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Precision and accuracy of stable isotope signals in otoliths of pen-reared cod (*Gadus morhua*) when sampled with a high-resolution micromill

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Abstract A high-resolution sampling technique for stable-isotope measurements in otoliths is now available, but little is known about the precision and accuracy of the results when using this technique. Otolith samples of pen-reared cod (*Gadus morhua* L.) with well-defined temperature history were obtained using this high-resolution sampling technique. Samples from different-aged cod otoliths were collected in two yearly increments corresponding to the calendar years 1994 and 1995, and the stable-oxygen ($\delta^{18}\text{O}$) and -carbon ($\delta^{13}\text{C}$) isotope compositions of the samples were measured. Otolith $\delta^{18}\text{O}$ values had a clear seasonal pattern in response to the seasonal water temperature. Otolith material deposited in the same calendar years of different-aged fish showed similar $\delta^{18}\text{O}$ values. High precision of the temperature estimate using otolith $\delta^{18}\text{O}$ values was obtained when comparing results from different otoliths. The accuracy of the temperature estimates, relative to measured mean monthly water temperature, was also high, but reduced sampling resolution in the otoliths significantly reduced the accuracy due to attenuation of the $\delta^{18}\text{O}$ signals. We found that the otolith $\delta^{13}\text{C}$ values had a cyclic pattern, roughly in phase with the $\delta^{18}\text{O}$ values. This was most likely caused by temperature-

increased metabolism. Indications of age-specific otolith $\delta^{13}\text{C}$ values are also presented.

Introduction

Fish otoliths are composed of CaCO_3 , with the inclusion of proteins, and formation of daily (Pannella 1971) and annual (Reibish 1899) growth zones has been used to age fish for more than a century. Analysis of the chemical composition of fish otoliths has also proven to be a valuable tool for fishery ecologists (Campana 1999). Since otolith material is not reabsorbed after deposition (Campana and Neilson 1985), the otoliths act as metabolically inert timekeepers and environmental recorders that make it possible to relate the chemical composition of the otoliths to the age of fish and the time of otolith precipitation (Campana 1999). Previous studies have shown that the stable oxygen isotope composition ($\delta^{18}\text{O}$) in otoliths can be used as a proxy of the ambient water temperature (Patterson et al. 1993; Thorrold et al. 1997; Radtke et al. 1996, 1998). Factors controlling the stable carbon isotope composition ($\delta^{13}\text{C}$) in otoliths are more complex than those controlling oxygen isotopes, since $\delta^{13}\text{C}$ is also influenced by fish metabolism and feeding pattern (Thorrold et al. 1997; Schwarcz et al. 1998; Høie et al. 2003).

Advances in microscale sampling techniques in otoliths have made it possible to extract environmental information, in high temporal resolution, concerning the life-history of fish (Wurster et al. 1999). Detailed measurements of otolith $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ composition are now possible, and are only limited by the constraints of the mass spectrometer. However, few studies exist that have critically examined the precision and accuracy of temperature estimates based on high-resolution micromill sampling techniques. Leder et al. (1996) showed that reduced sampling in corals led to underestimation of the true temperature range due to attenuation of the $\delta^{18}\text{O}$ signal, and modelling showed that an ontogenetic decrease in growth rate of biogenic carbonates causes the

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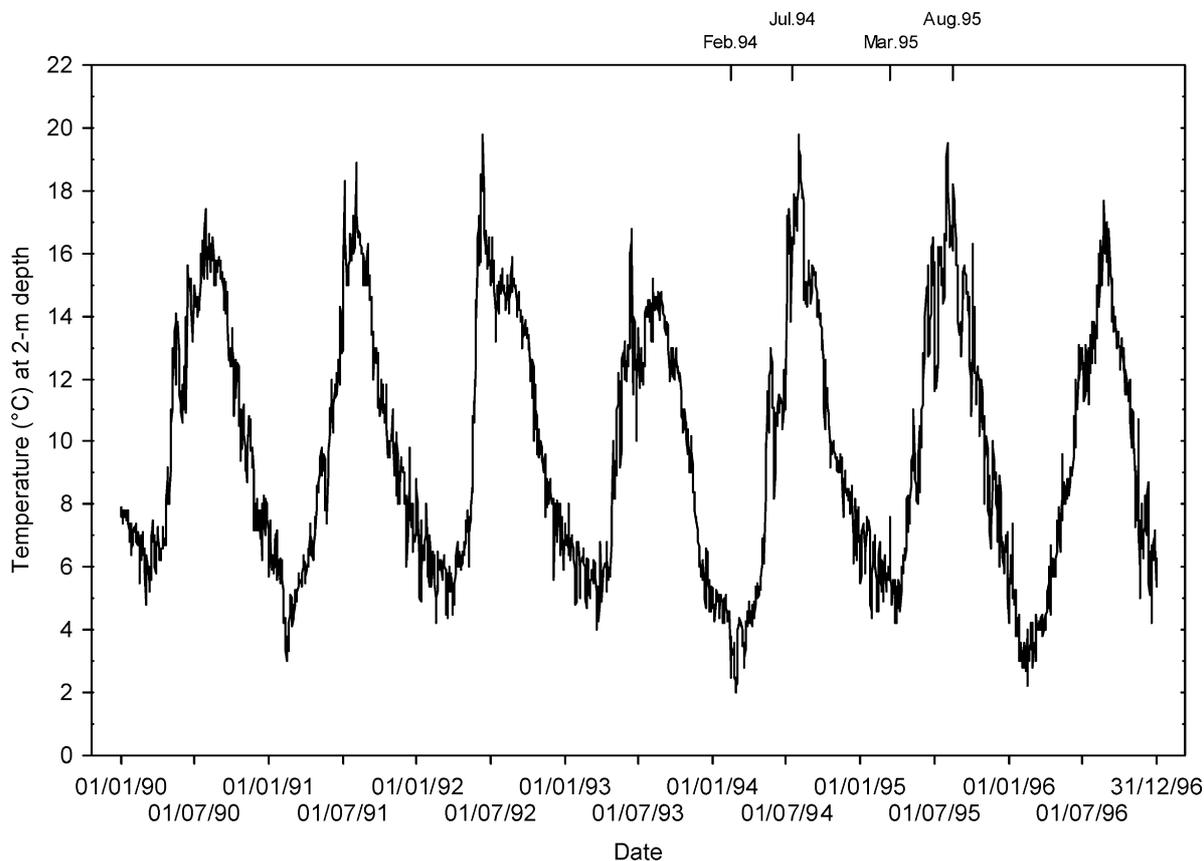
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annual isotope amplitudes to become progressively smaller when the annual temperature is held constant (Goodwin et al. 2003). To our knowledge, only one study has examined how accurate and precise temperature estimates based on otolith $\delta^{18}\text{O}$ are compared to measured temperatures (Gao et al. 2001). Gao and colleagues found that the isotope-based temperature estimates were generally in agreement with the measured summer temperatures, but overestimated the winter temperatures; low sampling resolution (two samples per otolith yearly increment) may have influenced the results.

In the present study we utilised a high-resolution micromill sampling technique and analysed otoliths from cod (*Gadus morhua*) that had been reared in pens in the sea for 4 and 6 years, while the water temperature was recorded on a daily basis. The objective of the study was to examine the variation in stable-oxygen and -carbon isotopes in otoliths between individual fish, and to study the precision and accuracy of water temperature estimates from otolith $\delta^{18}\text{O}$ values using micro-scale sampling techniques. We also aimed to describe the seasonal $\delta^{13}\text{C}$ signal in the cod otoliths and its relation to fish age.

Fig. 1 *Gadus morhua*. Recorded daily temperature at 2 m depth in the pen. The months of seasonal minimum and maximum temperatures, corresponding to the times the otolith material sampled was deposited, are provided on the upper frame of the graph



Materials and methods

The cod (*Gadus morhua* L.) in this experiment were reared in pens at Austevoll research station, Norway. One group was released in the pen in autumn 1990 and the other group was released in autumn 1992. Fish in both groups were transferred from a production pond when the fish were 6 months old, and were held in the pens until autumn 1996, when they were sampled at the ages of 6 and 4 years. All fish were fed in excess with commercial dry pellets, and all fish became sexually mature at age 2 years. Daily water temperature measured at 2 m depth from summer 1990 to autumn 1996 had a clear seasonal pattern, with minimum and maximum temperatures during spring and autumn, respectively (Fig. 1).

At the termination of the experiment, left and right sagittae were removed, carefully washed with fresh water, and stored dry in small envelopes until analyses in 2002. Weight and length of the fish were also measured. The stable-oxygen and -carbon isotope compositions of left sagittae from 12 fish were measured, 4 from the 1992 group and 8 from the 1990 group. From the 1990 group we chose the otoliths of 4 relatively small fish (6S group) and of 4 fish relatively large fish (6L group), in order to guarantee a large size span of fish (and of otolith yearly increments). Fish from the 1992 group are referred to as the 4L group, since we used otoliths from relatively large fish (Table 1).

Otolith preparation

Otoliths were mounted in polyester, and a 500 μm section in the transverse plane through the core was cut out using a slow-speed saw with a double-diamond blade. The otolith sections were ground and polished with P600–P4000 grit paper (FEPA standard) followed by 1- μm diamond suspension on one side; the thin sections were subsequently glued to a glass slide with the polished side

Table 1 *Gadus morhua*. Weight and length of fish in the three groups, and information on otolith weight and micron-sampling in the otoliths

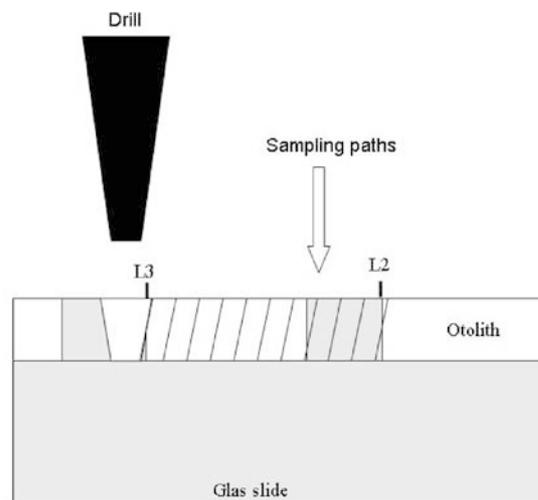
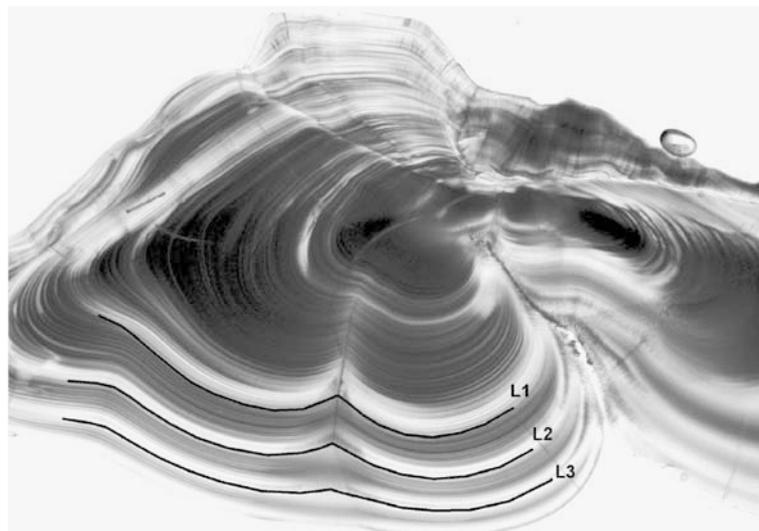
Group	Fish			Otolith						
	No.	Weight (g)	Length (mm)	Weight (mg)	Yearly increment sampled	Year deposited	No. of samples	Distance between scans (μm)	Sample volume, ($\text{mm}^3, \times 10^{-3}$)	
4L	1	3792	730	591.7	3	1994	10	50	13.5	
					4	1995	8	40	14.0	
	2	3054	630	556.1	3	1994	10	47	13.3	
					4	1995	8	42	14.7	
					3	1994	10	34	11.0	
					4	1995	8	34	12.2	
	4	6715	820	555.1	3	1994	10	45	12.5	
					4	1995	8	34	11.1	
6L	1	5738	845	790.8	5	1994	10	23	8.05	
					6	1995	8	25	9.18	
	2	5006	800	697.8	5	1994	10	22	8.44	
					6	1995	8	23	9.47	
					3	1994	10	21	8.11	
					6	1995	8	29	8.17	
	4	6155	815	665.0	5	1994	10	19	8.14	
					6	1995	8	28	8.54	
	6S	1	3050	640	438.3	5	1994	8	22	8.33
						6	1995	5	23	8.79
		2	2539	635	453.3	5	1994	8	25	8.06
						6	1995	5	33	9.39
3						1994	8	20	8.21	
6						1995	5	22	9.32	
4		2426	615	452.9	5	1994	8	22	8.12	
					6	1995	5	23	8.83	

facing down. The glass slides were then attached to a thin-section holder, and the other sides were ground and polished in the same way to a final thickness of the otolith sections of 140–160 μm .

Each otolith section was photographed with an Olympus C3040 camera at $\times 2.5$ magnification in a stereo microscope using transmitted light. The photograph was imported to an image analysing program, ImageJ (Rasband WS, ImageJ, National Institutes of

Health, Bethesda, Maryland, USA, 1997–2003), and the sampling paths were determined. We sampled yearly increments 5 and 6 and yearly increments 3 and 4 in otoliths from 6- and 4-year-old cod, respectively. Fish of the 4L group were then 2 and 3 years old, and fish of the 6L and 6S groups were then 4 and 5 years old when the otolith material we sampled was deposited (Table 1). The otolith materials in these yearly increments were deposited in 1994 and 1995. A yearly increment was defined as an opaque plus a translucent zone, when viewed with transmitted light. The border between two yearly increments was defined as the border between the translucent zone and the following opaque zone (Fig. 2). The carbonate was sampled on the distal side at the ventral margin of the otolith, where the yearly increments were largest, starting at the distal side and moving towards the nucleus (glossary used for describing position on otoliths is given in Panfili et al. 2002). Typically, from 3- to 4-mm-long sampling transects following the border between different yearly increments were selected. We

Fig. 2 *Gadus morhua*. *Left panel*: a transverse section (150 μm) of otolith of fish number 1 in the 4L group, viewed in transmitted light. Samples in yearly increment 3 were collected in the area between lines 1 and 2, and samples in yearly increment 4 were collected in the area between lines 2 and 3. *Right panel*: a schematic view of the sampling in yearly increment 4. A trench was first milled adjacent to line 3, and the ten samples were thereafter milled in the sampling paths interpolated between lines 3 and 2



marked three segmented lines that followed the growth zones in the otolith in the area between the translucent and opaque zones. Lines 1 and 2 defined the borders for otolith material deposited in 1995 (yearly increment 5 or 3), and lines 2 and 3 defined the borders of otolith material deposited in 1994 (yearly increment 6 or 4). The x and y co-ordinates of lines 1–3 were then imported to a Merchantek EO Micromill System and re-co-ordinated. The micromill software interpolated new lines between line 1 and line 2 and between line 2 and line 3, according to the number of samples we wanted to obtain. Samples were then collected by milling sequentially between each of the lines (Fig. 2). Milling depth was set to 125 μm . Ten and eight samples per yearly increment were sampled in yearly increments 3 and 4 and yearly increments 5 and 6 for fish in 6L and 4L groups, respectively (Table 1). For fish in the 6S group eight and five samples were collected in yearly increments 5 and 6, respectively.

The seasonal maximum and minimum otolith $\delta^{18}\text{O}$ values in the two yearly increments were related to time (month) by setting the seasonal otolith $\delta^{18}\text{O}$ sample with the lowest value to the month of highest temperature. The otolith sample with the seasonal maximum $\delta^{18}\text{O}$ was set to be deposited in the month of lowest mean temperature. Linear interpolation was used when distributing the estimated temperatures between the seasonal extremes. A similar procedure was used when distributing the estimated temperatures over the time series of measured temperatures for a single otolith sampled in its entirety (i.e. the otolith from fish number 2 in the 6L group); clear temperature peaks between the seasonal maxima and minima that could be estimated based on otolith $\delta^{18}\text{O}$ signals were also used for distribution to the measured temperatures for that otolith.

All isotope values are reported to standards by the International Atomic Energy Agency, Vienna. $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values of the otoliths are reported in standard δ notation relative to the Vienna Pee Dee belemnite (VPDB) reference standard:

$$\delta = \left(\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right) \times 1000 \text{ (‰)} \quad (1)$$

where R is the ratio $^{18}\text{O}:^{16}\text{O}$ or $^{13}\text{C}:^{12}\text{C}$ in the sample or standard. Results are reported with respect to the VPDB standard through calibration against NBS19 standards. The stable-isotope composition was analysed using a Finnigan MAT 252 mass spectrometer. Gas for isotope measurements was produced by reaction with orthophosphoric acid at 70°C in an automated on-line system with acid added to the sample in individual reaction chambers. Precision of the measurements was 0.07‰ for $\delta^{18}\text{O}$ and 0.06‰ for $\delta^{13}\text{C}$ (standard deviation of repeated measurements of the standard). The $\delta^{18}\text{O}$ precision corresponds to 0.3°C when estimating temperature using the relationship for inorganic calcite (Kim and O'Neil 1997). The mass spectrometer was set to handle small samples, and, based on pilot studies, we set the minimum sample size to $8 \times 10^{-3} \text{ mm}^3$ otolith materials, which corresponds to a sample weight of about 20 μg assuming the density of the otolith is 2.6 g cm^{-3} . The sample volume varied between 8.05×10^{-3} and $9.47 \times 10^{-3} \text{ mm}^3$ for otoliths of fish in the 6L and 6S groups. Since the widths of yearly increments 3 and 4 are larger than the widths of yearly increments 5 and 6, the sample volume varied between 11.0×10^{-3} and $14.7 \times 10^{-3} \text{ mm}^3$ for otoliths of fish in the 4L group (Table 1).

The isotope-based temperatures (T , °C) were estimated by using the equation for otolith aragonite of $\delta^{18}\text{O}_{\text{C}} - \delta^{18}\text{O}_{\text{w}} = 3.90 - 0.20T$ (Hoie et al., submitted). In order to relate the otolith $\delta^{18}\text{O}$ values to temperature, the isotope composition of the seawater ($\delta^{18}\text{O}_{\text{w}}$) must be known or estimated. We did not measure the $\delta^{18}\text{O}_{\text{w}}$ value in the experimental period, but Gao et al. (2001) measured 16 water samples in the same area in November 1996. We used their $\delta^{18}\text{O}_{\text{w}}$ value of -1.06‰ (corrected for VPDB-scale by -0.26‰) in our calculations.

Statistics

Repeated measurements analysis of variance (ANOVA) was used to test for group differences in estimated seasonal minimum and maximum temperatures, and to test for group differences in sea-

sonal minimum and maximum $\delta^{13}\text{C}$ values. This test uses multivariate criteria to simultaneously test the statistical significance of the two or more repeated measures that it contrasts. ANOVA was used to test for differences in isotope composition between groups and individual otoliths, and analysis of covariance (ANCOVA) was used to test for $\delta^{18}\text{O}$ -specific differences in $\delta^{13}\text{C}$ values of otoliths from different groups. Since the isotope values of different samples within otoliths could be dependent on each other, the degrees of freedom in the ANCOVA were reduced to the number of otoliths examined. Newman-Keuls post hoc comparisons were used to detect the significance of each group or individual fish if the ANOVAs indicated significantly differences between groups or individual fish otoliths. A significance level of $\alpha = 0.05$ was used in all tests.

Precision is defined as the closeness of repeated measurements of the same quantity to each other, while accuracy is defined as the closeness of a measured or computed value to its true value. Precision in this study refers to the closeness between temperature estimates based on otolith $\delta^{18}\text{O}$ values in the same months between individual fish and groups of fish. Also, the overall precision of one temperature estimate (95% confidence interval) based on seasonal minimum and maximum temperatures was estimated by computing the mean square of error of a one-way ANOVA, where the estimated temperature was the dependent variable and month was the categorical variable (fixed effect). Accuracy of the temperature estimates in this study refers to the closeness between the estimated temperature based on otolith $\delta^{18}\text{O}$ values and the measured temperature values.

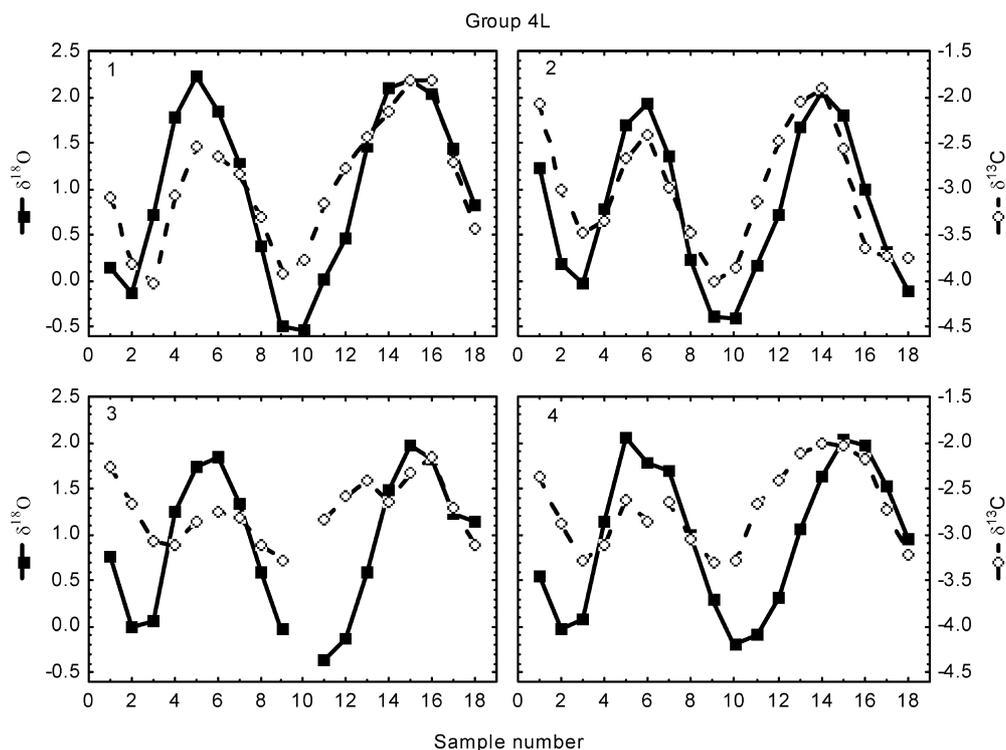
Results

Overall isotope composition of the otoliths

There were no significant differences in the overall mean $\delta^{18}\text{O}$ values of otoliths within and between each of the three groups (ANOVA, $F_{1,236} = 0.051$, $P > 0.05$). The time-span, in which the otolith samples could be related to time by relating to seasonal minimum and maximum otolith $\delta^{18}\text{O}$ values (February 1994–August 1995), had a mean recorded water temperature of 9.7°C. The mean estimated temperature in the same time span by using otolith $\delta^{18}\text{O}$ values, in which no samples were missing and the time span was covered by the otolith samples (five otoliths), was 9.7°C (range 9.5–10.1°C).

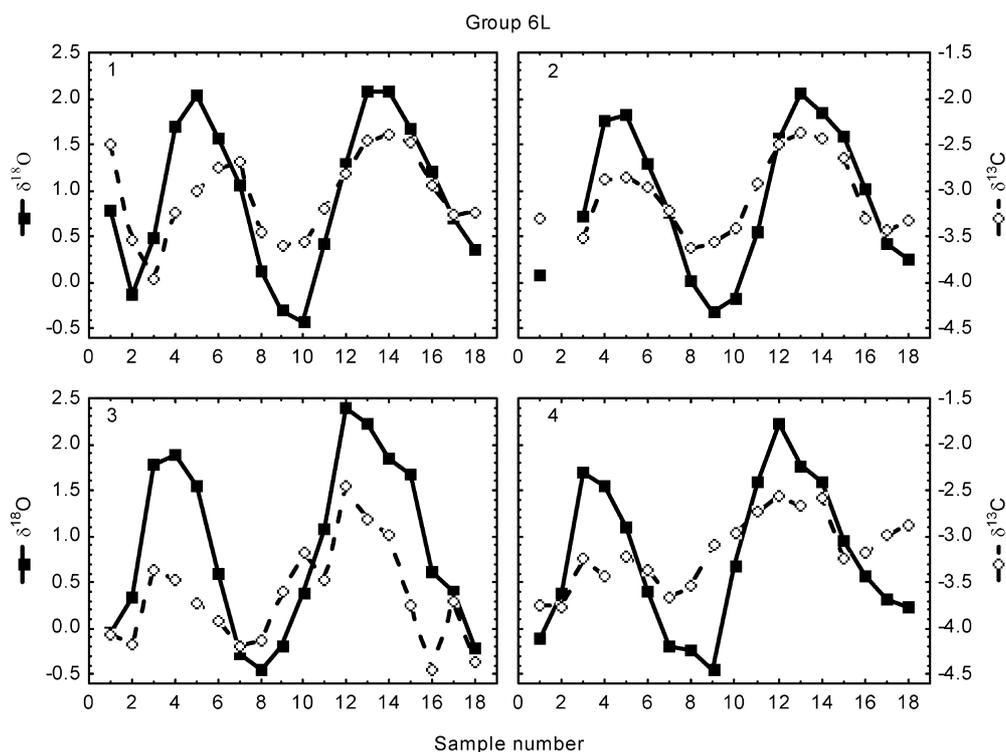
Both the otolith $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values showed seasonal cycles (Figs. 3, 4, 5). The $\delta^{18}\text{O}$ values in the 6L and 4L groups showed a seasonal range of about 2.5‰, while the $\delta^{13}\text{C}$ values showed a range of about 1.0–2.0‰. The exact times of the seasonal minimum and maximum otolith $\delta^{13}\text{C}$ values could not be established, since they were not directly linked to the seasonal otolith $\delta^{18}\text{O}$ minima and maxima. There was an overall significant difference between the 4L and 6L groups for the seasonal minimum (in 1994) and maximum (in 1994 and 1995) otolith $\delta^{13}\text{C}$ values, with the highest values at the seasonal maxima in 1994 and 1995 for fish in the 4L group (repeated measurements ANOVA, $F_{1,10} = 8.08$, $P < 0.05$). The $\delta^{18}\text{O}$ -specific increase in $\delta^{13}\text{C}$ was similar between groups when all measurements within the groups were pooled (homogeneity of slopes, $F_{2,6} = 1.59$, $P = 0.28$). Although there was a high degree of overlap between the three groups (Fig. 6), we found differences in $\delta^{13}\text{C}$ values at similar $\delta^{18}\text{O}$ values between the three

Fig. 3 *Gadus morhua*. Oxygen (filled squares) and carbon (open circles) isotope composition of otoliths of 4-year-old cod with large otoliths (group 4L). The otolith material was sampled in yearly increments 3 and 4, which correspond to years 1994 and 1995. Increasing sample numbers correspond to decreasing fish age when the otolith material was deposited. One otolith sample was lost for fish number 3



groups. Fish in the 6S groups had 0.55‰ and 0.19‰ higher otolith $\delta^{13}\text{C}$ values than fish in the 6L and 4L groups, respectively (ANCOVA, $F_{2,8} = 8.99$, $P < 0.05$). Significant, positive correlations between otolith $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values were found for all fish with r^2 values ranging from 0.52 to 0.87, except for fish 3 in the 4L group which had a non-significant r^2 value of 0.32.

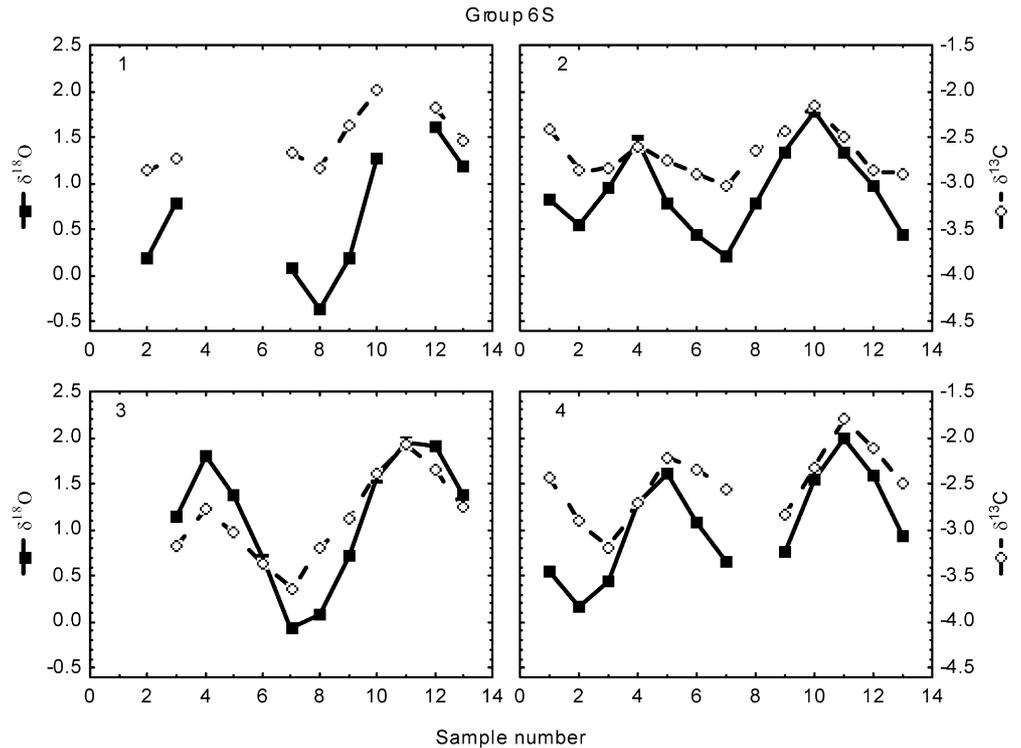
Fig. 4 *Gadus morhua*. Oxygen (filled squares) and carbon (open circles) isotope composition of otoliths of 6-year-old cod with large otoliths (group 6L). The otolith material was sampled in yearly increments 5 and 6, which correspond to years 1994 and 1995. Increasing sample numbers correspond to decreasing fish age when the otolith material was deposited. One otolith sample was lost for fish number 2



Precision of the temperature estimates

The precision of estimated seasonal maximum and minimum temperatures within the three groups varied, but no consistent trend was evident (Table 2). Highest precision within the 6L group was found at the maximum temperature in 1994, with a temperature range of

Fig. 5 *Gadus morhua*. Oxygen (filled squares) and carbon (open circles) isotope composition of otoliths of 6-year-old cod with small otoliths (group 6S). The otolith material was sampled in yearly increments 5 and 6, which correspond to years 1994 and 1995. Increasing sample numbers correspond to decreasing fish age when the otolith material was deposited. Five, two and one otolith samples were lost for fish numbers 1, 3 and 4, respectively



0.7°C, while a temperature range of <1°C was found both at the seasonal minimum in 1994 and the maximum in 1995 within the 4L group. Lowest precision, when using otoliths of fish from the 4L group, was found at seasonal maximum and minimum temperatures in 1994 and 1995, respectively, where the predicted temperature range was 1.6–1.9°C. The overall precision of one temperature estimate was estimated by calculating the mean

square error of a one-way ANOVA, where month was set as the fixed effect. The precision estimate when all 12 otoliths were included was ±1.1°C. When excluding the otoliths from the 6S group, the precision was ±0.6°C.

We found no differences in the estimated temperatures of the seasonal minima and maxima (February 1994, July 1994 and March 1995) between the 4L and 6L groups (repeated measurements ANOVA, $F_{1,10} = 1.41$,

Fig. 6 *Gadus morhua*. Relation between otolith $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ for the three groups (6S, 4L, 6L)

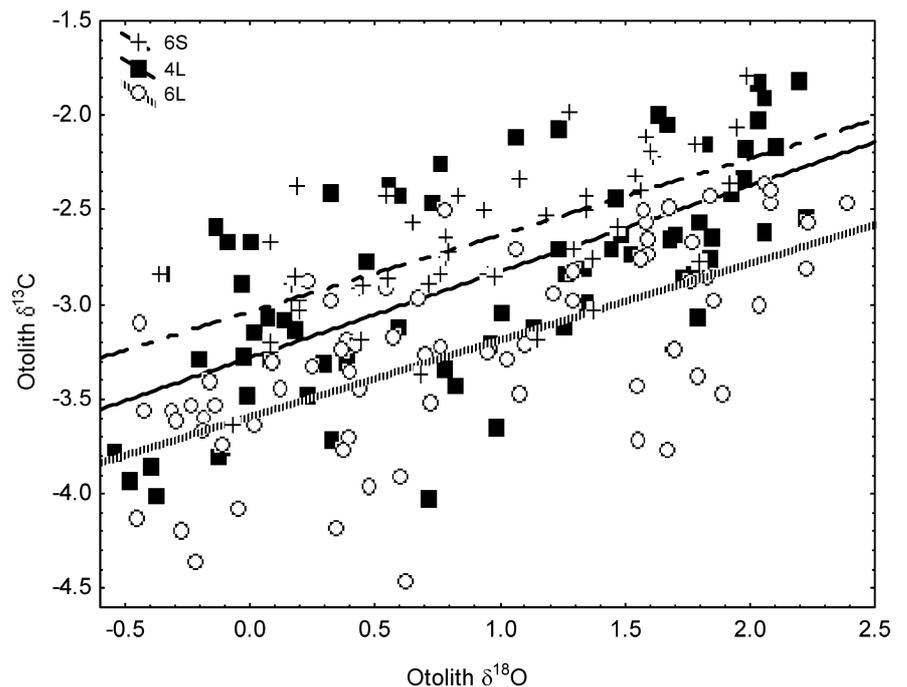


Table 2 *Gadus morhua*. Recorded temperatures and estimated temperatures using otolith $\delta^{18}\text{O}$ values of fish from the three groups

Group	Time	Recorded temperature ($^{\circ}\text{C}$)		Estimated temperature ($^{\circ}\text{C}$)	
		Mean	Range	Mean	Range
4L	Feb 1994	3.7	2.0–5.1	3.9	3.3–4.3
	Jul 1994	16.4	12.0–18.8	16.1	15.3–16.9
	Mar 1995	5.4	4.2–7.6	4.1	3.1–5.0
	Aug 1995	16.7	13.9–19.5	14.4	14.2–14.9
6L	Feb 1994	3.7	2.0–5.1	3.3	2.3–3.9
	Jul 1994	16.4	12.0–18.8	16.3	15.8–16.5
	Mar 1995	5.4	4.2–7.6	4.9	4.0–5.7
	Aug 1995 ^a	16.7	13.9–19.5	14.9	
6S	Feb 1994	3.7	2.0–5.1	4.7	4.3–5.3
	Jul 1994	16.4	12.0–18.8	13.6	13.2–14.5
	Mar 1995	5.4	4.2–7.6	6.1	5.2–6.9
	Aug 1995	16.7	13.9–19.5	12.4	11.5–13.4

^aOnly one measurement

$P=0.29$). Multivariate testing of the statistical significance of the four repeated measures showed no interaction effect between different months and groups ($P=0.28$). A comparison with the 6S group was not possible due to missing data.

Accuracy of the temperature estimates

Accuracy of the estimated temperatures at seasonal maxima and minima was calculated as the difference between the mean monthly measured temperatures and the estimated temperatures from otolith $\delta^{18}\text{O}$ values. For the 4L and 6L groups, 68% of the estimated

temperatures were within $\pm 1^{\circ}\text{C}$ of the mean monthly measured temperatures, and the mean values of estimated temperatures within each group were within $\pm 0.5^{\circ}\text{C}$ in two and three out of four occasions (Table 2). Lowest accuracy was found for otoliths of fish in the 6S group. The temperature was underestimated at both seasonal maxima by $> 2.5^{\circ}\text{C}$ for all otoliths except one, and the mean estimated temperature was underestimated by 2.8°C and 4.3°C (Table 2). The seasonal minima were also overestimated in both 1994 and 1995.

For fish 2 in the 6L group, otolith material was collected from the whole otolith. The $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values showed a seasonal pattern over the entire life span of the fish (Fig. 7). Estimated temperatures for all samples from seasonal minima in 1991–1995 were calculated (Fig. 8). The estimated temperatures were similar to the mean monthly temperatures in the second half of the fish's life, but both seasonal minima and maxima were shifted towards lower temperatures during the first half of the fish's life. However, the temperature range based on isotope values agreed well with the mean monthly measured temperatures. Furthermore, good correspondence was found between the highest estimated seasonal temperatures and the observed mean monthly temperatures. The ranking of the estimated temperatures at the seasonal maxima was the same as the ranking based on mean monthly temperatures, except for August 1991, which was 0.05°C lower than August 1995. The lowest estimated temperatures were also observed during the 2 years with the lowest average monthly temperatures. Also, note the relatively high $\delta^{13}\text{C}$ values at a distance of 3100–3400 μm from the nucleus, which were evidently higher than the other seasonal minima. This area in the

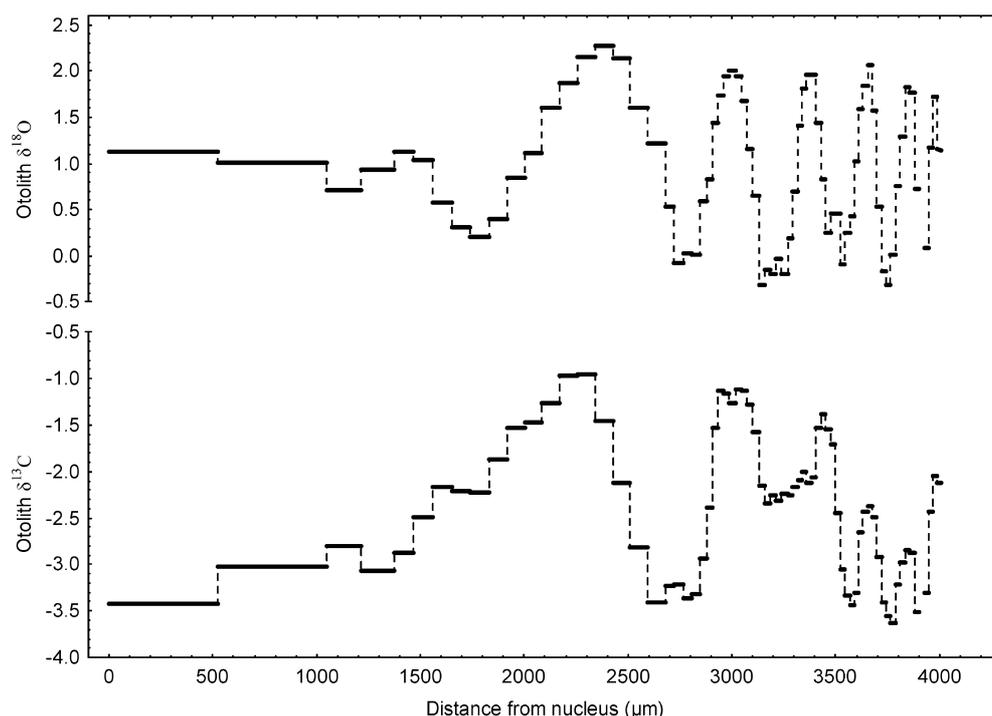
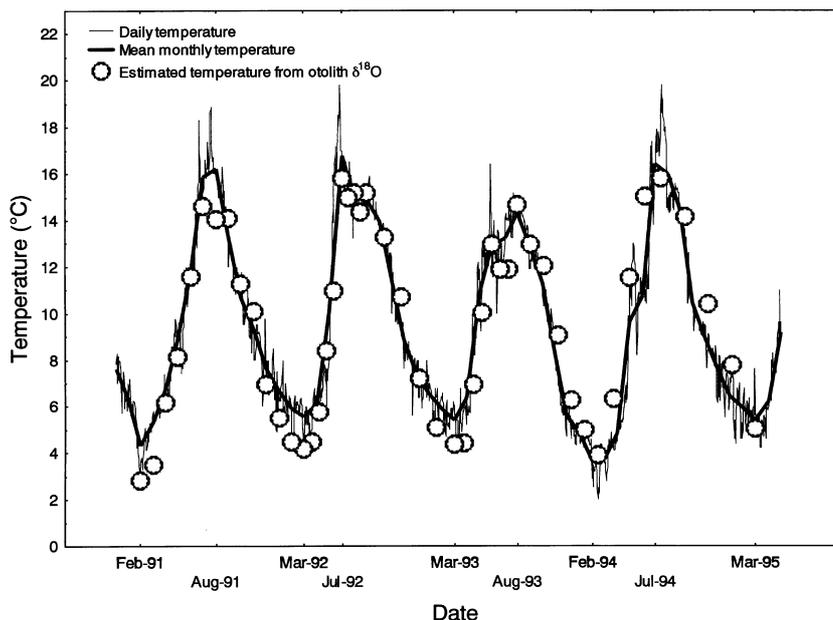
Fig. 7 *Gadus morhua*. Otolith $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values of fish number 2 in the 6L group, from the nucleus to the edge

Fig. 8 *Gadus morhua*. Estimated temperature using otolith $\delta^{18}\text{O}$ values of fish number 2 in the 6L group; discrete samples were milled from the edge to the core



otolith corresponds to the second summer of the fish, which was the first summer after the fish became sexually mature (spawned for the first time in early spring).

Discussion

Most of our estimates of ambient water temperatures at seasonal minima and maxima (using otoliths of *Gadus morhua*) were less than $\pm 1^\circ\text{C}$ different from the mean monthly measured temperatures. However, the accuracy decreased when fewer samples per yearly increment were collected (otoliths from the 6S group). Gao et al. (2001) also performed microsampling and stable-isotope analyses on cod otoliths from fish of known temperature history from the same area examined in the present study. They found the estimated temperatures to be close to the recorded summer temperatures early in the cod's life, but the winter temperatures were overestimated. The summer temperatures estimated from otolith accretion later in the fish's life were also underestimated. We believe that these differences can be explained by the sampling resolution in the otoliths in the same manner as Leder et al. (1996) showed that reduced sampling in corals led to attenuation of the $\delta^{18}\text{O}$ signals. The effect of shell growth and how it influences oxygen isotope signals when using micromill sampling techniques with constant sample size have been modelled for bivalve mollusc shells (Goodwin et al. 2003). In one of their models, daily increment widths varied throughout the year; they were largest at highest temperatures and decreased each year as the shell grew older. This type of growth pattern is typical for cod otoliths, as the growth rate is highest during times of high temperatures and high prey abundance (Campana 1996; Hüsey 2002; Otterlei et al. 2002), and the yearly increment thickness becomes progressively smaller for older fish (Smestad

and Holm 1996; Campana 2001). The model of Goodwin et al. (2003) predicts that the annual amplitude and maximum values of $\delta^{18}\text{O}$ decrease through ontogeny, due to reduced sampling resolution, while the minimum and average $\delta^{18}\text{O}$ signals remain nearly constant. These results support our finding that no difference could be determined in the overall otolith $\delta^{18}\text{O}$ signals between the small otoliths of the 6S group and the other two groups. The attenuation of the $\delta^{18}\text{O}$ signal in otoliths of the 6S fish was also mainly due to a reduction in the minimum $\delta^{18}\text{O}$ values and not the maximum $\delta^{18}\text{O}$ signal, as the model of Goodwin et al. (2003) predicts. This difference may be due to the higher numbers of samples per yearly increment in their models compared to our analyses. A small reduction in the minimum $\delta^{18}\text{O}$ signal was evident in the last year of the model by Goodwin et al. (2003), at which time the results were based on only five samples.

Our results also demonstrate that the true temperature range experienced by the fish cannot be extracted from otoliths if sampling is carried out at low sampling resolution. However, small otoliths, in which high sampling resolution is not possible with the current methodology, can still reveal important temperature information. The integrated temperature signal of the otolith $\delta^{18}\text{O}$ values over a relatively long time span correctly reproduced the mean temperature experienced by the fish in this study. The volume of otolith accretion below and above the mean temperature therefore seems to be very similar. This is reasonable since adult cod have their highest growth rates around the mean seasonal temperature (9.7°C) in this study (Jobling 1988).

Increasing the number of samples per yearly increment can result in increased accuracy of the estimated temperatures versus the ambient temperatures experienced by the fish, but not necessarily. The analytical capacities of the mass spectrometer set limits to sample

sizes; in this case, samples smaller than $8 \times 10^{-3} \text{ mm}^3$ resulted in low precision of the results. However, by expanding the sampling area in either length or depth more samples per yearly increment can be obtained. Our experience in sampling otoliths cut in the transverse section indicates that it is easy to follow the growth structures from the distal side towards the ventral side; the growth structures are more difficult to read on the dorsal side. Difficulties in recognising the structures increase the risk of sampling otolith material deposited at a different time, thereby causing the isotope temperature estimate to be integrated over a longer timespan. Expanding the sample area in depth is also possible, but using thicker sections ($> 160 \mu\text{m}$) will reduce the visibility of the growth structures in the otoliths. It will also increase the probability of sampling otolith material deposited over a longer time interval if the curvature of the otolith layers deeper in the otoliths is shifted relative to those on the surface of the section (Leder et al. 1996).

There are several possible sources of error when estimating the ambient temperature history experienced by fish according to the measured oxygen isotope composition of otoliths. We only had data available on the isotope composition of seawater ($\delta^{18}\text{O}_\text{W}$) on one occasion, November 1996 (Gao et al. 2001), and we used this value when calculating temperature based on otolith material deposited from 1991 to 1995. However, $\delta^{18}\text{O}_\text{W}$ values are probably not constant throughout the year (Frew et al. 2000). Interannual fluctuations are expected to occur, since evaporation during summertime concentrates ^{18}O , resulting in higher $\delta^{18}\text{O}_\text{W}$ values. Also, freshwater runoff from land, which is predominant during autumn and winter and during the spring snowmelt, adds ^{18}O -depleted water, resulting in lower $\delta^{18}\text{O}_\text{W}$ values. The magnitude of these interannual fluctuations in the area where the fish in this study were held is not known. There is no published isotope mixing line for that area, so an estimation of $\delta^{18}\text{O}_\text{W}$ by using salinity data is also inappropriate. Seasonal maximum and minimum isotope-based temperatures were close to mean monthly temperatures, except for August 1995. An overestimated $\delta^{18}\text{O}_\text{W}$ value would cause both seasonal minimum and maximum temperatures to be overestimated. Since the isotope-based temperatures minus the mean monthly measured temperatures are close to zero for otoliths in the 6L and 4L groups, it seems likely that the $\delta^{18}\text{O}_\text{W}$ used in this study was close to the true value.

Thermal stratification of the water masses could also explain why the isotope-based temperatures were underestimated on some occasions, especially for August 1995. During warm periods in summer and autumn the upper few metres of water masses can be several degrees Celsius warmer than the water masses below (Syvitski et al. 1987). The cod try to avoid the extremely high temperatures in summer, possibly since the optimal temperature for the growth of adult cod is $< 10^\circ\text{C}$ (Björnsson and Steinarsson 2002). The ambient temperatures experienced by fish during the warmest periods might therefore have been lower than those recorded at

2 m depth, if the fish actually exhibited such avoidance behaviour.

Relating the minimum and maximum cyclic $\delta^{18}\text{O}$ values of the otoliths to seasonal maximum and minimum temperatures relies upon the assumption that there is little or no time delay between exposure to a given temperature and a response in the depositing of otolith carbonate. The fine-scale temporal response to thermal changes of $\delta^{18}\text{O}$ values in otoliths is not known, but the temporal response of the thermal marking of otoliths has been thoroughly studied. Generally the otolith microstructure patterns respond to a thermal change lasting from 4 to 24 h (Volk et al. 1999). Since the fractionation of oxygen isotopes in otoliths is driven by temperature and is unaffected by the metabolic activity of the fish and by the otolith growth rate (Høie et al. 2003), the temporal $\delta^{18}\text{O}$ response in the otoliths is probably of the same magnitude as the response in microstructure changes.

The precision of the temperature estimate using otolith $\delta^{18}\text{O}$ values was generally high, and we found no differences in the estimated seasonal maximum and minimum temperatures when using otoliths of fish of different ages. This is further evidence that the ontogeny and physiological conditions of the fish have a minor influence on the otolith $\delta^{18}\text{O}$ values, and that there are no biological (vital) effects of the fractionation of oxygen isotopes on cod otoliths (Høie et al. 2003). Precise reconstruction of ambient temperature history experienced by the fish is therefore possible when sampling otolith material with high-resolution micromilling. However, there are still unexplained differences in the oxygen-isotope composition of otoliths from different fish species (Kalish 1991a; Patterson et al. 1993; Radtke et al. 1996, 1998; Thorrold et al. 1997). Co-rearing of several fish species under exactly the same environmental conditions, preferably in the same rearing tank, should be performed to investigate possible species-specific fractionation of oxygen isotopes in the otoliths.

The $\delta^{13}\text{C}$ values also showed a clear seasonal pattern, with values between -2‰ at the seasonal minimum temperature and -4‰ at the seasonal maximum temperature. The pattern of cyclic otolith $\delta^{13}\text{C}$ is also shown for wild cod (Weidman and Millner 2000) and for freshwater drum (*Aplodinotus grunniens*) (Patterson 1998), but otoliths of haddock (*Melanogrammus aeglefinus*) did not exhibit any clear $\delta^{13}\text{C}$ seasonal signal (Begg and Weidman 2001). The aragonite–bicarbonate enrichment factor in inorganic aragonite is independent of temperature (Romanek et al. 1992), so the cyclic otolith $\delta^{13}\text{C}$ values are not caused by a direct temperature effect. Weidman and Millner (2000) thoroughly discussed possible factors that could cause the cycling of otolith $\delta^{13}\text{C}$ values, and suggested seasonal changes in diet as a factor. The fish in this study were fed commercial dry food, so the cyclic pattern of otolith $\delta^{13}\text{C}$ values cannot be caused by the temporal differences in prey availability that wild cod can experience. Also, all the fish in this experiment became sexually mature at age

2 years, and were not fed in the spawning season from February to April. A more plausible explanation of the cyclic $\delta^{13}\text{C}$ values is a temperature-induced change in metabolism. These cod experienced large annual temperature cycles from 4°C to 18°C. Although optimum temperature for growth of adult cod decreases with increasing fish size and is generally < 10°C (Björnsson and Steinarsson 2002), the standard metabolism of fish increases with a temperature increase (Brett and Groves 1979). Since 20–35% of the carbon in otoliths is derived from metabolism (Kalish 1991a; Weidman and Millner 2000; Høie et al. 2003), and the metabolic carbon is depleted in ^{13}C compared to dissolved inorganic carbon (McConnaughey and McRoy 1979; Peterson and Fry 1987; Fry 1988), an increased metabolism will cause the otolith $\delta^{13}\text{C}$ to become more negative. This is also supported by experiments on juvenile cod: cod reared at 10°C had, on average, 0.7‰ lower otolith $\delta^{13}\text{C}$ compared to cod reared at 6°C (Høie et al. 2003). Several studies on gadoids have shown that the otolith $\delta^{13}\text{C}$ values increase through the first period of the fish's life, subsequently reaching a stable plateau or decreasing as the fish grow older (Schwarcz et al. 1998; Weidman and Millner 2000; Begg and Weidman 2001; Gao et al. 2001). This pattern has been attributed to the decreasing rate of size-specific metabolism by age and/or dietary shifts by age. Wild cod shift position in the food web with increasing age (Pålsson 1994), and there is a general increase of $\delta^{13}\text{C}$ in animals by 1‰ per tropic level (Peterson and Fry 1987), which should be reflected in the otolith $\delta^{13}\text{C}$ signals. Our results indicate that 3- and 4-year-old cod have higher seasonal maximum otolith $\delta^{13}\text{C}$ values than 5- and 6-year-old cod in the same calendar year. Also, the otoliths of fish in the 4L group had higher $\delta^{18}\text{O}$ -specific $\delta^{13}\text{C}$ values than the 6-year-old fish. This indicates a bell-shaped relation between otolith $\delta^{13}\text{C}$ values and fish age. Since cod of different ages were reared together and given the same food, the effect of diet can be ruled out, and the remaining age- and $\delta^{18}\text{O}$ -specific differences must be attributed to variations in fish age and/or fish ontogeny and not to a trophic level change by age. Changing otolith $\delta^{13}\text{C}$ by age of wild fish cannot therefore be explained by dietary shifts alone; rather, the age and/or fish somatic size-specific change in otolith $\delta^{13}\text{C}$ must also be considered. Sherwood and Rose (2003) showed that the activity level of fish influences otolith $\delta^{13}\text{C}$ values through altered metabolic activity. The higher activity of older fish could be an explanation for the reduced otolith $\delta^{13}\text{C}$ values in this study.

Although no conclusions can be made from a single otolith, it is interesting to note the change in the otolith $\delta^{13}\text{C}$ pattern at the time just after the fish became sexually mature, which might be related to spawning-induced physiological stress. Changes in the level of free Ca in the blood plasma and endolymph have been found in fish during periods when the gonadosomatic index was increasing (Kalish 1991b). The effect of physiological changes connected to spawning and its influence on

otolith $\delta^{13}\text{C}$ values should therefore be examined further.

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References

- Begg GA, Weidman CR (2001) Stable $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ isotopes in otoliths of haddock, *Melanogrammus aeglefinus*, from the northwest ocean. *Mar Ecol Prog Ser* 216:223–233
- Björnsson B, Steinarsson A (2002) The food-unlimited growth rate of Atlantic cod (*Gadus morhua*). *Can J Fish Aquat Sci* 59:494–502
- Brett JR, Groves TDD (1979) Physiological energetics. In: Hoar WS, Randall DJ, Brett JR (eds) *Fish physiology*. Academic, London, pp 279–352
- Campana SE (1996) Year-class strength and growth rate in young Atlantic cod *Gadus morhua*. *Mar Ecol Prog Ser* 135:21–26
- Campana SE (1999) Chemistry and composition of fish otoliths: pathways, mechanisms and applications. *Mar Ecol Prog Ser* 188:263–297
- Campana SE (2001) Accuracy, precision and quality control in age determination, including a review of the use and abuse of age validation methods. *J Fish Biol* 59:197–242
- Campana SE, Neilson JD (1985) Microstructure of fish otoliths. *Can J Fish Aquat Sci* 42:1014–1032
- Frew RD, Dennis PF, Heywood KJ, Meredith MP, Boswell SM (2000) The oxygen isotope composition of water masses in the northern North Atlantic. *Deep-Sea Res* 47:2265–2286
- Fry B (1988) Food web structure on Georges Bank from stable C, N and S isotopic composition. *Limnol Oceanogr* 33:1182–1189
- Gao Y, Schwarcz HP, Brand U, Moksness E (2001) Seasonal stable isotope records of otoliths from ocean-pen reared and wild cod, *Gadus morhua* L. *Environ Biol Fishes* 61:445–453
- Goodwin DH, Schöne BR, Dettman DL (2003) Resolution and fidelity of oxygen isotopes as paleotemperature proxies in bivalve mollusk shells: models and observations. *Palaios* 18:110–125
- Høie H, Folkvord A, Otterlei E (2003) Effect of somatic and otolith growth rate on stable isotopic composition of early juvenile cod (*Gadus morhua* L.) otoliths. *J Exp Mar Biol Ecol* 289:41–58
- Hüssy K (2002) Otoliths of juvenile cod (*Gadus morhua* L.): composition, microstructure and growth. PhD thesis, University of Copenhagen, Copenhagen, Denmark
- Jobling M (1988) A review of the physiological and nutritional energetics of cod, *Gadus morhua* L., with particular reference to growth under farmed conditions. *Aquaculture* 70:1–19
- Kalish JM (1991a) Oxygen and carbon stable isotopes in the otoliths of wild and laboratory reared Australian salmon (*Arripis trutta*). *Mar Biol* 110:37–47
- Kalish JM (1991b) Determinants of otolith chemistry: seasonal variation in the composition of blood plasma, endolymph and otoliths of bearded rock cod *Pseudophycis barbatus*. *Mar Ecol Prog Ser* 74:137–159
- Kim S-T, O'Neil JR (1997) Equilibrium and nonequilibrium oxygen isotope effects in synthetic carbonates. *Geochim Cosmochim Acta* 61:3461–3475
- Leder JJ, Swart PK, Szmant AM, Dogde RE (1996) The origin of variations in the isotopic record of scleractinian corals. I. Oxygen. *Geochim Cosmochim Acta* 60:2857–2870
- McConnaughey T, McRoy CP (1979) Food-web structure and the fractionation of carbon isotopes in the Bering Sea. *Mar Biol* 53:257–262
- Otterlei E, Folkvord A, Nyhammer G (2002) Temperature dependent otolith growth of larval and early juvenile Atlantic cod (*Gadus morhua*). *ICES J Mar Sci* 59:401–410

- Pálsson ÓK (1994) A review of the trophic interactions of cod stocks in the North Atlantic. ICES Mar Sci Symp 198:553–575
- Panfili J, Pontual H (de) Troadec H, Wright PJ (eds) (2002) Manual of fish sclerochronology. Ifremer and IRD, Brest, France
- Pannella G (1971) Fish otoliths; daily growth layers and periodic patterns. Science 173:1124–1127
- Patterson WP (1998) North American continental seasonality during the last millennium: high-resolution analysis of sagittal otoliths. Palaeogeogr Palaeoclimatol Palaeoecol 138:271–303
- Patterson WP, Smith GR, Lohmann KC (1993) Continental paleothermometry and seasonality using the isotopic composition of aragonitic otoliths of freshwater fishes. Geophys Monogr 78:191–202
- Peterson BJ, Fry B (1987) Stable isotopes in ecosystem studies. Annu Rev Ecol Syst 18:293–320
- Radtke RL, Showers W, Moksness E, Lenz P (1996) Environmental information stored in otoliths: insights from stable isotopes. Mar Biol 127:161–170
- Radtke RL, Showers W, Moksness E, Lenz P (1998) Corrigendum: environmental information stored in otoliths: insights from stable isotopes. Mar Biol 132:347–348
- Reibisch J (1899) Ueber die Einzähl bei *Pleuronectes platessa* und die Altersbestimmung dieser Form aus den Otolithen. Helgol Wiss Meeresunters 4:233–248
- Romanek CS, Grossman EL, Morse JW (1992) Carbon isotopic fractionation in synthetic aragonite and calcite: effects of temperature and precipitation rate. Geochim Cosmochim Acta 56:419–430
- Schwarzc HP, Gao Y, Campana S, Browne D, Knyf M, Brand U (1998) Stable carbon isotope variations in otoliths of Atlantic cod (*Gadus morhua*). Can J Fish Aquat Sci 55:1798–1806
- Sherwood GD, Rose GA (2003) Influence of swimming form on otolith $\delta^{13}\text{C}$ in marine fish. Mar Ecol Prog Ser 258:283–289
- Smestad OM, Holm JC (1996) Validation of back-calculated formulae for cod otoliths. J Fish Biol 49:973–985
- Syvitski JPD, Burrell DC, Skei JM (1987) Fjords, processes and products. Springer, New York Heidelberg Berlin
- Thorrold SR, Campana SE, Jones CM, Swart PK (1997) Factors determining $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ fractionation in aragonitic otoliths of marine fish. Geochim Cosmochim Acta 61:2909–2919
- Volk EC, Schroder SL, Grimm JL (1999) Otolith thermal marking. Fish Res (Amst) 43:205–219
- Weidman CR, Millner R (2000) High-resolution stable isotope records from North Atlantic cod. Fish Res (Amst) 46:327–342
- Wurster CM, Patterson WP, Cheatham MM (1999) Advances in microdrilling techniques: a new apparatus for acquiring high-resolution oxygen and carbon stable isotope values and major/minor elemental ratios from accretionary carbonate. Comput Geosci 25:1159–1166