

Gender differences in health and aging of Atlantic cod subject to size selective fishery

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Summary

We have analyzed health and physiological aging parameters in male and female Atlantic cod, *Gadus morhua*, captured in Kattegat, Skagerrak and in Öresund. Gender differences were clearly evident in a number of variables. Males had longer liver telomeres and higher catalase activities than females, while females had higher superoxide dismutase activity, liver somatic index and condition factor. Effects of age were found for males where levels of the antioxidant glutathione and telomere length declined with age, indicating physiological aging. Liver somatic index increased and percentage oxidized glutathione decreased with age. Between-site comparisons of males show that percentage oxidized glutathione and catalase were lowest in Kattegat, whereas protein carbonyls and condition factor were higher in Skagerrak. Females, on the other hand, showed no differences between sites or indications

of somatic aging or age-related effects in egg quality, indicating that older and larger female cod are healthy and show no changes in eggs with age. In contrast, males showed indications of physiological aging and lower condition than females. The results emphasize the importance of conserving old mature fish, in particular high egg-productive females, when managing fisheries.

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Key words: Telomeres, Oxidative stress, Glutathione, Gender differences, Predation effects, Egg quality, Fish

Introduction

In most animals, maximum somatic and reproductive performance at a certain age will be followed by a decline with advancing age (Bowen and Atwood, 2004; Young et al., 2006; Bonduriansky et al., 2008; Nussey et al., 2008; Rodríguez-Graña et al., 2010). This progressive decline in biological functions with time is caused by accumulation of damage, and insufficient repair and homeostasis in tissues and gametes (Dröge, 2002; Hulbert et al., 2007). Life history theory that addresses questions of mortality and reproduction, predicts that modulation of extrinsic mortality will after some generations also affect intrinsic mortality, reproduction and physiological aging pattern. This theory was first touched upon in the works of Weismann (Weismann, 1889) and Medawar (Medawar, 1952) and has been verified experimentally in a number of short-lived species including fruitflies (Stearns et al., 2000; Sarup et al., 2011) and fish (Reznick et al., 1990; Reznick, 1997). As an analogous example from the wild, commercial fisheries often target larger individuals within a population, leaving smaller individuals behind to have a chance to grow to maturity and to replenish stocks. Exploited stocks of for example cod are often skewed towards smaller, faster growing individuals with earlier age at maturity (Jackson et al., 2001; Olsen et al., 2004; Olsen et al., 2005; Wright et al., 2011) and may have undergone an evolutionary change in life-history. Moreover, intense fishery has further resulted in considerably smaller densities of cod in many

areas (Cardinale and Svedäng, 2004; Wright et al., 2011) which can potentially affect male–male competition and mate choice (Nordeide and Folstad, 2000). The consequences of these population structure effects on individual fitness and population survival are not well known but under intense debate. Concerns are rising regarding potentially reduced health, fitness and lifespan of the remaining fish in targeted areas, as predicted from theory (Stearns and Koella, 1986) and field data on Pacific cod populations (Ormseth and Norcross, 2009). Following the life history arguments, it has further been suggested that especially females of long-lived polycyclic species of fish, may not age biologically over time due to their indeterminate growth and the direct relationship between female body size and egg production (Elgar, 1990; Berkeley et al., 2004). Larger female fish produce not only larger quantities of eggs, but may even produce larger eggs and offspring which correlates positively to offspring survival (Longhurst, 2002). Basic knowledge concerning aging patterns by means of physiological aging markers in eggs or somatic tissues has, however, not previously been investigated in cod, but if fisheries may shift reproduction to younger ages and smaller sizes also accelerate female aging, this could have additional and so far overlooked negative consequences for stock restoration and survival.

In this study, we have analyzed health and aging patterns in wild cod populations where extensive fishing has resulted in a reduced and age truncated population structure (Cardinale and

Svedäng, 2004). We have compared male and female cod at different ages from populations that are heavily fished (Skagerrak and Kattegat) and from cod in Öresund where trawling has been banned since 1932 (Svedäng, 2010). While no genetic difference has been detected between Kattegat and Öresund, these cod appear to have separate site preferences including spawning grounds (Svedäng, 2010). We hypothesized that the largest and oldest individuals, and especially males, would show indications of physiological aging. We also considered it possible that cod from the heavily fished populations could show a faster ageing. Additionally, the decrease in size and age at maturation in female fish in these areas could result not only in fewer eggs (as determined by body size and gonad somatic index) but also in smaller eggs of lower quality (Walsh et al., 2006). To test these scenarios and to obtain a generally better understanding of aging and health patterns in cod, we measured a number of physiological markers associated with aging and health in other species, including antioxidant enzyme activity (catalase and superoxide dismutase activities) and molecular antioxidant levels (glutathione) that can vary over short as well as longer time frames but that commonly decline with advancing age resulting in oxidative damage (Hsu et al., 2008; Carney Almroth et al., 2010; Pamplona and Costantini, 2011). We also measured accumulation of oxidative damage (protein carbonylation and lipid peroxidation), telomere length, liver somatic index and condition factor that all indicate more long term changes in health and aging. Oxidative damage usually increases with advancing age, especially in males (Hsu et al., 2008; Rodríguez-Graña et al., 2010). The relationship between oxidative stress and aging was first addressed by Harman as the free radical theory of aging (Harman, 1956), which has been under some debate in recent years (Speakman and Selman, 2011). Telomeres protect chromosomes and long telomeres are associated with good health and long life expectancy (Pauliny et al., 2006; Bize et al., 2009; Atzmon et al., 2010), but telomeres commonly shorten with age (Chang and Harley, 1995; Kim, 2007; Hsu et al., 2008). Ethoxresorufin-o-deethylase activity (EROD) was included in the study as an indicator of possible exposure to common pollutants including dioxins and PAHs (Sarkar et al., 2006). These different markers were measured in livers from adults of both sexes as well as in stage four eggs where egg size was also included as a parameter.

Materials and Methods

Fishing areas and fish sampling

Fish were captured from three different populations in the North Atlantic off the west coast of Sweden: Skagerrak (ICES subdivision 20), Kattegat (ICES subdivision 21) and Öresund (ICES subdivision 23). Fishing for the current study was done during January and February of 2009 using trawl nets in connection with the Swedish Board of Fisheries yearly population monitoring. This period of the year is the mating period (Vitale et al., 2006). Fish were weighed, sexed, measured, and age estimated by otolith analysis. Weight and length parameters were used to calculate condition factor ($CF = \text{weight (g)} \times \text{length (mm)}^{-3} \times 100$). Liver tissue and eggs samples for biochemical analyses were removed and snap frozen in liquid nitrogen where they were stored until analyses. Egg samples of stage four (Vitale et al., 2006) were also placed in formalin (app. 1 g egg in 10 ml 10% buffered formalin solution containing 4% formaldehyde) for egg size measurements by microscopy. Eggs were photographed under 10× magnification with a Leica microscope and egg size was calculated using Adobe Photoshop.

In order to investigate cod of different ages and from the different populations Skagerrak, Kattegat and Öresund, we aimed to sample at least ten sexually mature males and ten females with mature eggs (stage 4), and of three different size classes which ranged between 30 and 110 cm (or larger). We captured and analyzed a total of 73 such cod, distributed as follows: Skagerrak – 10 males and

12 females, from Kattegat – 7 males and 7 females, and from Öresund – 23 males and 15 females. Due to low fish densities in Kattegat, the target was not achieved for this population. Males caught ranged in age from 2 to 6 years and females from 2 to 8 years, and age ranges were distributed well within each site.

Oxidative damage

Protein carbonylation was measured via a reaction with 2,4-dinitrophenylhydrazine (DNPH from Sigma) followed by trichloroacetic acid (TCA from Sigma) precipitation as described by Reznick and Packer (Reznick and Packer, 1994). Samples were homogenized in 50 mM phosphate buffer, pH 7.4, containing 0.1% digitonin and a protease inhibitor cocktail (Sigma, P8340), which were added immediately prior to use. Samples were then centrifuged at 10,000 g for 20 min at 4°C. Supernatants were incubated with 10 mM DNPH in HCl and then precipitated with TCA. Pellets were washed with ethanol-ethyl acetate (1:1) to remove any free DNPH and lipid contaminants. Proteins were resolubilized in 6 M guanidine hydrochloride solution and absorbance was read at 360 nm. Total protein concentration was measured using the BCA Protein Assay Reagent kit (Pierce, USA).

Samples for measurement of lipid peroxidation were homogenized frozen in 20 mM TRIS buffer, pH 7.4, containing 5 mM BHT, 10× volume:wet weight. Homogenates were centrifuged at 3000 g for 10 minutes at 4°C. Lipid peroxidation was quantified in liver and eggs using the LPO-586 kit (Bioxytech USA). The assay was performed according to the manufacturer's instructions.

Antioxidants

Tissues were homogenized in 0.125 M phosphate buffer, pH 7.4, containing 0.1% Triton X-100 and 200 µM phenylmethylsulfonyl fluoride (PMSF from Sigma). Samples were centrifuged at 13,000 g for 15 minutes, at 4°C. Supernatants were collected for analyses. These were stored at –80°C until use. Catalase (CAT) activity was measured using H₂O₂ as a substrate according to Aebi (Aebi, 1984). Results are presented as nmol/mg protein/min.

Superoxide dismutase (SOD) activity was measured using a kit from Assay Designs, USA, according to the manufacturer's instructions. This method is based on the conversion of xanthine by xanthine oxidase which results in formation of H₂O₂ and superoxide anion, and the conversion of WST-1 to WST-1 formazan by superoxide anion. Relative SOD activity is determined from the inhibition of the rate of formation of WST-1 formazan. Results are calculated from µg protein that cause 50% inhibition, and presented as units SOD/µg protein.

Total glutathione (tGSH) and oxidized glutathione (GSSG) were measured according to Baker et al., adapted to a microplate reader by Vandeputte et al. (Baker et al., 1990; Vandeputte et al., 1994).

Telomeres

Telomere length was measured using a method based on Cawthon (Cawthon, 2002). Genomic DNA was extracted from liver tissue using DNeasy Blood and Tissue kit (Qiagen). DNA concentration was determined using a nanodrop, and 0.05 ng were used for the qPCR reaction, which was run using KAPA SYBR FAST qPCR Kit (Master Mix Bio-Rad iCycler, Techtum, USA). Primer sequences were from Farzaneh-Far et al. (Farzaneh-Far et al., 2008) as follows:

Forward: CGGTTTGGTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT

Reverse: GGCTTGCCCTTACCCTTACCCTTACCCTTACCCTTACCCTTACCCT

Concentrations used for the forward and reverse primers were 100 nM and 200 nM, respectively. Telomeres were amplified using the following protocol: 3 min at 95°C followed by 25 cycles of 15 sec at 95°C and 1 min at 56°C. Finally, 81 cycles of temperature increases from 55°C to 95°C were performed to generate a melt curve. Results are presented as relative length, showing the CT value relative to ng of DNA, as calculated from a standard curve of pooled samples diluted from 5 ng to 0.0025 ng.

Ethoxresorufin-O-deethylase (EROD) activity

Livers were homogenized in sodium phosphate buffer and S9 fractions were prepared via centrifugation at 9,000 g for 20 minutes at 4°C. EROD activity was measured according to Förlin et al. (Förlin et al., 1995).

Statistics

The putative effects of the health and aging variables were tested against the factors gender, site and age, using the multivariate statistical program PRIMER 6 together with PERMANOVA (Anderson, 2001; McArdle and Anderson, 2001; Anderson, 2005). Permutation of residuals was under a reduced model and with 999 permutations. Each data set to be tested was adjusted for missing values and transformed by Log(x+1). Euclidean distance measure was used to construct the similarity matrices. Data distribution graphs (CAP analysis) were used to visualize potential associations and proportional significance of each variable to explain the association pattern. Health and age variables analyzed were condition factor (CF), liver somatic index (LSI), liver protein carbonyls (PC L), liver total glutathione (tGSH L), liver oxidized glutathione (GSSG L), ratio GSSG L/tGSH L (% GSSG

L), liver lipid peroxidation (MDA, 4-HNE L), liver catalase (CAT L), liver superoxide dismutase (SOD L), liver relative telomere length (telomeres). Egg variables were excluded when both males and females were included, but included when only females were tested. EROD measurements on males from Kattegat versus Öresund were compared using ANOVA (Statistica).

Results

Gender differences and effects of age and site for males

Multivariate comparison (PERMANOVA) of the somatic variables revealed significant effects of age (pseudoF_{5, 43}=2.5, P=0.004) and gender (pseudoF_{1, 43}=5.6, P=0.001), but not site.

There were no significant interactions between age, gender or site. Subsequent correlation analysis (two-tailed significance of the correlation coefficient *r*) of CAP scores against variables for the first order CAP axes revealed that the difference between genders was explained by higher LSI, CF and SOD L, and lower levels of hepatic catalase and shorter telomeres in females compared to males ($r > 0.25$, $df = 61$, $P < 0.05$) (Fig. 1).

Further multivariate analyses were performed on males and females respectively. Males showed significant effects for age (pseudoF_{4, 25}=1.8, P=0.019) and site (pseudoF_{2, 25}=2.8,

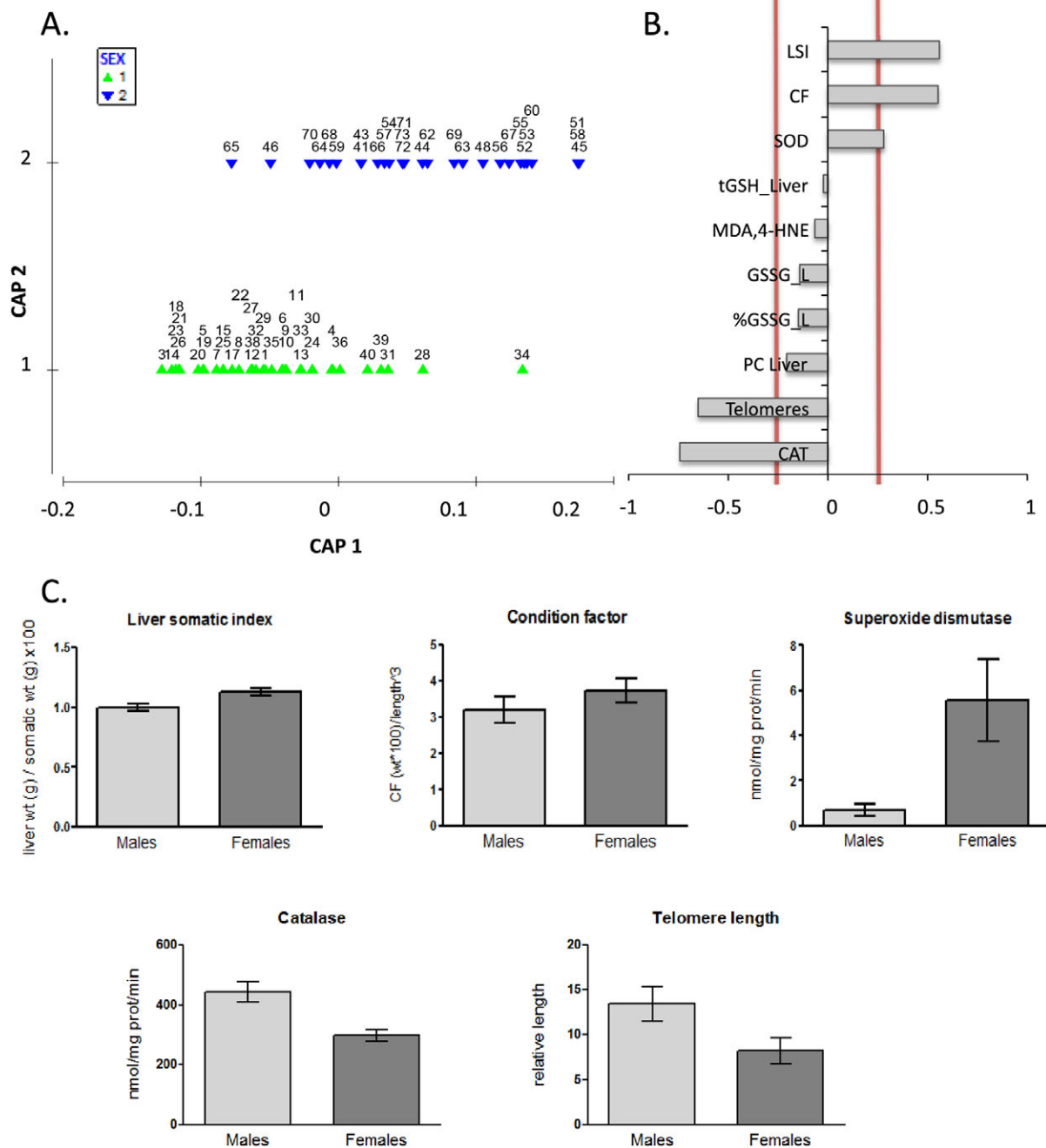


Fig. 1. (A) CAP analysis on sex (1=male, 2=female) for all cod. (B) Correlation analysis for CAP axis 1. Level of significance ($df = 61$) ± 0.25 is indicated by red line. Levels of liver somatic index (LSI), condition factor (CF) and superoxide dismutase (SOD) are higher in females, while catalase (CAT) and telomeres are lower, in comparison to males. PERMANOVA, pseudoF_{1, 43}=5.6, P=0.001. (C) Results of variables found to differ significantly, illustrating differences between the sexes.

$P=0.003$) but there was no interaction between these two factors. CAP analysis showed weak clustering, but indicated differentiation for age and site for males along the first order axis. Subsequent correlation analysis of CAP scores against variables for the first order CAP axes revealed that LSI contributed positively and telomeres, tGSH L and % GSSG L negatively to the clustering of age groups of males ($r>0.349$, $df=34$, $P<0.05$) (Fig. 2; supplementary material Table S1). CAP analysis showed clustering for site comparisons among males and indicated differentiation along the first order axis (Fig. 3A). Subsequent correlation analysis of CAP scores against variables for the first order CAP axes revealed that % GSSG L, CAT L, PC L and CF varied significantly between sites ($r>0.349$, $df=34$, $P<0.05$) (Fig. 3B). Between site comparisons show that % GSSG and CAT L were lowest in Kattegat as illustrated for four year males in Fig. 3C, and similar pattern was found also when data from all ages were included (not shown). There were, however, no differences in EROD activity between males from

Öresund and Kattegat (not shown). Protein carbonyls and condition factor were lower in both Kattegat and Öresund (Fig. 3C).

For the analyses of female data, female gonad variables were also included (supplementary material Tables S2, S3). In contrast to males, females showed no effects for either age or site (not shown).

Discussion

In this study, we investigated evolutionary life history of Atlantic cod, predicting that female cod may not age biologically, as well as that higher extrinsic mortality pressures by fisheries may have resulted in faster physiological aging. We did this by measuring somatic health and aging parameters as well as egg quality indicators in Atlantic cod from Skagerrak, Kattegat and Öresund.

In order to investigate fish of different age classes, we collected both males and females, ranging between 30 and 100 cm in length. Multivariate tests showed differences in health

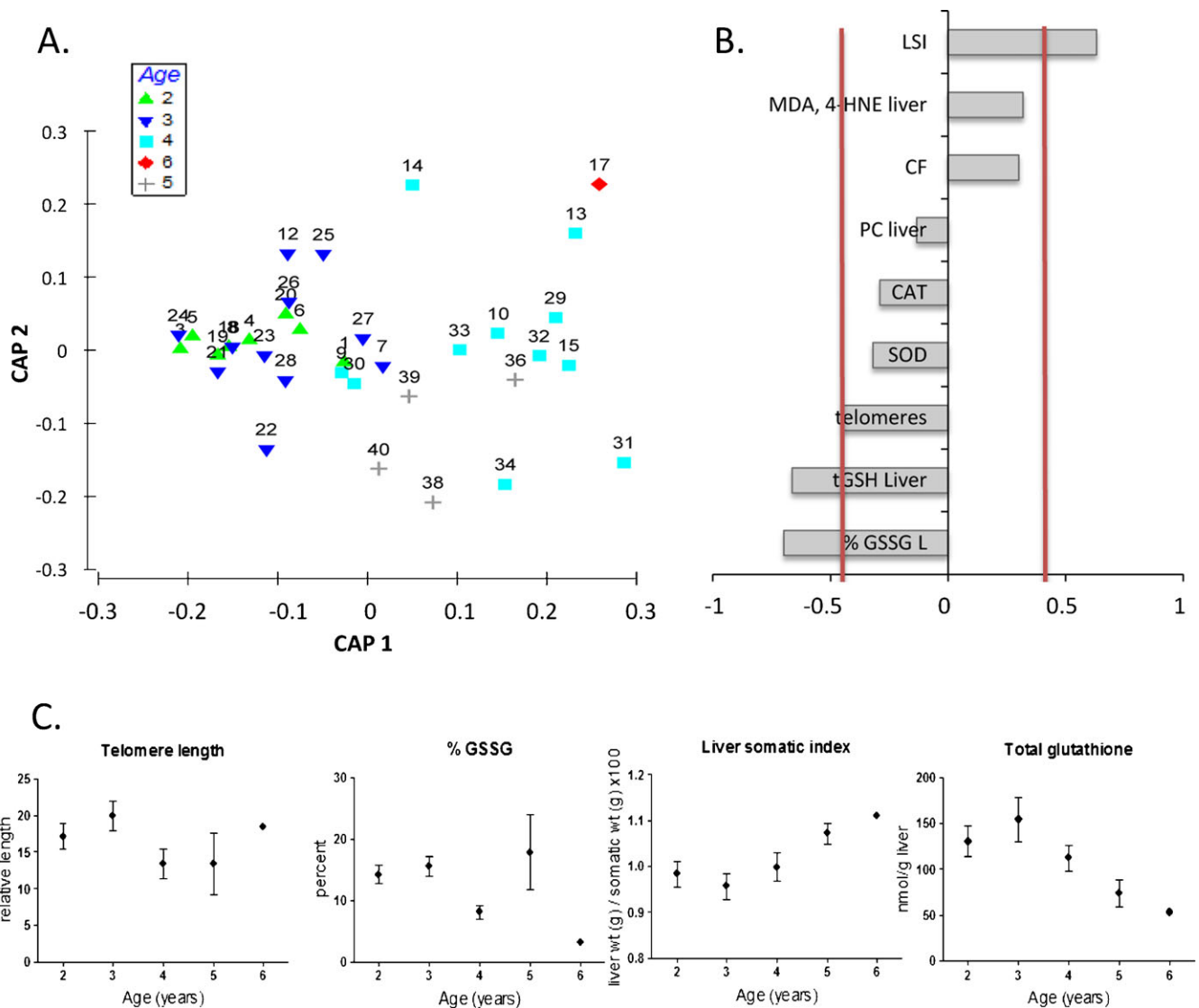


Fig. 2. (A) CAP analysis on age for male cod (years). (B) Correlation analysis for CAP axis 1. Level of significance ($df=34$) ± 0.349 is indicated by red line. Telomeres, total glutathione (tGSH) and percent oxidized glutathione (%GSSG) decrease with age while liver somatic index (LSI) increases significantly. PERMANOVA pseudo $F_{4, 25} = 1.8$, $P=0.019$. (C) Results of variables found to differ significantly, illustrating differences with age in male cod.

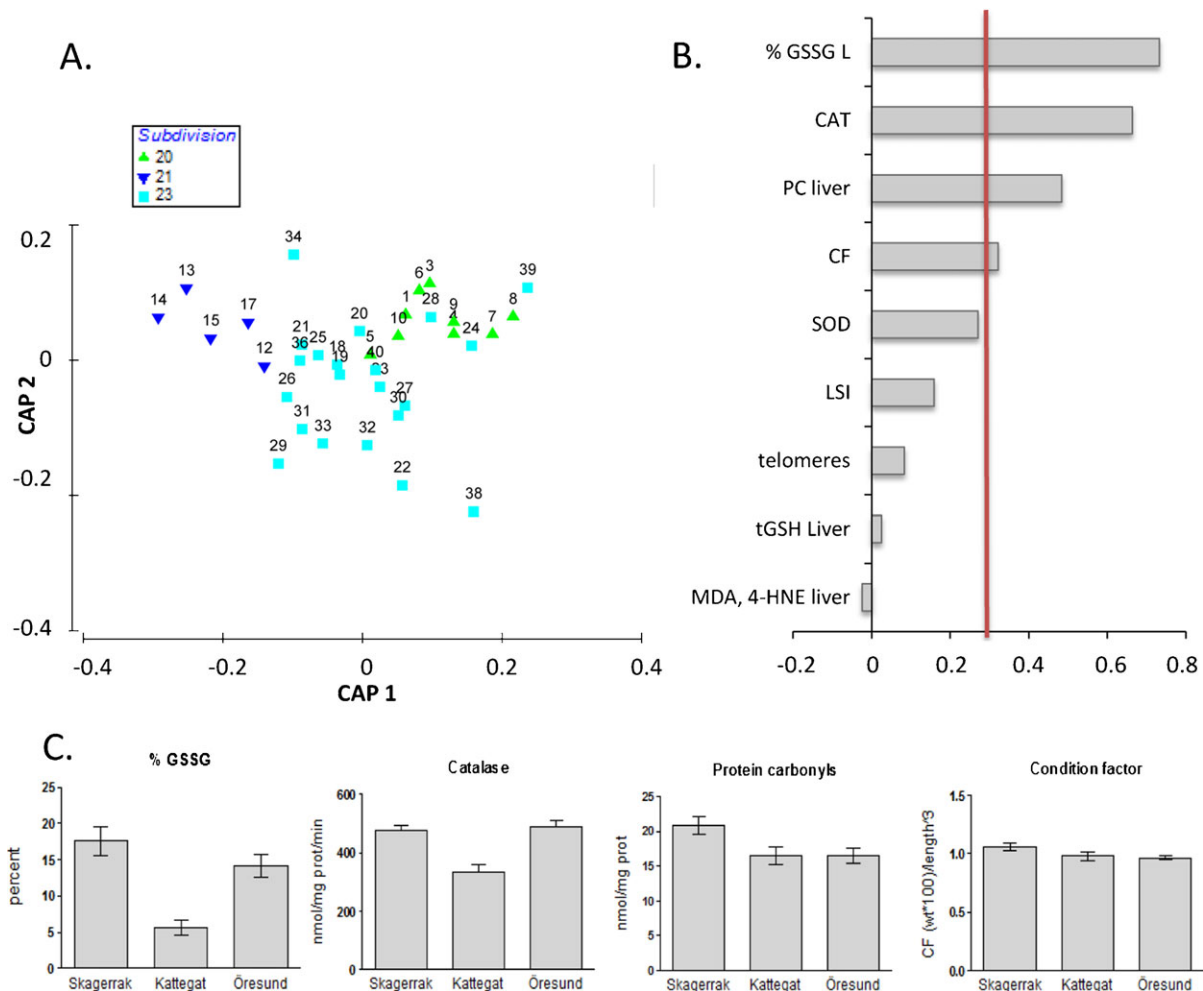


Fig. 3. (A) CAP analysis on sites (ICES subdivision 20 Skagerrak, 21 Kattegat and 23 Öresund) for male cod. (B) Correlation analysis for CAP axis 1. Level of significance ($df=34$) 0.349 is indicated by red line. Levels of percent oxidized glutathione (%GSSG), catalase (CAT) and protein carbonyls (PC) are lower in the Kattegat compared to the other areas. PERMANOVA pseudo $F_{2, 25}=2.8$, $P=0.003$. (C) Results of variables found to differ significantly, illustrating differences between sites using data from four year old males.

and aging parameters between males and females, and further, differences in male fish between ages and between sites. In the females, where we also investigated variables of egg quality, there were no significant effects of age or site. In general terms, this means that cod females within the range of 2–8 years appear not to age in terms of somatic or gonadal decline. It also means that cod females from Skagerrak and Kattegat are not significantly different in health and aging pattern than those from Öresund. Our results confirm the importance of older/larger fish since females indicated no increase in damaged molecules (proteins and lipids) in eggs as they age chronologically. The fact that we do not find this in Atlantic cod is consistent with previous results indicating that long-lived, slow growing fish including cod show no decline, and even increases, in reproductive success (Berkeley et al., 2004), which is in contrast to so many other species (Bonduriansky et al., 2008). Our results thus provide further arguments for protection of larger and older female cod in order to restore exploited populations.

We had predicted that commercial fisheries would have effects on physiological aging and reproduction in females, resulting from fisheries-induced changes in population structure and

possible also life history. However, we did not find any differences between females from different sites. In males, no overall shift in aging pattern was found in Skagerrak and Kattegat in relation to Öresund either. We consider it possible that the cod in Kattegat and Skagerrak may not have undergone a significant change in health and aging pattern in relation to Öresund despite differences in population structures between these sites. This conclusion is supported by previously reported lack of differences in growth rate in cod from Öresund and Kattegat (Svedäng, 2010). While genetic differences among Skagerrak populations have been found (Knutsen et al., 2003), Kattegat and Öresund populations are genetically similar to each other but separated geographically (Svedäng, 2010). In our study, we observed higher catalase and % GSSG levels in Öresund males compared to Kattegat, suggesting that Öresund male cod may be more stressed at a short term basis. This potential stress does not seem to be related to exposure to organic pollutants that may arrive from the coast, as indicated by EROD measurements, but could instead be related to density-related stress and/or intraspecific male–male competition for females during the mating period (Schreck, 2010; and references therein). Since

differences between sites were found for males but not females, there may be gender differences in mating and or migration behaviors as well as physiology influencing this pattern.

Although female cod did not show signs of decline with age, we identified age related changes in some parameters starting approximately from year three in males. Telomere length and total glutathione decreased, which is indicative of molecular onset of aging. A simultaneous decline in % oxidized glutathione (GSSG) and elevation of LSI imply that the older males may still be in relatively good health in comparisons to their younger rivals. In surveys aiming to investigate potential life-history changes in future times or in other populations, it may thus be relevant to include analysis of telomere length, glutathione levels and % GSSG in livers of males. Differences in aging between males and females were not surprising, since males of most species have shorter life spans than females (Bowen and Atwood, 2004; Austad, 2006; Bonduriansky et al., 2008; Rodríguez-Graña et al., 2010). Most physiological explanations for this difference are associated with differences in costs related to reproductive behavior as well as less estrogen in males (Bowen and Atwood, 2004). Estrogen reduces ROS production, and increase levels of antioxidant enzymes in female rats compared to males (Viña et al., 2003). Vitellogenin, a yolk protein precursor which is also induced by estrogen, functions as an antioxidant in a number of species including fish (Goto et al., 1999). Estrogen has also been shown to induce telomerase (Kyo et al., 1999) which suppresses telomere shortening. In line with this, telomere length and glutathione declined with age in cod males but not in females. On average, however, our results indicate that male cod have higher catalase activities and longer telomeres than females, which was surprising since females of different species often have longer telomeres than males. High levels of catalase have been associated with increased longevity (Larsen, 1993), and telomere length is also correlated with longevity in a number of species (Hausmann and Mauck, 2008; Atzmon et al., 2010).

In summary, we have found gender differences in ageing patterns where males but not females up to eight years show signs of physiological ageing. Of special interest was the reduction in glutathione levels and telomere length with age in males, suggesting these parameters as potential ageing markers for future investigations. By comparing the extensively fished sites of Kattegat and Skagerrak with the Öresund that has been protected from trawling since 1932, we found site differences among males that may relate to density-dependent behavioral effects but we did not find signs of an evolutionary shift towards earlier onset of ageing in the extensively fished populations. We cannot exclude that a survey based on a larger sample size that takes into consideration the variation we have here observed, may show that an evolutionary shift has occurred, however. Physiological ageing patterns have previously not been investigated in cod, but are valuable for better knowledge on life history processes in this species. We encourage further research on this matter, including plasticity of ageing at the individual level, using both controlled laboratory or mesocosm experiments and field observations.

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Competing Interests

The authors have no competing interests to declare.

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