiegand 1996). Egg lipids are derived from dietary fatty acid and fatty acid mobilized from live reserves. This involves selective incorporation of EFA into yolk lipids and discrimination against incorporation against others during maturation (Wiegand 1996). In the present study, the proportion of total PUFA in ovarian PL of Baltic cod increased significantly during development from undeveloped stages MII+MIX to prespawning MIV. The proportions of DHA, EPA and n-3 PUFA increased in ovary PL during maturation, while the proportion of ARA and n-6 PUFA significantly decreased. DHA increased significantly in ovary PL and TAG as well as in liver PL, reaching on average 33 %, 24 % and 38 % respectively in MIV while proportion remained low in liver TAG i.e. averaging 18 %. By contrast, ARA levels decreased significantly in ovary PL and TAG as well as liver PL, and remained constant in liver TAG, however the average proportion in ovary PL i.e. 2.0 % was higher than in ovary TAG 1.8 %, liver PL 1.5% and liver TAG 0.8%. EPA also increased in ovary PL during maturation but the increase was less pronounced i.e. 13% compared to 12 %, 12% and 10% in ovary TAG and liver PL and TAG respectively. The results suggest that DHA, EPA and ARA were retained selectively in the ovary PL and to some extent TAG during maturation in spite the decrease in ARA. The proportion of ARA during development was significantly lower than in North Sea cod and seemed to be depleted during oocyte maturation in Baltic cod.

The low proportions of ARA in Baltic cod affected the relationships between DHA and ARA as well as EPA and ARA. If a unique value of the ratio is important, it indicates that the fatty acids within the organism should be present in a certain proportion for optimal structure and/or function (Parrish 2009). ARA and DHA enzymatically compete (Sargent et al. 1999b, Sargent et al. 2002, Tocher 2003) and similarly EPA competes with the enzyme systems producing eicosanoids from ARA, thus exerting a modulating influence over the quantity and efficacy of ARA-derived eicosanoids (Bruce et al. 1999, Sargent et al. 1999b). While DHA and EPA are the main components of the phospholipids in cell membranes, and important for the structural and functional integrity of the cell membranes, ARA acts as main precursor for the eicosanoids responsible for final oocyte maturation and ovulation control in ripening female (Stacey & Goetz 1982, Sorbera et al. 1998, Sorbera et al. 2001, Henrotte et al. 2011b). Hence low level of ARA and high EPA/ARA

in MIV Baltic cod can be very critical for the completion of oocytes development considering the important role of ARA as main precursor to the eicosanoids (Sargent et al. 2002). This is further supported by the relatively constant ratios in Baltic cod compared to increased ratios in North Sea cod with increasing available lipid levels. The high lipid energy in Baltic cod and high ratios of DHA/ARA and EPA/ARA indicate ARA limitations in the Baltic food web.

Ecosystem differences: Trophic imprint

The study of FA in aquatic food webs often focuses on their broad use as biomarkers in trophic transfer studies (e.g. Dalsgaard et al. 2003, Parrish 2007, 2009). By contrast, the study of fatty acids in aquaculture usually centres on only two or three essential fatty acids i.e. DHA, EPA, and, to a lesser, extent ARA and their importance as essential dietary nutrients for female broodstocks and fish larvae (e.g. Parrish 2009). However recently, scientists studying ecology started to consider DHA, ARA and other fatty acids as key factors understanding ecosystem dynamics (Arts et al. 2001, Ahlgren et al. 2005, Parrish et al. 2007, Parrish 2009). The present study confirmed studies reporting an imbalance in DHA and ARA at the base of the food web of the Baltic Sea (Ahlgren et al. 2005) transferred via zooplankton (Peters et al. 2006) to the clupeid prey of Baltic cod (Røjbek et al. 2012). Other lipid markers included high proportions of 22:1(n-11) and 20:1(n-9) in the North Sea cod. These FAs relate to calanoid copepods rich in these fatty acids and abundant in the North Sea (Sargent & Falk-Petersen 1988), which do not exist in the Baltic Sea.

Implications on spawning time and recruitments success

The shift in Baltic cod spawning time from peak egg abundance during late April-early June in the 1970's and1980's to June-July in the 1990's (Wieland et al. 2000), persisted into the 2000's. Changes in spawning time of Atlantic cod in different areas have been attributed to temperature, stock demography, food availability and condition or a combination of these factors for different stocks (Kjesbu 1994, Hutchings & Myers 1994, Wright & Trippel 2009). Wieland et al. (2000) identified water temperature during the period of gonadal maturation, size of the spawning stock and food availability as factors influencing the spawning time of Baltic cod, however, without being able to explain the magnitude of the observed shift in spawning time.

Our results on ovarian and liver FAC in Baltic cod suggest that high fecundity related to ample liver lipid reserves and low nutritional quality of prey with shortage of ARA required for egg production may contribute to prolonged oocyte development period delaying the spawning time of cod. As

ARA acts as main precursor for the eicosanoids responsible for stimulation of final oocyte maturation and ovulation control (e.g. Sorbera et al. 2001, Henrotte et al. 2011) specific threshold levels may be required. Consequently the suboptimal DHA/ARA ratios, reflected in low ARA proportions in liver and ovarian FAC may delay the maturation process until sufficient quantities of ARA can be gathered. The imbalance in EFA ratios, as evidenced by our results, may compromise cod egg quality and reproduction success, as early larvae depend critically on the supply of EFAs from the yolk. This would support the theory that capital breeders such as Baltic cod with determinate fecundity and a long maturation period are more sensitive to environmental changes than income breeders such as sprat being an indeterminate batch spawner with a short maturation period and better opportunities to regulate egg production and egg quality (Jaeger et al. 2008).

CONCLUSION

The present study demonstrated that Baltic cod was not energy limited, but on the contrary exhibited high liver lipid levels during the entire ovarian maturation, while some North Sea cod approached critically low energy levels in the prespawning stage. A detailed FAC analysis confirmed studies reporting an imbalance in fatty acid composition in the Baltic Sea food web, which we found imprinted in cod at the upper trophic level. In particular, the proportions of DHA were significantly higher in Baltic cod ovaries and livers than in North Sea cod, while ARA proportions were significantly lower skewing the ratio of DHA/ARA and EPA/ARA. High food abundance and energy reserves in the Baltic may lead to high cod fecundity, while low nutritional quality of the diet may hamper completion of the oogenesis and lead to reduced egg quality. This may have contributed to the delay in spawning time as well as to the reproduction failure observed throughout the 1990's and 1st half of the 2000's.

LIST OF ABBREVIATIONS

ARA = arachidonic acid (20:4n-6), B = Baltic Sea, DHA = docosahexaneoic acid (22:6n-3), EFA = essential fatty acids, EPA = eicosapentaenoic acid (20:5n-3), FA = fatty acid, FAC = Fatty acids composition, GSI = Gonadosomatic index, HSI = Hepatosomatic index, MII = Maturity stage II: immature in late primary growth stage, MIII = Maturity stage III: early vitellogenesis, MIV = Maturity stage IV: late vitellogenesis i.e. prespawning, MIX = Maturity stage IX: regeneration, MUFA = Monounsaturated fatty acids, N = North Sea, N^a = North Sea subdivision IVa, N^b = North Sea subdivision IVb, NL = Neutral lipids, PCA = Principal component analyses, PL =

Phospholipids, PUFA = Polyunsaturated fatty acids, SFA = Saturated fatty acids, TAG = triacylglycerols.

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Paper IV

Effects of dietary fatty acids on production and quality of eggs and larvae of Atlantic cod (*Gadus morhua* L.)

by Maria C. Røjbek, Josianne G. Støttrup, Charlotte Jacobsen, Jonna Tomkiewicz & Edward A. Trippel

Illustration modified from image by Glynn Gorick for the ICES/GLOBEC project: Cod and Climate Change

Effects of dietary fatty acids on production and quality of eggs and larvae of Atlantic cod (*Gadus morhua* L.)

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ABSTRACT

This study investigated the effect of dietary levels of the essential fatty acids on egg production and egg and larvae quality. Farmed cod broodstock were fed diets 6.5 month prior to spawning with a low or high level of arachidonic acid (ARA) and/or eicosapentanoic acid (EPA) resulting in three diets with increasing EPA/ARA ratios. Broodstock fish were divided into triplicate tanks during the spawning period and total egg production was measured. All fish were stripped three times: at the beginning, peak and end of spawning. Lipid composition and egg and larval quality of 34 crosses of individual females and males were investigated. The results indicated that ARA uptake into eggs from broodstock diet was highly efficient. The diet with the lowest EPA/ARA ratio had significant highest egg production. Eggs from fish fed diet with high ARA level (2.5% of total fatty acids) had significant higher probability of fertilization success and survival to 8 days post hatch (DPH) than fish fed low levels of ARA (0.8%). High EPA resulted in additional enhancement of fertilization success and larval survival; however this was not significant and did not compensate for a reduced egg production. These results clearly demonstrate benefits of ARA supplementation and low EPA/ARA ratio of cod broodstock feeds in terms of improved number of viable larvae per kg female.

INTRODUCTION

The global production of Atlantic cod (*Gadus morhua*) in aquaculture has increased dramatically in recent years from 169 t in 2000 to 22,729 t in 2009 (FAO, 2009) due to declines in catches of cod in commercial fisheries. One of the most significant challenges for successful commercialization of cod mariculture involves reducing juvenile production costs (Penney et al. 2006). Egg quality is a major factor influencing hatching success and the subsequent survival rate of fish larvae (Kjørsvik et al. 1990) and in the fish farming industry, good-quality eggs are usually defined as those exhibiting high fertilization and hatching success with viable offspring (Bromage et al. 1992). Factors governing good egg quality in farmed fish include broodstock diet, physiology and hormonal status of the broodstock, genetic influences and husbandry (Brooks et al. 1997).

Dietary lipid and fatty acid composition have been identified as major factors determining successful reproduction and survival of offspring in fish (Izquierdo et al. 2001). Especially the fatty acids docosahexaenoic acid (22:6n-3 = DHA), eicosapentaenoic acid (20:5n-3 = EPA) and arachidonic acid (20:4n-6 = ARA) are important for maturation and egg quality (Sargent et al. 1999,

Tocher 2003). These polyunsaturated fatty acids (PUFA) have a general role in phospholipids (PL) of membrane bilayers to help maintain the structural and functional integrity of cells. EPA and ARA furthermore have a more specific role as precursors of eicosanoids e.g. prostaglandins (Bell et al. 1986, Sargent et al. 1995, Tocher 2003), which have a wide range of physiological actions including oocyte maturation and ovulation in fish (Goetz & Garczynski 1997, Sorbera et al. 2001, Lister & Van der Kraak 2008, Planas J.V. & Swanson 2008). Eicosanoids produced from ARA are generally more active than those produced from EPA; however EPA competitively interferes with eicosanoid production from ARA and competes for the same membrane receptors (Sargent et al. 1999, Sargent et al. 2002). Thus, eicosanoid action is determined by the ratio of EPA/ARA in cellular membranes this in turn being determined by the dietary intake of PUFA (Sargent et al. 1999, Tocher 2003) as there is a highly significant relationship between feed oil and liver oil percentage of DHA, EPA and total n-6 fatty acids in cod (Jobling & Leknes 2010).

Proportions of ARA are generally higher in eggs of wild fish than in farmed fish and egg quality is often found to be higher in wild than farmed broodstock (Pickova et al. 1999, Salze et al. 2005, Pickova et al. 2007). Further, supplemental dietary ARA improved spawning performance in Japanese flounder (*Paralichthys olivaceus*) where total egg production, percentage of buoyant eggs, hatching success, larval survival and normality of larvae was higher when the proportion of dietary ARA was increased from 0.6 to 3.6% of total fatty acids (Furuita et al. 2003). An enhanced reproductive performance has also been reported in Atlantic halibut (*Hippoglossus hippoglossus*) in terms of higher fertilization rates, blastomere morphology scores and hatching rates with supplemental ARA (Mazorra et al. 2003). Furthermore the proportion of viable eggs was improved in sea bass (*Dicentrarchus labrax*) when fed trash fish with high ARA content compared to dry pellets with four times lower ARA level (Bell et al. 1997).

Studies on dietary effects of ARA on egg production and quality in cod have, however, shown inconsistent results. In one study on the supplementation of ARA in cod diet affected realized fecundity (Norberg et al. 2008) but in another study it did not (Sawanboonchun 2009). High ARA levels in eggs were correlated with high fertilization success, blastomere symmetry and survival in a study comparing farmed and wild Atlantic cod from Scotland (Salze et al. 2005) and with blastomere symmetry and hatching success comparing cod from Skagerrak and the Baltic Sea (Pickova et al. 1997). In contrast high fertilization and hatching success were not correlated with higher ARA levels of Norwegian wild broodstock compared to farmed cod (Lanes et al. 2012). In a

study of Atlantic cod fed diets coated with three different oils and ARA levels of 0.1, 0.2 and 0.8 % of total lipid no differences in egg production, egg size and fertilization success were found between diets (Lie et al. 1993). Egg quality and lipid composition in broodstock fed the same diet, no correlation between FAC and blastomere morphology, fertilization success and hatching success was found (Penney et al. 2006). Neither was hatching success affected in a study with cod fed a diet supplemented with ARA (3.2% of total fatty acids) compared to a control (0.6%) (Sawanboonchun 2009).

Feeding duration is of great importance as cod has a long vitellogenic period (Kjesbu et al. 2010) and stores lipid in the liver prior to spawning (Kjesbu et al. 1991). In a study on the effect of diet on total body fat composition in cod, body lipids changed significantly after 3 weeks and did not change further with longer feeding period (Kirsch et al. 1998). FAC of cod eggs have in contrast been remarkably conserved irrespective of diet in some studies (Silversand et al. 1995, Pickova et al. 1997) and ARA levels of diet were only partially reflected in eggs after one to three months of feeding on a diet with high ARA level (Sawanboonchun 2009) or two months on a diet with low ARA level (Salze et al. 2005). However ovarian ARA levels (0.5, 1, 2 or 4% of total fatty acids) were highly correlated to diet after a minimum eight months of feeding and a net accumulation of ARA compared to other essential fatty acids was observed (Norberg et al. 2008). Six and a half months feeding duration was applied in the present study to ensure dietary effects during the entire vitellogenic period. Ovaries typically mature for 5 to 6 months before initiation of spawning (Kjesbu et al. 2010).

All studies on dietary effect of ARA on egg quality have so far been based on natural spawning with mixtures of eggs from several females. In contrast the studies on Atlantic halibut, Japanese flounder and sea bass, where a correlation between fatty acid composition and egg quality was found, were based on eggs from single females. This study presents for the first time results on the effect of different ARA and EPA levels on egg quality of individual females in cod. The aim of this study was to investigate effects of a high (2.5%) and low (0.8%) level of dietary ARA in combination with a high (11%) and low (7.5%) level of EPA on production and egg and larvae quality of Atlantic cod.

MATERIALS AND METHODS

Broodstock

Adults were offspring of wild broodstock collected on Georges Bank produced in 2008, hatched and raised at the Biological Station, St. Andrews, New Brunswick, Canada. Total length (TL, cm) and bodyweight (BW, g) of cod were measured monthly from April 2010 to January 2011. Prior to start of the feeding trial, cod were scanned using ultrasonography to assess fish gender (Karlsen & Holm 1994, Martin-Robichaud & Rommens 2001). To focus the feed trial on first time spawners, a few females that showed signs of spawning in the winter 2010, were excluded from the study.

Diet

The experimental diets applied in this study were formulated based on analyses of each feed ingredient. The diets were produced as 10mm dry pellets by Northeast Nutrition, Canada. Three different fish meals, four different fish oils and fungus oil were applied to produce the diets with three different EPA/ARA ratios and at the same time maintain DHA constant and as high as possible (Table 1). Astaxanthin was added (78 mg/g) to all diets because it has been shown to enhance egg quality in Atlantic cod (Sawanboonchun et al. 2008) and because the high PUFA level can otherwise be harmful due to peroxidation (Sargent et al. 2002). Basal feeds were coated with fish oil EPAX 1050® (EPAX AS, Norway) with 50% DHA and 10% EPA and Vevodar® (DSM, Food Specialties) produced by means of fermentation of a fungus with 40% ARA. Coating with oil supplements was carried out every second month at St. Andrews Biological Station and feed was stored at -20°C to reduce the rate of oxidation. Samples of each batch were stored at -80°C for later lipid analyses.

Table 1. Feed ingredients of the three experimental diets. The proportion of ARA, EPA and DHA in the ingredients (% of total fatty acids) and the proportion of each ingredient in diet A, B and C (% of wet weight) are given.

	Ingredient composition			Diet composition		
Feed ingredients	ARA	EPA	DHA	A	B	C
Fish meal, N.S.	0.3	6.7	11.0	24.7	14.4	14.8
Fish meal, S.A.	1.2	14.8	19.0	0.0	33.7	34.5
Fish meal, CPSP	0.7	5.4	8.0	26.7	0.0	0.0
Wheat gluten meal	0.0	0.5	0.5	9.9	9.6	9.9
Wheat	0.0	0.1	0.4	15.7	15.1	15.4
Corn meal	0.0	0.0	0.5	6.4	6.3	6.4
Krill meal	0.6	12.4	7.5	4.9	4.8	4.9
Poultry tall	0.3	0.1	0.1	1.0	0.0	0.0
Fish oil, N.S.	0.2	7.2	5.8	4.3	2.3	3.5
Fish oil, S.A.	1.0	18.4	7.7	0.5	8.7	6.7
Fish oil, EPAX 1050	2.8	13.7	48.9	2.1	1.5	1.1
Fungus oil, Vevodar	41.3	0.1	1.7	0.9	0.8	0.0
Vitamins & minerals*				2.1	2.0	2.1
Astaxanthin				0.8	0.8	0.8

*Vitamin A, D, E and choline, calcium and phosphorus. N.A. = North America, S.A. = South America, CPSP = Fish protein concentrate.

Feeding regime and tanks

Fish were maintained in one circular tank (3.9 m diameter, 15 m^3) and fed grower feed (EWOS, Canada) until July 2010. On July 8th 2010, 100 cod were

divided into three circular tanks (2.4 m diameter, 5 m^3) and the fish in each tank were fed one of three experimental diets for 6.5 months until close to spawning. Feeding ended the 19th of January 2011. Unexpected high mortality in diet A and B resulted in an uneven number of fish among diets at the end of the feeding period: Diet A = 17 fish, Diet B = 15 fish and Diet C = 27 fish. These fish were divided into smaller circular tanks (1.9 m diameter and 3.5 m^3) inside a tent building. Fish from diet A and C were divided into triplicate tanks while in diet B, where only five males survived, were divided into duplicate tanks. Five to eight cod were placed in each of the experimental tanks ensuring minimum variation of average body weight of both females and males among replicates (Table 2). All tanks operated on a flow-through seawater system under ambient salinity (33 ppm), which filtered the sea water through a sand filter (20µm). Tanks were under ambient temperature from the start of the experiment and until mid February when spawning had started in all tanks. During this period, the temperature decreased from an average of 4°C to between 2 and 3°C in all tanks. During the main spawning period, water temperature was held around 4°C by adding a constantly adjusted amount of hot seawater to each tank. The facility had a natural photoperiod and tank cover to limit light intensity. Oxygen and temperature were measured daily with an Oxyguard®. During the entire period the temperature was also monitored with submersible data loggers (Data Storage Tag milli temperature and SeaStar software, version 4.3.9, Star Oddi, Reykavik, Iceland).

Table 2. Average total length (TL), average body weight (BW) and total body weight (TBW) of female and male cod from diet replicates (Rep.). Values represents mean ± standard deviation, n = 8 tanks.

	Females				Males			
Rep.	n	TL (cm)	BW (kg)	TBW (kg)	n	TL (cm)	BW (kg)	TBW (kg)
A1	3	44.3±3.4	1.5±0.6	4.6	2	46.9±4.6	1.6±0.5	3.3
A2	3	46.3±2.8	1.6±0.2	4.7	2	43.0±10.0	1.4±0.9	2.7
A3	4	45.3±2.3	1.5±0.2	6.1	3*	43.4±0.5	1.4±0.2	4.1
B1	5	45.3±4.5	1.5±0.5	7.5	3**	46.4±2.4	1.5±0.3	4.5
B2	5	44.9±3.2	1.5±0.2	7.6	2	45.3±2.9	1.5±0.1	3.0
C1	4	48.1±2.4	1.8±0.3	7.3	3	47.2±2.5	1.5±0.2	4.6
C2	4	46.6±1.6	1.9±0.2	7.4	3	45.1±4.5	1.5±0.2	4.5
C3	4	47.8±1.6	1.9±0.1	7.4	3	45.3±3.0	1.5±0.4	4.5

*One male died in replicate A3 before spawning started, hereafter; 2 males and TBW = 2.7 kg. ** One male died during spawning in replicate B1, hereafter; 2 males and TBW = 2.7 kg.

Egg production

Each of the small tanks had a two part egg collector system developed at the St. Andrews Biological Station and described in detail in Thorsen et al. (2003). Briefly a surface collector collected eggs from the surface layer and eggs that settled to the tank bottom were collected through a drain

collector. Eggs were collected from both surface and drain collectors daily at the same time of day during the spawning season. The eggs were transported to a cold room (5.5°C) for further analyses. The volume of floating and sunken eggs was measured with a graduated cylinder with mesh in the bottom. To calculate the egg density the spawned eggs per ml were estimated from average egg diameter (see method below) by using the packing density equation $1222*D^{-2.71}$ (Kjesbu 1989).

Gamete collection and egg and sperm density

Cod were stripped over three to five days every second week during the spawning season. Fish were anesthetized with MS222 for a few minutes to minimize stress on broodstock during stripping of gametes. Sperm was obtained by applying slight pressure on the abdomen and eggs were collected of females exhibiting signs of ovulation by applying slight pressure to the abdomen along the abdominal cavity towards the opening of the genital duct. Samples of sperm and eggs were immediately stored in a cooler with ice and brought to a cold room at 5.5°C. Samples of 5-10 ml from each egg batch were taken shortly after stripping and drained for ovarian fluid with a mesh (200 µm mesh size) and dried outside the mesh with Kimwipes® and stored in cryovials at -80°C until lipid analysis.

The number of eggs per ml was determined by counting three replicates of 200 µl samples per female under a microscope immediately after stripping (Butts et al. 2009). Sperm concentration was estimated by measuring spermatocrit (the percentage of milt occupied by spermatozoa). Spermatocrit was measured by drawing semen into two heparinized micro-hematocrit capillary tubes (Butts et al. 2009). One end of each tube was sealed with Critoseal and the tubes were centrifuged for 10 min at 7500 RPM (model Centra CL3, Thermo IEC). The mean of the two measurements per male was used for sperm density calculation. Spermatozoa density was calculated from the sperm density and spermatocrit relationship using the formula: $(2680+148*\text{Spermatocrit \%}) \times 10^5$ (Rakitin et al. 1999).

Fertilization success and egg measurements

Stripped eggs were dry fertilized (no exposure to seawater prior to activation) for each female separately with sperm from a single male from the same tank or from another replicate of the same diet to maximize the number of independent crosses. Five replicates of 200 eggs were placed in a

Petri dish using a 1.0 ml syringe with the tip cut off to prevent the eggs from being compressed (Fig. 1, Ia). The volume of milt needed from each male was calculated using the spermatozoa density and ratio of 100.000 sperm per egg ensuring both a reasonable fertilization rate and a fertilization challenge (Butts et al. 2009). The milt was added to 200 ml of UV filtered seawater (31 ppt.) and stirred with a sterile glass rod for 5 s. 40 ml of the sperm-seawater solution was added to the eggs in each Petri dish. After 5 min of gamete contact time excess sperm were rinsed with an ample quantity of UV sterilized water and transferred to a clean Petri dish for incubation in a temperature controlled room with temperature kept constant at 5.5°C (±0.5°C) for 20-30 hours.

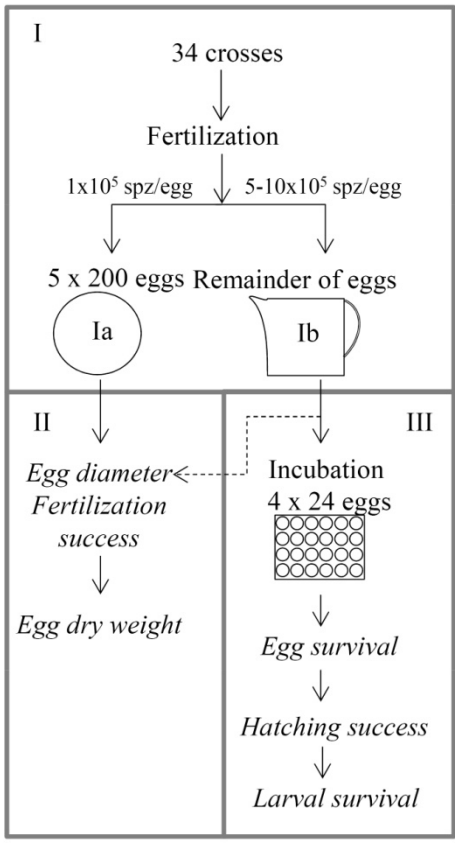

Fig. 1. Flow diagram of analysis of eggs from crosses. Boxes and roman numbers represent different steps in the procedure. Black arrows are the main procedure and the broken arrow is an alternative procedure. Text in italics represents measurements.

Overall fertilization rate was derived from observations of eggs from the five replicates 20-30 hours post fertilization (Fig. 1, II). Images of embryos were recorded using a dissecting microscope (Leica) equipped with a digital camera (Qimaging MicroPublisher 3.3 RTV camera) enabling quantification of fertilized eggs (symmetric blastomeres) and egg diameter. Egg diameter was measured on fertilized eggs from each replicate (Fig. 1, Ia). Diameters of fertilized regular eggs were measured with ImageJ software using the plugin Object J (http://simon.bio.uva.nl/objectj) and a template designed for fish eggs (examples: fish eggs + Hough transform, developed by the Institute for Marine Research, Norway). The eggs from each replicate were then drained for water on a small piece of mesh (200 μm mesh size) and weight was measured before and after freeze drying for 48 hours (± 0.00001 g). The eggs were not rinsed in distilled water as this could create variation due to removing salt from PVS (Thorsen et al. 2003).

Hatching success and survival of egg and larvae

Eggs were fertilized with a ratio of 500.000-1.000.000 sperm per egg from the same male (Fig. 1, Ib). A little UV sterilized seawater was added and stirred gently for 5 seconds and then sea water

was added to give a 1:250 semen-seawater dilution to give optimal fertilization (Trippel & Neilson 1992). After 3 minutes UV sterilized sea water was added to a total amount of 2.5 liter and set for 5 minutes. Afterward the eggs were washed gently and transferred to covered beakers containing UV sterilized sea water. A subsample was taken out for photos the following day (see method above). In batches with low fertilization rate egg diameter was measured from these photos in addition to the photos from the fertilization trial (Fig. 1, Ia, Ib). The volume of sunken eggs was measured 24 hours after fertilization and the volume of floating eggs estimated as total eggs minus the sunken eggs.
20-30 hours after fertilization 96 fertilized eggs from each batch (or less in batches with very few fertilized eggs) were transferred to four 24-well microplates (Fig. 1, III) for single egg incubation (Shields et al. 1997, Panini et al. 2001, Unuma et al. 2004). Each well contained 2 ml of UV sterilized and filtered seawater (0.2 μm mesh size) and eggs were transferred individually with an eye pin to each well. Each microplate was labeled and stored in a temperature controlled room with temperature kept constant at 5.5°C (± 0.5°C). Mortality was registered for each plate every second day until day 15 post fertilization (DPF) and subsequently mortality and hatching were registered every day until 8 days post hatch (DPH). Calculations of survival, hatching success were based upon the average of the 4 microplates. Viability was calculated as fertilization success x hatching success x larval survival at 8 DPH. At 8 DPH larvae were anesthetized with MS222 (75 mg/L) and sacrificed before starvation.

Analyses of lipid content and fatty acid methyl esters

Lipids were extracted from 10-15 g diet and 5-15 g eggs with a homogeneous mixture of chloroform, methanol and water (2:2:1.8) following the method of Bligh & Dyer (1959). Lipid content was determined by gravimetry after evaporation of chloroform and is expressed as percentage of wet weight of fish sample throughout the paper. Lipid extracts were used for the preparation of fatty acid methyl esters using AOCS Official Method Ce 2-66 (1998) and 5 mg C23:0 methyl ester was used as internal standard. Fatty acid methyl esters (FAME) were analysed on gas chromatograph (Agilent Technologies 7890A, Denmark A/S) equipped with a DB-WAX (10 m x 0.100 mm x 0.10 μm) column (Agilent Technologies) using AOCS method Ce 1b-89 (1998). The oven temperature programme was: initial 160°C, 10.6°C min^{-1} to 200°C, hold 0.3 min and 10.6°C min^{-1} to 220°C, hold 1 min, and 10.6°C min^{-1} to 240°C, hold 3.8 min. A split ratio of 1:25 was used. Fatty acids were identified by comparison of retention times with a mixture of standards containing all the fatty acids identified in this study. Each fatty acid was quantified by calculating

its peak area relative to the total peak area or relative to the peak area of the internal standard. These values are referred to as fatty acid proportion of total fatty acids (%) or absolute content per gram sample (mg g^{-1}) throughout the paper.

Statistical analyses

Statistical differences between eggs from broodstock fed different diets (A, B, C) and different periods (early, mid or late in the spawning period) were calculated with R version 2.12 or AD-model builder (Fournier et al. 2011). Statistical differences in lipid and fatty acid content in diets and eggs as well as total egg production were tested with ANOVA. The data were checked for normal distribution with a residual analysis. Statistical differences in egg diameter and egg dry weight were tested with a normal linear mixed model, fitted by restricted maximum likelihood. The random variation due to different females, males, crosses (female and male combinations) were included. The full model tested was: $y_i = \gamma(diet_i, time_i) + F(female_i) + M(male) + C(cross_i) + \varepsilon_i$, where y_i is the measured diameter or weight, and where $F(female_i) + M(male) + C(cross_i)$ and ε_i are assumed to follow independent normal distributions with separate variances. Statistical differences in fertilization and hatching success and larval survival were calculated with logit-linear binomial models with random effects (ADMB). The random variation due to different females, males, crosses (female and male combinations) and replicate handling were included. The model used was $y_i \sim Binomial(N_i, p_i)$ where y_i = no. of eggs fertilized/hatched/survived out of N_i. The full model tested was: $logit(p_i) = \gamma(diet_i, time_i) + F(female_i) + M(male) + C(cross_i) + R(replicate_i)$ where $F(female_i) \sim N(\sigma, \sigma^2_F)$, $M(male_i) \sim N(\sigma, \sigma^2_M)$, $C(Cross_i) \sim N(\sigma, \sigma^2_{F+M})$ and $R(replicate_i) \sim N(\sigma, \sigma^2_R)$ which were all independent. The model takes into account that eggs within each cross were not independent. Models were reduced using likelihood ratio tests. A significance level (p) of 0.05 was applied in all tests.

RESULTS

Diet and broodstock

The fatty acid compositions of the diets are presented in Table 3. The proportion of ARA was significantly higher in diet A and B compared to diet C and the proportion of EPA was significantly lower in diet A compared to diet B and C. The experimental diet was successfully applied to the broodstock cod and after a two week weaning period, all broodstock cod were feeding on the experimental diets and had a normal growth. Broodstock cod had an average total body weight of around 670 g at the beginning of the feeding period in July 2010 and increased to an average weight around 1500 g for diet A and B while cod fed diet C increased to around 1700 g (Table 2). At the end of the spawning period the average weight of females was 1100, 1250 and 1400 g in diet A, B and C respectively and males weighed around 1200 g in diet A and B and 1300 g in diet C. Fish mortality was relatively low during the experiment. One male died in tank A3 before the spawning had started for that tank and one male died in tank B1 during spawning. Four females (tank A3, B2, C1 and C2) became egg bound and died at the end of the spawning season.

Fatty acid composition of eggs

All fish were stripped three times; early in the spawning period season (Time 1: early February), mid spawning (Time 2: late February) and late spawning (Time 3: mid March). Fatty acid compositions (% of total FA) of stripped eggs from individual females fed diet A, B or C are given in Table 3. The significantly higher proportion of ARA in diet A and B compared to diet C was reflected in the eggs. The proportion of EPA in eggs was significantly different between all diets. The proportion of 16:0, 18:1n-7, ARA, 22:5n-3, EPA, DHA and the sum of PUFA was higher in eggs than in the diet. ARA in eggs from cod fed diet A and B was 32-33% higher than in feed while it was 84% higher in the diet C group. EPA was 42-76% higher and DHA was 155-173% higher in eggs than in the three diets. The remaining fatty acids were lower in eggs than in diet and in particular the reduction of the proportion of long chained MUFA; 20:1n-9, 22:1n-11 and 22:1n-9 was striking. The absolute content of ARA was significantly lower in diet C than diet A and B ($p < 0.05$) and correspondingly ARA content was significantly lower in egg from fish fed diet C than from fish fed diet A and B ($p < 0.05$) (Fig. 2a). The absolute content of ARA tended to decrease with time (Fig. 2b) however this was not significant ($p = 0.13$). The decrease in ARA was concurrent with a decrease in total fatty acids (mg g^{-1} ww) in eggs ($p = 0.08$).

Table 3. Lipid content (% of wet weight) and fatty acid composition (% of total fatty acids) of total lipid extracted from diet A, B and C and total lipids of eggs from females fed diet A, B or C. Egg / diet represents proportions (%) of FA in eggs in relation to diet FA in diet. Values represents mean ± standard deviation of duplicate analysis of all feed batches produced of each diet (diet A: n = 6, diet B: n = 8 and diet C: n = 6) and of egg samples from 31 crossings (diet A: n = 11, diet B: n = 9 and diet C: n = 11). Values with a different superscript letter within rows of diet composition and egg composition respectively are significantly different ($p < 0.05$).

	Diet composition			Egg composition			Proportions egg / diet		
	A	B	C	A	B	C	A	B	C
Lipid	21.6 ± 1.2	21.2 ± 1.4	20.6 ± 0.8	0.7 ± 0.1 ^{ab}	0.7 ± 0.1 ^{a}	0.6 ± 0.1 ^{b}	3	3	3
14:0	4.6 ± 0.4 ^{a}	5.0 ± 0.2 ^{a}	5.5 ± 0.3 ^{b}	1.4 ± 0.1 ^{ab}	1.2 ± 0.1 ^{a}	1.4 ± 0.2 ^{b}	30	25	26
16:0	13.7 ± 0.3 ^{a}	17.9 ± 0.7 ^{b}	17.4 ± 0.6 ^{b}	18.4 ± 0.4 ^{a}	19.6 ± 1.0 ^{b}	19.8 ± 0.5 ^{b}	134	110	114
18:0	3.2 ± 0.2 ^{a}	4.2 ± 0.3 ^{b}	3.4 ± 0.2 ^{a}	2.3 ± 0.3 ^{a}	2.6 ± 0.4 ^{ab}	2.8 ± 0.3 ^{b}	73	61	80
SFA	22.1 ± 0.4 ^{a}	27.9 ± 0.9 ^{b}	26.9 ± 0.6 ^{b}	21.3 ± 1.0 ^{a}	22.7 ± 1.4 ^{b}	23.0 ± 0.8 ^{b}	97	81	86
16:1(n-7)	4.8 ± 0.2 ^{a}	5.9 ± 0.2 ^{b}	6.1 ± 0.1 ^{b}	2.3 ± 0.2	2.2 ± 0.2	2.4 ± 0.2	48	37	39
18:1(n-9)	14.8 ± 0.4 ^{a}	12.9 ± 0.5 ^{b}	11.5 ± 0.4 ^{c}	13.5 ± 0.5	11.9 ± 0.4	11.7 ± 0.6	92	92	102
18:1(n-7)	2.5 ± 0.2	2.6 ± 0.2	2.7 ± 0.1	3.1 ± 0.2	3.3 ± 0.3	3.2 ± 0.2	125	128	117
20:1(n-9)	7.3 ± 0.4 ^{a}	4.1 ± 0.3 ^{b}	5.6 ± 0.4 ^{c}	2.0 ± 0.2 ^{a}	1.1 ± 0.1 ^{b}	1.4 ± 0.1 ^{c}	28	26	26
22:1(n-11)	9.7 ± 0.8 ^{a}	5.0 ± 0.6 ^{b}	7.4 ± 0.7 ^{c}	0.6 ± 0.2 ^{a}	0.2 ± 0.0 ^{b}	0.4 ± 0.1 ^{c}	6	5	6
22:1(n-9)	0.9 ± 0.1 ^{a}	0.6 ± 0.0 ^{b}	0.7 ± 0.1 ^{c}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0	0	0
24:1(n-9)	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	0.2 ± 0.0 ^{a}	0.2 ± 0.1 ^{b}	0.2 ± 0.0 ^{ab}	43	30	34
MUFA	40.9 ± 1.4 ^{a}	31.8 ± 0.7 ^{b}	35.0 ± 1.2 ^{c}	21.9 ± 1.3 ^{a}	18.9 ± 1.1 ^{b}	19.3 ± 1.3 ^{b}	53	59	55
16:3(n-4)	0.6 ± 0.1 ^{a}	0.8 ± 0.0 ^{a}	1.1 ± 0.3 ^{b}	0.2 ± 0.0 ^{a}	0.3 ± 0.0 ^{b}	0.3 ± 0.0 ^{b}	42	37	28
16:4(n-1)	0.7 ± 0.2 ^{a}	1.2 ± 0.2 ^{a}	1.1 ± 0.1 ^{b}	0.1 ± 0.0 ^{a}	0.1 ± 0.0 ^{a}	0.1 ± 0.0 ^{b}	14	8	13
18:2(n-6)	6.4 ± 0.3 ^{a}	5.5 ± 0.2 ^{b}	4.4 ± 0.2 ^{c}	3.5 ± 0.2 ^{a}	3.0 ± 0.2 ^{b}	2.5 ± 0.1 ^{c}	54	55	56
18:3(n-3)	1.3 ± 0.1 ^{a}	0.8 ± 0.0 ^{b}	0.8 ± 0.0 ^{b}	0.5 ± 0.0 ^{a}	0.3 ± 0.0 ^{b}	0.3 ± 0.0 ^{b}	38	41	41
18:4(n-3)	1.3 ± 0.2	1.3 ± 0.1	1.5 ± 0.1	0.3 ± 0.1 ^{ab}	0.3 ± 0.0 ^{a}	0.3 ± 0.0 ^{b}	23	20	23
20:4(n-6)	2.6 ± 0.2 ^{a}	2.4 ± 0.3 ^{a}	0.8 ± 0.0 ^{b}	3.4 ± 0.4 ^{a}	3.3 ± 0.4 ^{a}	1.4 ± 0.1 ^{b}	132	133	184
20:4(n-3)	0.6 ± 0.0 ^{a}	0.6 ± 0.0 ^{b}	0.6 ± 0.0 ^{b}	0.4 ± 0.0 ^{a}	0.3 ± 0.0 ^{b}	0.3 ± 0.0 ^{b}	62	56	58
20:5(n-3)	7.5 ± 0.6 ^{a}	10.7 ± 0.5 ^{b}	11.0 ± 0.6 ^{b}	13.2 ± 0.5 ^{a}	15.2 ± 0.8 ^{b}	17.6 ± 0.7 ^{c}	176	142	160
22:5(n-3)	1.5 ± 0.0 ^{a}	1.8 ± 0.0 ^{b}	1.8 ± 0.0 ^{c}	2.0 ± 0.2	2.0 ± 0.1	2.1 ± 0.3	136	115	116
22:6(n-3)	10.8 ± 0.6	10.3 ± 0.4	10.2 ± 0.4	27.6 ± 0.9 ^{ab}	28.1 ± 0.8 ^{a}	26.9 ± 0.8 ^{b}	255	273	265
PUFA	34.4 ± 1.6 ^{a}	36.5 ± 1.1 ^{b}	34.5 ± 1.3 ^{a}	52.3 ± 2.8 ^{a}	53.8 ± 2.8 ^{b}	52.9 ± 2.6 ^{c}	152	148	153
DHA/EPA	1.4 ± 0.1 ^{a}	1.0 ± 0.1 ^{b}	0.9 ± 0.0 ^{b}	2.1 ± 1.9 ^{a}	1.9 ± 1.0 ^{b}	1.5 ± 1.2 ^{c}			
EPA/ARA	2.9 ± 0.2 ^{a}	4.4 ± 0.7 ^{b}	14.4 ± 0.3 ^{c}	3.9 ± 1.2 ^{a}	4.7 ± 2.3 ^{a}	12.5 ± 5.5 ^{b}			
DHA/ARA	4.2 ± 0.3 ^{a}	4.2 ± 0.4 ^{a}	13.3 ± 0.5 ^{b}	8.1 ± 2.4 ^{a}	8.6 ± 2.2 ^{a}	19.2 ± 6.9 ^{b}			

SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, ARA = arachidonic acid (20:4n-6), EPA = eicosapentaenoic acid (20:5n-3) and DHA = docosaheaxaenoic acid (22:6n-3)

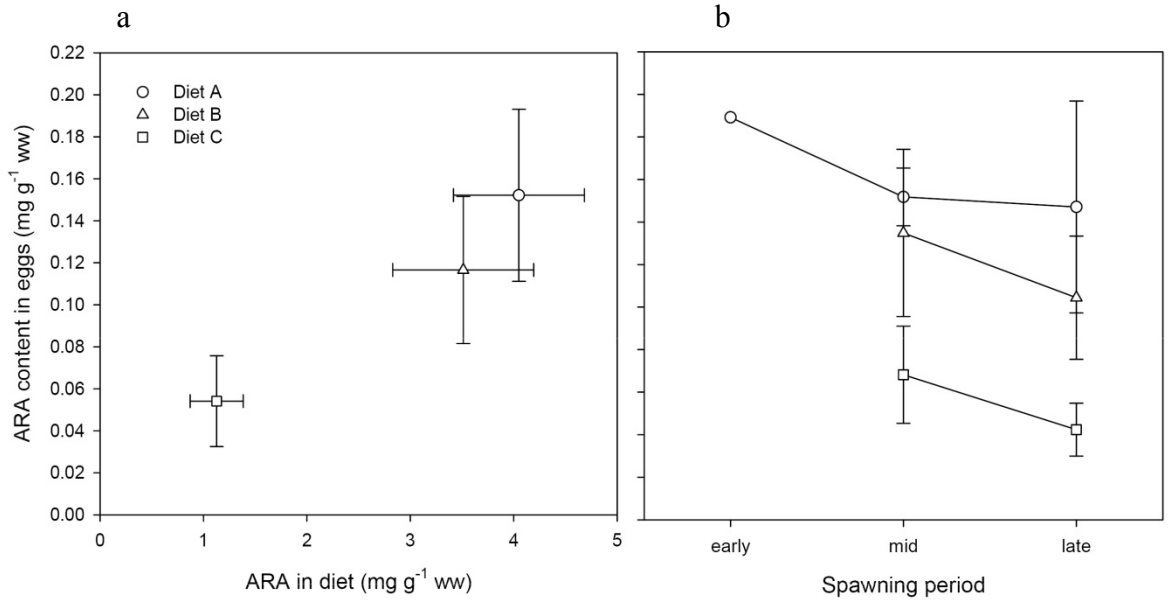

Fig. 2. ARA content in eggs from fish fed diet A, B and C in relation to ARA content in diet (a) and time of spawning (b). Number of diet samples; diet A: n = 6, diet B: n = 8 and diet C: n = 6 and number of egg samples; diet A: n = 11, diet B: n = 9 and diet C: n = 11. Fitted line in Fig. 2a: y = 0.0169 + 0.0313x.

Realized fecundity

The cumulative total realized egg production per kg female body weight in the eight tanks is shown in Fig. 3. Data in this figure includes floating eggs from surface collectors and sunken eggs from the drain collectors as well as the eggs from stripping. Stripped eggs constituted 1-10% of total eggs spawned in diet A, 6 - 20% in diet B and 8 - 13% in diet C. Females in all tanks had started spawning in mid February and the main spawning period was from end of February to end of March. By mid March 50 - 66% of total eggs were spawned except for A1 and A3 which only had spawned 40% at this point and had high

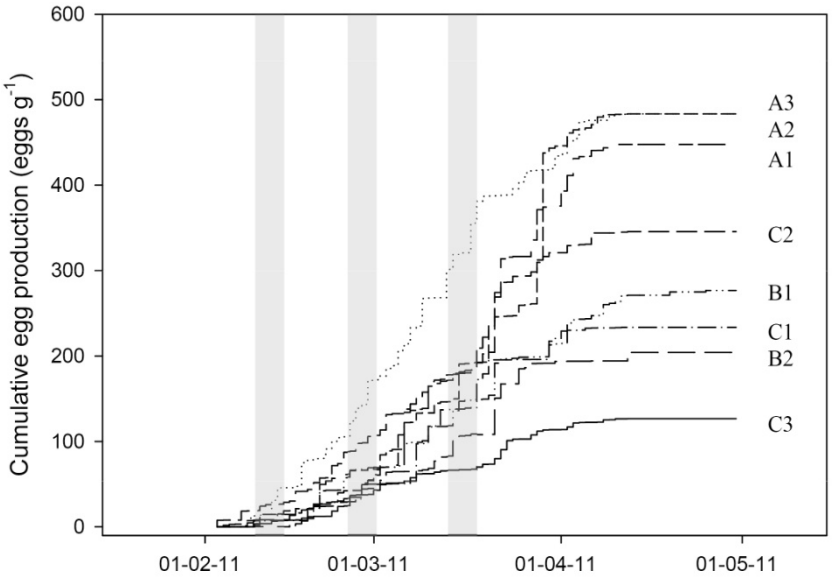

Fig. 3. Cumulative daily egg production (eggs g-1 female) in tanks from January to May 2011, including eggs from stripping. Grey boxes indicate the periods when fish were stripped early, mid and late in the spawning period.

spawning in end of March. Total egg production was significantly higher ($p = 0.02$) in tanks with cod fed diet A, all producing on average 472 eggs g^{-1} female compared to tanks with cod fed diet B and C which produced around 242 and 235 eggs g^{-1} respectively (Table 4).

Table 4. Average total and viable egg production per g female in tanks fed diet A, B or C. Numbers include eggs from natural spawning and stripping. Values represents mean ± standard deviation, n = 8 tanks. Values with a different superscript letter within columns are significantly different ($p < 0.05$).

	Egg production (egg g^{-1})	
Diet	total	viable
A	471.5 ± 20.6[a]	71.3 ± 63.4
B	242.0 ± 53.8[b]	32.5 ± 3.8
C	235.2 ± 109.5[b]	4.0 ± 2.7

Total numbers are estimated from average egg diameter using the equation $1222 \times D^{-2.71}$ from Kjesbu et al. (1989). Viable eggs are estimated as total egg production x fertilization success x hatching success x larval survival 8 DPH for each tank.

Egg diameter and dry weight

In total 34 crosses were analyzed; 12 from diet A and C and 10 from diet B (Table 5). Some females and males were crossed two times in different periods but each female-male combination was unique. Average egg diameter varied between crosses from 1.43 to 1.58mm. The final model for egg diameter was $Diameter_i = \beta(time_i) + F(female_i) + M(male_i) + \varepsilon_i$. There was no effect of diet on egg diameter but the diameter early in the spawning period was significantly bigger than the diameter in mid and end of spawning ($p < 0.01$). Average egg dry weight varied between 0.110mg egg^{-1} and 0.160mg egg^{-1}. The final statistical model for egg dry weight was $DW_i = \alpha(diet_i) + \gamma(diet_i, time_i) + F(female_i) + \varepsilon_i$. The model showed a significant effect of diet but it was not the same in the different periods. The relationship between egg diameter (D, mm) and egg dry weight (DW, µg) for the three diets combined was: $DW = 254.4D - 247.9$, $r^2 = 0.44$. The relationship was strongest for diet A, $r^2 = 0.89$, weak for diet B, $r^2 = 0.22$ and not existing for diet C, $r^2 = 0.00$.

Egg and larval quality

Average fertilization success varied highly between crosses in all diets from 0-45% (Table 5). Average egg survival and hatching success varied between 22 and 100%, and larval survival varied between 73-100% in diet A and B but down to 8% in diet C. Average survival of eggs and larvae from 1 days post fertilization (DPF) until 8 days post hatch (DPH) in diet A, B and C is shown in Fig 4.

Table 5. Egg diameter of fertilized eggs, dry weight (DW) and average fertilization success of 5 replicates of around 200 eggs and hatching success and larval survival (hatch to 8 days post hatch) of 1-4 replicates of 24 eggs from each cross fed the three different diets. Viability is calculated as fertilization success x hatching success x larval survival. Diet replicate, sampling period and ID of females and males from each cross are given. Values represent mean ± standard deviation and underlined values are means of all replicates within a diet.

Diet	Samp.	Females	Males	Eggs					Larvae			
Rep.	Period	ID	ID	n	Diameter (mm)	n	DW (µg)	Fertilization (%)	n	Hatching (%)	Survival (%)	Viability (%)
A1	late	A-F1	A-M1	274	1.45 ± 0.02	974	109.6 ± 4.3	43.7 ± 14.2	96	93.8 ± 4.2	78.0 ± 7.5	32.0
A2	early	A-F2	A-M2	30	1.58 ± 0.03	837	n.a.	2.1 ± 2.1	96	83.3 ± 3.4	96.3 ± 4.8	1.7
A2	mid	A-F2	A-M3	151	1.53 ± 0.01	700	140.3 ± 3.9	16.6 ± 10.7	96	92.7 ± 2.1	85.3 ± 13.1	13.1
A2	mid	A-F3	A-M2	183	1.45 ± 0.02	954	114.7 ± 2.3	20.0 ± 5.3	96	93.8 ± 2.4	93.4 ± 4.3	17.5
A2	late	A-F3	A-M3	158	1.44 ± 0.02	939	112.3 ± 2.0	19.8 ± 6.3	72	98.6 ± 2.4	95.8 ± 4.2	18.7
A2	late	A-F4	A-M4	0	n.a.	156	156.0 ± 16.7	0.8 ± 1.4		n.a.	n.a.	n.a.
A3	early	A-F5	A-M5	140	1.52 ± 0.01	988	149.0 ± 8.8	14.7 ± 4.7	96	83.3 ± 5.9	98.8 ± 2.5	12.1
A3	late	A-F5	A-M1	177	1.49 ± 0.02	0	n.a.	n.a.	72	100.0 ± 0.0	97.2 ± 2.4	n.a.
A3	mid	A-F6	A-M6	50	1.56 ± 0.03	1075	153.8 ± 4.7	5.0 ± 2.3	96	85.3 ± 13.8	88.7 ± 2.6	3.8
A3	late	A-F6	A-M2	167	1.53 ± 0.01	0	n.a.	n.a.	72	92.8 ± 2.7	84.9 ± 9.6	n.a.
A3	late	A-F7	A-M5	3	1.49 ± 0.04	355	141.9 ± 20.1	0.3 ± 0.8		n.a.	n.a.	n.a.
A3	late	A-F8	A-M6	24	1.50 ± 0.03	806	134.8 ± 4.7	2.2 ± 1.4	72	65.3 ± 10.5	86.3 ± 12.0	1.2
					1.49 ± 0.05		134.2 ± 19.4	12.3 ± 14.3		88.9 ± 10.6	90.4 ± 9.1	12.7 ± 10.0
B1	early	B-F1	B-M1	51	1.53 ± 0.01	675	160.1 ± 7.2	19.1 ± 6.4	96	90.6 ± 5.2	93.3 ± 5.8	16.2
B1	mid	B-F1	B-M2	29	1.51 ± 0.03	909	137.2 ± 3.6	1.5 ± 1.0	72	87.5 ± 8.3	72.7 ± 9.0	1.0
B1	mid	B-F2	B-M3	263	1.50 ± 0.02	1127	126.5 ± 2.2	42.6 ± 5.3	96	90.6 ± 2.1	88.6 ± 9.5	34.2
B1	late	B-F2	B-M4	201	1.50 ± 0.02	782	115.5 ± 6.7	27.0 ± 12.4	72	88.9 ± 4.8	95.2 ± 5.0	22.9
B1	late	B-F3	B-M4	151	1.43 ± 0.02	297	127.7 ± 14.3	2.8 ± 1.8	96	94.8 ± 2.1	97.8 ± 2.5	2.6
B1	late	B-F4	B-M2	39	1.46 ± 0.03	925	117.9 ± 2.9	1.9 ± 1.2	96	79.2 ± 12.3	85.8 ± 7.4	1.3
B2	mid	B-F5	B-M5	79	1.52 ± 0.01	976	129.1 ± 4.6	17.2 ± 6.3	96	69.8 ± 8.6	93.7 ± 9.5	11.2
B2	late	B-F5	B-M6	5	1.52 ± 0.03	861	125.7 ± 5.8	0.1 ± 0.2	18	22.2 ±	100.0 ±	0.0
B2	mid	B-F6	B-M6	219	1.47 ± 0.03	1118	123.7 ± 2.6	31.6 ± 15.7	96	77.1 ± 11.0	96.1 ± 2.7	23.4
B2	late	B-F7	B-M5	226	1.53 ± 0.01	861	131.3 ± 2.7	45.3 ± 11.3	96	89.6 ± 5.4	94.4 ± 5.4	38.2
					1.49 ± 0.04		128.8 ± 12.4	18.1 ± 17.5		83.4 ± 14.7	91.5 ± 9.1	16.6 ± 13.6
C1	mid	C-F1	C-M1	16	1.49 ± 0.03	746	141.6 ± 5.7	1.0 ± 0.8	48	62.5 ± 17.7	88.9 ± 15.7	0.6
C1	late	C-F1	C-M2	10	1.49 ± 0.03	776	142.4 ± 2.0	0.0 ± 0.0	48	87.5 ± 5.9	84.1 ± 22.5	0.0
C1	late	C-F2	C-M3	0	n.a.	638	127.8 ± 2.9	0.0 ± 0.0		n.a.	n.a.	n.a.
C1	late	C-F3	C-M1	87	1.51 ± 0.01	763	139.7 ± 5.0	11.8 ± 5.1	48	91.7 ± 5.9	84.0 ± 4.2	9.0
C2	mid	C-F4	C-M4	8	1.49 ± 0.04	677	136.7 ± 5.4	0.4 ± 0.9	23	56.5 ±	7.7 ±	0.0
C2	mid	C-F5	C-M5	71	1.52 ± 0.02	825	139.7 ± 4.4	10.2 ± 5.8	96	30.2 ± 11.0	31.0 ± 11.5	1.0
C2	late	C-F6	C-M6	18	1.53 ± 0.02	829	136.9 ± 7.1	1.1 ± 0.7	72	91.7 ± 8.3	80.0 ± 10.5	0.8
C2	late	C-F7	C-M4	111	1.51 ± 0.02	891	131.1 ± 3.1	15.6 ± 8.0	96	20.8 ± 6.8	42.6 ± 19.2	1.4
C3	early	C-F8	C-M7	49	1.49 ± 0.04	987	137.0 ± 6.5	1.9 ± 2.3	96	78.1 ± 9.2	89.5 ± 3.5	1.3
C3	late	C-F8	C-M8	13	1.48 ± 0.02	633	135.8 ± 3.4	0.3 ± 0.4	96	72.9 ± 5.4	59.6 ± 17.9	0.1
C3	mid	C-F9	C-M9	17	1.49 ± 0.03	852	135.8 ± 1.1	0.6 ± 1.0	96	73.7 ± 5.2	76.2 ± 11.5	0.3
C3	mid	C-F10	C-M10	39	1.52 ± 0.03	853	136.4 ± 2.8	2.5 ± 2.7	48	85.4 ± 2.9	80.5 ± 0.7	1.7
					1.51 ± 0.03		136.9 ± 5.6	3.4 ± 5.5		65.3 ± 25.9	66.2 ± 25.7	1.3 ± 2.1

The average survival at 8 DPH was 84% in diet A, 73% in diet B and 50% in diet C. At 20 DPF over 50% of eggs had hatched. The survival decreased in diet C after hatching. Viability refers to fertilized egg that hatched and survived to 8 DPH and varied between 0 and 34% (Table 5). Average fertilization success and larval survival were lowest in diet C and hence the estimated number of viable eggs would be 4 eggs g^{-1} female for this diet compared to 71 eggs g^{-1} for diet A (Table 4).

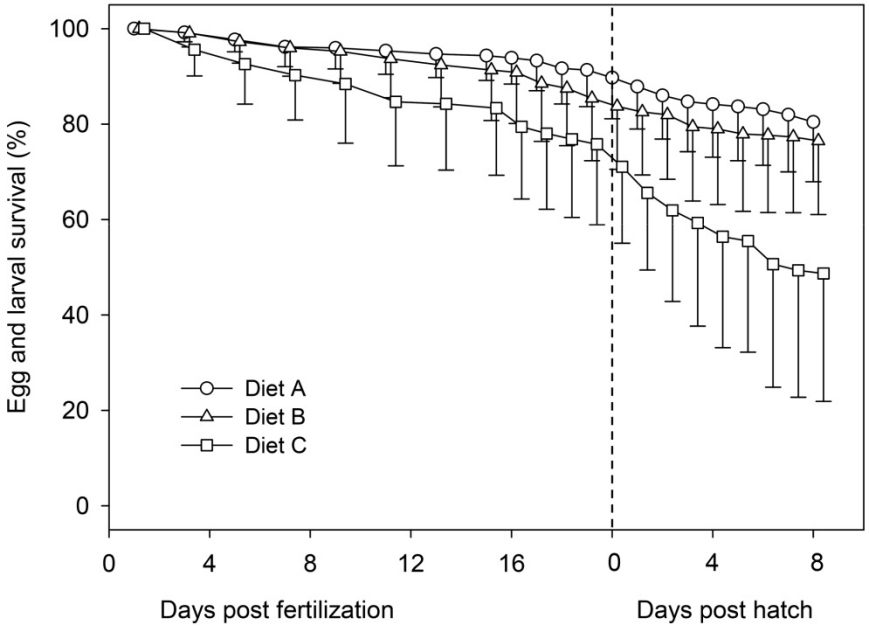

Fig. 4. Average embryonic and larval survival of eggs from 1-4 replicates of 24 eggs of 31 crosses of the diet A, B and C. The dotted line 20 days post fertilization represents the time when 50 % of eggs had hatched. Diets are displaced slightly for visual clarity. Values represent mean ± standard deviation of replicates. Diet A: n = 33, diet B: n = 35 and diet C: n = 32.

Average fertilization success is shown in Fig. 5a for the three diets in the different periods. Fertilization success varied greatly particularly in diet A and B where fertilization rate between 0 – 65% were observed. The final statistical model for fertilization success was: $\text{Logit}(p_i) = \alpha(\text{diet}_i) + \beta(\text{time}_i) + F(\text{female}_i) + M(\text{male}_i) + R(\text{replicate}_i)$. The odds ratios of fertilization success were significantly different being 14 times higher in diet B than in diet C. Similarly the odds of fertilization success for diet A was six times higher than C, although this was not significant (Table 6). The probability of fertilization success was highest with diet B at all three times tested during the spawning period and for all three diets the probability of fertilization success was highest in the middle of the spawning season.

Average hatching success was lowest in diet C in mid and late spawning (Fig. 5b). The final statistical model for hatching success was: $\text{Logit}(p_i) = \gamma(\text{diet}_i, \beta\text{time}_i) + F(\text{female}_i)$. The odds of hatching success in mid spawning season were significantly lower in diet C than in diet A and B (Table 6). The probability of hatching was between 70 and 95% for all diets and time combinations

except for diet C in mid spawning with only 57% probability of hatching (Table 6). Larval survival at 8 DPF for the three different periods is shown in Fig. 5c. The final statistical model for larval survival was: $Logit(p_i) = \alpha(diet_i) + \beta(time_i) + F(female_i)$. The odds of survival to 8 DPH was significantly different, being six times higher in diet B that in diet C and diet A was 4 times higher than diet C (Table 6). Odds for larval survival were 4 - 5 times higher in the beginning of the spawning season compared to later in the season. The probability of survival to 8 DPH was between 88 and 98% except for in diet C in mid and late spawning (Table 6). The average viability for the three diets and periods are shown in Fig. 5d. The probability of viability to 8 DPH was highest in diet B early and mid in the spawning period (Table 6). There was no clear relationship with female body weight and the different egg quality measures.

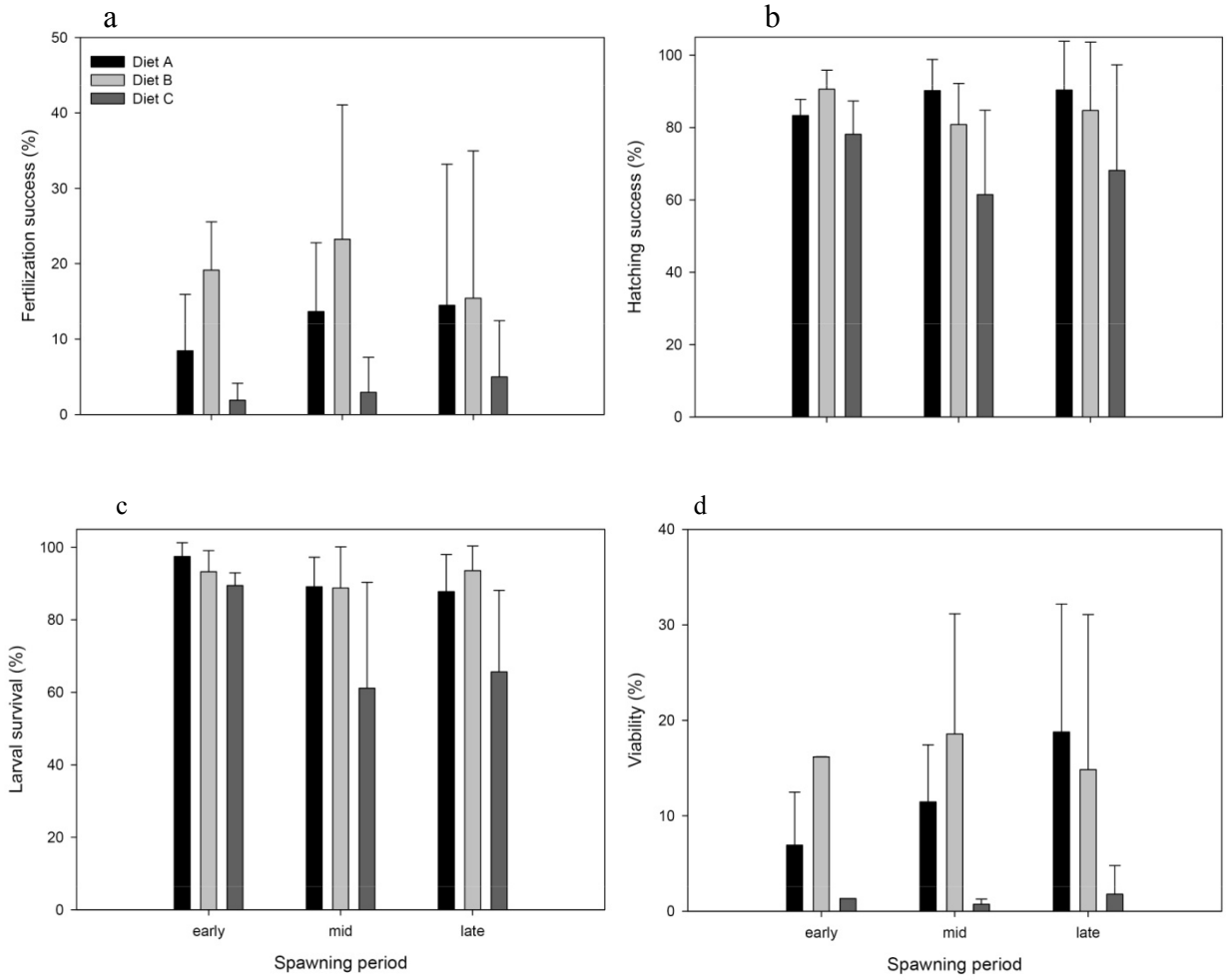

Fig. 5. Average fertilization success (a), hatching success (b), larval survival 8 days post hatch (DPH) (c) and viability calculated as fertilization success x hatching success x larval survival (d) of gametes stripped early, mid or late in the spawning season from fish fed diet A, B or C. n values see Table 5.

Table 6. (a) Model results of odds ratios and probability of fertilization rate, survival 8 DPH and hatch of gametes stripped early, mid or late in the spawning season from fish fed diet A, B or C. (b) Model results of estimates of fertilization rate, survival 8 DPH and hatch of gametes stripped early, mid or late in the spawning season from fish fed diet A, B or C.

		Odds ratios (OR)		
Variable		Fertilization	Survival	Hatch
Diet	A / B	0.5	0.7	
	A / C	6.5	4.4*	
	B / C	12.6*	6.4*	
Time	1 / 2	0.7	5.3*	
	1 / 3	3.9	4.3*	
	2 / 3	5.3*	0.1	
Diet, time	A1 / A2			0.3*
	A1 / A3			0.1*
	A2 / A3			0.4*
	A2 / C2			5.2*
	A3 / B3			4.7*
	A3 / C2			13.8*
	A3 / C3			4.3*
	B1 / C2			9.2*
	B2 / B3			2.3*
	B2 / C2			6.8*
	C1 / C2			4.2*
	C2 / C3			0.3*

		Probability (%)		
Diet/Time		Early	Mid	Late
Fertilization	A	10	13	3
	B	18	23	5
	C	2	2	0
Hatch	A	70	87	95
	B	92	90	79
	C	85	57	81
Survival	A	98	88	90
	B	98	92	93
	C	90	63	67
Viability	A	7	10	2
	B	16	19	4
	C	1	1	0

Estimates

Var.	Comp.		Fertliz.	Hatch	Survi.	Diam.	DW
Diet	A	α_A	-2.20		3.67		
	B	α_B	-1.54		4.05		
	C	α_C	-4.07		2.19		
Time	1	β_1	0.00		0.00	1.53	
	2	β_2	0.32		-1.66	1.49	
	3	β_3	-1.35		-1.47	1.49	
Diet, time	A, 1	γ		0.84			x
	A, 2	γ		1.91			x
	A, 3	γ		2.9			x
	B, 1	γ		2.49			x
	B, 2	γ		2.18			x
	B, 3	γ		1.35			x
	C, 1	γ		1.71			x
	C, 2	γ		0.27			x
	C, 3	γ		1.44			x
Indepen.	Female	σ_F	1.68	1.1	0.84	x	x
	Male	σ_M	1.17			x	
	Cross	$\sigma_{F,M}$					
	Replic.	σ_R	0.55				

Footer: * Statistically significant ($p < 0.05$).

DISCUSSION

The results of the present study show that FAC of eggs was strongly influenced by that of the diet after 6.5 months of feeding and that there was an accumulation of ARA in eggs as a function of broodstock diet composition. This is in agreement with Norberg et al (2009) who also found a strong correlation between dietary and ovarian FAC after feeding diets with four different ARA levels for over 8 months. In the present study, it is striking that the proportion of ARA in eggs compared to diet was approximately 30% higher in diet C than in diet A and B. This indicates that ARA is highly essential and that cod are able to secure disproportionately greater amounts for oocyte development when dietary content of ARA is low. However, the proportion and absolute content of ARA in diet C was still less than half the level of diet A and B. The dietary influence of ARA levels in cod eggs are in contrast to the findings of Pickova et al. (1997) where four different Swedish broodstock were compared: Skagerrak cod fed 2 months and Baltic cod fed 2, 7 or 24 months a diet consisting of commercial dry feed and sprat (*Sprattus sprattus*). ARA levels in ovary phospholipids were significantly lower in Baltic than in Skagerrak cod irrespective of the feeding period of Baltic cod and the authors concluded that lipid composition was mostly related to stock

origin (genetic) rather than to diet. In the present study, FAC of egg lipids were not analyzed in phospholipids and neutral lipids separately but total lipid is highly influenced by phospholipids as they constitute approximately 70% of total lipids (Tocher & Sargent 1984). However, FAC of diet was not investigated by Pickova et al (1997) and it is possible that dietary ARA was low and the feeding period of Skagerrak cod was too short to reduce ARA level in eggs. A slow decrease in ARA level is indicated in the study of Salze et al. (2005) who compared FAC in eggs from three different broodstock of Atlantic cod; farmed/fed 2years, wild/fed 2-8 months and wild/unfed. ARA level in total lipid from eggs of farmed/fed broodstock was similar to that of the diet and significantly lower than in wild/unfed and wild/fed with the latter having an intermediate ARA level.

The significantly higher realized egg production in the triplicate tanks given diet A in the present study indicates that an EPA/ARA level around 3 is superior to a ratio around 4.5 (diet B) or 14.5 (diet C) in terms of realized fecundity in Atlantic cod. Difference in EPA/ARA ratio between diet A and B was smaller than planned but it showed to be more difficult than expected to decrease EPA level with the available ingredients. In this study the combination of natural and strip spawning on the same fish may imply a risk of disturbing the natural spawning, but the effect was similar in all tanks and there was no sign of irregular spawning in the tanks in periods after stripping. The results are in agreement with Sawanboonchun (2009) where egg production was highest in groups with ARA supplementation (EPA/ARA = 2) compared to a control without ARA supplementation (EPA/ARA = 11), however there were no replicates in her study and the differences were not significant. The results are in contrast to a study investigating the supplementation of dietary ARA in Atlantic cod in duplicate sea cages where realized fecundity was higher at EPA/ARA around 7 compared to a ratio around 2 (Grethe Rosenlund, pers. comm.). Lie et al (1993) did not observe an effect of different ARA levels (0.1, 0.2 and 0.8% of lipid), but this could be due to the much lower ARA level tested here in that study and/or the high EPA/ARA levels (16 to 45). Dietary supplemented ARA to 7.3% of total fatty acids reduced total egg production in Japanese flounder (*Paralichthys olivaceus*) compared to 3.6% ARA (Furuita et al. 2003) indicating a negative effect of high ARA levels or EPA/ARA ratios under 1. In the present study, diet A was significantly superior in terms of egg production but because only three ratios were tested and due to differences in ingredients among diets it remains unclear if this was solely due to low EPA/ARA ratio.

To the author's knowledge, the present study is the first to show a positive effect of dietary ARA and EPA on fertilization success and larval survival in broodstock cod. The odds ratios between diets indicate that high levels of essential n-3 (EPA) and n-6 (ARA) fatty acids are equally important for fertilization success and both fatty acids are also important in terms of larval survival but here ARA is of higher importance than EPA. ARA seems only to have a positive effect on hatching success during mid spawning. The lack of significant difference could be due to the low number of crosses in the early period and due the great variation in hatching success in diet B in the late period. It is likely that the lack of effect of ARA in previous studies of cod with similar origin on fertilization success and hatching success (Lie et al. 1993, Penney et al. 2006, Sawanboonchun 2009) was due to the assessment on tank level (no replicates) instead of individual fish level as in the present study. The assessment of relationships between egg biochemistry and quality on individual fish in the present study was very successful. However, it is very important to ensure that eggs are only collected when females are ready to spawn.

In addition to the dietary effect, a significant effect of females on fertilization success, hatching success and larval survival was evident while paternal effects were only significant in fertilization success in the present study and there was no significant effect of interactions between parents. A maternal effect in embryonic survival has previously been reported for Baltic cod during the first 4 days while male-female interaction was evident 5 to 9 days after hatch (Trippel et al. 2005). A low and very variable fertilization success observed in the present study has previously been reported in first-time spawners of young cod of wild origin from the northwest Atlantic (Trippel 1998). In the latter study, wild fish caught in the Bay of Fundy were maintained on a mixed diet of Atlantic herring, northern shortfin squid and Aesop shrimp. Egg diameter in the present study was comparable, but the average slightly higher, than the first-time spawning cod from north-west Atlantic (Trippel 1998). Farmed cod broodstock has previously been reported to exhibit extremely variable fertilization success (Kjesbu 1989) and lower than in wild stock (Salze et al. 2005) indicating that diet is of prime importance and the composition of extruded diet was not optimal. The number of hatched larvae g^{-1} female (first-spawners) was in the study of Trippel (1998) on average 19 which was higher than diet C in the present study but lower than in diet A and B, indicating that diet A and B may be at least comparable and maybe even better than natural diet. In the Baltic Sea, ARA level and EPA/ARA ratio observed in main prey species of cod; sprat and herring (Røjbek et al. 2012) was comparable to the level in diet C. Low ARA levels in cod eggs

(Pickova et al. 1997) and the results from the present study indicate that FAC of prey species in the Baltic Sea may not be optimal for egg production, fertilization success and larval survival in Baltic cod.

The results from the present study show that the dietary level of ARA and the EPA/ARA ratio have a major impact on spawning performance. It would therefore be wise to consider the level of these fatty acids in broodstock diet of cod. FAC differs between types of fish meal and oil and some fish oils and oil derived from Fungus had the potential of reducing EPA/ARA ratio in the present study. Fish meal is a limiting factor in fish diet production (Jobling & Leknes 2010) and it is therefore of interest to include oils from fungus and algae in fish feed to increase ARA and decrease EPA levels. However, the economics of using special oils in diet production must be examined in details before implementation can be recommended on a commercial scale.

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