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Report of the workshop on age estimation in beluga: Beaufort, North Carolina, US 5-9 December 2011

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ABSTRACT

A workshop convened by C. Lockyer and A. A. Hohn to examine variation among readers in estimating beluga ages was held in Beaufort, North Carolina, US. Terms of Reference for the workshop included the following:

1. Provide a guide as to acceptable levels of accuracy and precision for age reading that will enable ages to be used in population models.
2. Conduct an inter-reader/laboratory comparison for calibration and standardization of age readings from GLG counts among all readers/laboratories.
3. Provide information on validation that will enable GLG counts to be translated to real age.
4. Produce a manual of guidelines for the preparation and reading of GLGs in beluga teeth.

Presentations by participants are abstracted here. Then we report on the processes used to compare sections, images, and interpretation, and generate guidelines for best practices in beluga age estimation. A comparative study quantified differences among readers and found that precision of experienced readers was good, higher than reported for other odontocetes. Participants agreed that counting GLGs using well prepared thin sections was preferred because they are simpler to prepare than stained sections and there was more agreement among readers compared to using half sections. Examination of teeth from captive beluga as both untreated sections and stained sections and did not clarify the reading of wild beluga teeth. This Workshop concurred with Workshop 1 (Tampa 26-27 November 2011) that interpreting one GLG as an annual record is irrefutable. Guidelines for best practices were developed.

INTRODUCTION

At its meeting in February, 2009, the Joint Scientific Working Group on Narwhal and Beluga (JWG) of the North Atlantic Marine Mammal

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Commission (NAMMCO) and Joint Canada-Greenland Commission on Narwhal and Beluga (JCNB) supported the initiative of a workshop to produce a report on age estimation in belugas and narwhal; in particular to examine the criteria used in counting Growth Layer Groups (GLG) in teeth of belugas with a view to producing a manual to guide researchers. The JWG also recommended that a Steering Committee (SC, chaired by Lockyer and including Hobbs, Hohn, and Stewart) work inter-sessionally to scope the problems and produce draft terms of reference for one or more workshops.

In 2010, NAMMCO noted the need to standardize ages using growth layers with new methods and recommended that a workshop on age estimation be held to review age estimation methods, and discuss how to standardize ages using GLG counts with new methods such as Aspartic Acid Racemisation (AAR). NAMMCO encouraged the SC to proceed with the workshop's organisation following the direction provided by the NAMMCO/JCNB JWG. The SC determined that two separate workshops were required: one on marine mammal age estimation with a special focus on monodontids and a workshop to address the specific issue of beluga age estimation based on teeth. Workshop 1 was held during the workshop sessions of the 19th Biennial Conference of the Society for Marine Mammalogy in Tampa, Florida, in the US (NAMMCO 2013, Hohn et al *submitted*). This report documents the proceedings of the second workshop.

To guide the focussed workshop, the SC developed the following Terms of Reference:

1. Provide a guide as to acceptable levels of accuracy and precision for age reading that will enable ages to be used in population models.
2. Conduct an inter-reader/laboratory comparison for calibration and standardization of age readings from GLG counts among all readers/laboratories.
3. Provide information on validation that will enable GLG counts to be translated to real age.
4. Produce a manual of guidelines for the preparation and reading of GLGs in beluga teeth.

The Workshop comprised three parts: a pre-meeting reading of images by several readers; the meeting itself at which reading results, methods, images, and sections were discussed; and a post-meeting reading of new images, the analysis and results of which are reported upon in a separate paper in this volume (Coggins *in prep*.). The meeting was held at the Beaufort Laboratory of the US National Oceanic and Atmospheric Administration (NOAA) in Beaufort, North Carolina, from 5-9 December, 2011 (Appendix 1 – Agenda; Appendix 2 - List of participants). It consisted of general discussions with short presentations, laboratory work to examine images and physical specimens with comparisons to pre-workshop reading results, and further discussions about what had been learned during the laboratory session. A background document (Stewart 2012) provided to the workshop included the basic biology of tooth development and growth of beluga teeth. Readers are specifically directed to Stewart and Stewart (2014).

BEAUFORT WORKSHOP PRESENTATIONS

Participants from labs currently engaged in beluga age estimation presented brief oral summaries of their protocols, which are summarized below (see Appendix 1a-g for details). When possible, most participants chose one or more large straight teeth, usually from the mid-posterior area, and most participants used one side of the jaw routinely. Two labs used milling machines, the others a low speed saw, but all used diamond wafering blades. Only Alaskan labs routinely used a 2-blade gang to cut a section in a single pass. Most labs chose the optimal line through recurved teeth, although in one lab highly curved teeth were bisected and the two parts sectioned, each along its own optimal line. Most sections were stored wet and all were viewed wet, using a dissection microscope. Transmitted light was used commonly while reflected or polarized light was used for added clarity of problematic sections. In most labs, the sections were read multiple times, sometimes by multiple readers. One lab routinely prepared stained sections and another used this as a supplementary technique when required to clarify ultra-structural details.

3 or 5 Blind Replicates?

Rob Stewart reported that, since 1993, Department of Fisheries and Oceans, Canada (DFO), Central and Arctic Region (C&A) have been estimating beluga ages by reading thin sections in 3 to 5 blind replicates, meaning 3-5 reading-sessions would be used to produce an age estimate without reference to previous age estimates. The reader would stop when 3 identical readings were recorded or when the section had been read five times. Outliers were removed based on Maximum Normed Residual test (Snedecor and Cochrane 1967) and the final estimate based on the median. Of 1,788 teeth from Arctic Canada, 1,244 needed 5 readings, so there is potential time-saving if 3 readings will suffice. Stewart compared final age estimates based on 3 and 5 readings of the same teeth. Three readings (Final3) accurately predicted the age relative to 5 readings (Final5) (Final5 = 0.19 + 0.999*Final3, $R^2 = 0.995$, n=1,788) and the age distribution did not differ by method (age distribution $\chi^2 = 1.67$, p=0.999). However, the Average Percent Error suggested researchers may want to use 5 readings if their question involved very young or older age classes (Fig. 1). For about half the age classes, errors were small (<2.5%) but young (0-4) and old (50-54, 55-59, 65+) age classes had larger errors.

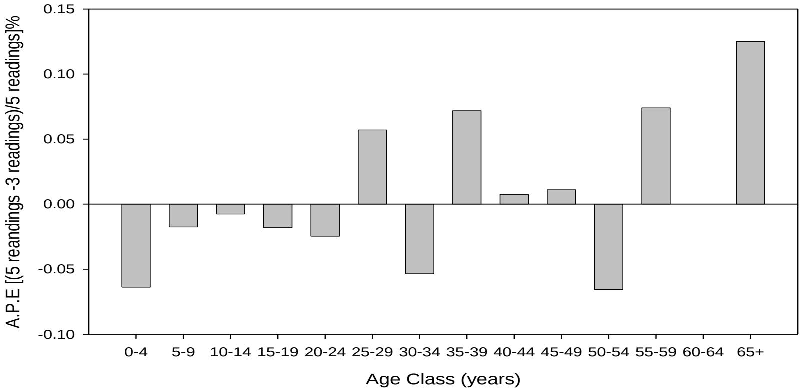

Fig. 1. Average Percent Error (A.P.E = ((Final5-Final3)/Final5)x100%) by age class. Negative values indicate 3 readings placed more whales in that age class than did 5 readings.

Inter-lab Comparison Using Physical Specimens and Images at DFO
Rob Stewart reported that two DFO Regions (C&A and Quebec) initiated a comparison of aging methods. Counts in teeth from C&A were made by examining thin sections. Counts in teeth from Quebec were from ½-tooth sections viewed with reflected light and also digital images of these sections. The first comparison used the preferred method of each Region for 39 belugas from the Beaufort Sea stock. Images were prepared and ages estimated using a ½-tooth and reflected light (see Morin Appendix A3-d) using teeth from the right mandible, second or fifth tooth from the mandibular symphysis (MNR2, n=39) or MNR5 (n=38); ages were estimated by Reader 1. These estimates were compared with thin sections (see Appendix 3 A3-c) of various remaining teeth MNL2 (n=17), MNL5 (n=18), MNL3 (n=2), MNL4&6 (n=1 each); ages were estimated by Reader 2. A subsequent comparison used thin sections of teeth from 30 whales which were read according to the C&A protocol (Reader 2) then sent to Quebec where images were prepared and counts made using these images by a third Reader.

Comparisons of final age estimates based on images of ½-tooth and thin sections from different teeth indicated no significant differences (Image = 1.42 + 0.94*Section R^2=0.96, n = 39; Intercept not significantly different from 0; Slope not significantly different from 1, Wilcoxon Signed rank test not significant (p=0.65), Image median = 29, Section median = 30). However, when the same material was examined as an image rather than as a physical specimen, the median ages differed significantly (Wilcoxon Signed rank test: p=0.003, Image median = 21, Section median = 25.5) and the regression fit less well (Image = -1.26 + 0.93*Section, R^2=0.85) although the intercept was not significantly different from 0 and the slope was not significantly different from 1. Results are confounded by the logistic requirement to use different teeth in the first comparison and to change readers of images in the second, but suggest image reading might under-estimate ages. Future studies should reduce the number of variables by using the same teeth, the same readers and either the same stock or a larger sample to test for stock differences.

Inter-laboratory comparison using physical specimens and images for this workshop

Aleta Hohn and Karen Altman introduced the intent and design of the pre-meeting experiment. The general objectives of the pre-workshop experiment were to collect a pre-treatment (*i.e.* pre-workshop) sample of age estimates to measure reader performance to compare to a post-treatment (*i.e.* post workshop) sample, and to examine the pre-treatment and post-treatment within- and between-reader aging precision.

The overall design of the experiment included (1) reading of tooth images prior to the workshop by a subset of participants, (2) discussion of results at the workshop for the purpose of comparing (a) counts among readers, and (b) counts from images to counts from the physical specimen, and, then, (3) the reading of tooth images after the workshop to evaluate whether the workshop discussions resulted in increased precision and agreement relative to readings prior to the workshop. Readers used their experience to highlight areas of agreement and disagreement in interpreting lines, fostering informed discussion at the workshop.

Prior to the workshop, tooth samples and images were submitted from 60 different animals representing stocks from 6 locations, including animals in captivity, and comprising individuals very young to very old; 8 were from Point Lay, Alaska; 22 from the Gulf of St Lawrence, Canada; 4 from Dikson, Russia; 14 from Greenland; 6 from Cook Inlet, Alaska; and 6 had been captive (primarily originally from the Churchill area of Canada). These samples included three different preparation methods: untreated half tooth sections (12), untreated thin sections (28) and decalcified, histologically sectioned and stained (20) thin sections. Stained sections mounted on slides and untreated thin sections (placed between two slides while moist) were scanned using a Nikon Super Coolscan 2000™ (http://www.nikonusa.com/index.page) slide scanner, and saved both as high-resolution jpeg and tiff images. Half-tooth specimens originating from St Lawrence, along with some of the samples from Greenland, were received as images to be included in the study. Each of the 60 images was replicated three times, for a total of 180 images, to allow for measures of within-reader variation. The images were arranged in three replicate sets of 60, randomly sequenced within sets 2 and 3 to ensure image sequence changed among sets, and then given a unique code ranging from

001-180. The sequencing and coding was conducted by an individual (KA) not participating in the reading experiment.

Coded images were distributed to participants along with a spreadsheet on which to record their age estimates and comments. There were four specific instructions provided: 1) Provide your best estimate of age for each image; 2) Mark the GLG you counted on the images (with ink on hard copy is OK) and bring marked copies to workshop; 3) Read in the sequence provided; 4) Do not return to previously read images once a reading is done. Readers were given approximately 3 weeks to complete their readings and submit their age estimates. Hohn and Lockyer also scored images on GLG distinctiveness, tooth preparation quality, and image quality. Completed age estimates from the seven readers were compiled in an Excel™ spreadsheet with ages from each replicated image listed together along with other associated data. An additional spreadsheet was created to capture the reader's comments on each sample.

Inter-laboratory comparison of physical specimens and images: statistical analysis

Lew Coggins presented the results and conclusions from analysis of the readings prior to the workshop. A set of graphical procedures (*e.g.* Figure 2) was used to highlight differences and similarities among estimated ages from the various readers. In general, mean age was similar among readers with the exception of Reader 2 who tended to consistently estimate greater age than the other readers. Additionally, Reader 3 demonstrated higher precision among replicated readings than other readers. The data were also analyzed using a multinomial logistic model to explore which factors (reader, sample preparation, and stock) best predicted the probability that a modal age would be found among 3 replicate readings. The results of this analysis suggested that the probability of obtaining a mode was significantly higher for Reader 3 ($p<0.01$) than for the other readers. Additionally, the probability of obtaining a modal age was significantly higher for untreated samples than for half tooth or stained samples ($p<0.01$). A caveat on these findings was that the design was not well balanced among stocks and sample treatments. In particular, no stocks contained all sample preparation types and some stocks (Point Lay, Dixon, and Captive) used stained sample preparation nearly exclusively. Additionally, only the St Lawrence stock contained half-tooth preparations. Thus, it was difficult to singly evaluate the effect of sample preparation or stock.

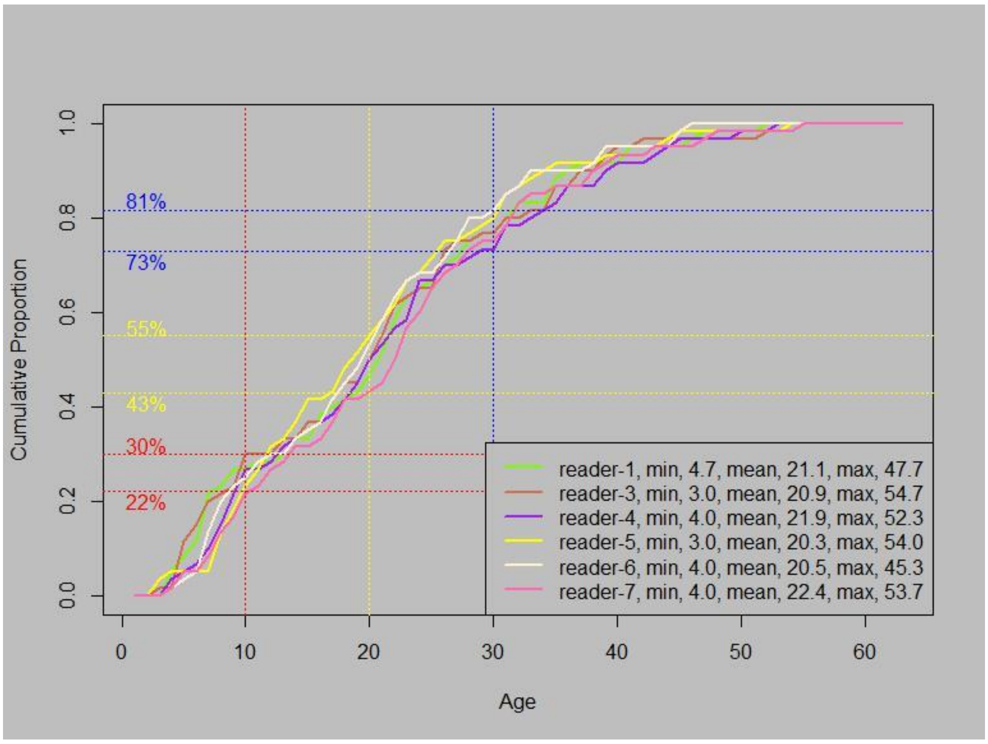

Fig. 2. Similarities in age composition among 6 of 7 readers (Reader 2 excluded) in the pre-workshop reading experiment.

Perhaps most notably, estimated age compositions among the 6 of the 7 readers were nearly indistinguishable (excluding Reader 2) suggesting that while readers may have differed in the age estimated for particular whales, the age composition of the sample (arguably the information most useful to researchers and managers) would not have varied much among readers. For example, even among the most disparate readers the difference in the estimated sample proportion less than age-10 (22% versus 30%) and less than age-30 (73% versus 81%) differed by only 8%. This observation raises the question: what level of age estimation precision is required by the users of whale age data (*e.g.* researchers conducting inference based on age data or managers contemplating alternative conservation strategies based on age data)? While no criteria appear to exist to evaluate the procedures currently used to estimate whale age, these results should prompt examination of whether expending effort to further increase aging precision is warranted considering the users of whale age data. The aging precision observed in this study was slightly better than that published for sperm whale (*Physeter microcephalus*; Evans *et al*. 2002) and spotted dolphin (*Stenella attenuata*; Reilly *et al.* 1983), possibly providing another comparison useful to evaluate the aging precision of beluga whale.

For the post workshop comparison (see Coggins *in prep*.), the number of variables will be reduced by limiting analysis to thin sections to standardize preparation technique and by scanning the sections to create digital images at one location to standardize image quality. All specimens selected will have putative ages roughly ≤ 30 years of age to focus comparisons on the most biologically sensitive portion of the population. Also to reduce the number of variables, only three stocks, all with readily available thin sections, will be examined: Hudson Strait (combined stocks), Cook Inlet and West Greenland (replaced with Baffin Bay samples if necessary). Samples will be selected to be representative of the overall collection, including distinctiveness of GLG and tooth characteristics, and proportional to their original contribution, 10-20 samples from each stock.

RESULTS FROM LAB SESSION

The group viewed projected images of half-teeth, untreated thin-sections, and decalcified and stained thin (*i.e.* <0.01 mm) sections to discuss areas of agreement and areas for further discussion. This review resulted in three specific hypotheses regarding using the images as adequate substitutes for the physical specimens:

> Hypothesis 1: H_o deviations of 1-2 GLGs among the experienced readers (pre-experiment) were largely the result of ambiguity in the first (neonatal line (NNL) and fetal dentine (FD) are absent) and/or last GLG which could not be resolved by examining the physical specimen.
>
> Hypothesis 2: H_o deviations of 1-2 GLGs in images of half-tooth sections were the result of ambiguity in the first and/or last GLG of the polished tooth surface as it appeared in the image, which could not be resolved by examining the half-tooth.
>
> Hypothesis 3: H_o large deviations in GLG counts that could not be attributed to the previous factors were not due to ambiguity in the interpretation of GLGs.

The group compared the circulated images to the physical specimen using appropriate microscopic magnification and lighting. Examples were selected (Table 1) to address the hypotheses above.

Table 1. Examples of specimens evaluated to determine whether images were adequate substitutes for physical specimens under three hypotheses.

H_o	Coded ID Number	Source Location	Specimen Number	Sample Preparation	Pre-Workshop Image Capture	
					Method	Light
1	28	Cook Inlet	10_07_07 L5	thin section	slide scanner	transmitted
1	15	Greenland	2327_V4	stained section	slide scanner	transmitted
1	20	Cook Inlet	11_12_03 L1	thin section	slide scanner	transmitted
1	19	Cook Inlet	10_15_07 R6	thin section	slide scanner	transmitted
2	40	Gulf of St Lawrence	DI-127-1986H	half section	flatbed scanner	reflected
3	11	Russia	Dikson 3	stained section	slide scanner	transmitted
3	24	Gulf of St Lawrence	DI-104-2006H(5)	half section	flatbed scanner	reflected
3	22	Gulf of St Lawrence	DI-102-2002H	half section	flatbed scanner	reflected

Hypothesis 1

H_o deviations of 1-2 GLGs among the experienced readers (pre-experiment) were largely the result of ambiguity in the first (NNL and FD absent) and/or last GLG which could not be resolved by examining the physical specimen.

Material Examined and Results

ID#28 - (Fig. 3.)

The initial age estimate was 24-27. When the section was evaluated under the microscope during the workshop, consensus was that in reflected light the section was better than the image for identifying the first GLG (no NNL or FD present). However, there was some uncertainty about the last GLG due to a possible edge effect. Using transmitted light, it was agreed the last GLG was much clearer (no change in first GLG) and the consensus age was 26.

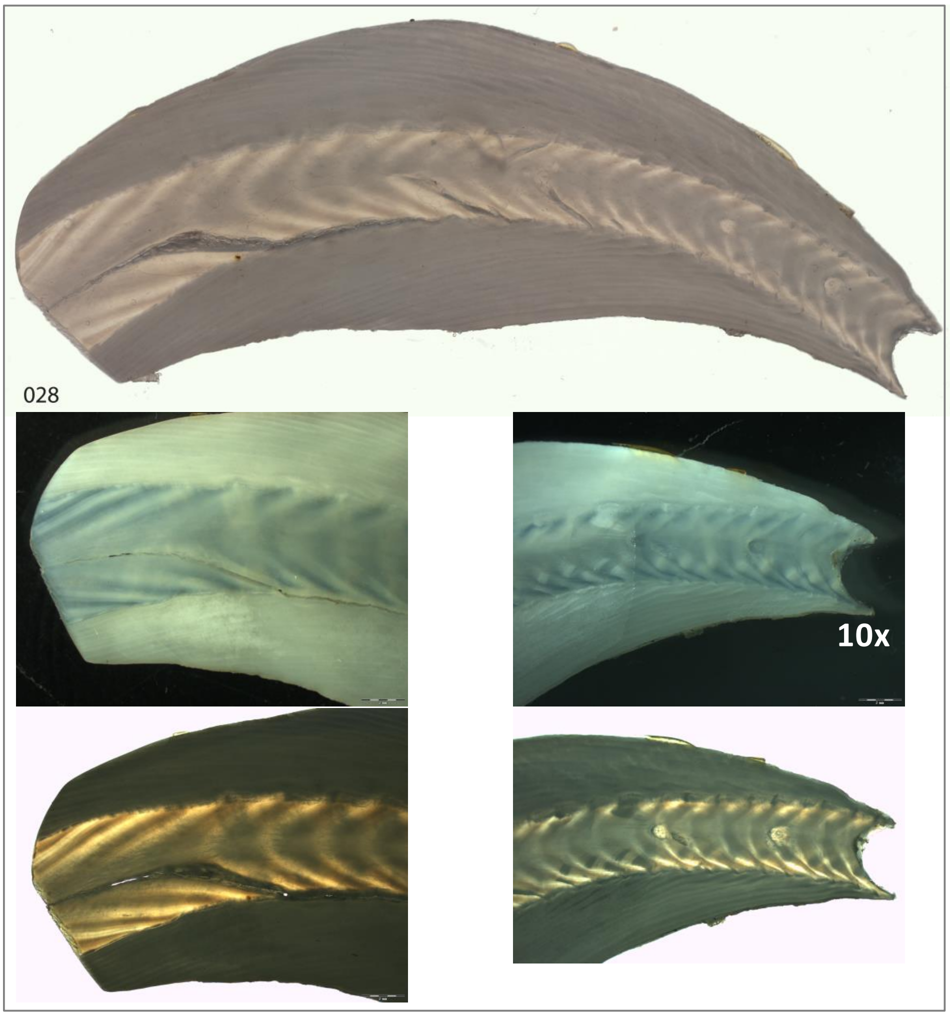

Fig. 3. The slide-scanned image used in the reading experiment along with higher magnification images of the crown and root ends taken using a dissection microscope, with both reflected (upper pair) and transmitted light (lower pair).

ID#15 (Fig. 4.)

The initial age estimate was 39-43. Consensus was that the image and slide were equivalent. Participants moved between dentine and cementum using the slide at different magnifications and lightings; the image was not manipulated in a similar manner. No consensus was reached on age.

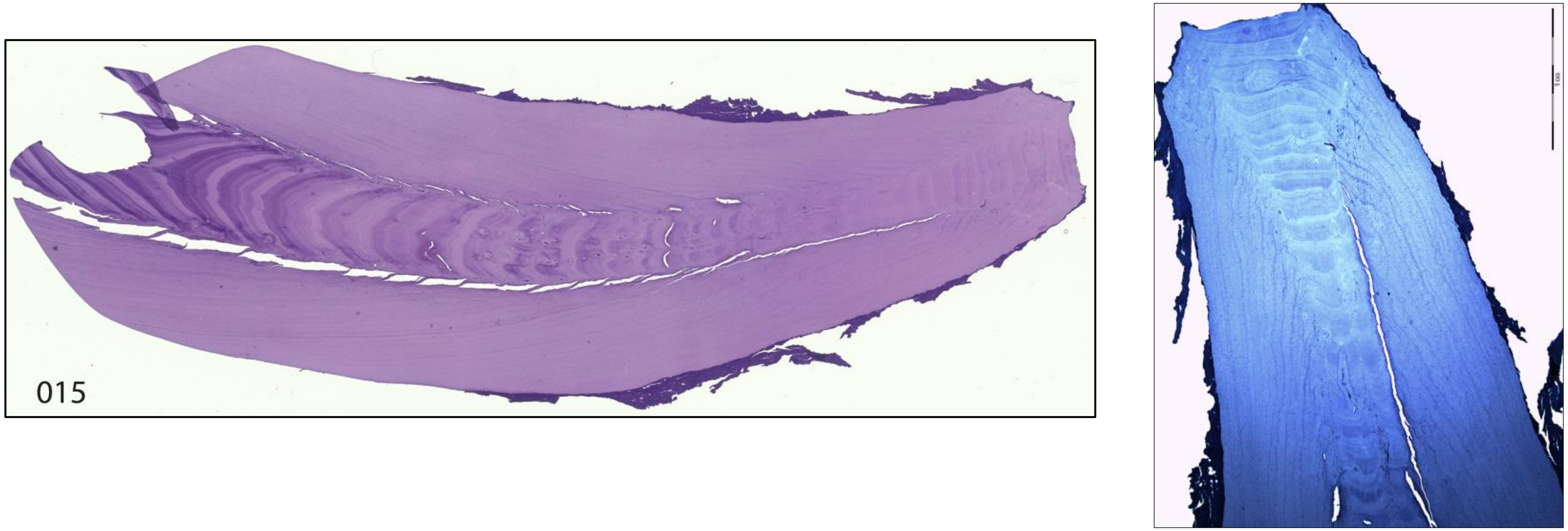

Fig. 4. The image of the whole section from the slide scanner (left) included in the reading experiment. The higher magnification image of the root end was taken using a compound microscope (right) to illustrate the small GLGs not readily visible in the scanned image.

ID#20 (Fig. 5)
This sample was given an initial age estimate of 18-19 years. Consensus was that the image was less clear than the section for evaluating the last GLG. Consensus age was 18 because the last GLG was not fully formed.

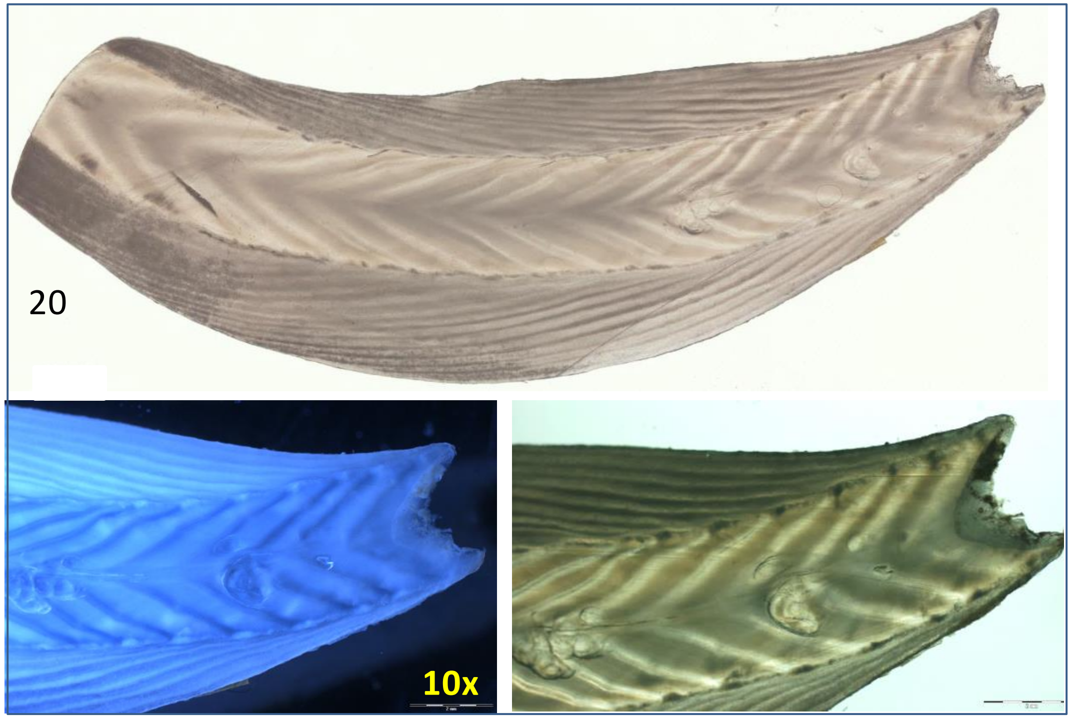

Fig. 5. The slide-scanned image used in the reading experiment along with higher magnification images of the root end taken using a dissection microscope, using both reflected (left) and transmitted (right) light.

ID #19 (Fig. 6)
This sample was given an initial age estimate of 28-29 years. Use of reflected light on the physical section at lower power was unacceptable because it emphasized tool marks from the sectioning process, obscuring the GLGs. With transmitted light, notwithstanding problems getting a uniform bright field, the medial surface showed a clear GLG-1 on both sides of the crown while in the image it was seen only on one side of the crown. With the physical specimen, the magnification can be increased to provide better resolution of questionable areas, such as the whether there is prenatal dentine or the first GLG is present. Consensus was that the section was clearer than the image and the age was 28, because the last GLG was not completely formed.

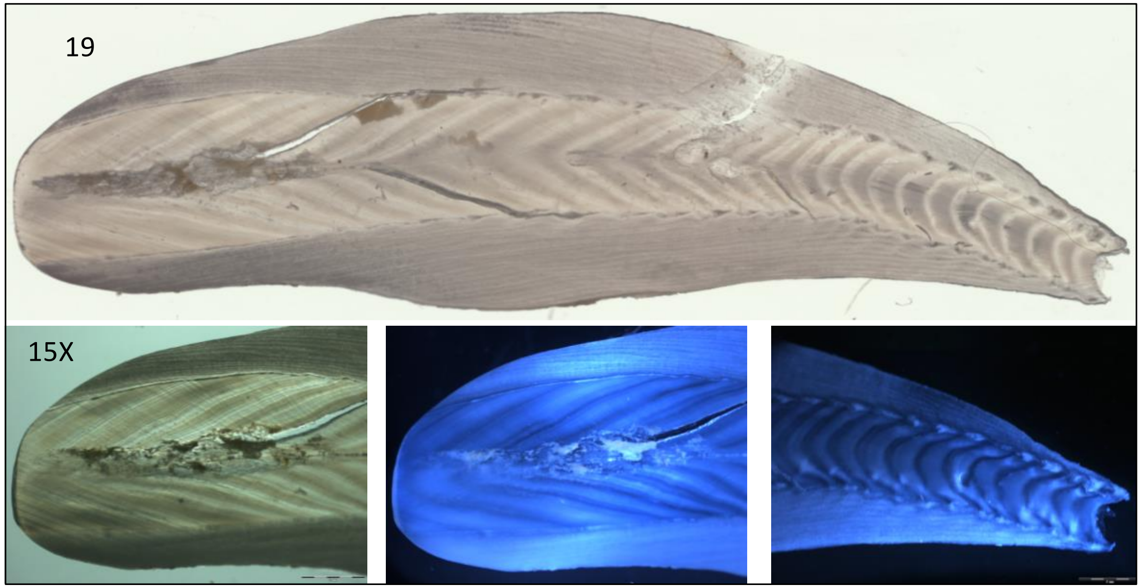

Fig. 6. The scanned section (top), transmitted (left) and reflected light (center) images using a dissecting microscope illustrating differences in detectability of GLG-1, and equivalent reflected light image of the root (right).

The group then discussed adopting a convention that if prenatal material is missing, one could include a partially formed final GLG on the assumption that the count would already be a minimum and counting a full GLG at the beginning might produce a better estimate. There were two sources of concern: 1) this practice then introduces a small positive bias which would complicate the statistical analysis; and 2) because the GLGs in the first few years are rather elongated it is possible that the partial GLG counted at the edge of the tooth may represent a complete age record so that the added GLG would result in an over-estimate. An example would be some of the teeth from Cook Inlet where the NNL/FD appeared to be missing when the GLG count for the tooth was low (*e.g.* 10) and the initial GLG were elongated V-shapes. Further discussion focussed on the possible stock-specific nature of this issue since tooth growth and wear is probably dependent on diet and annual growth cycle which vary by stock. Further research is required, but readers need to be explicit about how they counted the first and last GLG.

Conclusions:

- Hypothesis is rejected: examination of the physical specimen allowed deviations to be resolved.
- When prenatal material is absent, the thin section specimen is preferable to images for identifying the first GLG.
- The thin section specimen is preferable to images for identifying the last GLG formed.
- Best practice would use images for convenience and documentation but refer to the thin-section to resolve the ends of the teeth. It would also be helpful to take higher magnification images of the tip and root to help resolve those GLGs.

Hypothesis 2

H_o small deviations in readings of half-tooth images were the result of ambiguity about the end of the polished surface, which could not be resolved by examining the half-tooth.

Material examined and Results

<u>ID#40 (Fig. 7)</u>

This sample was given an initial age estimate of 29-32 years. Consensus was reached on the first GLG but the root end was less clear. Consensus age was 30 and most agreed that they preferred to use the half-tooth over the image because the former allowed more variety in lighting and angles of viewing, and, hence, was more helpful for resolving confusing lines near the root. Some image analysis systems are available to alter the contrast/brightness of the image and it would be possible to re-image regions of the 1/2-tooth that were not well represented, but this was not fully explored.

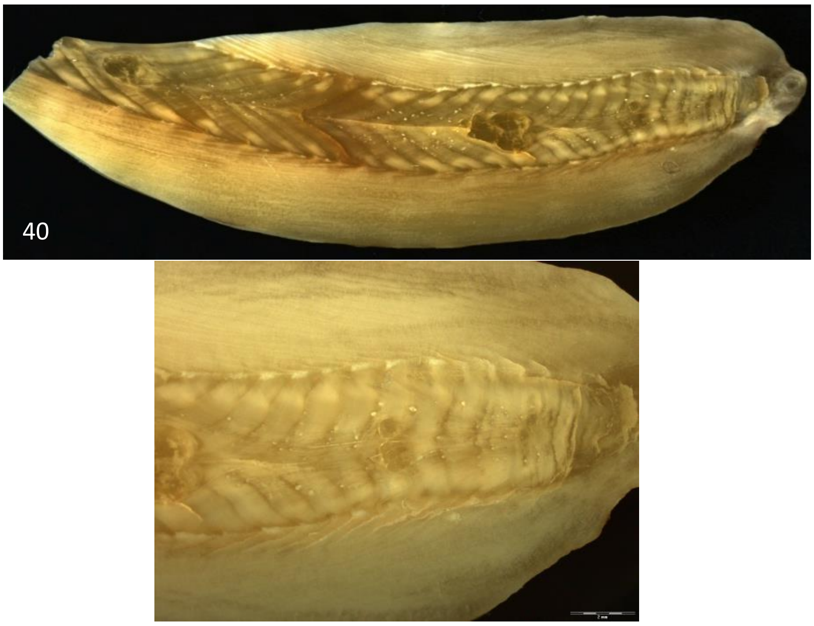

Fig. 7. Image of tooth half-section (top) used in the reading experiment and higher magnification image of the root taken with a dissecting microscope and reflected light (bottom).

Conclusions:

- Hypothesis is not rejected: examination of the physical specimen did not resolve the source of deviations of 1-2 years.
- All agreed the image and half-tooth would produce similar age estimates but the physical specimen was more easily manipulated for optimising lighting and contrast.
- Good uniform image quality was more difficult with the half-tooth because of the need for even, unidirectional reflected light.

Hypothesis 3

H_o large deviations in GLG counts that could not be attributed to the previous factors were not due to ambiguity in the interpretation of GLGs.

Material examined and Results

ID#11

This sample was given an initial age estimate 6-17 years. The stained section was examined using a binocular compound microscope. The NNL was equally identifiable in both the image and slide and did not contribute to the range of estimates. No one counted a cluster of lines near a large pulp inclusion, eliminating this apparently obvious source of deviations (*i.e.* accessory lines). Consensus was that there were poorly defined lines both mid-tooth and near the pulp cavity that were difficult to count. Participants manipulated the slide of the mounted stained section under the microscope and used both cementum and dentine to arrive at an age range of 10-11. The image was not manipulated as much as the slide but consensus was that the slide was a better source. There was also agreement that for this animal, if possible, another tooth should be sectioned.

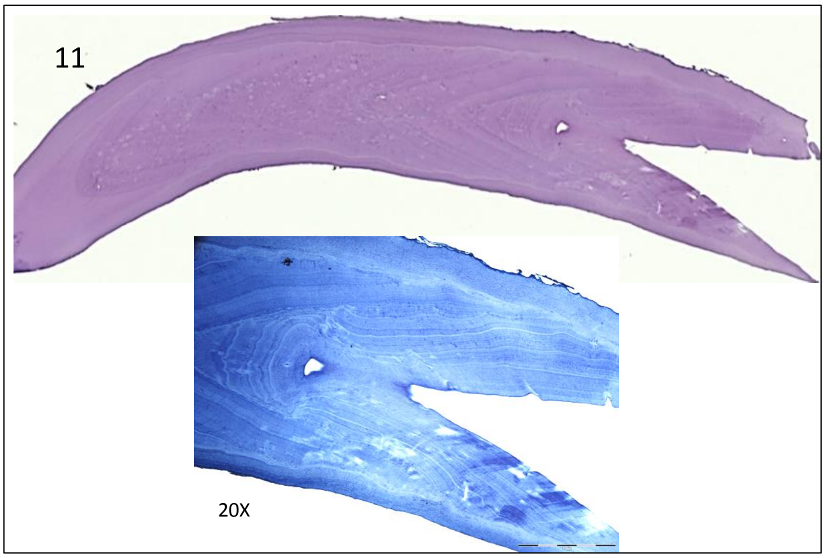

Fig. 8. The scanned image used in the reading experiment (top) and a higher magnification image of the cluster of lines around the pulp inclusion. The neonatal line and prenatal zone are clear in the crown of this tooth.

ID#24 (Fig. 9.)

This sample was given an initial age estimate of 6-26 years. The thin section was viewed using a dissecting microscope. Estimates made during the workshop using the thin section ranged from 6 to 16 years. The participants agreed that this was a challenging tooth, but by relying heavily on cementum, consensus was that there were 8-10 GLGs. It was noted that not all microscopes available were suitable for counting cementum lines. Two participants used polarized light to view this section and both obtained counts of 10 GLGs. Overall, the main source of deviations appeared to be the inclusion of accessory lines in the initial counts.

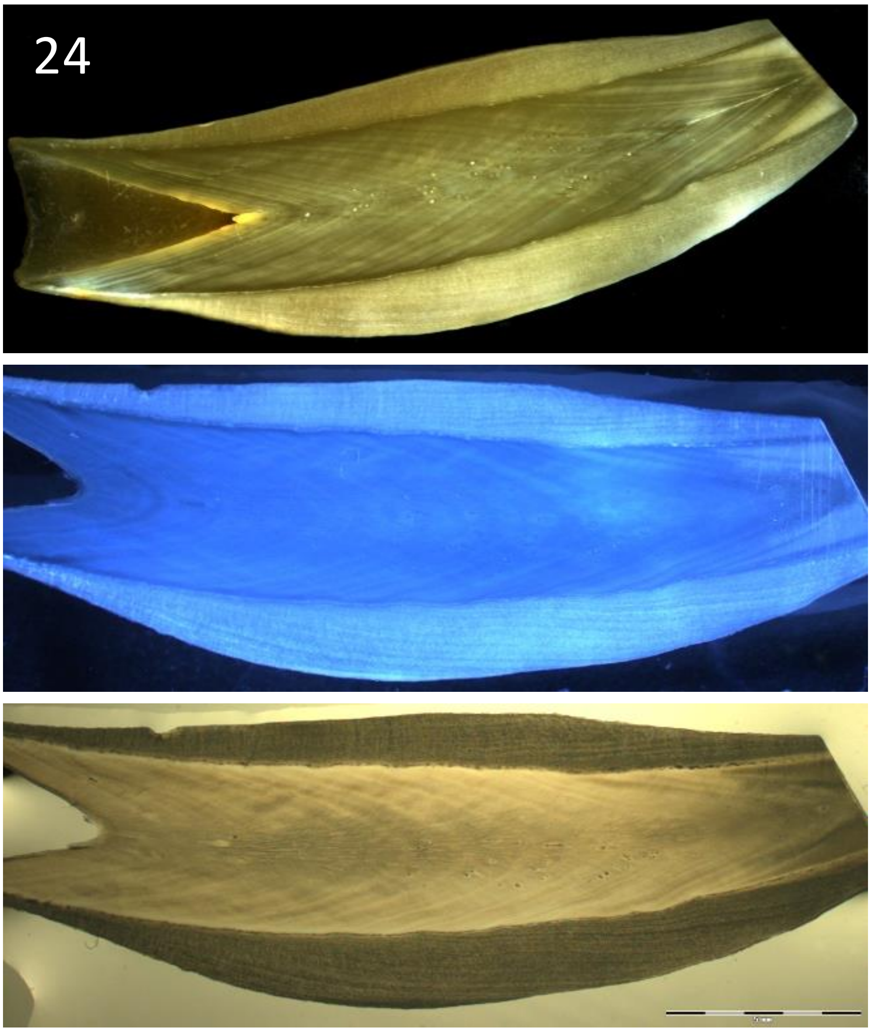

Fig. 9. Images of scanned half section used during the reading experiment (top) and thin sections imaged using dissecting microscope with reflected (middle) and transmitted (bottom) light.

<u>ID#22 (Fig. 10)</u>

The initial age estimate was 27-56 years. The thin section was viewed using a dissection microscope. This tooth had a completely closed pulp cavity and several pulp stones, both of which made identifying GLGs difficult. Using the thin section, consensus was that there were 54-55 GLGs, relying heavily on cementum lines in areas where the dentine was unclear. The thin section allowed 47 dentinal GLGs to be counted whereas the image became indistinct at about 42 GLGs. A suggestion was made that in this case a very thin (*e.g.* 10 µm) stained with toluidine blue specifically for cementum lines might be useful.

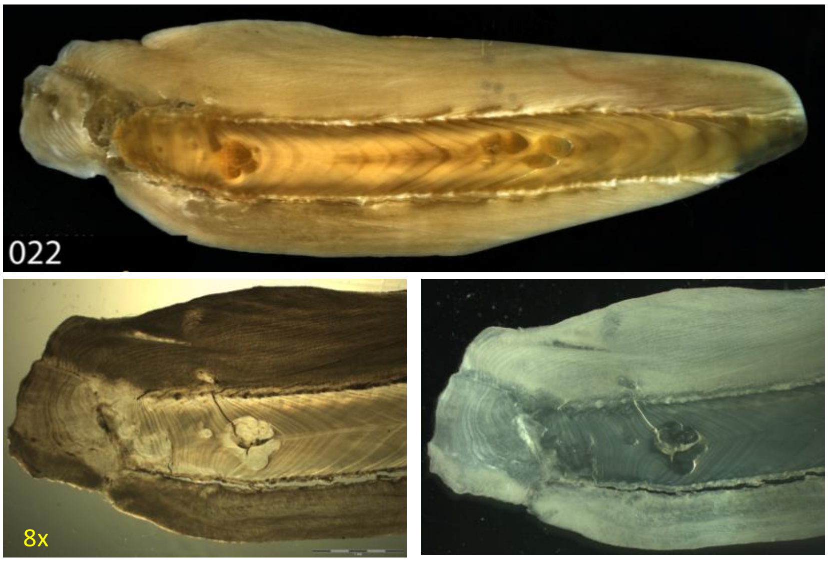

Fig. 10. Image of half tooth used during reading experiment (top) and higher magnification images of the root end of thin sections taken with transmitted (left) and reflected (right) light.

Discussion

The group refrained from an 'if and only if' statement of when an optical band in a tooth constitutes a GLG, an accessory line, or an artifact. Instead, it acknowledged that some sections are exceedingly difficult to read and interpret and offered a series of approaches to enhance GLG visualization in less than ideal sections:

1. Use untreated thin sections whenever possible.
2. Adjust the microscope to maximum GLG resolution by manipulating the light source (reflected, transmitted, polarized, diaphragm settings), magnification and focus (sometimes being slightly out of focus helps, as this may minimize the prominence of accessory lines).
3. Examine the cementum for legible GLG and the dentino-cemental nodes for patterns.
4. Trace lines up and down the tooth to move to areas of greater clarity.
5. Print an image to help keep track of landmarks.
6. Use land marks in adjacent sections to move between legible areas of two sections.
7. Prepare a new section, especially if problems arise from an off-centre section.
8. Section a different tooth which may be straighter.
9. Do not guess the count, and admit 'no data' if a count cannot be agreed.

Conclusions

- Hypothesis rejected: the largest deviations among readers appeared to be related to how well lines were defined.
- There is no easy solution.

Table 2. Summary of various methods of preparing beluga whale teeth for age estimation.

Preparation Type	**Thin section, unstained, transmitted light**	**Thin section, unstained, reflected light**	**Histological section, stained 25 µm**	**Half-tooth, unstained**
Storage	• Dry or in glycerin, H_20, or ETOH solution	• Dry or in glycerin, H_20, or ETOH solution	• Sealed microscope slide • Dry, cool, dark space	• Very stable, can be stored wet or dry. If in glycerin, re-polish to remove glycerin residue
Archive	• Dry - may crack • Wet - may lose detail after a few years	• Dry - may crack • Wet - may lose detail after a few years	• Some stains fade over years (especially if mounted in glycerin rather than with resin); must be re-stained if this occurs	• Very stable can be stored wet or dry • Re-polish to read
Viewing	• Dissecting microscope, in water or wet with option of polarized light	• Dissecting microscope, in water or wet with light source from variable angles	• Compound microscope or with camera on computer	• Dissecting microscope, in water with black background, light source from variable angles
Image assessment	• Transmitted light better resolved GLG-1 and the completeness of the last GLG than the images	• Sections with transmitted light better resolved GLG-1 and state of completeness of the last GLG than images of those sections	• No direct comparisons of image and physical specimen made because for the group analysis, the physical specimen was displayed on a computer monitor, mimicking the image	• There were too few sections with matching and half-teeth available to test.
Pros	• Easy to prepare • Easily viewed • Easily manipulated • Most precise readings • Allows use of various light sources		• High level of ultrastructure detail • Slides are easily stored	• Easy to section • Easily viewed • Easily manipulated
Cons*	• Storage of sections in vials can become voluminous • If stored in alcohol, vials MUST be inspected and topped-up every couple of years • Alcohol storage must meet applicable safety standards		• Labour intensive • Requires additional equipment (freezing microtome/cryostat) • Transmitted light only • Requires decalcifying agents, stains, etc. • Relatively costly	• Less precise • Must be polished before each viewing • Cannot be viewed from flip-side • Reflected light only • Storage can become voluminous

*For all sectioning methods, it is difficult to section along the central line in teeth that curve in two planes.

Captive beluga whales

Teeth were available from two animals. The group noted the value of these samples as examples of known-history animals but acknowledged that tooth development depends on diet and annual feeding cycle of the individuals so that GLGs might not be as distinct as in free-ranging animals.

ID#55 (Fig.11)

Sample ID#55 (SW_DL_7903), was captured at 262 cm and held for 7 years and 11 months. A stained section was examined, and was given an initial age estimate of 11-24 years. This animal was tetracycline-marked 4 years prior to death and a previously published paper (Lockyer *et al.* 2007) identified a tetracycline mark 4 years prior to death.

Fig. 11. Stained thin section from captive beluga whale.

ID#Tiqa (Fig. 12)

The sample from Tiqa was not available prior to the workshop. In September, 2011, a captive beluga whale (Tiqa) held at the Vancouver Aquarium died at age 3 years and 3 months. Heart failure was the proximal cause and more detailed necropsy data were not available at the workshop. The Aquarium made teeth available to the DFO-Central and Arctic lab and the NOAA – Beaufort NC lab. During the workshop, images of thin sections and stained sections were made available courtesy B. Stewart and A. Hohn, respectively.

The teeth were soft. They broke easily and the Isomet™ (www.buehler.com) diamond-embedded saw blades took less time than expected to cut through them. The teeth were all heavily worn to the level of the gingiva despite the young age and, macroscopically, the dentine appeared darker than normal.

In both the image of the untreated thin and section and the physical specimen stained section, fetal dentine and a neonatal line (in both dentine and cementum) were apparent. GLGs in the dentine were ill-defined and only slightly better defined in cementum. Pulp stones hanging like a string of beads were present in the tooth used for stained sections but were damaged during sectioning. The presence of pulp stones as a mineralization anomaly in such a juvenile animal is very rare (C. Lockyer pers. observation), perhaps indicative of physiological stress associated with health (Manzanilla, 1989; Lockyer 1993).

Consensus was there were 3 fully formed GLGs in the dentine and a partial fourth.

Fig. 12. Stained (upper) and untreated (lower) thin sections of teeth from Tiqa. Scale bar is associated with the stained section, which was photographed during the workshop and images stitched. The stained section was provided by A Hohn and the untreated image was provided by B. Stewart.

GUIDELINES FOR BEST PRACTICES

Based on all the foregoing and additional discussion presented below, the group developed guidelines for best practices (see also Stewart and Stewart 2014) that could be proposed to the two sponsoring organizations, JCNB and NAMMCO. The group recognizes that "best practice" is dependent on the intended use of the age estimates and that deviations can be expected. However, participants strongly urge authors who deviate significantly from these general guidelines to detail their methods and rationale. We provide here a summary of "best practices" for routine age estimation of beluga, sharing information (including training) about age estimation of specific samples, and preparation of sections for more detailed, including cytological, studies. The physical specimens of thin (untreated) sections are preferred for routine age estimation; images for information sharing; and stained sections for detailed ultrastructure analysis.

Routine Age Estimation in Beluga Whales

Routine age estimation comprises four broad steps: (1) selecting a tooth to be sectioned, (2) sectioning the tooth, (3) viewing a section to count GLGs, and (4) recording the data.

1. Tooth Selection

Tooth selection can be done for two different, but overlapping, reasons. One is to select the most readable tooth; the other is to select a tooth that shows the maximum growth layer groups. But ultimately, a readable tooth with the maximum growth layer groups is needed. Teeth that are deformed, have compound curves, or are in a spiral shape are difficult to read and individual researchers may choose not to age them.

An ideal tooth is either straight or curved in only one direction so that it can be cut close to the central line through the length of the tooth ("on-center" see #24, Figure 9). It will have a complete record of growth with minimum distortion and clear GLGs. Larger teeth usually have clearer lines, simply because they are more widely spaced. Large, straight, unworn, undamaged teeth are, therefore, the best candidates.

The numbers of teeth in the jaws varies among stocks and among individuals but generally teeth near the front of the jaw are highly recurved and difficult to section. Teeth at the back of the jaw may have convoluted root tips.

Examination of beluga teeth taken by Burns and Seaman (1986) showed great variation in size and wear from an individual and among individuals. The two or three largest mandibular teeth were selected for sectioning and aging. Hohn and Lockyer (1999) used tooth numbers 6-7 and 8-9 from two captive whales in their study. Finley *et al*. (1982) stated that the second and fifth teeth are routinely sectioned for age estimation; a convention adopted by DFO C&A (Wainwright and Walker 1988). Other factors that may affect tooth selection are tooth straightness, degree of wear (Marsh 1980), and occlusion of the pulp cavity (Hohn 1980).

Vos (2003) compared the number of growth layer groups of 348 teeth of known position. An ANOVA showed the means of GLG counts, based on tooth position, were significantly different (F=2.349, p=0.018), with tooth number eight showing the most growth layer groups. Vos (2003) recommends aging the last three posterior teeth in a lower jaw based on his finding that they exhibited the least wear, greatest frequency of neonatal caps, and the highest GLG counts.

R. Stewart (1986 unpublished) examined tooth sizes in 44 belugas from three stocks. Generally, maximum tooth length and mass occurred at positions 5-6 and only 48% of the whales examined had more than 8 teeth in row. Differences in tooth size associated with stocks were noted.

Benjamins (1999) discussed the tooth crown wear relative to age estimation in tooth sequences of Greenlandic belugas, and reported that wear was generally greatest in the front teeth. Teeth from the mid-jaw were deemed best, *i.e.* position 5-7 from the front, for both dentinal and cemental GLG counts. Examples of age estimated relative to expected maximum age are shown for tooth specimens in Fig. 13.

<u>Conclusions</u>
Age estimates can vary among teeth within a mouth, depending on tooth crown wear, compactness of GLGs in the root, and other reasons. For each stock, an initial assessment should be made to identify the tooth position that, on average, provides the clearest material and highest GLG count, to be adopted as the standard for that stock. The average number of teeth present in each example (Vos, Stewart, Benjamins) varied, but it appears that more posterior but not the last teeth in the tooth row might be preferred. Vos selected #8 from tooth rows that averaged 10 teeth; Stewart #5 or #6 out of 8 teeth; and Benjamins #5-7 out of 9 teeth with average.

2. Tooth Sectioning
The tooth should be examined carefully to identify the major direction of curvature, the end points of the mid line and any cracks in the tooth. The mounting of the tooth for sectioning will depend on the saw available; however the group noted that this requires experience with the teeth of the study species, so practice cuts should be done first on dispensable samples. The tooth should be mounted so that one cut surface will fall on the mid-line of the tooth. It is often easier to determine the midline at the root and start cutting from there. Sections should be thin enough to be translucent but thick enough to maintain physical structure (0.1-0.3 mm) with consistent thickness throughout. For teeth from an apparently older (>25 y) animal or teeth with unusual curvature, two or more sequential sections may be required so that the sawyer should plan cuts accordingly. It is often helpful to mark the desired cutting line on the tooth with pencil before cutting, and also to note that because of the grinding process, the cut should be a little to the side of this line to allow for the grinding away of tissue.

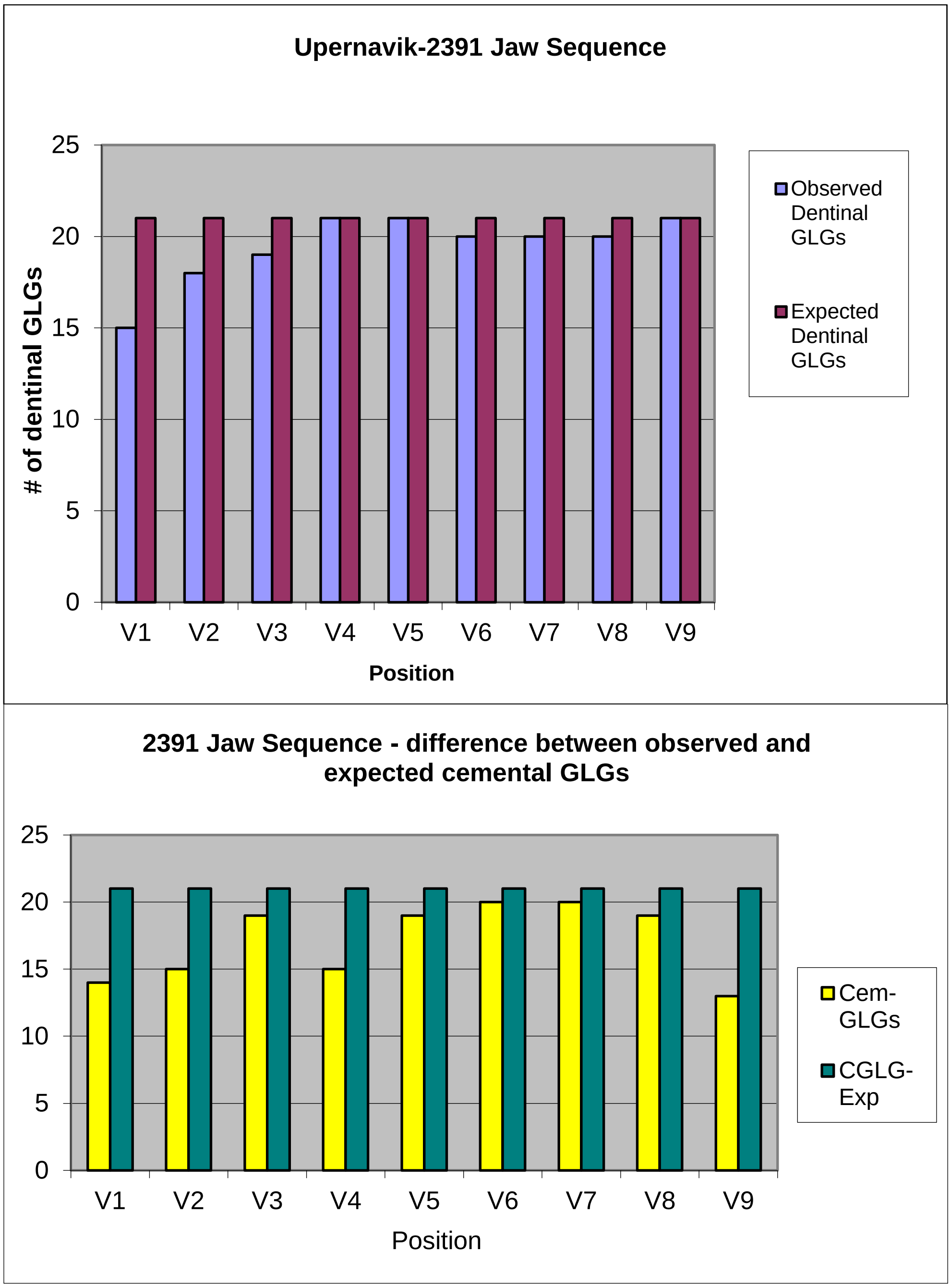

Fig. 13. Example from beluga teeth from W. Greenland, showing that the most complete ages are determined from the mid-jaw and back for dentine (left) because of crown wear at the front of the jaw. Cementum may be variable (right), but generally provides a lower age estimate than dentine. The teeth in this example are from the left jaw and numbered sequentially from the front to back (after Benjamins 1999).

A frequently used mounting method is to align the tooth in the correct plane and angle on a small wood block designed specifically to sit firmly within the vice or mounting chuck on the saw. Methods of fixing the tooth to the wood block are dental wax or quick-setting glue using a glue gun with glue sticks. The latter is especially easy to remove after use when the block is soaked in water for a few minutes.

Airborne dust from teeth can be hazardous to your health. Sawing with a water or oil reservoir for the blade reduces this hazard. Also, sawing in water or oil will cool the tooth and reduce the tendency of the section to warp or burn or the blade to catch and shatter.

After sectioning, thin sections and remaining sides of the tooth (cut-offs) are stored. Several different storage methods were used by different labs for thin untreated tooth sections.

1. Dry samples stored well with no deterioration of readability. Dry thin sections may warp, but can be rehydrated in water for later reading. Dry sections may crack and are more vulnerable to breakage than wet sections when compressed between two slides for viewing or imaging. They may need to be re-hydrated before being read or imaged.
2. Samples stored in 70% isopropyl alcohol or ethanol showed no deterioration in readability, no bacterial growth, and retained their flexibility. Sections stored in glycerin – alcohol mixture and then ETOH only showed no adverse effects of storage (B. Stewart, *pers. comm.*) for almost 30 years. The AREP87 tooth illustrated in the glossary (Appendix 4) was stored in this manner for approximately 24 years.
3. Samples stored in water showed no deterioration in readability. No bacterial growth was noted by the one lab that stored samples in this manner.
4. Researchers found that samples stored in a mixture of 5% glycerin /35% alcohol / 60% water, lost some readability. The growth lines became less defined over time. No bacterial growth occurred.

It appears that dry storage and alcohol storage present the most viable methods of storage. Water may also be viable if no bacterial growth occurs. It is recommended that a glycerin mixture not be used to store samples due to deterioration in readability. In addition, glycerin is oily and can be messy to work with.

3. Viewing Sections and Estimating Age

During periods when the sections are being read, wet storage is recommended because the sections distort less. As a result, the section should be kept moist during viewing because the optical properties change as the section dries and the section can twist and bend. Also, sections viewed wet under a cover slip or in water generally appear clearer, because liquid fills cracks, voids and saw marks, thus reducing the number and contrast of reflective and refractive surfaces that obscure the more subtle pattern of GLGs. While transmitted light may generally result in the best resolution of GLGs, using reflected light alone or with a polarizing filter may also be helpful.

Reading of images has some inherent level of imprecision, as has been documented across a wide range of taxa. As a result, it is necessary to conduct independent readings of each specimen. For beluga whales, either 3 or 5 readings have generally been used and found suitable for most specimens.

Some end users may insist on a Final Age Estimate. On the basis of prior analyses and the current analysis by Coggins for this workshop, participants adopted the mode as the single final age estimate although attaining a mode may require burdensome blind readings.

Stewart and Lavigne (1979) introduced the use of a statistical test for outliers, a protocol adopted by DFO C&A (Stewart 2012). Briefly, 3 to 5 blind readings were made. If three identical readings were obtained, that reading was taken as the final age estimate (mode). If there were not 3 identical readings, the 5 readings were examined using the Maximum Normed Residual (MNR) test (Snedecor and Cochran 1967). This approach protects against the inclusion of blatant errors, such as digit inversion (recording 21 when 12 was the intended number). If no statistical outliers were present, the median of the 5 readings was selected. If an outlier was removed, the median value was selected randomly from the middle two readings, if necessary.

While the group did not come to a consensus on a particular treatment for sections with no mode after several readings, it was generally acknowledged that a standard that requires multiple readings should be adopted. The preferred number of readings appears to be 3 to 5. Options to be considered include:

- mode (uses as few as 3 readings)
- MNR examination and median
- MNR examination, replacement reading for deleted outliers, and median
- simple median
- consensus readings from more than one reader
- additional readings (within limits) to attain a mode

Inter-reader and inter-laboratory readings are also encouraged to prevent drift and ensure that age estimates are comparable.

Conclusions

Every effort should be made to optimize viewing of the sections. Then, sections should be viewed **at least 3 times in blind replicates**, recognizing that **each viewing will entail several counts until a best count for that viewing** is obtained.

4. Recording Data

Here we provide guidelines for the most stringent data use; individual researchers may settle for less but should be aware that some data cannot be regenerated later from handled specimens, should they become required.

Recorded information should include:

- condition of crown and root (*e.g.* worn tip, occluded root);
- presence/absence of prenatal dentine or cementum;
- presence of unreadable subsections (subsections for which no clear reading of the GLG is possible or where GLGs are ambiguous), due to preparation quality or intrinsic characteristics of the tooth;
- summary of counts of GLGs either as a series from landmark to landmark (crack, pulp stone, node, *etc.*) or as a complete count;
- results from multiple counts with best, high and low results. "Best" can be the mode from entire counts for clear teeth, or the sum of best counts from subsections including the readers preferred count from unreadable sections; the method should be stated. High and low should account for the uncertainty (maximum and minimum) of the unreadable subsections.

It is recommended that the end user, *e.g.* the population dynamics modeller, receive all the data to decide whether or how to incorporate variation in readings.

Participants adopted Stewart's (2012) data sheet (see Appendix 5 and Stewart and Stewart 2014) with the following addition:

- Include a brief description or narrative with drawings or attached images to indicate how GLG or marker lines were identified for each viewing;
- Append an annotated printed image whenever possible, indicating GLGs counted;
- When data are entered into a database, link the photographs to the database.

Images

1. Image Capture

Images are useful for sharing, for training, and for creating a record of identified GLGs. Both the initial comparison reported by Stewart (above) and the more comprehensive one reported by Coggins (above) demonstrated that most readers were counting the same things but that counting on images often produced under-estimates compared to counting on the section itself. Images can be used to provide sufficient background for new readers before refining the details of age estimation techniques on thin sections. Marks made on images using digital layers can be hidden for blind experiments and displayed for comparative purposes. A series of images representing easy to hard-to-read thin sections, with examples of specific problems, can be collated for training and electronic sharing. Sharing image files can be done using internet FTP or cloud sharing sites or mailing of external hard drives, DVD or USB drive for inter-lab comparisons. Size limits on emailing large files restrict the sharing capabilities of larger image files, but may be an option based on the image resolution and file size.

Most importantly, good images require good thin sections. Then, for any imaging approach, the most important step is to get the best quality tooth image (contrast, focus, image resolution) at the time of the image capture by optimizing camera or software settings for the device available. High resolution images are recommended because they maintain resolution even when magnification is increased to better examine specific areas in an image.

While a flatbed or platen scanner is useful for scanning images of half-teeth, for the preferred preparation method, *viz* a thin section, a slide scanner is preferred. A slide scanner is also appropriate for scanning stained sections. Such a scanner uses transmitted light; hence it best represents the physical specimen on a microscope.

Other options were reviewed briefly. Microscopes with computerized camera combinations directly connect the microscope to a computer and use software associated with the microscope camera to capture an image of a tooth section. If using lower magnification (on a compound or dissecting microscope) the entire tooth can be captured in one image. Capturing a good image with digital SLR and macro lens has been problematic but using a digital SLR with microscope adaptor has not been explored. In general, these methods require manipulating image quality before image capture to maximize the quality of the image. Variables include focus, microscope or lens settings, and camera exposure (depth of field) settings as well as variables in the software tools of the camera. Digitally stitching several images together (*e.g.* using software programs such as Adobe Photoshop™ or Microsoft Powerpoint™) can cause distortion, disturbance of colour balance and lack of clarity at the joint. The process is also labour intensive. However, it might be the best option for large teeth or when higher magnification is needed to produce details in a section. Specific higher magnification, high-resolution images can supplement the scanned images to provide, for example, better documentation of the tip or root or of marker lines.

2. Image Format

It is highly recommended that Raw format (unprocessed data file) or Tiff file formats (no compression) are acquired to maximize image quality for fine scale analysis image. Since Jpeg format uses compression to save the image file, Jpeg formats should not be used in case further analysis is needed at a later time. Name the image file of the tooth to include the basic sample information.

3. Image (Data) Storage

The internal hard drive of a computer can be used for analysis but longer term archiving of image files should include multiple locations. External hard drives are an affordable option for archive purposes and have the added potential for sharing large datasets (non-archival copies). Original image files **should be protected at the folder level by designating the folder as "read only"** forcing users to save a copy to another location for analysis that might alter the image and image quality. Use of computer cloud sharing sites may also protect the files and allow sharing without having to email files.

Metadata (saved within the original image file) have potential for maintaining basic information (sample number, location) with the original image and any subsequent copies. EXIF metadata are embedded with the image from the original down to marked up copies or saved versions. Sharing of image files with associated metadata helps maintain basic information about the image sample outside of the basic filename.

Sharing image files can be done using internet FTP or cloud sites or mailing of external hard drives, DVD or USB drive for sharing of images for inter-comparisons. Size limits on emailing large files restrict the sharing capabilities of larger image files, but may be an option based on the image resolution and file size.

4. Image Processing

Various software options are available including but not limited to: Photoshop CS, Photoshop Elements (cheaper alternative), or GIMP.org (freeware, but may require plug-ins). The main purpose of the software is to help manipulate the image (contrast, lighting, colour curves) to help with GLG detection and mark the layers to help keep track of GLG layers. One benefit of using

Photoshop (CS or Elements) is the ability to mark GLG in “layers” that can be turned on and off for multiple readings, allow an analyst to track differences in counts over a period of time and compare counts with other analysts, when the image file is saved as a “psd” file which does not compress the different “layers”. The psd files can be opened by other users in Photoshop and GIMP, and the marked GLGs over the image viewed or the layers can be turned “off” for their own analysis. Care should always be taken not to make changes to one’s original image but to work with a “working” copy so as to maintain the unmarked original image file in high resolution.

SPECIAL INVESTIGATIONS

Teeth are occasionally acquired from captive belugas that have experienced clinical tetracycline antibiotic treatments, or have been deliberately dosed with tetracycline drugs for the purpose of “time-marking” GLG in the teeth, as part of an age validation investigation. If teeth are to be examined for tetracycline time-marking, the tooth should NOT be formalin-fixed before sectioning, but simply frozen until sectioned. The cutting procedure is similar to that described above under Routine Age Estimation *2. Tooth Sectioning.*

The section can be examined wet under water using a dissecting microscope but the light source is reflected UV, and the room where the tooth is examined should be dark to enhance the visibility of fluorescing mark(s). The age can be estimated by examining the section in the usual manner as described above under Routine Age Estimation *3. Viewing*. In suspected or known tetracycline-marked teeth, an additional stained section is often prepared.

CONCLUSIONS

NAMMCO recommended and convened an Age Estimation Workshop to review age estimation methods and discuss standardizing ages using growth layers with new methods. The resulting Terms of Reference set four objectives:

1. Provide a guide as to acceptable levels of accuracy and precision for age reading that will enable ages to be used in population models.
2. Conduct an inter-reader/laboratory comparison for calibration and standardization of age readings from GLGs among all readers/laboratories.
3. Provide information on validation that will enable GLGs to be translated to real age.
4. Produce a manual of guidelines for the preparation and reading of GLGs in beluga teeth.

With respect to these objectives:

1. Accuracy and precision (objective 2 is a specific action item aimed at addressing objective 1)
   a. A comparative study was initiated and quantification of differences presented here. The resulting training process for calibration and standardization is underway.
   b. Precision of experienced readers was better than that reported in the literature for sperm whale (Evans *et al.* 2002) and spotted dolphin (Reilly *et al.* 1983) age estimation.
   c. Efforts to continue to improve precision should be tempered by the need for better precision in the application of age data; example applications are analyses of catch age structure, age of maturation, body growth, and age-structured population models.
   d. Good thin sections are recommended for relative ease of preparation compared to stained sections and agreement among readers (precision) compared to half sections.

2. See above.

3. Translate GLGs into ages
   a. Workshop 1 (Tampa 26-27 November 2011) concluded that the evidence for interpreting one GLG as an annual record is irrefutable.
   b. Teeth from captive beluga were particularly problematic as both untreated sections and stained sections and did not clarify the reading of wild beluga teeth.

4. Guidelines = Best Practices
   a. <u>Routine Age Estimation</u>: The working group recommends using thin medial sections from teeth known to yield, on average, the most complete GLG record, viewed wet under a dissecting microscope with transmitted light. Detailed data should be recorded on a standardized form that will include an annotated image. End-users should be provided with all the data but the default reduction would be the mode of at least 3 readings or median of at least 5 readings, with statistical outliers replaced.
   b. <u>Imaging</u>: The working group recommends using a high-resolution slide scanner to make images of thin sections. Images should be stored in Raw or Tiff format, protected from changes and securely archived with complete metadata. Processing that allows hidden layers of data is preferred.
   c. <u>Stained Sections</u>: The working group recommends using stained sections for microstructure, particularly difficult teeth, in support of tetracycline marking calibration, or for comparative purposes.

Otherwise, age estimation from untreated thin sections is preferable because section preparation is easier and faster.

Other General conclusions

1. Sections of teeth suitably decalcified, and then stained with haematoxylin, allow examination of microstructure better than untreated sections.
2. Reading half-tooth sections was more difficult than thin sections for counts of 30 GLGs or more. These sections were also more labour intensive than thin sections because the surface must be polished every time it is examined.
3. Images were generally less satisfactory than the physical specimen.

Recommendations

For presenting age estimates to the JCNB and NAMMCO, authors should adopt the relevant Best Practice outlines here or provide a detailed rationale for deviating from it.

1. Continue to explore new methods and technologies to clarify or enhance GLGs to increase precision.
2. New approaches should use, as the "control", the Best Practices for section preparation and reading to quantitatively calibrate the new method.
3. A reference collection of thin sections with high quality digital images should be prepared for training new readers, for refreshing experienced readers, and for use in inter-lab comparison studies and standardization.
4. Inter-lab comparisons of precision are necessary especially when data are to be combined or results compared.

RESEARCH TOPICS

1. Accuracy would be improved by a better understanding of why GLG lines form. Examination of life-history correlates associated with GLG and accessory lines, the season of light and dark band formation, isotopic changes from wild to captive conditions, and known-age (or bio-marked) wild whales could all contribute.
2. Accuracy may improve with a better understanding of how many lines are lost through occlusal wear.
3. Accuracy may improve with the investigation of a method that may compensate for the loss of GLGs resulting from crown wear. During the Tampa Workshop preceding this workshop, a method was proposed by Brodie (in NAMMCO 2013, p.505-6) that involves the changes in angle of deposition of GLGs as an animal ages.
4. Variation exists among stocks and a comparison of readability among stocks could guide the development of stock-specific methods. Topics

could include characterization of the GLGs, tooth growth and selection, and differences in seasonality.
5. Identifying GLGs in juvenile belugas remains a challenge and further research on the life-history correlates, the season of formation, isotopic changes associated with weaning, and known-age wild whales would be useful. Specifically, the influence of the protracted season of birth on the relative width and general characteristics of the early formed GLGs would assist in their interpretation.
6. Investigation of cementum lines and development of associated techniques are desirable. In some teeth the cementum lines are very clear and as detailed as the dentinal lines. Comparison of counts of both tissues would be useful to verify the comparability when cementum counts are used in place of dentine or difficult to read teeth.
7. Maximize the use of teeth from captive animals including tetracycline marking or diet studies

The previous research ideas were generated during discussions. Additional suggestions and ideas are presented in Stewart (2012), which was tabled but not discussed, and included:
1. Deciduous dentition has been seen in a few beluga (Stewart 2012) and the issue of monodonty/diphylodonty should be investigated by x-ray analysis of fetal jaws. Establishing the time of formation and resorption of deciduous teeth and the development of permanent teeth relative to the time of birth would assist interpretation of GLG counts, confirming that a full complement of GLGs is present at birth.
2. Interpretation of the neonatal line would be improved by a better understanding of the detailed characteristics of the fetal cementum-postnatal cementum interface.
3. A better understanding of eruption dynamics is required to fully appreciate how the stages of eruption (embedded, partially erupted, fully erupted) relate to GLG count.
4. A detailed characterization of occlusal wear patterns and progression *i.e.* shape of cusp and tissues present, may provide a numerical approach for relative age analysis.
5. Similarly, detailed characterization of the changes in pulp cavity/root tip shape may provide a numerical approach for relative age analysis.
6. Beluga teeth vary in size, shape, dimensions of dentine and cementum growth layers, wear patterns, and the quantity and nature of inclusions. A better understanding of these stock differences may allow stock-specific approaches to interpreting GLGs. (see also 4 above).

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LIST OF APPENDICES

1. Workshop Agenda
2. Workshop Participants
3. Detailed methods for participating labs
   a. Age Dynamics - Denmark
   b. Alaska Beluga Whale Committee - North Slope Borough, US
   c. Fisheries and Oceans Canada – Central and Arctic Region, Canada
   d. Fisheries and Oceans Canada – Quebec Region, Canada
   e. Makivik Corporation – Quebec, Canada
   f. NOAA – Anchorage, US
   g. NOAA – Beaufort, US
4. GLOSSARY
5. Data sheet adopted by Workshop

Appendix 1

Workshop Agenda

Programme for Monodontid Workshop 2, 5-9 December 2011, Beaufort

Monday a.m.

- Introductions - housekeeping; who does what and where; Terms of Reference for Workshop
- Rapporteuring among whole membership of workshop, coordinated by C. Lockyer and M. Acquarone
- Discussion on reading experiment – design and execution
- Presentations of how to prepare and read teeth from different labs including images and demonstrating techniques of reading GLG
- Introduction of B. Stewart's 2012 report
- Short Presentations
  - R. Stewart: Number of replicates and inter-lab comparison (with Lesage).
  - A. Hohn: Reading of best and worst tooth samples using microscope and images
- Markup of teeth. Focus on ca 15 teeth

Monday p.m.

- L. Coggins: Report on statistical analysis of reading experiment
- Lab
  1. Comparisons of digital images and actual specimens - in lab
  2. Microscope and image projection and inter-reader trials - in lab
  3. Discussion - Method comparison – thin untreated sections, decalcified stained thin sections, ½-tooth sections; transmitted light and polarised light
- Discussion - general

Tuesday a.m.

- Lab-based - Comparison of images and actual specimens, continued

Tuesday p.m.

- Discussion on results.

Wednesday a.m.

- Outline report (R. Stewart and R. Hobbs)
- Discussion

Wednesday p.m.

- Lab
  1. GLG patterns
  2. How to address crown wear
  3. Geographical variation

Thursday a.m.

- Selecting a reference set of teeth (images) to illustrate points in the workshop report

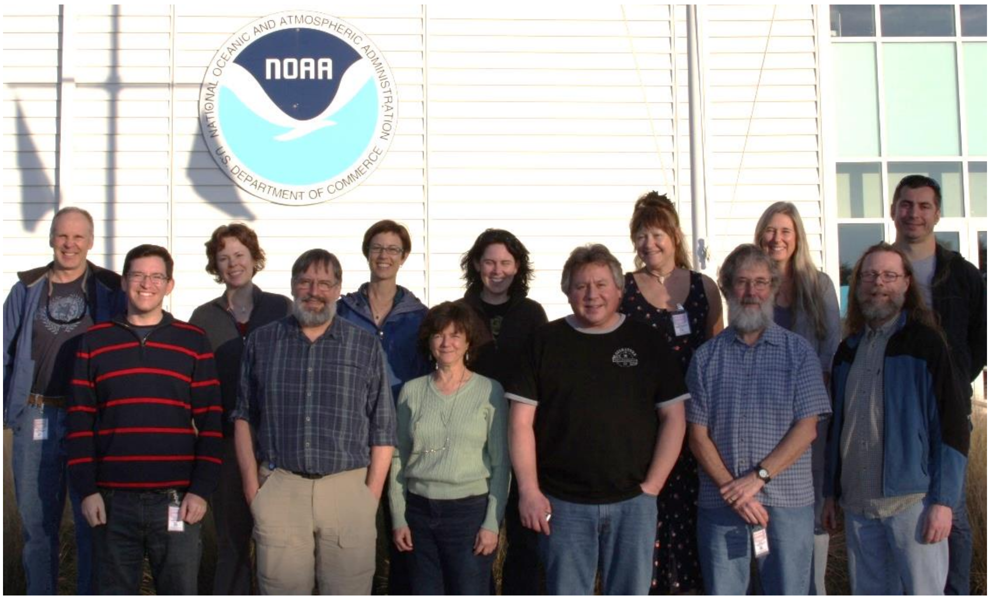
Thursday p.m.

- Discussion on whether it is important to get real ages for very old animals
- Precision and accuracy issues.

Friday all day

- Report writing
  1. Agreeing and recommendations
  2. Best practices.

Appendix 2 Workshop Participants



Front row: Barrie Ford, Rod Hobbs, Aleta Hohn, Peter May, Rob Stewart, Dan Vos. Back row: Yves Morin, Christy Sims, Linda VateBrattstrom, Heather Smith, Christina Lockyer, Karen Altman, Mario Acquarone

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Appendix 3

Detailed methods for participating labs

(If the author and presenter differ, the author is listed first)

A3-a: Age Dynamics (C. Lockyer)

Usually the tooth is supplied without reference information as to position in the jaw, as contract work. If there is a choice, position 5th -7th back from the lower jaw tip is preferred based on a comparison of results of aging from entire tooth series to see the potential difference in total GLGs, readability and tooth wear with tooth position (Benjamins 1999). The rearmost teeth may be curved, and the forefront teeth may be most worn. Several methods are employed in preparation, depending on what the focus is.

An Isomet Buehler slow speed saw is used for obtaining untreated sections for estimating total age. The tooth is mounted on a wood block with resin glue that melts when warm using a glue gun, with the tooth bearing down on the circular diamond blade, beneath the wood block and mounting vice (Figure A3-a1).

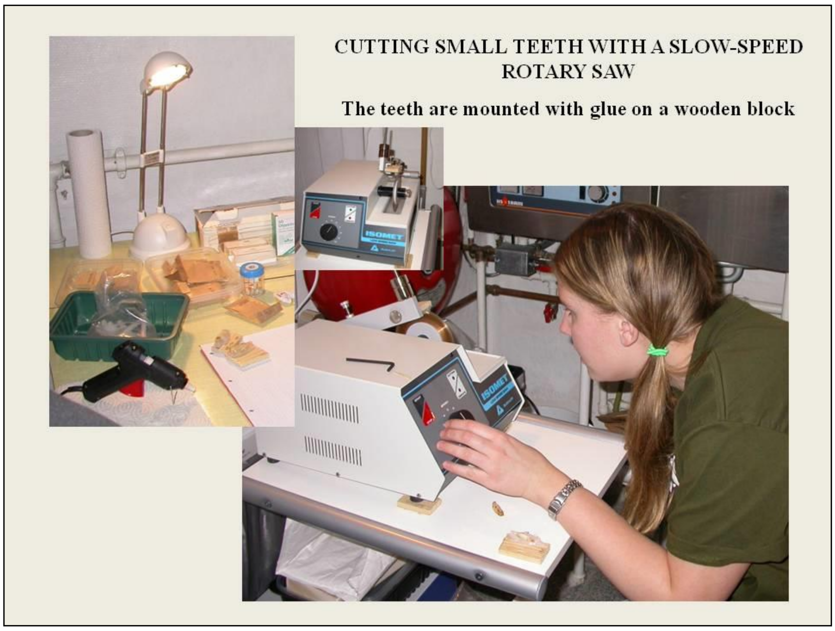

Fig. A3-a1. Set up for cutting and sectioning teeth on a low-speed circular diamond saw.

The first section is offset slightly to the side of a pencil line drawn along the tooth as a cutting guide through the crown and root along the midline. Up to 2 sections per tooth are usually cut at 100-150 micron. The lubricating fluid used with the Isomet is water with a drop or two of liquid soap added to reduce foaming by reducing surface tension.

If the focus is ultra-structure and mineralisation anomalies, either a half tooth or a thick wafer at 2.5 mm is cut around the centre of the tooth, and decalcified in RDO (Apex Engineering, Illinois, USA). Pre-treatment, the teeth are fixed in 10% neutral buffered formalin and subsequently rinsed well in water and rinsed well before the decalcification process. If teeth are to be examined for tetracycline time-marking, the tooth is NOT formalin-fixed, and is examined only in untreated thin section. If teeth are curved / twisted, the tooth is cut in two and then each half sectioned in different planes in order to get the entire centre. The subsequent sections are then married when read.

Decalcified tooth segments (using RDO) are sectioned at 20-25 micron close to the midline running from crown through the pulp cavity using a freezing microtome (Figure A3-a2).

Figure A3-a2. Sectioning a decalcified tooth on a freezing microtome with a fixed freezing platform and movable blade.

Stain (Ehrlichs haematoxylin) is then applied to the sections for a period long enough to stain the teeth adequately (10-15 min. in ripened stain). The sections are then rinsed and mounted wet under water on 5% gel-coated slides, dried and then mounted permanently with DPX under a cover slip.

All sections are examined under a dissecting microscope using plain transmitted light, and sometimes using polarizing filters for untreated sections. Turning the section through different angles can help with reaching a consistent count. Projection of microscope images via video camera onto a colour TV monitor can be helpful to enlarge the image.

Usually a tooth section is counted up to 10 times at one time before committing to an age result. The modal count is preferred to the average in all counts. Digital images are usually taken for each tooth as a double check on counts and for mark-up. The tooth is counted again later as a double check referring to the digital images. Comments on readability, presence of anomalies, accessory lines, neonatal line (NNL) and crown wear, tooth condition – cracked or whatever, are recorded.

All age readings are generally done by one person. The final age reading does not reflect missing layers and there is no age adjustment; rather a '+' suffix indicates that the NL is absent and some crown wear. If there are problems with the root, they are also noted. Untreated sections are stored dry in sealed glass jars. Stained sections are stored in the dark.

Reference

Benjamins S (1999) Defining populations of the beluga (*Delphinapterus leucas*) using morphometry and ultrastructure of teeth. Thesis presented to University of Gröningen, Netherlands, as part of a Master degree program. 54pp.

A3-b: Alaska Beluga Whale Committee - North Slope Borough (R. Suydam and H. Smith)

Age is estimated by counting growth layer groups (GLG) in thin sections of teeth. Either the right or left half of the lower jaw was collected from each beluga. Jaws were boiled, and teeth were extracted and scrubbed clean with a wire brush. The three largest and least-worn teeth from each beluga were selected. Jaws often included up to 9 teeth, and largest and least-worn teeth selected for sectioning were often from positions 5-8. Teeth were mounted on a small wooden block and thinly sectioned using a Buehler IsoMet low-speed saw equipped with a diamond wafering blade (Figure A3-b1). Tooth sections are stored in vials of water.

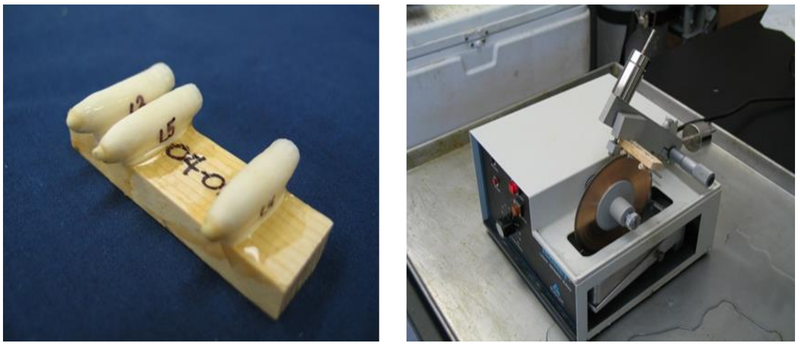

Fig. A3-b1. (Top) Three beluga teeth from the left mandible, positons 3, 5, and 4, mounted on a wooden block for sectioning. (Bottom) mounted teeth being sectioned on an Isomet saw.

In 2007, teeth were initially attached to the wooden block using a hot glue gun. In 2010, teeth were initially attached to the wooden block using double-sided upholstery tape until the orientation of the tooth on the block was satisfactory. Then teeth were firmly mounted to the block using epoxy.

In 2007, thin sections were made using successive cuts with the saw but in 2010, two blades were mounted on the saw separated by a plastic spacer (cut from the lid of a yogurt container) that yielded tooth sections of a thickness in which GLG were most easily visible. The thickness of the spacer was determined by trial and error and is about 0.63 mm microns thick. This two blade approach was modeled after the setup developed by Steven Campana's lab at the Bedford Institute of Oceanography (Dartmouth, NS, Canada).

For each beluga, GLGs in at least two teeth were counted a minimum of twice each by two readers. Tooth sections were read on a dissecting scope and light source was manipulated to give best contrast. Tooth sections were placed directly on the stage, and water was only wiped off the tooth section when it produced distracting reflections.

If needed, additional teeth were counted until an agreed-upon final count for each animal was reached by both readers. Actual age was estimated for animals when the neonatal line of the tooth was visible, while minimum age was estimated for older animals when the neonatal line had been worn away. In accordance with the findings of Stewart *et al*. (2006), one GLG was assumed to form annually.

Reference

Stewart REA, Campana SE, Jones CM and Stewart BE (2006) Bomb radiocarbon dating calibrates beluga (*Delphinapterus leucas*) age estimates. *Can. J. Zool.* 84:1840-1852.

A3-c: Fisheries and Oceans Canada - Central and Arctic Region (B. Stewart and R. Stewart)

Mandibles are collected in the field, frozen, and then shipped to the FWI where they are stored prior to processing. Records are made of the number of teeth present (erupted and embedded), supernumerary, missing or damaged teeth. Preliminary tooth selection evaluates two teeth from standard positions in the tooth row to be removed. Typically MNR-2 and MNR-5 are removed based on Finley *et al* (1982). Substitute teeth are selected if MNR-2 or MNR-5 is missing, damaged or excessively worn. After immersion of the right mandible in a hot water bath, selected teeth are removed and a substitute removed if necessary.

Teeth are mounted onto wooden blocks using hot glue, which allows for manipulation of the tooth to its best orientation. Teeth are aligned for median, longitudinal sectioning such that the root tip makes first contact with the rotating blade during sectioning (Figure A3-c1). The root tip is often more symmetric than the cusp of the tooth and the centre of the pulp cavity (= the median zone) may be more obvious, facilitating alignment.

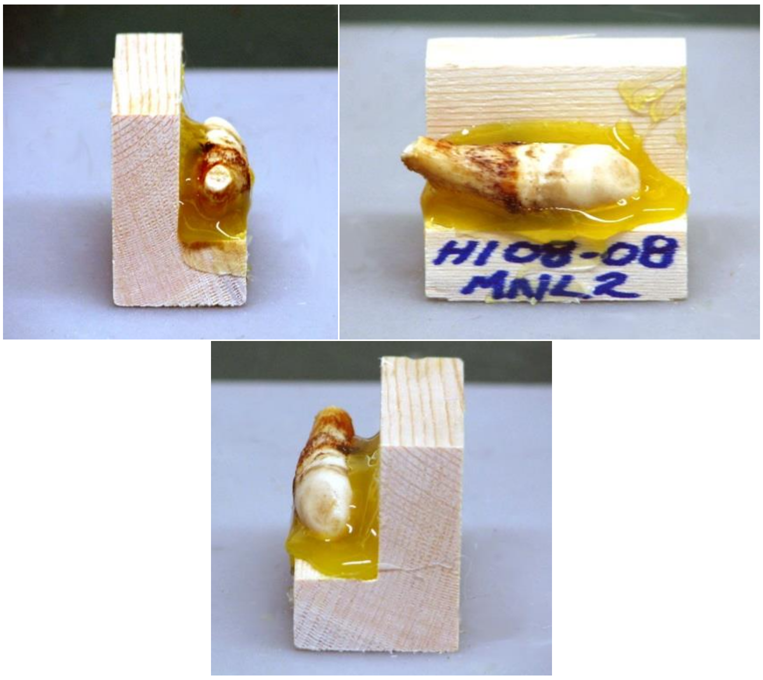

Fig. A3-c1. Tooth mounted on block.

Mounted teeth are sectioned using diamond wafering blades (Figure A3-c2) which are water cooled during operation of the thin sectioning machine or tooth saw. The sections produced (~0.3 mm) are thick enough to be free from mechanical sheering or tearing and thin enough to view all GLGs with transmitted light (wet).

Figure A3-c2. Block carrying mounted tooth through the saw (milling machine).

Two or three median, longitudinal sections are cut for each tooth, size permitting, then one optimum thin section is selected for assessment through light microscopy. The final age is determined by counting dentine GLGs. All of the attributes in the tooth section are recorded to allow for a comprehensive, holistic assessment (see Appendix 5).

Age estimates from three to five viewings are made without reference to the previous readings. When three identical readings are obtained, that value is presented as the final age estimate. If, after five readings, there are not three identical values the five readings are tested for statistical outliers (Maximum Normed Residual - Snedecor and Cochran 1967) and outliers deleted. Without outliers, the median of the five readings is accepted as the final estimate. Deletion of an outlier leaves four values and the median was selected from the two middle values randomly (coin toss).

References

Finley KJ, Miller GW, Allard M, Davis RA and Evans CR (1982) The belugas (*Delphinapterus leucas*) of northern Quebec: distribution, abundance, stock identity, catch history and management. *Can. Tech. Rep. Fish. Aquat. Sci.* 1123: v + 57 p.

Snedecor GW and Cochran WG (1967) *Statistical methods.* Sixth Edition. Iowa State University Press, Ames, Iowa. 593 pp.

A 3-d: Fisheries and Oceans Canada – Quebec Region (V. Lesage and Y. Morin)

This protocol is used to determine age in beluga whales using tooth longitudinal cut and reading the GLG.

Special equipment: Low speed saw Buehler IsoMet, Buehler Diamond wafering blade 4'' 15HC, sealing wax, Leister hot air blower with temperature control, dremel tool 5,000 to 35,000 rpm with felt polishing wheel no 414, modelling clay, digital camera Leica DFC 480 (Leica Camera AG, http://us.leica-camera.com/) mounted on a binocular Wild M3C (Wild Heerbrugg AG http://www.wild-heerbrugg.com/index.html), Photoshop CS software.

Reagents: Storage solution (Ethanol: Glycerol: Water 1:1:1)

Figure A3-d1: Fixing the tooth on the plastic block.

Jaws are simmered in water and left for 10-15 min. or overnight. Teeth are extracted with tooth extractor, cleaned with a scrub sponge and water and stored in storage solution until ready to cut.

Figure A3-d2: Taking photos of the section.

For cutting, the tooth is removed from the storage solution and dried. It is fixed, longitudinally, on the plastic block using sealing wax with hot air blower and then cooled (Figure A3-d1). The plastic block is mounted in the Buehler low speed saw and adjusted so the cut is off the center of the tooth on the exterior side. After the first cut, the position is adjusted so the slice will be 50 units on the micrometric scale of the saw (at about the center of the tooth). The second cut is made and all parts are kept in the storage solution until ready to read.

To photograph the tooth section, the tooth slice is placed between two microscope slides, held together with clamps, and placed on the dissecting microscope (Figure A3-d2). The focus is the center of the tooth and, using transmitted light, serial photos are taken starting at the top using a Leica camera. Images are saved in Tiff format. To photograph a ½-tooth, a Dremel (http://www.dremel.com/en-us/Pages/default.aspx) tool and felt polishing wheel are used to polish the flat surface of the tooth (Figure A3-d3). The half section of the cut tooth is placed, face-down, on the scanner, with a dark cardboard or black glove over it and scanned (Figure A3-d4).

Photoshop is used to make a copy of the image using Duplicate layer so the original image of the tooth is preserved. Working with the duplicate image, the brightness, contrast, level and color are adjusted to get the best image of the GLG. Then a new layer is created for each count and the GLG marked using the tools of the software; a different color for each count. Layers of previous counts are hidden and three counts of the same tooth are made at different times. After three readings, the counts are compared and if they are different, one or two additional counts are made and compared. Finally, all layers of the tooth are displayed to identify differences and make a final count from all the readings. The process is repeated by a second reader and the results compared.

Figure A3-d3. Polishing tooth surface.

Figure A3-d4. Scanning tooth half section.

A3-e: Makivik Corporation (P. May)

Makavik receives extracted teeth and not the jaw, so there is no possibility for determining location of tooth in jaw. An Isomet saw is used to section a tooth mounted on a wood block with jeweler’s wax. Sections of 65 micron or less are cut on low to medium speed. Water is used as coolant when cutting. One blade only is used, as opposed to a pair to cut a wafer. Age estimation is done with the tooth submerged in water using dissecting microscope with light transmitted or reflected. Scanned images are made using dry tooth section. All estimates are made by one reader who reads the section.

A3-f : NOAA – Anchorage Lab (D. Vos)

To prepare the teeth for aging, the jaws are thawed and boiled in water. Generally about 15 to 20 minutes of boiling time loosens the teeth enough to be pulled from the mandible. Care is taken not to boil the jaws so much that the teeth fall out, thereby losing the position of each tooth in the jaw.

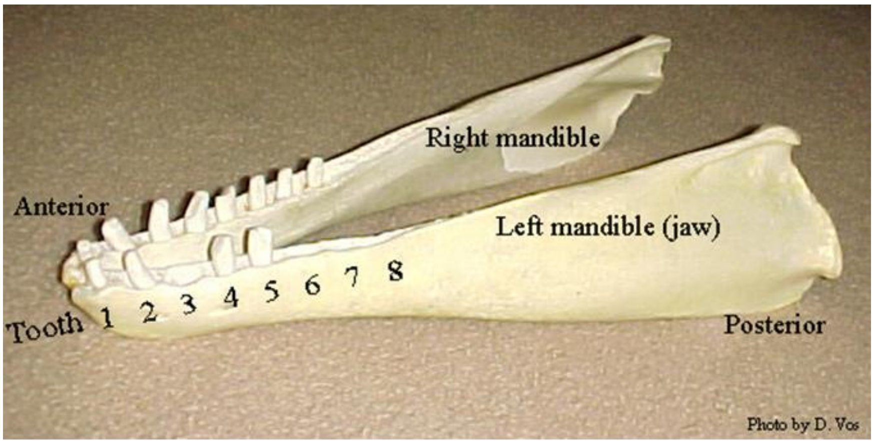

Figure A3-f1. Beluga mandible with 8 teeth on the right and 6 on the left.

Teeth are immersed for about two minutes in 50% hydrogen peroxide and then scraped clean. Mandible quadrant is recorded as left or right, and teeth are numbered consecutively, starting with 1 and generally going up to 8 in a full jaw (sometimes as many as 10), starting at the anterior and going toward the posterior of the mandible (Figure A3-f1).

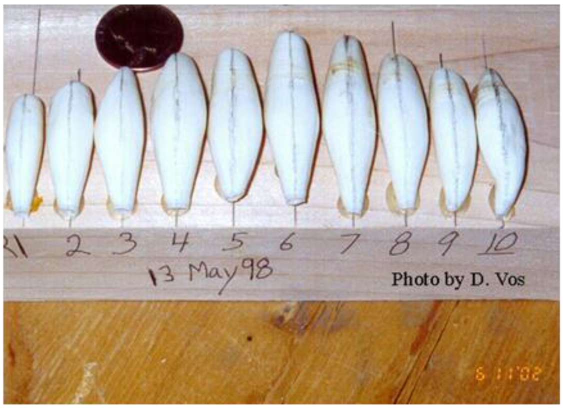

Figure A3-f2. Beluga teeth extracted from the right mandible, arranged in positional order and marked to indicate the center line for sectioning.

To mount the teeth for cutting, each tooth is first marked with a longitudinal centerline on the outside of the curve. Wooden blocks 1 ½" thick x 3" wide x 8" long (3.8 x 7.6 x 20.3 cm) are marked with lines perpendicular to the long edge (Figure A3-f2). Teeth are aligned to these marks, with the inside curve of the tooth down, and spot glued into place using a hot melt glue gun, taking care to align the tooth both vertically and horizontally. A masking tape dam is placed around the teeth on top of the block, and two-part epoxy is mixed and poured around the teeth, inside the dam, covering about one-half to three-quarters of each tooth, securing the teeth in an epoxy cast on top of the block.

Teeth are cut on a Sherline Model 2000 milling machine (http://www.sherline.com/index.html), using two MK-303 diamond impregnated lapidary saw blades separated by shims that determined the width of the medial longitudinal section. The block is held in a machinist clamp and auto fed into the blades at about 0.5 cm per minute. Mineral oil is used as a cooling lubricant to prevent burning of the teeth and blades. Optimal section thickness is between 0.30 and 0.50 mm thick. Teeth are stored in water until read and then stored long-term in a 5% glycerin/35% alcohol/60% water mixture. Most tooth sections needed no other preparation; however, some sections had saw marks removed by wet sanding with 600 grit silicon carbide sandpaper placed on a hard flat surface auto fed into the blades at about 0.5 cm per minute.

Sections are viewed wet on a variable powered dissecting microscope using transmitted and/or reflected light. Two readers read each tooth section and recorded: 1) the number of GLG, 2) presence or absence of a neonatal cap, 3) if the last layer next to the root was complete or partial, and 4) the quality of the tooth. Tooth quality was defined as: good=GLG range of 0 with multiple readings (a mode was selected); fair=range of 1 GLG; poor=range of 2 to 3 GLG; unreadable=range greater than 3 GLG, or differences between readers not resolved. Readers did blind readings, not knowing the whale identification, length, gender, or tooth position. After the individual readings, the results from reader one and reader two were compared. If the readings agreed, it was recorded as the final agreed reading. Differences between the first two readings were cooperatively reviewed by both readers reexamining the tooth, and sometimes looking at other teeth from the same animal. If a final result could be agreed upon, it was recorded. If the readers could not reach agreement, the tooth was considered unreadable and was discarded.

A3-g: NOAA – Beaufort Lab (A. Hohn)

Cleaned (not boiled), whole teeth are mounted cut on a wood block with hot glue from a small glue gun of the type commonly used for arts and crafts. The tooth is aligned such that a cut will produce a mid-longitudinal section in a buccal-lingual plane. The block is attached to a chuck that is screwed into the arm of an IsoMet saw. The IsoMet is fitted with a single blade and the outer portion of the tooth is cut off, the arm shifted to the other side of the mid-plane and then the middle of the tooth cut off. Water with a drop or two of regular liquid soap is used as a lubricant. The thickness of the mid-longitudinal section depends on whether the section will be used as an untreated thin section or will be decalcified, thin-sectioned and stained.

Figure A3-g1. Frozen section on sliding microtome.

For untreated sections, the tooth is cut such that the thin section will be approximately 75-150 um thick, although thicker sections may be cut for animals with a potential tetracycline mark. The section may be permanently attached or loose. Sections are stored dry. For decalcified and stained thin sections, the mid-longitudinal section will be 2-3 mm in thickness. That section will be fixed in formalin from a few hours to overnight, rinsed in tapped water, then placed in a commercial decalcifying agent called RDO. Once fully decalcified, the 2-3 mm section will be rinsed in tap water from a few hours to overnight. It is then sectioned on a sliding microtome with a freezing stage (Figure A3-g1) to 25 μm. The center-most sections are rinsed in tap water, stained in Mayer's hematoxylin using a shaking plate (Figure A3-g2), rinsed again, blued in a weak ammonia solution, then rinsed again. These sections are soaked in a 50:50 water:glycerin solution for at least 30 min then transferred to 100% glycerin. For mounting, these sections are transferred to a petri dish with fresh glycerin and then the best section or two selected and placed on a microscope slide in the glycerin. A cover slip is placed on the slide, with the edges sealed with a permanent mounting medium. Slides are stored in the dark to reduce fading. Extra sections are stored in a glass vial in glycerin in the dark.

Although the tooth number is important for beluga whales, often the samples received are opportunistic and do not include location of the tooth in the tooth row. Sections will be viewed with transmitted light using both a dissecting microscope and a compound microscope with low magnification lenses. Generally, counts are made three times per specimen, with each series of counts separated by sufficient time to help prevent recollection of previous age estimates. Each count includes examining each side of the tooth section, and multiple tooth sections when stained, and using all available characteristics to inform about the age estimate.

Figure A3-g2. Staining thin sections in hematoxylin.

Appendix 4
ILLUSTRATED GLOSSARY

(Reprinted from Stewart (2012) with the author's permission.)

The following definitions are based on Bloom and Fawcett (1975) and International Whaling Commission (1980) terminology. A labeled photograph of a thin section follows to illustrate some key terms.

Alveolus: the socket in the alveolar bone of the maxilla or mandible in which the root of a tooth fits.

Anterior face: the side of the tooth which is closest to the front of the mandible or maxilla.

Cementoblasts: cells which produce cementum.

Cementum: a calcified tissue that may be cellular and which forms the outer layer of a tooth below the *gingiva*.

Cusp: the distal end of a tooth; seen as the tip of the visible portion of an erupted tooth.

Dentine: a cellular tissue that forms the inside layer of a tooth; it is covered by enamel above the *gingiva* and by cementum below the *gingiva*.

Diphyodont: possessing two sets of teeth – the deciduous and permanent dentition.

Distal: situated away from the point of origin or attachment (opposite of proximal).

Enamel: acellular tissue that forms the outside layer of a tooth above the *gingiva*.

Eruption: migration of a tooth to a position external to the gingival surface (above the gum line).

Fetal cementum: cementum that is deposited in teeth *in utero*.

Fetal dentine (prenatal dentine; predentine): dentine that is deposited in teeth *in utero*.

Gingiva: the gums of the mouth; tissue that is attached to the bones of the jaw and surrounds/supports the bases of the teeth.

Growth layer: incremental band or line occurring parallel to the formative surface of a hard tissue such as dentine, cementum, or bone.

Growth layer group (GLG): a repeating or semi-repeating pattern of adjacent incremental growth layers within the dentine, cementum, or bone which is defined as a countable unit. Such a unit must involve at least one change, *i.e.* from transparent to opaque, light to dark, ridge to groove etc.

Homodont: a series of teeth with the same morphology.

Inclusions (pulp stones): globular masses of secondary dentine that form in the region of the pulp cavity.

In utero: referring to the time when a fetus is developing in the uterus.

Longitudinal section: a section that is cut along the long axis of a tooth; opposite to a cross-section which is a transverse cut.

Mandible: the lower jaw; has a left and right half.

Mandibular symphysis: the joint between the two halves of the mandible.

Maxilla: one of the two major bones in the upper jaw; the other bone is the pre-maxilla.

Median: (anatomical) situated in or pertaining to the middle; the plane dividing the tooth into two equal halves; (statistical) the middle value in a distribution.

Monophyodont: possessing only one set of permanent teeth, *i.e.* having no deciduous precursors.

Neonatal line: a well-defined growth layer situated in the interface between the prenatal dentine and postnatal dentine; thought to be the associated with physiological changes at birth and for a short period postnatally.

Occlusion of pulp cavity: the condition in which the pulp cavity has become filled with dentine and/or the deposition of primary dentine has stopped.

Odontoblasts: cells which produce dentine.

Periodontal ligament (periodontal membrane): the fibrous connective tissue that surrounds the root of a tooth, separating it from and attaching it to the alveolar bone, and serving to hold the tooth in its socket.

Posterior face: the side of the tooth which is closest to the rear of the mandible or maxilla (closest to the throat).

Proximal: situated close to the point of origin or attachment (opposite of distal).

Pulp: richly vascularized and innervated connective tissue inside the pulp cavity of a tooth.

Pulp cavity (pulp chamber): the pulp-filled central chamber in the tooth.

Pulp stones (inclusions): globular masses of secondary dentine that form in the region of the pulp cavity.

Root: the part of the tooth typically covered with cementum and contained within the tooth socket below the gingival surface.

Root tip: the proximal end of the tooth.

References

Bloom W and Fawcett DW (1975) *A textbook of histology.* Third Edition. WB Saunders, Philadelphia, Pennsylvania. 1033 p.

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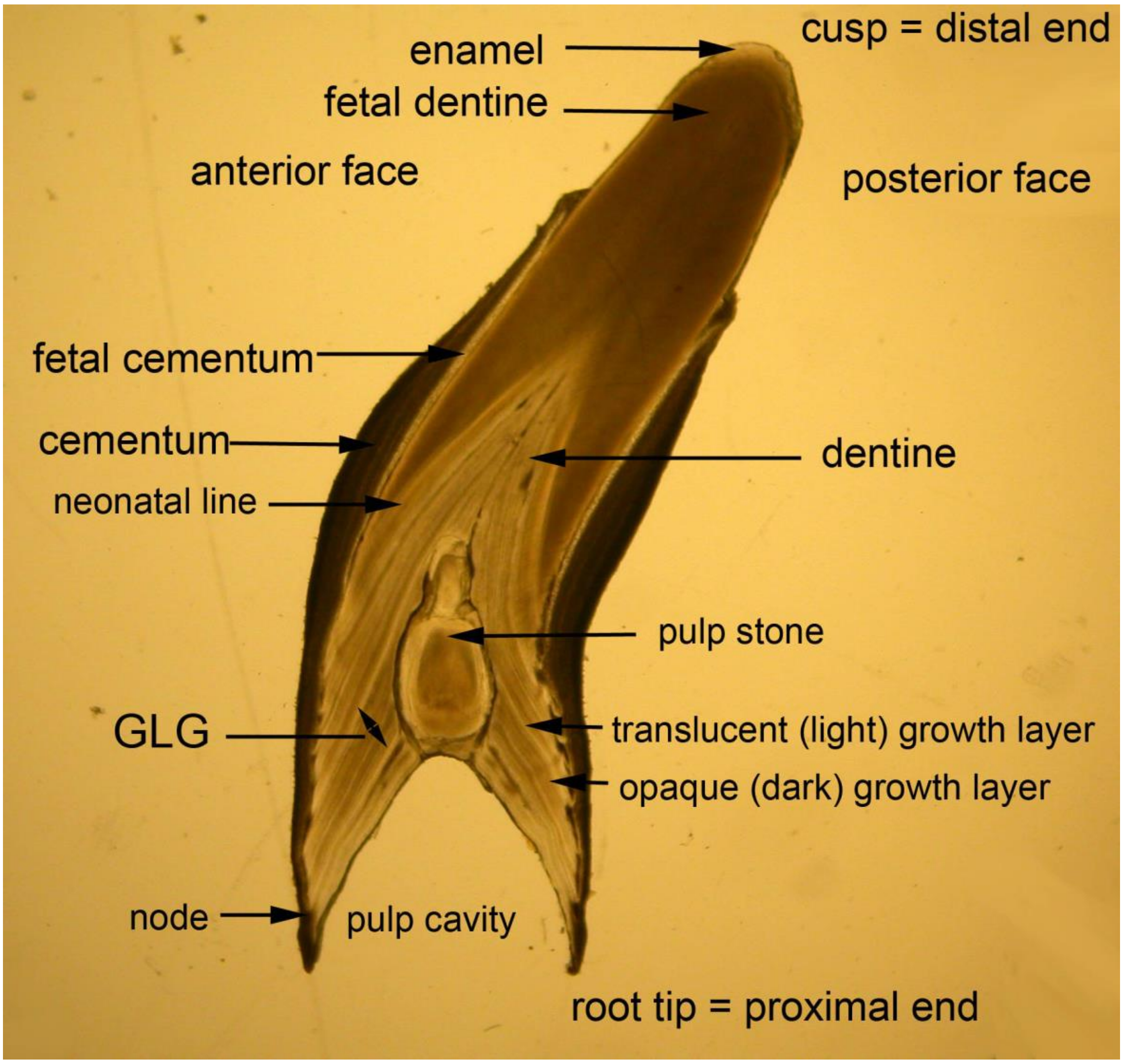
Stewart BE (2012) A technical report on methods for tooth preparation and age estimates of beluga (*Delphinapterus leucas*). Can. Tech. Fish. Aquat. Sci. 3020:xi+85 p.



Specimen AREP87-11, MNR-5 showing important features.

Appendix 5

Data sheet

Workshop participants adopted the datasheet (next page) used by Fisheries and Oceans Canada, Central and Arctic Region, presented here from Stewart (2012) with the author's permission. The workshop suggested notes be included for each reading identifying problems or issues and a digital image be appended to each data sheet.

Reference

Stewart, B.E. 2012. A technical report on methods for tooth preparation and age estimates of beluga (*Delphinapterus leucas*). Can. Tech. Fish. Aquat. Sci. 3020:xi+85 p.

Beluga (*Delphinapterus leucas*) age estimate data sheet. Part 1.

Specimen No.:_____________ Reader: _______________ Date: ____________

TOOTH SECTION:

Mandibular (MN); left (L) or right (R) ; no. in tooth row: ______

Is this section a good median section? Y N
Viewed wet with transmitted light? Y N

Sketch the cusp: Sketch the root tip:

AGE ESTIMATES:

Dentine reading no.

1. _____
2. _____
3. _____
4. _____
5. _____

FINAL: _____

Is this a minimum estimate? Y N

If yes, due to: a. wear of cusp

b. unreadable portion

Cementum reading no.

1. _____
2. _____
3. _____
4. _____
5. _____

FINAL: _____

Is this a minimum estimate? Y N

If yes, due to: a. wear of cusp

b. unreadable portion

OBSERVATIONS

Dentine

Fetal dentine present? Y N

Resorption or damage? Y N

Nodes at dentine-cementum interface? Y N

Nodes used for dentine GLG counts? Y N

Pulp stones/inclusions present? Y N

a. Distribution:
diffuse clumped regular other

b. Shape(s):

c. Estimate of the dentine surface area
that the inclusions occupy: _____ %

Clarity of growth layers:

Cementum

Resorption or damage? Y N

Other details/notes:

Clarity of growth layers:

poor good excellent

Last growth layer:

opaque (dark) / translucent (light)

poor good excellent

Last growth layer:

opaque (dark) / translucent (light)

Beluga (*Delphinapterus leucas*) age estimate data sheet. Part 2.

Reading No.: 2 3 4 5

Age estimate reader: ________________________ Date: _______________

	SPECIMEN. NO.	TOTAL GLG COUNT DENTINE	CEMENTUM	NOTES
1.				
2.				
3.				
4.				
5.				
6.				
7.				
8.				
9.				
10.				
11.				
12.				
13.				
14.				
15.				
16.				
17.				
18.				
19.				
20.				