Increased precision of growth data gained by reading multiple scales from each individual of Atlantic salmon (*Salmo salar*)

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The precision of growth estimates based on fish scales often remains uncertain because of within-individual variation in scale size and scale patterns, and also due to measurement errors. Based on scale readings of Atlantic salmon, we show that errors decreased with number of times and number of scales read per fish. The annual number of scale circuli was not constant, but positively correlated with annual specific growth rates. Number of circuli deposited after the last winter correlated positively with sampling date. There was no significant relationship between mean inter-circuli distance and the total circuli number during the first and second year at sea. For growth estimation in scientific studies, we recommend the use of 4-5 scales per fish. Consideration should be given to both circuli number and inter-circuli distances when estimating growth rate instead of relying on inter-circuli distances only.

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INTRODUCTION

Fish scales are typically used for age determination and retrospective individual growth analyses (Marco-Ruis *et al.* 2013). For correct estimation, the sampling of a high number of scales from each fish is recommended (Jonsson & Stenseth 1976; Shearer 1992). This is to decrease measurement errors such as those documented by Jonsson & Stenseth (1976) when scales of brown trout *Salmo trutta* Linneaus, 1758 were used for growth estimation, both as a function of number of times read and number of scales measured. Despite such recommendations the majority of scale analyses are frequently performed on only one scale from each individual. It should be noted that developments in the equipment for scale image analysis have made it possible to improve the accuracy during recent years (e.g. Peyronnet *et al.* 2007), making it easier to

measure distances within each scale than in early studies of the methodology such as Jonsson & Stenseth (1976). Even with such technical improvements, the measurement uncertainties remain an important source of error, which should be estimated, and errors due to variation in scale size will not be influenced by the improved scale reading equiment.

As fish increase in length, circuli are deposited at the scale margin (Wootton 1998). In salmonids, scale circuli formation rate and inter-circuli distances have been positively correlated with growth rate (e.g. Pearson 1966; Fisher & Pearcy 2005). Thus, number of circuli and inter-circuli distances in anadromous species like Atlantic salmon *Salmo salar* Linneaus, 1758 scales may reflect the variation in growth rate during the years at sea (Peyronnet *et al.* 2007). Friedland *et al.* (1993) provided an estimate of the scale circuli depositon rate for a

group of tagged fish and found that that approximately four circuli per month were formed during the first spring and summer at sea, and two circuli per month were formed in the autumn and winter when the individual growth rate is low. Based on these assumptions, Friedland *et al.* (2005) calculated indices meant to represent early growth of salmon at sea. A modification of this method was used in Friedland *et al.* (2009 a, b). Implicit in these later studies was the recognition that the circuli deposition rate is not fixed, but varies among individuals within populations and life stages (Dietrich and Cunjak 2007).

Aims of the present study were to calculate growth from the number of circuli, and by measurements of inter-circuli widths in scales of anadromous Atlantic salmon to estimate errors of sampling and measurement in the growth analysis, and thus provide recommendations regarding the number of scales that should be used and number of times each scale should be measured. We hypothesized that (i) inter-circuli distances, but not number of scale-circuli in the first marine growth zone increased with growth-rate of the fish, and that (ii) number of scale-circuli in the growth zone following the last marine winter annulus increased with date of sampling.

MATERIAL AND METHODS

During 2004-2010 fishing seasons, Atlantic salmon (n=245, Table 1) on their spawning migration were caught with a bag net at Stumpodden (58°01′N; 7°44′E), situated one-kilometre south-west of the outlet of the River Mandalselva on the Skagerrak coast of Norway. A large portion of the captured individuals is probably hatched in Mandalselva, and heading for their home river to spawn. The dominant age-group in the catches were 2SW (1SW: 9%, 2SW: 67%, 3SW: 3%, previous spawners: 20%, escaped farmed salmon: 1%).

The fish were caught for human consumption, but before processed, the total length was measured (mm), and approximately 50 scales per individual were taken from a restricted area above the lateral line, midway between the posterior edge of the dorsal fin to the anterior edge of the anal fin, as recommended by Dannevig & Høst (1931). The scales were stored dry in paper envelopes. For each individual, a random sample of ten scales was used for further analyses. Only scales with small central plates were used in order to avoid missing the first annulus (Jonsson & Stenseth 1976). The scales were cleaned in a Petri dish with soapy water and rubbed clean between two fingers (Shearer 1992), and thereafter placed between two glass slides and dried overnight. An impression of each scale was pressed onto a cellulose acetate slide by a scale press. A stereoscopic microscope (Leica MS5, 16 x magnifications) with digital camera (Leica DFC320, 0.63x magnification) was used to record an image (2088 x 1550 pixels) of each scale impression. Using the program Adobe Photoshop Elements 2.0, the quality of the scale images was improved (increasing contrast) and scale measurements taken by use of the program Image pro express 6.3. All distances, measured in pixels, were made along the longest axis of the scale, from the
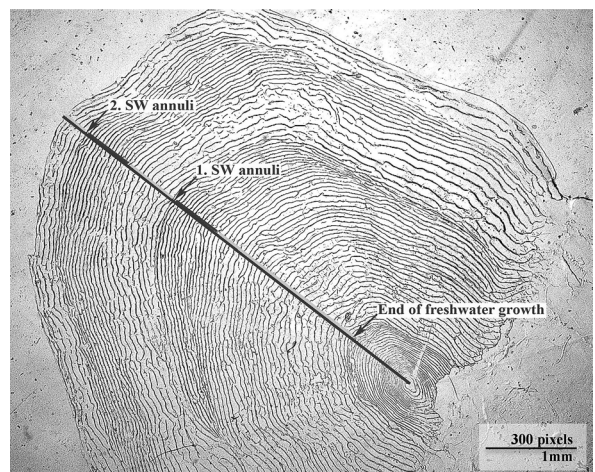

Figure 1. Scale from 2 SW Atlantic salmon illustrating: End of freshwater growth, summer growth (grey line), winter growth (black line at sea) and the annual zones during growth at sea (picture resolution 2088x1550 pixels).

Table 1. Bag net catches of Atlantic salmon at Stupodden (58°01′N; 7°44′E) , situated one-kilometre south-west of the outlet of River Mandalselva, Norway.

Year	1 SW	2 SW	3 SW	4 SW	Previous spawners	Not aged	Escaped farmed salmon	Sum
2004	8	24	2		6	1		41
2005	7	25			12		1	45
2006	4	31	1		14			50
2007	1	40	2	1	7	1	1	53
2008		24			2	1	1	28
2009		11	1		5			17
2010	2	6	1		1	1		11
Sum	22	161	7	1	47	4	3	245

focus to the scale margin (Fig. 1).

The end of the freshwater growth zone was identified by a substantial increase in inter-circuli distances. The sea-winter bands of the scales were identified by short inter-circuli distances and forking of the circuli (Wootton 1998). The annual zone is a theoretical line running between the last of the narrow spaced circuli of a winter band and the first of the wide spaced circuli of the following summer band, indicating that one year of growth is completed (Shearer 1992). The start of an annual growth zone was identified by the first continuous circulus cutting over the narrow spaced, incomplete circulus and/or the first widely spaced circulus after the winter band. As growth is reduced during homeward migration, narrow inter-circuli distances may be formed at the edge of the scale (Shearer 1992). Accordingly, this was not regarded as part of the last sea-winter band.

The inter-circuli distances of the last four circuli in the freshwater growth zone as well as all inter-circuli distances in the marine phase were measured. The software recognized the inter-circuli distances by analysing the contrast between the dark circuli and the light spacing. Dust on the camera lens or on the scales led to some incorrect identifications by the program. Due to this all scales were controlled manually, and errors corrected. The total number of circuli deposited during the years at sea was recovered from the measurements.

To quantify sampling error, two measurements (total radius and radius at smolting) were made along the longest radius axis of 10 scales from each of 36 randomly chosen individuals. Each scale was measured once. In order to quantify measurement errors, 10 randomly selected scales were measured 20 times each in random order. The ratio between the freshwater growth zone and the total scale length was used in the analysis. All measurements were carried out by a single observer to minimize error created by different observers having a slightly different technique or interpretation of where certain events occur (e.g. end of fresh water growth) on the scale.

In order to allow for quantifying the relationship between fish growth and circuli deposition rates, annual growth rates were back-calculated from the scale annuli. By assuming proportional growth of scales and fish body length (e.g., Jonsson & Stenseth 1976; Dietrich & Cunjak 2007), the annual specific growth rate during first year at sea could be back-calculated from $\ln(R_t)-\ln(R_{t-1})$, where R_t and R_{t-1} are scale annuli radius at age t and t-1, respectively. Here, t-1 represents age at smolting. First-year sea growth was used for this purpose as number of observations was highest for this growth interval, and thus allowing for the most powerful statistical analyses.

The statistical tests were carried out in R (R version 2.12.0 (2010-05-31), R Development Core team 2010). In order to access the 95% confidence limits of the measurement errors, the data were re-sampled 1000 times using a non-parametric bootstrapping routine (Manly 1997). The coefficient of variation was used to estimate the measurement error compared with the numbers of repetitive measurements. For quantifying the

within-individual sampling error, the difference (bias) between respective individual scale measurements and mean of those found for 10 scales (assumed to represent the correct value) were calculated. The overall sampling error was estimated as the mean of individual-biased coefficient of variation estimated from 10 scale readings. In order to predict fish length from scale radius, linear least squares regression based on ln-transformed fish length and scale radius was used. When testing and quantifying the effect of various exogenous (water temperature) and endogenous (spawning history and sea-age) factors on the various circuli responses (number and distances), ID (unique identification for scale) was used as a random factor in linear mixed model (LME) analyses, accounting for size variation among scales sampled from the same fish (sampling error). An ordinary linear model approach was used to predict number of circuli deposited as a function of the annual specific growth rate. Because there were multiple scale measurements and only one growth measurement per individual, it was not possible to use ID as random factor in these analysis. Therefore, the mean number of circuli per individual, weighted by the inverse of the variance for the multiple scales measured per individual was used as response. LME models were fitted using the lme-procedure available from the nlme package in R (Pinheiro *et al.* 2015; R Development Core team 2010), and linear models were fitted using the lme-procedure (stat package). Model selection was based on Akaike`s information criterion (AIC) following procedures available in Zuur *et al.* (2009). An approximate determination coefficient (R^2) for the mixed models (Nagelkerke 1991) was also derived.

RESULTS

Measurement errors and sampling error

Scale measurement errors were low, on average 0.12% ± 0.03% (SD) for the total scale radius and 0.61% ± 0.15% (SD) for the scale radius at smolting (Fig. 2A). The slope of the regression of measurement error over number of readings declined with increasing number of scales read, but stabilized after ten readings. There was a positive, but not significant relationship between the scale size and the measurement error ($R^2 = 0.50$; $P>0.05$). The within-individual sampling error declined at a decreasing rate when more scales were measured (Fig. 2B). The average sampling variation coefficient for ten different scales was 4.57 ± 1.65 %.(± SD, n=36).

Scale size in relation to fish length

Total individual length was positively correlated with the scale radius ($R^2 = 0.62$, Fig. 3A, Table 2a). Previous spawners added a slightly negative, but not statistically significant effect on the relationship between scale radius and fish length (Table 2a), Despite the non-significant effect from pre-spawners on scale radius, we decided to exclude such individuals in the remaining

analyses as many of the scales belonging to this group were difficult to read. We found no significant sea-age (SW) or catch-date effect on the scale radius - fish length relationship.

Circuli formation rate

The specific growth rate of Atlantic salmon during the first year at sea was positively correlated with the corresponding number of scale circuli ($R^2 = 0.67$, Fig 3B). An additive model including sea-age and day of year to the catch date (day number) received the highest AIC support (Table 2b) in predicting the number of circuli deposited after the last sea-winterband ($R^2 = 0.87$, Fig. 4). 1SW fish deposited a larger number of circuli (e.g., at day 180

the estimated number of circuli was 10.95) than the 2SW (5.60 circuli) and 3SW (3.94 circuli).

The relationship between circuli number and inter-circuli distances

There was no significant correlation between circuli number and mean inter-circuli distance for 1SW and MSW Atlantic salmon during the first year at sea ($R^2 = 0.101$; $P > 0.05$). Furthermore, there was no significant correlation between circuli number and mean inter-circuli distance for second-year sea growth groups ($R^2 = 0.079$; $P > 0.05$).
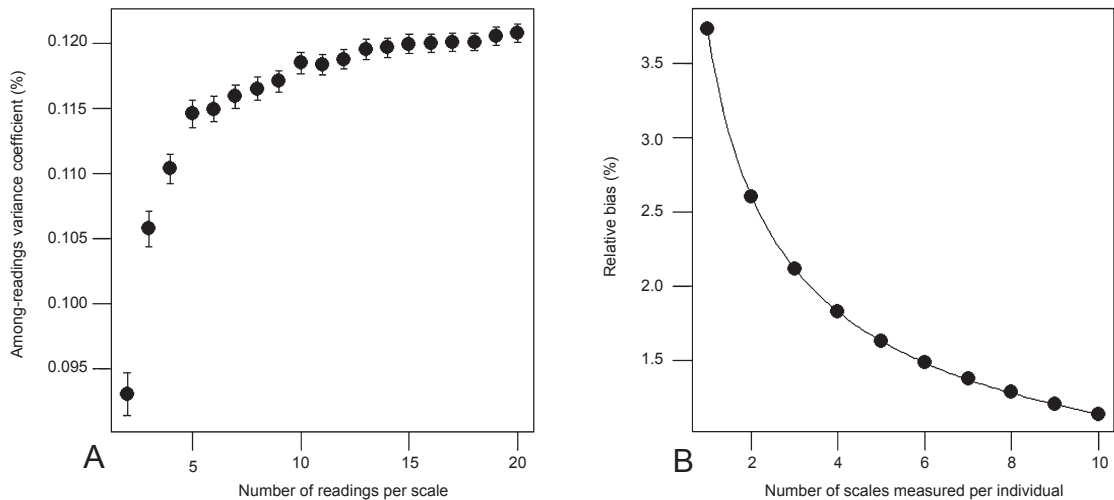

Figure 2. (A) Error due to measurement, CV %, as a function of number of times the radius (total scale radius) of one scale was read. (B) Error due to sampling as function of number of scales read. ln(relative bias) = -3.29 – 0.51*ln(number of scales).
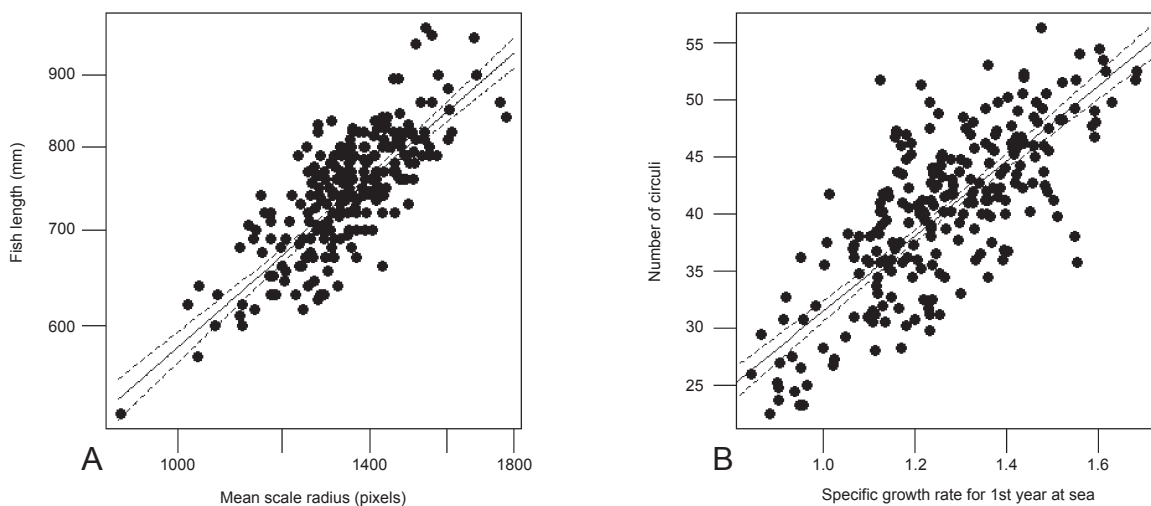

Figure 3. (A) Total body length ($L \pm$ S.E., mm) and mean scale radius (X, pixels). $\ln L = 0.788(\pm0.2969) + 0.807(\pm0.041)\ln X$, $P < 0.001$. (B) Mean number of scale circuli deposited during the first growth year at sea (C_{SW1}) as function of specific growth rate (g_{SW1}). $C_{SW1} = -1.353(\pm1.914)+32.833(\pm1.516)\ g_{SW1}$, $P < 0.001$. Dashed lines show 95% confidence intervals of the predicted linear model (solid line) in both cases.

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Table 2. (a) Model selection based on the effect of previous spawners on scale radius/fish length relationship with parameter estimate and ANOVA table for the selected model, (b) the effect of the number of circuli deposited after the last winter-band at sea with parameter estimate, with ANOVA table for the selected model, and (c) the effect of sea-age groups and circuli number on mean inter-circuli distances, with parameter estimate and ANOVA table for the selected model

Model							
(a) Response: Scale radius						AIC	ΔAIC
Scale radius vs Fish length + Previous spawner						11552.75	
Scale radius vs Fish length * Previous spawner						11554.92	2.17
Scale radius vs Fish length						11560.24	7.49

Parameter estimate			*ANOVA Test statistics*			
Parameter	Value	SE	Term	df	*F*-value	*P*-value
Intercept	317.78	57.02	Fish length	1	330.19	<0.0001
Fish length	1.38	0.07	Previous spawn	1	2.40	0.12
Previous spawn	-21.42	13.82				

(b) Response: number of circuli deposited after the last winter-band at sea	AIC	ΔAIC
Sea age + day number	3737.55	
Sea age * day number	3742.31	4.76
Sea age + number of days >7°C at Lindesnes	3750.38	12.83
Sea age * number of days >7°C at Lindesnes	3751.72	14.17
Sea age + number of days >8°C at Lindesnes	3755.16	17.61
Sea age * number of days >8°C at Lindesnes	3757.77	20.22

Parameter estimate			*ANOVA Test statistics*			
Parameter	Value	SE	Term	df	*F*-value	*P*-value
Intercept	-8.65	3.35	Sea age	3	28.28	<0.0001
Sea age 2	-5.35	0.88	Day number	1	43.66	<0.0001
Sea age 3	-7.01	1.06				
Sea age 4	-8.90	1.90				
Day number	0.11	0.01				

(c) Response: Mean inter-circuli distances	AIC	ΔAIC
sea-age groups (1SW, MSW) * circuli number	746.28	
sea-age groups (1SW, MSW) + circuli number	747.20	0.92
circuli number	751.73	5.45

Parameter estimate			*ANOVA Test statistics*			
Parameter	Value	SE	Term	df	*F*-value	*P*-value
Intercept	16.91	1.23	Sea-age groups (1SW, MSW)	1	3.11	0.079
Sea-age groups (1SW, MSW)	1.57	1.31	Circuli number	1	24.06	0.001
Circuli number	-0.01	0.03	Interaction	1	2.89	0.091
Interaction	-0.05	0.03				

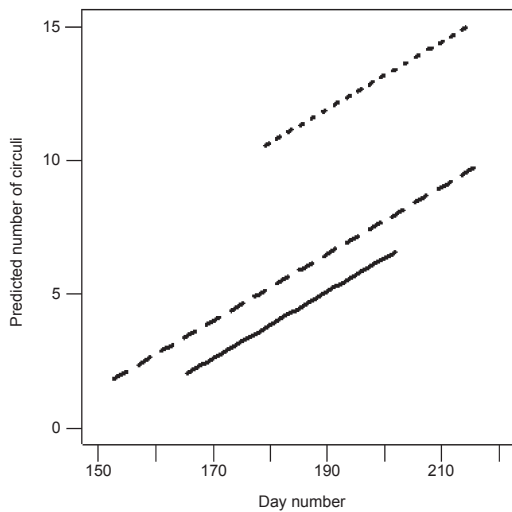| *Parameter estimate*			*ANOVA Test statistics*			
Parameter	Value	SE	Term	df	*F*-value	*P*-value
Intercept	12.767	0.400	Maximum circuli number (MSW)	1	19.416	0.001
Maximum circuli number (MSW)	0.068	0.016				

Figure 4. Predicted number of circuli deposited after the last sea-winter band on scales of Atlantic salmon 1SW (– – –), 2SW(— —) and 3SW(——) as a function of days after 1 January, to the catch date (day number). Lines represent model predictions from the most supported LME model presented in Table 1b.

DISCUSSION

The measurement errors from the scale readings in this study were small (<0,1 - 0,6%). Consequently a single reading of each scale was enough to obtain an accurate measurement. Measure accuracy is, however, dependent on the observer, and whether reading has been carried out according to an established common procedure. This may illustrate an improved accuracy in the equipment used for scale image analysis in recent years compared to those obtained by Jonsson and Stenseth (1976). In our study, measurment errors were estimated for total scale radius and radius of freshwather growth. For other annuli, and especially for the freshwater-phase annuli, the measurment errors most probably are larger owing to their less clear appearances.

The present study demonstrates that sampling error declines with number of scales read. This illustrates the problem by using only one scale per fish for retrospective growth estimation. When choosing the optimal number of scales for analysis, the trade-off between the reduction in sampling error by including another scale and the time and resources spent measuring the scale has to be considered, depending on the purpose and funding of the project. In this study, 4.6 scales fish[-1] gave the optimal balance, assuming a linear increase in time used (i.e., same amount of time used per scale) with number of scales included and an exponentially decreasing bias with number of scales used. Accordingly, it may be recommended that more than one scale per fish is incorporated in the analysis, and that 4-5 scales appears to balance the sampling effort and sampling error if minimizing error due to measurement is a goal. Clearly,

considerations on the relevance of the accuracy, depends on the study topic. For instance, the estimated sampling error in our study system yield mean back-calculated smolt size estimates between 139 mm and 150 mm for various subset of one-scale readings, where the grand mean from multiple-scale readings provides an estimate of 144 mm. The relevance of this variation clearly depends on the study topic addressed and on the magnitude of the effects that one wants to reveal. If a multiple-scale reading approach is undertaken, the among-scale variance should be quantified in the statistical analyses by including the fish ID as a random factor, using for instance linear mixed effects models (e.g. Marco-Rias *et al.* 2013). The relatively low number of individuals used for sample error estimation (n=36) in our study may have biased the sample error estimate upwards. As a consequence, the recommended number of scales used for multiple-scale analyses (4-5 scales) may be too high. As shown in Newman and Weisburg (1987), a large sample size reduces the number of scales needed per fish to maintain the within-class level of variance for a given annulus. The relationship between sample size and number of scales needed to maintain within-class variance probably varies among study systems, but can be retrieved from intra-class correlation estimates (i.e., among-fish variance/(among fish variance + among scale variance) for a given class). Also, collecting a high number of scales from each fish may allow for more error by collecting from a larger region on the fish (e.g., Dannevig and Høst 1931). In many study situations, number of scales sampled per fish should be kept at a minimum. Collecting a large number of scales from living fish that are released back in nature may expose the fish to subsequent infections and disease. Clearly, under such conditions animal welfare aspects must be prioritised over scale-analysis precision. If feasible, a sub study applied on sacrificed individuals may be conducted for estimation of measurement precision and bias. Based on the error estimates from the sub study the power of results found in the main release study may be assessed.

Since there was no significant relationship between mean inter-circuli distances and total circuli number during the first and second growth year at sea, circuli formation rate and inter-circuli distance appear to offer differing prediction potential of individual growth rate. However, both inter-circuli distance and circuli formation rate increased with individual annual growth rate, but varied among individuals and year-classes of Atlantic salmon. As a consequence, high individual growth rate can result in wide inter-circuli distances and/or higher total circuli numbers, and likewise low growth rate may result in narrow inter-circuli distances or/and low circuli formation rate. At very slow growth, there may be no circuli deposition at all, or even absorption of existing circuli (Shearer 1992). In brown trout, no new growth band was formed when the annual length increment was less than 1.5 cm and the fish length were 25-30 cm (Jonsson 1976, 1977). These facts highlight the importance of considering both circuli number and inter-circuli distance when estimating Atlantic salmon growth rather than

to rely on inter-circuli distances only (McCarty *et al.* 2008). Consequently, future retrospective growth analysis using scales should be based on both circuli formation rate and inter-circuli distances to analyse aspects of within-year individual growth rate. The mechanisms behind circuli formation rate and inter-circuli distance remain enigmatic.

In conclusion, there is large individual variation in number of circuli deposited during the first year at sea, but as hypothesized the circuli deposition rate during the first year at sea was positively correlated with individual growth rates, and furthermore the number of circuli deposited after last sea annulus was positively correlated with catch date. To minimize sampling error in scale analysis of Atlantic salmon, mean values of 4-5 scales from each fish should be used.

ACKNOWLEDGEMENTS

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