ICES CM 2001/J: 34

A comparison of variability and bias when ageing Northeastern Atlantic minke whales (Balaenoptera acutorostrata) by counting growth layer groups in the mandible and bulla tympanica

By: Erik Olsen, H.J. Skaug, A. Leithe, B. Bergflødt, K.A. Fagerheim

Abstract

The age of 43 minke whales (*Balaenoptera acutorostrata)* was estimated by counting growth layer groups (GLGs) GLGs in 500µm thick haemotoxylin stained transverse sections of left and right mandible. The same whales were also aged by counting GLGs in 150µm unstained sections of left and right *bulla tympanica.* The staining and preparation methods were also used to prepare and stain mandible sections of a sperm whale and the GLG count of this was the same as the GLG count of a longitudinal section of a tooth from the same animal. Minke whale mandible age estimates had higher CV (63% on average) than the bulla age estimates (36% on average). Comparing the age estimates with the number of ovulations revealed that both methods underestimated the true age of the whales.

Olsen, Erik, Marine Mammals Division, Institute of Marine Research, PO Box 1870 Nordnes, N-5817 Bergen, Norway [tel: +47 55 23 86 06, fax: +47 55 23 86 17, e-mail: eriko@imr.no]

Introduction

Age determination of baleen whales has due to their lack of teeth been less straightforward than ageing other mammals. Many methods have been attempted, and most species are now routinely aged by counting annual Growth Layer Groups (GLGs) in the wax-like ear-plug (Christensen 1992). Such ear-plug seldom form in the North Atlantic minke whales (*Balaenoptera acutorostratai)* (Christensen 1992)and these have been aged by counting GLGs in the periosteal layer of the tympanic bulla (Christensen 1981). In sperm whales annual GLGs have been found in the mandibular walls, and these correlate well with the age estimate from counting GLGs in the teeth until the attainment of physical maturity (Laws 1958; Nishiwaki and Ohsumi 1961). Similar GLGs are found in the mandible of many other mammals and birds (Klevezal, G.A. and Kleinenberg, S.E. 1967). (Klevezal and Mitchel 1972) attempted to determine the age of fin- (*Balaenoptera physalus*) and sei whales (*Balaenoptera borealis*) from counting mandibular laminations, but failed as they were unable to detect clear growth zones. However, this method had not been attempted on minke whales, and it was conceivable that in this short-lived species mandibular GLGs were formed. In addition, during the three decades since (Klevezal and Mitchel 1972) study the advent of high-resolution digital cameras and state-of-the-art image analysis software had given us new tools to identify possibly diffuse growth zones in minke whale mandibles.

Our main objective was therefore to attempt to detect annual GLGs in the mandibles of minke whales, and compare the age estimate and associated variance of this method with the bulla age estimates. A secondary objective was to develop the techniques and skills necessary to detect and count mandibular GLGs, and verify these techniques by employing them on samples from species with validated ages.

Materials And Methods

The samples used in this study were collected in 1999 from commercial whaling vessels operating in the Norwegian Economic Zone along the coast of Northern Norway, in the North Sea and in the Norwegian Sea east of Jan Mayen island. Trained personnel aboard 4 whaling vessels collected the samples. While the scientific personnel was aboard six whales were caught in Northern Norway, 42 in the North Sea and 17 in the Norwegian Sea. Of these two were lost when the harpoon line broke when the whales were hauled to the side of the boat.
We collected both bulla, and the whole mandible while the whale was flensed on deck. Bulla were retrieved by prying loose the exposed bulla with a screwdriver, while the mandibles were cut loose at the jaw joint, and the anterior 50 cm of both mandibles were cut off using a saw. The whaling vessels we were aboard are were all less than 9 m wide, and when the whale was pulled in across the vessel the head was left hanging over the side of the ship. This made it unsafe to retrieve the mandibles when the sea was rough as the person had to hang over the side of the vessel to fix a rope to the jaw. Therefore for 22 whales we have no mandible samples.

After sampling the bulla were stored raw on the vessel, while the mandibles were frozen at –23ºC. At the laboratory on land the bulla were cleaned and three thin (150μm) segments were cut from each bulla with a dual-bladed saw according to the procedures described in (Christensen 1981). These were in turn mounted on microscope slides and three different readers aged each segment independently two or three times. From these multiple age readings of a specific animal a single age estimate was estimated using General Linear Mixed Model regression as described in (Olsen and Skaug 2001).

Mandible ageing

The mandibles were thawed, and the bone cleaned of excess tissue. Using the same dual-bladed saw as in the bulla work 200-500 μm segments were cut of the lateral wall of both mandibles about 45 cm posterior of the tip of the lower jaw. It was in this area we had observed what appeared to be GLGs in a pilot-study of the mandibles of two whales caught in 1997. This study also indicated that the lateral region 40-50 cm from the tip of the jaw was where the bone was most ossified. Most of the mandible of baleen whales consists of a highly spongiose bone matrix filled with fat, and with an outer edge of highly ossified bone, and even this ossified bone has a high fat content. The high fat content prevented our first attempts of staining the sections, but this was improved by soaking the segment in concentrated HCl for about 30 seconds. The bone segments were then rinsed in water, followed by ethanol and lastly immersed in a glycerin solution to for storage. Each segment measured in excess of 5x3 cm, larger than the available microscope slides, and we therefore opted to store the segments in glycerol. One such segment was prepared from both mandibles of all whales except two because one of the mandibles were lost. For six whales an additional segment was prepared when the technicians were unsatisfied with the quality of the first segment cut. An additional

four segments was prepared from 13 whales to investigate if the same GLG pattern found in one segment could be identified further along the mandible. These extra segments were cut within 5 cm of each other and 5cm posterior of the first one.

Visible light analysis

Due to their size it was difficult to examine the mandible segments in the limited field of view of our microscopes. Instead we placed the segments on a light table and took a picture of each using a Nikon Coolpix 990 digital camera. Each picture was taken at maximum resolution (2048×1536 pixels) in color mode, and stored as TIFF files for conservation of all image information. Each picture was later analyzed using ImagePro 4.0 software. In the image-analysis we attempted enhance the contrast and clarity of the pictures using several different filters and techniques. However, in the end we only manipulated the brightness and contrast of each color channel (Red Green and Blue) separately to achieve best contrast of the GLGs (Figure 1). Following the image-enhancement two people cooperated in determining where we thought the potential GLGs were placed in the segment, and marked and measured these using the software's tools. After adjusting contrast and brightness the clarity (how easily the GLGs were to detect) was rated as either "good" or "poor". Prior to the analysis all image files had been renamed by an independent observer to prevent the two readers from using additional knowledge or remembering how many GLGs had been counted in previous sections from the same whale.

The GLG counts of all segments from a single whale was used to estimate mean age, the coefficient of variation and percentage agreement between the age estimates. Estimating both CV and percentage agreement may seem redundant, but we chose to include the latter as it has been used as a routine measure in other age estimation work, and allowed for comparison of variation with previously published studies.

Use of mammographic x-ray.

An alternative way of studying ossified structures has been by x-ray imaging. We therefore examined the x-ray images of the mandible section from four whales (K13, K21, K22 and K27), This pilot-study was carried out using a human mammography x-ray apparatus at Haukeland Hospital in Bergen. The pictures were taken using Kodak x-ray film, and after some trial and error we found highest contrast at 30 kV and 2.9 mAs settings of the apparatus. Ordinary (higher intensity) x-ray technique was attempted as well as ultra-sound imaging was attempted, but the resolution of these were too low to discern any GLGs or fine structure in the mandible. In addition the ultrasound did not sufficiently penetrate the bone Using mammography x-ray we able to achieve much the same resolution as using the when the segments were examined using visible light and digital camera (Figure 3). However, this method was only employed on mandible segments from four whales, which is a too small sample to evaluate the accuracy and precision of the method.

Control of mandible aging using mandible and tooth from sperm whale

During the course of our work a sperm whale (*Physeter catodon*) stranded on a beach in Sola in southwestern Norway, and we were able to attain a section of both the mandible and teeth of this animal. As GLGs have previously (Nishiwaki and Ohsumi 1961) been found in the mandible this sample allowed us to verify if our preparation and examination techniques were appropriate to identify the GLGs in the mandible. We prepared and stained three segments of the sperm whale mandible in the same way as the minke, and cut one tooth longitudinally and polished the surface to verify if the mandible GLG count corresponded with the tooth GLG count. Two readers independently examined the two sides of the tooth and mandible segments visually using only magnifying glass and a light source.

Bulla ageing

Two independent readers examined the bulla segments (Figure 4) according to the procedures described in (Olsen and Skaug 2001). From the age estimates mean age was modeled for each whale using General Mixed Model Regression according to the methods in (Olsen and Skaug 2001).

Analysis of age estimates

The reading of bulla and mandible segments yielded two sets of mean age estimates for which we could compare CV and percentage agreement. In addition we plotted the age estimates against the total body length and number of ovarian scars, two independent parameters positively correlated with the true age. Body length would show an

logistic growth with age leveling out around a sex-specific maximum body length, which we modeled with a von Bertalanffy growth equation(Olsen and Sunde 2001). Like other animals, minke whales show individual differences in growth, but since length is the only independent measure of age available to evaluate age estimates of males we opted to use it.

Most species of *Mysticeti* have been shown to have a regular ovulation and birth cycle, giving birth to one young every 1-3 years depending on species. (Olsen 1997; Christensen 1981)have shown minke whales to have an annual ovulation rate, and mature female North Atlantic minke whales give birth annually. Ovarian scars from ovulations (*Corpus lutea*, and *Corpus albicantia*) have been shown to persist for life (Laws 1958), and the number of these are positively linearly correlated with true age. To evaluate the precision of the ovary examinations both ovaries from 22 of the females included in the study were examined by two readers experienced in examining ovaries. These did their examinations independently, and without any accessory information about the animals examined. Body length, on the other hand was measure aboard the vessels. This may have introduced some error in the precision as the whales are generally wider than the boat, and are placed on the side while flensing. This would introduce some random error to the length measurement.

Results

True color analysis of the mandible segments yielded age estimates ranging from 1 to 23 years, mean age estimates ranged from 1.5 to 18.5 years. Mean CV based on one reading was calculated from the standard deviation of all readings to 63%. Similarly mean modeled bulla age ranged from 0.5 to 14 years with a mean CV from one reading of 36%. The CVs were calculated based on one reading to allow comparison of variation between the mandible and bulla ageing methods (see Table 1). Analysis of the sperm whale mandible (Figure 2) and tooth both yielded counts of 14 to 15 GLGs based on the readings of two readers. This agreement between sperm whale teeth and mandibular GLG count indicated that the methods for preparation and staining mandible sections we employed were appropriate. Also, Figure 2 show similar growth layers to those shown in Figure 1A and B.

The analysis of ovarian scars revealed that there were some inconsistencies between the readers. The readers agreed 100% when analyzing ovarian pairs with less than 7 scars, above this some variation in interpretation became apparent. The CVs from counting ovarian scars of the animals with more than 6 scars ranged from 6% to 61%, with a mean of 10%. However, we have reason to believe that there had been some mix-up in the analysis, and that the high CV of 61% for K8 and 40% for K12 should be treated with much caution. Excluding these from the analysis, mean CV dropped to 9% while CV for older females with more than 6 ovulations dropped from 22% to 12%. We also analyzed the readings of the single ovaries using Principal Component Analysis, and in a plot of the single ovaries it was apparent K8 (right) and K12 (left) were outliers in relation to how the other ovaries had been interpreted by the three readers.

Comparing the mandible and bulla age estimates with number of ovulations showed no apparent positive increase in number of ovulations with age for either method (Figure 5). Attempts to fit linear regression models showed a very low correlation (0.16 for mandible age – ovulations and 0.023 for bulla age – ovulations). The age estimates for a given number of ovulations spanned 5 to 10 years. Similar comparisons of the age estimates with length (Figure 6, Table 2) show a poor fit with the growth model for both mandible and bulla age estimates. The model parameters (Table 2) showed differences in L_{MAX} between the two methods, where as for mandible age L_{MAX} for males was estimated to 753.6 cm it was estimated to 885.6 cm when using the bulla age estimates. Similarly, the female L_{MAX} was estimated to 841.0 and 802.4 cm using the mandible and bulla age estimates respectively. Similarly, the growth rate (k) and t_0 varied with the sexes and ageing methods, with the difference between the sexes being most pronounced using the bulla age estimates.

Discussion

Using GLGs in the mandible of minke whales proved to be more difficult and time-consuming than first anticipated. We were unable to prepare and stain the sections in a manner that brought forth the faint and elusive GLGs with such clarity and distinctiveness that we were satisfied ourselves. Our experiments with the sperm whale mandible and tooth (Figure 2) and harbour porpoise (*Phocoena phocoena*) teeth and mandibles (E. Olsen unpublished results) showed us that our technique was not at fault. Rather, it appeared that minke whales minke whales like their larger cousins (fin-, and sei whales) (Klevezal, G.A. and Kleinenberg, S.E. 1967)do not form GLGs in the mandible which

are clear and distinct under visible light. However, our examinations of minke whale mandibles clearly show that they are thicker in larger animals, and that the highly ossified outer wall where GLGs were found in sperm whales and harbour porpoise is thicker in larger than in small animals. Also, in some minke whale mandibles clear GLGs were found, and these were structurally very similar to GLGs in the sperm whale mandible examined. We therefore believe that there is an annual growth in minke whale mandibles, but that the GLGs are not formed as clearly as in the *odontocetes*, and possibly only in some minke whales.

Our reading of minke whale mandibles under visible light does however show that this method of examination is unfruitful. Mandible age estimates had significantly higher CV than the bulla age estimates, which in turn were much higher than precision when ageing sablefish (*Anoplopoma fimbria*) (Heifetz et al. 1998) or Greenland halibut (*Reinhardtius hippoglossoides)* (Bowering and Nedraas 2001), species which are considered difficult to age. Comparing the age estimates with the number of ovulations and length also indicated that counting mandibular GLGs under visible light presents underestimation bias to the age estimates. Had the age estimates been unbiased in relation to true age we would have seen a linear increase in age with increase in number of ovulations. Similarly, all four plots in Figure 6 show very poor agreement between the plotted length/age data and the von Bertalanffy growth equation. However, these plots are based on very few data points, and it is possible that a larger sample size would yield a better fit. Still, the wide spread in length at a given age indicates that either the age or length data were severely biased. We admit that there is some room for random error associated with the length data, but we find it highly unlikely that this is so large and biased that it could explain the poor fit of the length/age plots. We therefore interpret the poor fit as indicative of a large and unknown bias in both bulla and mandible age estimates.

This conclusion is further strengthened by the comparison of numbers of ovulations with age. North Atlantic minke whales are assumed to have an annual ovulation rate and 98% of sexually mature female minke whale examined in the Northeastern Atlantic in 1972-1979 were pregnant (Olsen 1997). In our sample 7 of 18 (39%) of the sexually mature females had a higher or same number of ovulations as both mandible and bulla age estimates, and two whales with no ovulations were estimated to more than 10 years using both methods. These findings are worrying as they do not correspond with our current knowledge of the reproductive cycle of the species. One interpretation of these results is that there is large variations in ovulation rate for the species, while another is that the age estimates are biased. We believe that neither explanation should be ruled out, and more in-depth analyses of ovaries and reproductive data are needed to determine the ovulation rate of the species. However, even if ovulation rate is found to vary, this does not refute our conclusions drawn from Figure 5, for even with a vary varying ovulation rate (and even reduced ovulation rate with increased age) we would expect an increase in age with an increase in ovulations where only the slope and form of a fitted regression line would be affected.

Our ovary reading experiment did show that there was some variability in the way ovaries were interpreted by the three readers. As expected the readers agreed completely when analyzing ovaries with few *corpora*, while the variability increased with increase in *corpora* count. Unfortunately our data set included too few animals with high *corpora* counts to accurately determine the nature of this increase. Still, our crude examination of CV found a CV of 12% for the mature animals (excluding two outliers). We believe much of the reason for this variability is caused by differences in how readers interpret small *Corpus albicans* and *Corpus artretica*. These two forms are sometimes hard to distinguish, and in addition one or more of the readers may have overlooked some small corpora. Further analysis on a larger data set is needed to determine if this variability is just a random error, or if there is some bias involved. The observed variability in corpora counts is however too small to have any major implications on our comparison with age. Estimated 95% confidence intervals averaged only ±2.4 years for the females where the readers did not agree on the *corpora* count.

We had hoped in this project to find a way to use mandibular GLGs to determine the true age of minke whales. Our experiment using visible light examination did not yield sufficiently precise or unbiased age estimates. However, our pilot-study using mammographic x-ray imaging shows promise. We will therefore analyze a larger sample of mandible sections using this technique in the hope that it will reveal the elusive GLGs in the mandibles of minke whales.

Acknowledgements

This project was funded as part of Norwegian Research Council project number 127211/120.

References

Bowering, W. R. and Nedraas, K. H. 2001. Age validation and growth of Greenland halibut (*Reinhardtius hippoglossoides*): A comparison of populations in the Northwest and Northeast Atlantic. Sarsia. **86**:53-68.

Christensen, I. 1981. Age determination of minke whales, *Balaenoptera acutorostrata,* from laminated structures in the tympanic bulla. Rep. Int. Whal. Commn. **31**:245-253.

Christensen, I 1992. Age determination of baleen whales. North Atlantic Studies. **2**:32-35.

Heifetz, J., Anderl, D., Maloney, N. E., and Rutecki, T. L. 1998. Age validation and analysis of ageing error from marked and recaptured sablefish, *Anoplopoma fimbria*. Fish. Bull. **97**:256-263.

Klevezal, G.A., and Kleinenberg, S.E. 1967. Age determination of mammals from annual layers in the teeth and bones. Israel Program for Scientific Translation. Jerusalem, Israel.

Klevezal, G. A. and Mitchel, E. 1972. On the annual layers in the bones of whalebone whales. Zoologicheckii Zhurnal . **50**:1114-1116.

Laws, R. R. 1958. Recent investigations on fin whale ovaries. The Norwegian Whaling Gazette. **47**:225-254.

Nishiwaki, M. and Ohsumi, S. 1961. Age characteristics in the sperm whale mandible. The Norwegian Whaling Gazette. **50**:499-507.

Olsen, E. 1997. A study of the reproductive potential of North Atlantic minke whales (*Balaenoptera acutorostrata*, Lacépède 1804) in the period 1972-79. Master Thesis. University of Bergen, Bergen, Norway, 76 pp. English.

Olsen, E. and Skaug, H. J. 2001. Methods for variance decomposition of age estimates applied to age estimates of North Atlantic minke whales. Can. J. Fish. Aquat. Sci. **In prep**.

Olsen, E. and Sunde, J. 2001. Age determination of minke whales (*Balaenoptera acutorostrata)* using aspartic acid racemization technique. SARSIA. **Submitted**.

Tables

Table 1 Age and growth (length, number of ovulations) for minke whales for which age had been estimated by counting growth layers in the mandible. Bulla age for the same animals are also shown, and coefficient of variation (CV) and number of readings (n) for age estimate is also shown. Body length was measured in centimeters.

		Mandible			Bulla			Ovarian scars		Body
Whale	Sex	Mean age	CV [B]	n	Mean age	CV [B]	n	Count	CV	Length
F1	1	7.0	0 %	3	missing bulla			*	*	620
F2	1	9.3	11 %	3	8.1	36 %	6	1.0	0 %	760
F7	1	8.0	65 %	3	5.7	43 %	12	0.0	0 %	760
F9	1	8.3	24 %	3	11.6	30 %	12	3.0	0 %	780
K1	1	4.0	n.a.	1	8.5	34 %	11	12.0	0 %	857
K2	1	11.0	36 %	2	10.2	32 %	12	8.7	6 %	840
K3	1	9.0	33 %	3	12.2	30 %	12	0.0	*20 %*	810
K4	1	11.8	107 %	6	11.1	31 %	12	14.3	8 %	885
K5	1	15.8	104 %	6	10.2	32 %	12	0.0	*16 %*	870
K6	1	5.7	101 %	6	9.4	34 %	12	3.0	0 %	776
K7	1	7.5	120 %	6	8.1	36 %	12	13.7	16 %	855
K8	1	6.6	161 %	8	7.2	38 %	12	0.0	*61 %*	801
K9	1	14.0	88 %	6	8.1	36 %	12	9.0	44 %	868
K10	1	11.0	73 %	6	10.0	33 %	12	5.0	0 %	845
K11	1	11.5	61 %	2	10.4	32 %	6	0.0	0 %	705
K12	1	10.0	118 %	6	6.7	40 %	12	11.7	40 %	802
K13	1	18.5	56 %	6	7.5	38 %	12	18.0	*12 %*	855
K14	2	10.0	80 %	2	10.4	32 %	12	n.a.		730
K15	1	7.8	118 %	6	11.3	31 %	6	4.0	*0 %*	858
K17	1	5.0	0 %	2	10.2	32 %	12	0.0	*0 %*	760
K18	2	10.0	40 %	2	6.8	40 %	12	n.a.		769
K19	2	4.5	67 %	2	7.1	39 %	12	n.a.		762
K20	2	7.5	40 %	2	8.7	35 %	12	n.a.		730
K21	1	8.0	75 %	2	9.7	33 %	12	0.0	[A]	675
K22	1	14.5	7 %	2	12.8	29 %	12	0.0	0 %	725
K23	1	5.0	69 %	3	13.7	28 %	12	0.0	0 %	726
K24	2	2.0	0 %	2	0.5	141 %	12	n.a.		485
K25	2	4.5	111 %	2	14.0	28 %	12	n.a.		840
K26	2	5.0	40 %	2	10.8	32 %	12	n.a.		740
K27	1	6.5	165 %	6	13.1	29 %	12	6.0	0 %	760
K28	1	8.2	108 %	6	12.2	30 %	12	10.0	[A]	835
K29	2	9.0	0 %	1	11.4	31 %	12	n.a.		832
K30	1	8.2	136 %	6	9.9	33 %	12	4.0		822
K31	2	1.5	67 %	2	10.8	32 %	12	0.0		820
K33	2	6.5	15 %	2	7.8	37 %	12	0.0		700
K34	1	7.0	70 %	6	9.0	35 %	12	6.0	0 %	788
K35	2	3.5	86 %	2	9.6	33 %	12	n.a.		845
N9	2	5.5	18 %	2	*			n.a.		640
N10	1	3.5	29 %	2	*			0.0	[A]	580
U1	2	7.0	29 %	2	6.7	15 %	3	n.a.		701
U2	1	5.5	91 %	2	4.7	43 %	3	0.0	[A]	593
U3	1	11.5	9 %	2	5.0	35 %	3	3.0	[A]	788
U4	1	7.5	13 %	2	4.3	23 %	3	2.0	[A]	822
Average		8.0	63 %		9.1	36 %		4.3	10 %	768

A) Ovaries examined only once by one reader

B) CV calculated as if based on one reading to allow comparison in variability between mandible and bulla age estimates.

Table 2 Model parameters for von Bertalanffy growth model fitted to plots in Figure 6

Parameter	Mandible		Bulla	
		α		α
L_{MAX}	753.6	841.0	885.6	802.4
k	0.42	0.22	0.14	0.76
t_0	-4.56	-3.93	-5.10	1.12

Figures

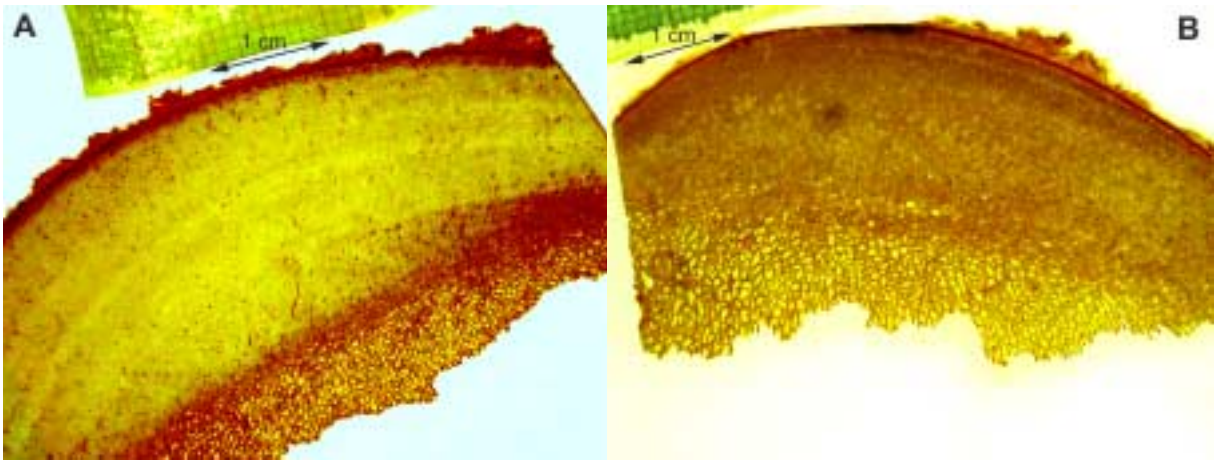

Figure 1 Images of right outer wall of mandibles from two female minke whales (A:K2 and B:K27). The color balance has been manipulated in both images to enhance the contrast of possible growth layers.

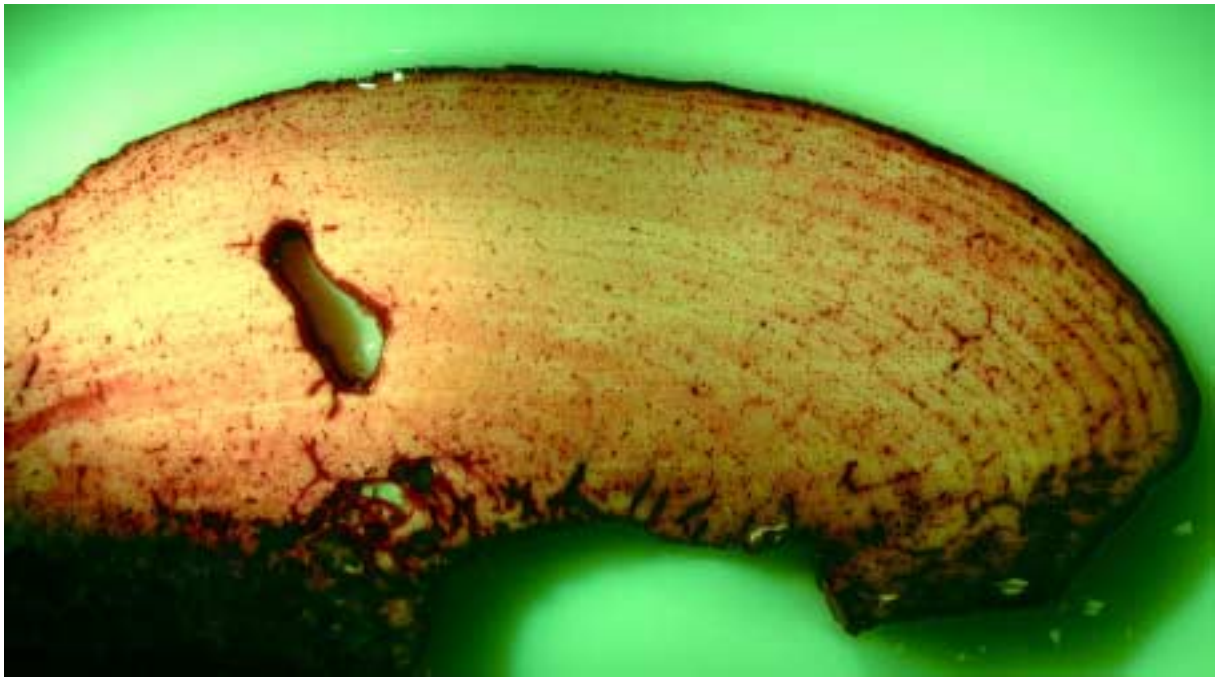

Figure 2 Color enhanced image of haemotoxylin stained section of sperm whale mandible.

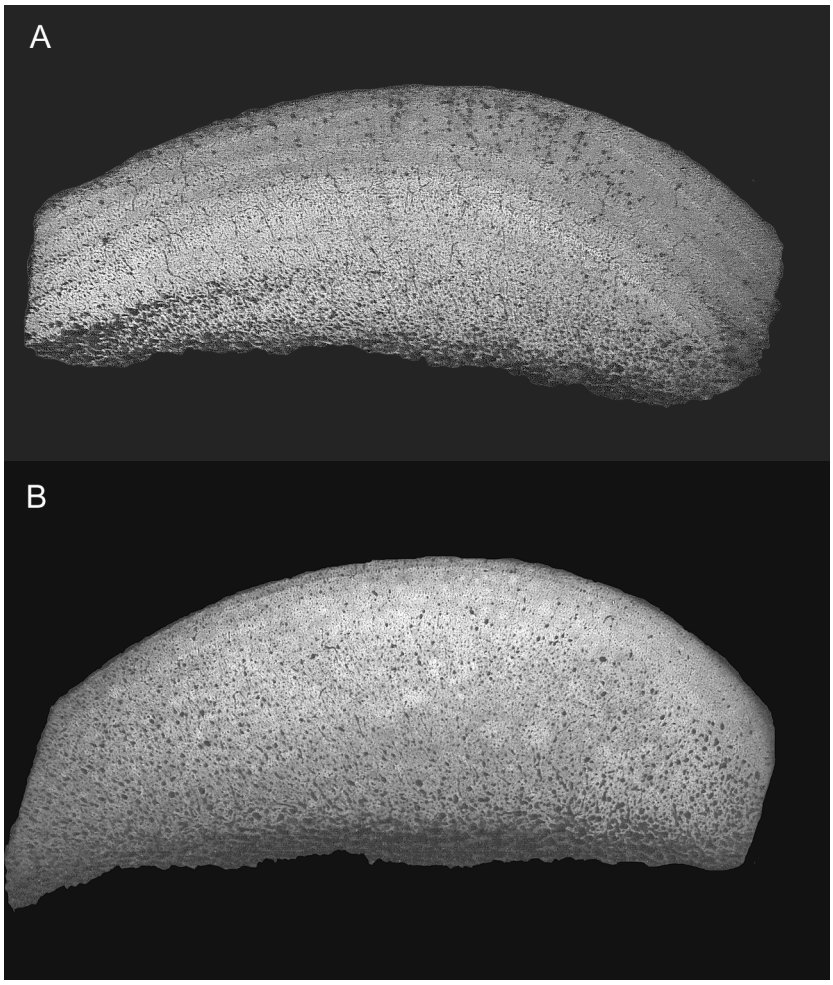

Figure 3 Scanned X-ray (mammographic equipment) of outer wall of right mandible from a female minke whale (K2). The image was photographed at 30 kV and 2.9 mAs settings using Kodak X-ray film. Growth layers can be clearly seen and followed through the length of the segment. Picture B is a similar image of whale K 27.

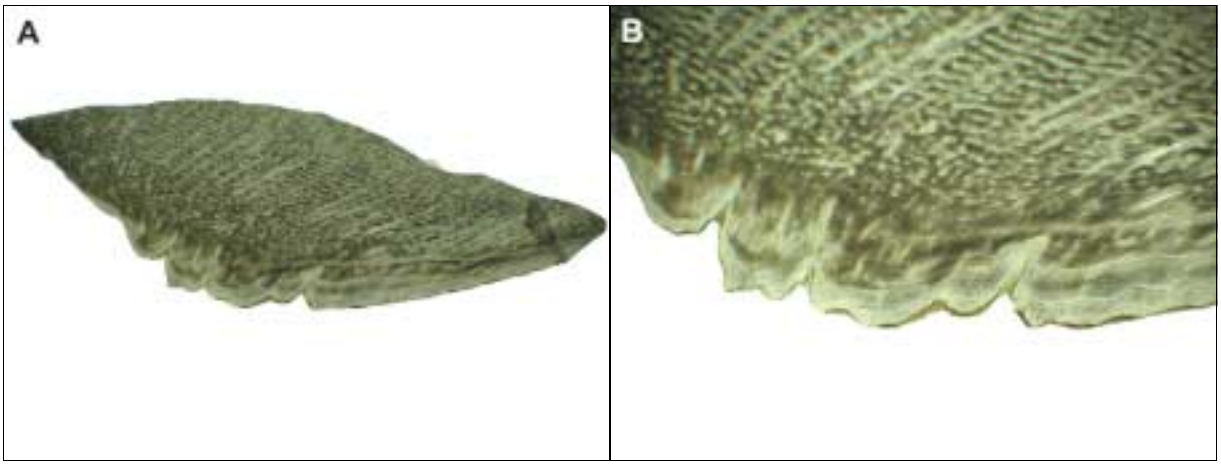

Figure 4 Images of whole bulla segment (A) and close up (B) of potential growth layers in the periosteal layer of right *bulla tympanica* of a female minke whale (K2).

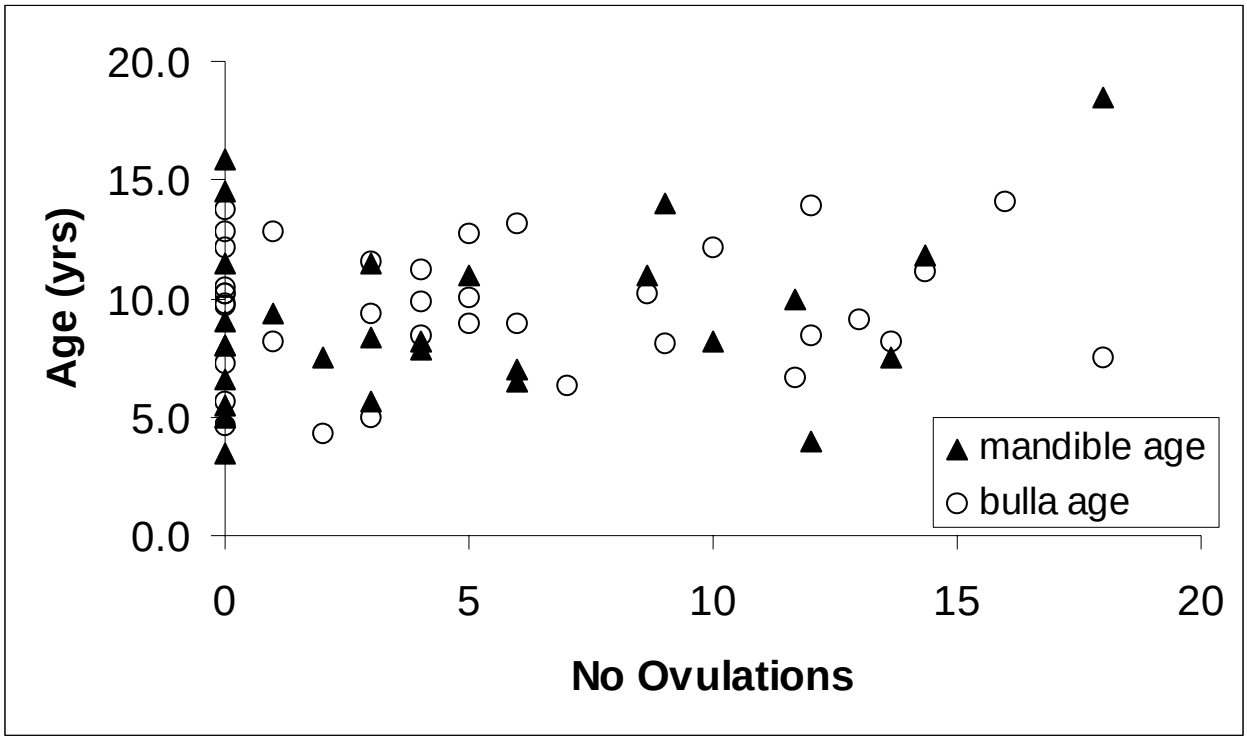

Figure 5 Plot of number of ovulations versus age estimates based on counting GLGs in the mandible wall, and in the periosteal layer of the tympanic bulla. Correlation was very low (<0.16) between age and number of ovulations for both plot and the regression line is therefore not shown.

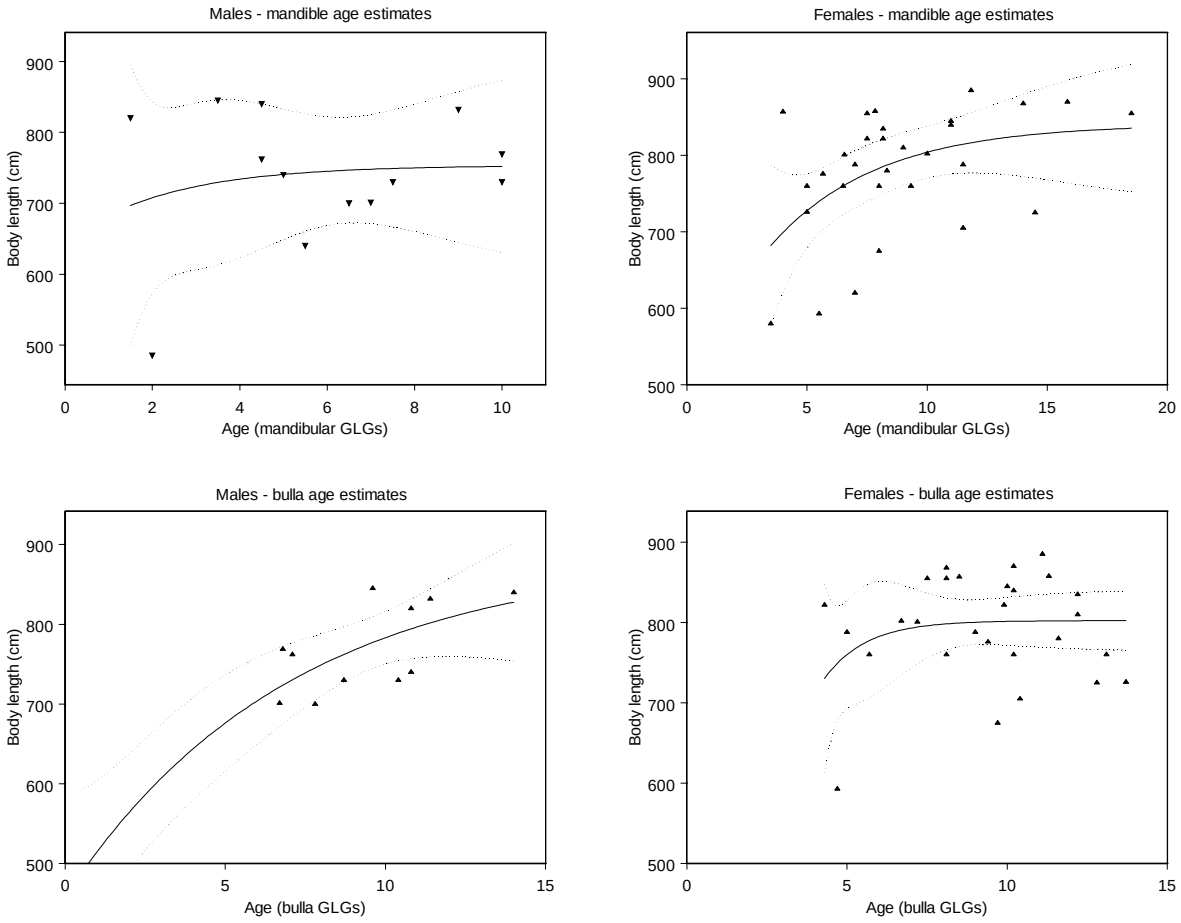

Figure 6 Plot of age estimates versus body length for male and female minke whales. A von Bertalanffy growth model is fitted to the plot in each figure.

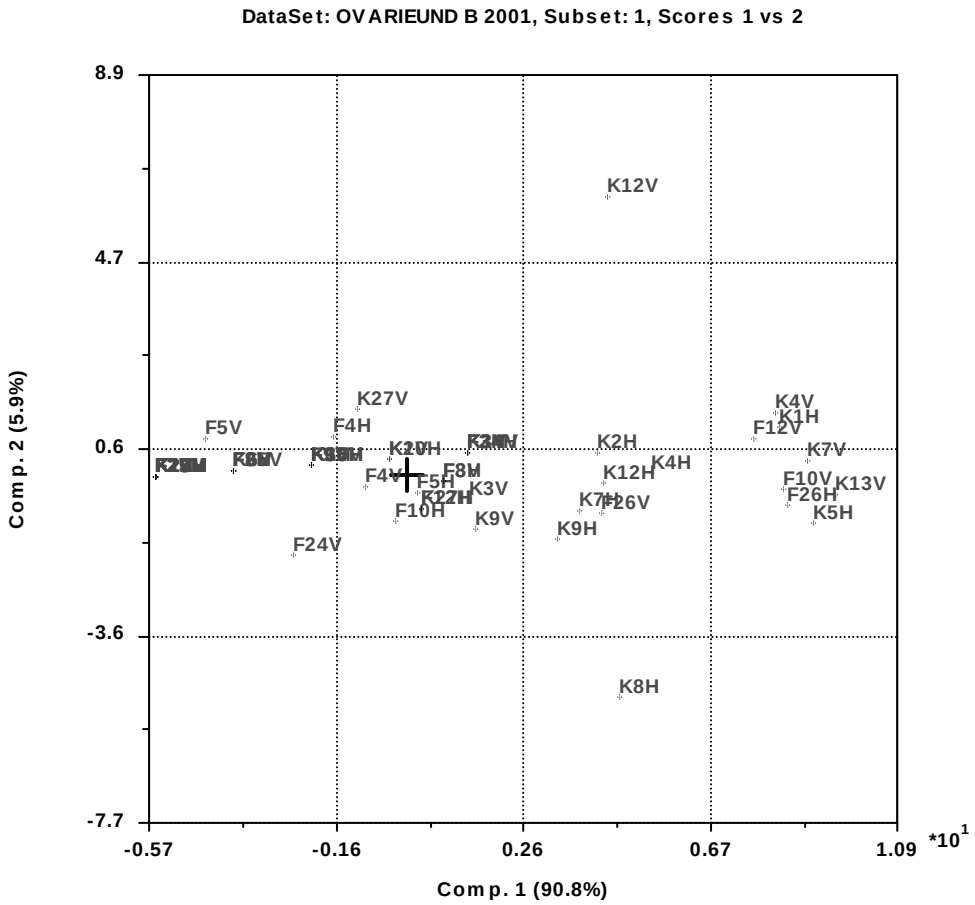

Figure 7 PCA plot of counts of ovarian scars (*Corpora lutea* and *Corpora albicans*) of single ovaries from female minke whales. Right and left ovaries are identified by a H (right) and V (left) following the whale id-number.