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Report of the Workshop on Age Estimation in Monodontids

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EXECUTIVE SUMMARY

The workshop was a 2-day event organised immediately before the Society for Marine Mammalogy biennial conference in Tampa. Funding from the North Atlantic Marine Mammal Commission (NAMMCO) enabled participation of 4 invited experts and also supported the organization and logistics associated with the workshop. The breadth and depth of the workshop presentations made it clear that most issues concerning monodontid age estimation are not unique. Many researchers investigating many taxa have considered a diversity of methods and tissues to reveal biological records of age. Aside from the biological materials, accuracy and precision of the counts or metric have been considered, as well as their interpretation.

Relative age can be estimated using biological or chemical changes if the rate of change is known. Attempts to use **genetic telomere length** to estimate relative age show telomere lengths provide a measure of individual body fitness and condition rather than age, as environment, migration, health and reproduction affect telomere length. The method has potential but is still under investigation. Reviews of **aspartic acid racemization (AAR) aging techniques** on eye lenses from harp seals (*Pagophilus groenlandicus*), fin whales (*Balaenoptera physalus*), harbour porpoises (*Phocoena phocoena*), and bowhead whales (*Balaena mysticetus*), indicated potential for producing relative ages but warned that the presence of cataracts in the eye lens could seriously bias the age estimation upward. In narwhal (*Monodon monoceros*), tusk growth layer groups (GLG) correlated well with AAR age. The AAR method is relatively accurate, but species-specific racemization rates are essential for accurate age estimation. Age models using **endogenous fatty acid (FA) ratios** have been successfully derived for killer whales (*Orcinus orca*), and humpback whales (*Megaptera novaeangliae*). Preliminary results using a single FA ratio for Cook Inlet belugas (*Delphinapterus leucas*) correlated with age from tooth GLG for physically immature animals. Future

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work using two FA ratios in belugas is expected to provide more precision in relative age. It may be possible to use bone density as an indicator of relative age in beluga and narwhal flippers. The method would need to be calibrated with reference to GLG in beluga teeth and validated AAR ages in narwhal.

The recording of **historic hunting artifacts** recovered in bowhead whales in Alaska has presented an opportunistic and potentially remarkable insight into longevity of this species (which may exceed 100 years if the interpretation of the age of the artifact is correct).

Micro-CT scanning demonstrated potential for investigating internal structure of teeth and other hard tissue specimens. Because there is no destruction of the specimen and 3-D viewing is possible, this technique could be applied to specimens that are difficult to interpret from thin sections are rare, and therefore, not possible to section.

Counts of presumed annual markers provide a more accurate (absolute) estimate of age than other tissues which show gradual changes with age. Hard structures that show **regular episodic growth** are the most commonly used tissues to investigate for records of age that can be estimated from **Growth Layer Groups (GLG).** Tissues including bones, otoliths, claws, and ear plugs although teeth are most widely used. Undamaged **ear plugs in baleen whales** provide a permanent record of total age from GLG therein. Apart from longevity, life-history parameters of age at sexual maturation and possibly physical maturation can be identified from the GLG patterns. Such patterns might exist in some teeth and should be investigated. Ear plug extraction from carcasses of minke whales (*Balaenoptera acutorostrata*) is facilitated by a new method **using injected gelatin** which increases the possibility of extracting whole and undamaged ear plugs. This method should be evaluated for bowhead whales in which ear plugs are soft and fragile.

Teeth GLG are commonly used to age carnivorous mammals, including marine mammals. Techniques for preparing teeth vary. All are directed at obtaining the most complete record of clear laminae, key to which are tooth sections with correct orientation to display all the laminae. A review of aging in sirenians indicated there are many internal similarities between dugong (*Dugong dugon*) tusks and beluga teeth, and also perhaps narwhal tusks. The GLG deposition rate in dugong tusks is annual.

In belugas, counting **GLG in dentine,** as seen in medial longitudinal sections of teeth, is the standard method and consistent with methods used in other taxa. The most suitable method is using a thin untreated section (ca 150-200 microns). Counting **GLG in cement**, using the same medial sections, may be useful for belugas where cement is thick and especially when the dentine is

worn down at the tooth crown. Cement is not useful for most cetaceans. GLG patterns in sperm whales and belugas are very similar.

Precision and accuracy are essential in age estimation from GLG counts. The repeatability of the GLG counts (precision) and accuracy (whether or not the GLG counts indicate the correct age) are not the same. **Quality control** is essential for both and there must be regular monitoring of an aging programme. The best measures of age precision are coefficient of variation (CV), average percent error (APE) and index of dispersion (D), while the least reliable is percent agreement among readers which is usually the most commonly used assessment. A permanent reference collection of aging materials, *e.g.*, known-age beluga teeth, is the key to effective quality control. In an investigation of precision and bias in beluga tooth age data, it was concluded that errors arising in estimation of biological parameters can be both negatively and positively biased with varying degrees of variance. In turn these translate to errors in estimation of growth rates. Efforts should be made to quantify bias and precision.

One of the most persistent debates in age estimation of the beluga has been about the accurate translation of GLG counts into time units (years). The **measurement of radiocarbon, ^{14}C,** in laminated hard structures of animals has been a precise and successful method for validating age in many species, including **belugas where GLG deposition rate was found to be unquestionably annual.** The most direct age estimation technique is that of following recognisable individuals through time. **Long-term photo-ID monitoring** and surveys of the Gulf of St Lawrence belugas resulted in an abundance of reliable data on life history, age, reproduction, growth and colour change. Teeth collected during necropsies on recovered known-age and known-history belugas have validated an annual GLG deposition rate.

Investigation of age from teeth of known-history captive belugas, together with data on **tetracycline time-marking of teeth** generally also supported an annual deposition rate of GLG. However, GLG definition was unclear in some specimens, particularly in the juvenile phase. Several other studies confirmed the value of long-term monitoring of known animals for validation of age. **Information on growth and reproduction** of Cumberland Sound belugas that was presented in support of a deposition rate of 2 GLG per year was criticized on a number of counts and was not accepted by participants.

Future research was identified in several areas to fine tune our understanding. One potential technique for estimating total age from worn beluga teeth using **the angle of the boundary layers relative to the pulp cavity edge** appeared promising and should be followed up. Of a broader nature is the potential to understand the ecological correlates to lamina formation. **Laser ablation**

(ICPMS) for trace elements in beluga tooth GLG indicated some elements show periodic oscillations. Investigation of **stable isotope ratios** ^{13}C and ^{15}N in beluga teeth were also promising. The point of weaning can be identified from the ^{15}N depletion up to this point. Oscillations of elements in the teeth may be linked to ecology and movements associated with feeding and migration, although these may not be annual, and thus cannot be used as an age proxy presently.

A number of **specific recommendations for monodontids** were made at the workshop. Comparisons of aging methods using samples and data from free-living known-age, whales would be instructive. The number of samples of known-age captive beluga from which teeth can be collected should be augmented with comprehensive sampling of other materials useful for age estimation. A focus on the immature phase of growth in teeth in beluga with reference to captive animals to determine GLG patterns is desirable. Reference collections (hard parts) should be established and digital image exchange for calibration and training among labs be considered. Quality control routines should be established and should include periodic exchanges among laboratories and inter-laboratory calibration for all aging techniques. Comparison of tooth preparation methods among labs is desirable. A new study to estimate crown wear from angles of boundary layers into the dentin-cement junction in beluga teeth to estimate the maximum number of GLG that have disappeared should be initiated. Chemical time-marking for age calibration of hard parts and bomb radiocarbon validation of hard parts and eye lenses is encouraged. A study comparing GLG in teeth to GLG in ear bones for beluga, and if successful, evaluating ear bones as a method for obtaining direct estimates of age in narwhals, is encouraged. A comparison of GLG structure among stocks (free-living and captive) is desirable.

In conclusion, the workshop members agreed on several aging methods which are or may be applicable to monodontids, including potential new methods which, depending on the type of tissue required for analysis, may be applicable both to living and dead animals.

Overall, tooth GLG are judged to be the best and most precise method. Presently, tooth GLG are only useable in belugas, but the AAR technique is very promising in narwhals. More work needs to be undertaken on embedded tusks of young narwhal to help calibrate the AAR rate in narwhals. GLG in ear bones should be compared to results from the other methods. The AAR method should also be applied to beluga eye lenses to provide a correlation with beluga tooth GLG. Such a study might provide more reliability on the narwhal AAR work presently done.

Currently, **bomb radiocarbon** is the method that is most accurate and that can be used for calibration of alternative aging methods**.** However, the main limitation is that at least some of the teeth or hard tissues must come from animals that were born before the fallout commenced, *i.e.*, pre-1958.
The workshop agreed that an annual deposition rate of tooth GLG was to be the accepted standard in belugas.

Finally, it was agreed to publish the proceedings from the workshop in a volume of the NAMMCO Scientific Publication Series, entitled *Age estimation in marine mammals with a focus on monodontids which* is now online at http://septentrio.uit.no/index.php/NAMMCOSP/issue/view/236. The editors comprise the members of the Steering Committee for this workshop in addition to the technical editor, Mario Acquarone.

MAIN REPORT

Opening, Welcome and Introduction

The workshop opened with a welcome by Christina Lockyer, General Secretary of the North Atlantic Marine Mammal Commission (NAMMCO), who presented the other members of the steering committee responsible for planning and convening the workshop. The steering committee, appointed by the Joint Scientific Working Group (JWG) of the NAMMCO-JCNB (Joint Canada Greenland Commission on Narwhal and Beluga), was Aleta Hohn (NOAA, Beaufort, North Carolina, USA), Roderick Hobbs (NOAA, Seattle, Washington, USA), and Robert Stewart (DFO, Winnipeg, Manitoba, Canada) in addition to Christina Lockyer. Mario Acquarone, Scientific Secretary of NAMMCO, was appointed general rapporteur for the meeting.

Lockyer stated that the focus of the workshop was on monodontids although contributions on all marine mammals, and even other organisms, that had possible relevance to methods applicable for monodontids would be welcomed. Many contributions had already been offered and registered, and a booklet of abstracts of most presentations was available to participants at the workshop.

Workshop Background, Basis and Objectives

Approval for the workshop came from the JWG of the NAMMCO-JCNB, and a budget was subsequently approved by NAMMCO Council under the work of its Scientific Committee.

Initial publications on age estimation in odontocetes used tooth growth layer groups (GLG) were codified and defined by Klevezal (1980) and Perrin and Myrick (1980). A GLG is a group of incremental layers which may be recognised by virtue of cyclical repetition. Spacing of GLG is usually

constant or changes in a regular, systematic manner, usually diminishing with age, and a GLG must involve at least one change between light and dark incremental layers. Hohn (2009) provides a good overview of aging in marine mammals.

From a historical perspective, publications on age estimation in belugas (*Delphinapterus leucas*), date from the pre-1970s (Sergeant 1959, Brodie 1982) when 2 GLG per year were anticipated by comparison with the then supposed deposition rate in sperm whales (*Physeter macrocephalus*; Gambell and Grzegorzewska 1967). When this assumption for sperm whales was subsequently amended to an annual deposition rate (IWC 1969; 1980; Best 1970; Gambell 1977), a more general assumption was made for all odontocetes, except for belugas (Heide-Jørgensen *et al.* 1994). The assumption of an annual GLG deposition rate in odontocetes was also supported by other publications on a variety species for which validation of age estimation was possible, *e.g.*, Myrick *et al. (*1984, 1988), Hohn *et al.* (1989), and Lockyer (1993).

The question of GLG deposition rate was raised again for belugas at the 51st IWC SC (IWC 2000) by Hohn and Lockyer (1999) referring to the examination of two captive known-age, known-history belugas with tetracycline time-marking of teeth. Although no conclusions were reached at this time, there was sufficient evidence to cast doubt on the interpretation of 2 GLG per year in belugas.

Subsequently, a workshop, supported by NAMMCO and the NOAA laboratory in Beaufort, USA, was held in 2001 focusing on interpreting age from teeth of 10 known-age and known-history belugas (Lockyer *et al*. 2007). Recommendations included further monitoring of known-age captive belugas, trials of other aging methods, *e.g.*, aspartic acid racemization (AAR), and, not least, standardisation of GLG counting among researchers.

At the meeting of the JWG held February 2009 in Winnipeg, Canada, participants expressed broad support for a workshop to address age estimation in monodontids (beluga and narwhal). They noted, for example, the value of cross-laboratory calibration, standardisation of methods, and the use of AAR of eye lenses relative to growth layers in small, embedded tusks of narwhal. It was suggested that consideration should be given to how the insights on age estimation developed at the workshop(s) will be incorporated into model input. Better life-history data based on known-age animals will improve the reliability of population assessments. Finally, interest was expressed in having new methods of age estimation (*e.g.*, using fatty acids) explored in a workshop context.

NAMMCO indicated a willingness to convene and organize the workshop(s) and that selection of the venue(s) would be critical. For the practical components, it would be necessary to hold the workshop(s) in an appropriately and adequately equipped laboratory.

Recognising that there are a number of problems with age estimation for both the monodontid species, and that these need to be studied in more detail, the JWG recommended that a steering group (chaired by Lockyer and including Hobbs, Hohn, and Stewart) work inter-sessionally by e-mail, to scope the problems and produce draft terms of reference for one or more workshops. Terms of Reference (TOR) were developed by the steering group, and subsequently approved by NAMMCO which also approved a budget for the workshop(s). The two TOR provide the following guidance:

1. To standardise tooth GLG reading methods for age estimation in beluga and narwhal where feasible, and calibrate against other techniques such as using AAR, and produce a manual as a guide to tooth reading in the above species.
2. To draw together traditional and new techniques for determining age in marine mammals, where these methods may be applicable to belugas and narwhals, by holding a workshop of experts in this field, and produce a report.

At this stage it became clear that there should be two different workshops: one where new ideas and techniques could be presented and discussed (TOR 2), and another which focused purely on laboratory preparation, examination, interpretation and validation of teeth (TOR 1). Two workshops were planned in association with the biennial Society for Marine Mammalogy (SMM) conference in 2011 when there would be the opportunity to bring as many experts together as possible. TOR 1 was addressed at a laboratory workshop at the NOAA Beaufort laboratory following the SMM Conference (Lockyer *et al*. 2016).

For the current workshop, held in Tampa, the following TOR were derived from TOR 2 (above):

1. Review current methods of age estimation in marine mammals with a focus on monodontids.
2. Recommend the method(s) most suitable for monodontids; and trials of any new techniques that are as yet untried in monodontids.
3. Compile previously unpublished papers submitted to the workshop and relevant to age estimation in monodontids in a publication volume 10 entitled "*Age estimation in marine mammals with a focus on monodontids*" of the NAMMCO Scientific Publication Series.

Invited participants (Appendix 1) discussed a diversity of studies as noted in the workshop agenda (Appendix 2). The following sections present each author's abstract, a summary of the presentation materials, and discussions that followed each presentation. The workshop was attended by a number of researchers and many students interested in age estimation. Several attendees offered to present their recent findings to the group. These presentations from the floor are reported, in the same manner, after the invited papers.

Presentation 1: Age estimation methods applicable in mammals with special emphasis on marine mammals and especially monodontids – Fiona L. Read

ABSTRACT: *Accurate age estimates are fundamental for understanding and interpreting many aspects of mammalogy. Age has traditionally been used to understand the biology of a species at an individual and population level and further study the dynamics of the population and the need for accurate and precise ages has increased over time due to changes in research interests. Age estimation can be defined as absolute and relative age. Absolute ages are achieved by counting growth layer groups (GLG) in hard structures such as teeth, ear plugs, baleen, bones and claws. Relative age can be obtained by methods such as aspartic acid racemization of the eye lens, telomere length, bone mineral density, fatty acid signatures etc. The present work provides a review of methods for age estimation in marine mammals, including the pros and cons and accuracy of each method. Methods for validating age estimations will be discussed. Furthermore, the unresolved discrepancies of aging monodontids (narwhals and belugas) (mainly 1 or 2 GLG per annum) with special emphasis on recommendations for overcoming these problems and the application of newer methods, e.g., telomere length, will be discussed. Finally, concluding with the main objectives that future age estimation studies should focus on.*

The presentation covered a variety of materials (Fig. 1) and methods used for estimating age in marine mammals, with an appraisal of each type. During her presentation the difference was noted between absolute and relative ages. The concept of a Growth Layer Group (GLG) was also explained as a repeating pattern that equates to a period of time. The structure of teeth was discussed in relation to the dentine originating from the pulp cavity and external cementum originating from the gum tissue, as well as the significance of the neonatal line at birth.

Preparation methods:
Direct methods
In summary, for teeth:

- Untreated sections: are good for dentinal layers in some species, and can be used for cementum. The method is time and cost effective. The method is less reliable for some species as GLG are not prominent and the pulp cavity becomes occluded with age.
- Stained sections: are best for small teeth, and are successful for several species but are time consuming to prepare and require additional specialized equipment.
- Acid etching of half teeth: is a simple and inexpensive method, but is not very satisfactory for teeth from small animals.
- Scanning electron microscopy (SEM): produces a 3D image with high clarity, which is very readable, but is not good for small species and is very expensive in time and resources.
- Microradiography: is a method that is non-destructive, unlike many others mentioned. For determining older GLG, high precision is needed.

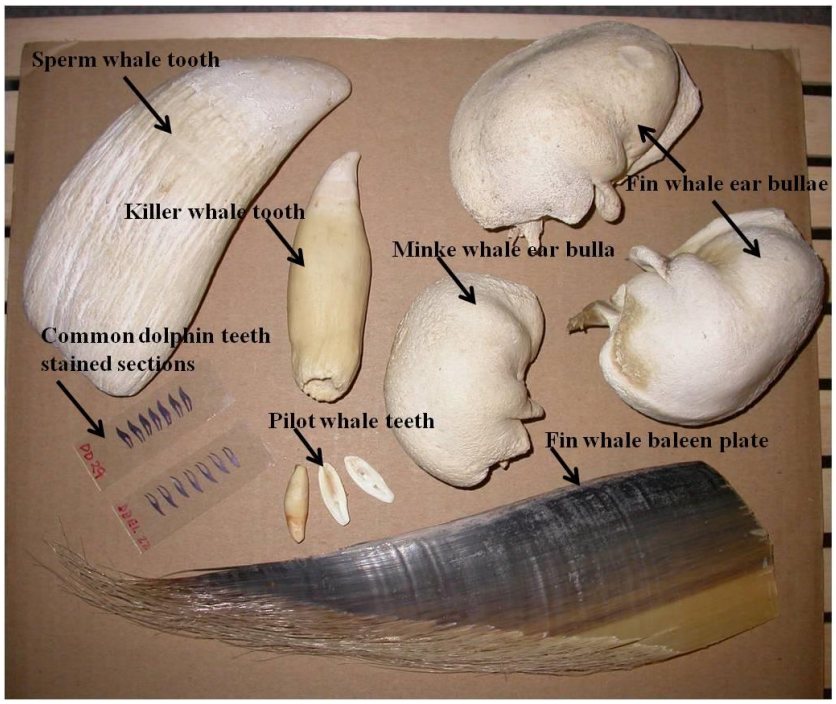

Fig. 1. Hard parts including teeth, ear bones and baleen plates that can be used for age estimation (Photo: Christina Lockyer, Age Dynamics, Denmark.).

Other materials than teeth employed for aging include:

- Claws: a method useable for seals, which is quick, easy and inexpensive. The method is only applicable for young animals with less worn claws.

- Baleen plates: this method is useable for baleen whales, but as with claws, only in young animals with unworn baleen.
- Ear plugs: this is a method useable for baleen whales. Total age and age at sexual maturity can be estimated, but the ear plugs are challenging to collect.
- Ear bones (tympanic bullae): thin untreated sections provide GLG counts lower than ear plugs, perhaps because of bone resorption with age. The method is limited to a few mysticete species.
- Periotic bones: this method is used in manatees where teeth (molars) are changed throughout life and cannot be used, but maybe be useful for other species, as well. Sections are decalcified and stained.
- Tusks: found in narwhals and walruses, as well as male dugongs, are often worn down and provide an incomplete age.
- Other bones, mainly mandibles and ribs, are sometimes useable but the methods are very species-specific, and to be used as a last resort when other means fail.

Mineralization anomalies in teeth can be used to identify environmental variations or unusual events (El Niño, life-history events – Manzanilla 1989). Presence of anomalies can be a source of potential misinterpretation of age. Misinterpretation of the GLG and poor preparation of the sections can lead to inaccuracy in counting. These issues may be overcome by some standard routines. There are also differences in GLG between young and old animals, which may lead to age inaccuracies. The former have accessory lines, while old animals may have tightly packed GLG that are hard to differentiate.

Indirect methods

Such methods provide relative age and are mostly unpublished and/or are unsuccessful.

- Bone density: a non-invasive, fast and relatively inexpensive method. However, it requires basic age data for the model and specialized equipment. The method can be used from live to badly decomposed animals, both sexes.
- Genetic telomere: a method based on measurement of the average telomere length. Only two studies on marine mammals are currently published and are not unequivocally promising. Validation and calibration are necessary and the results may only produce age class information.
- Aspartic acid racemization (AAR): a method based on the D/L ratios of enantiomers, requiring stable temperatures. The method is better for old animals and species for which other methods are difficult, sensitive, and complicated, but it needs species-specific data for calibration and more precision.

- Fatty acid signatures (FA): the method can use the FA composition of the outer blubber layer. It has been successful with some species and is minimally invasive, as biopsies can be taken. Presently the method cannot be used for estimating longevity and is not comparable across labs. The underlying biological factors are unclear.
- Ovarian corpora: a method that is easy and inexpensive, but requires dead animals and applies only to females. To calibrate age, life history data are needed to provide age at first parturition.
- Baleen plates in baleen whales: can be analysed for isotopic patterns due to seasonal dietary changes. The method has been successful in bowhead whales but may only be useful in younger animals when other methods are more reliable.
- Dental colour: this method requires a standard colour reference guide for interpretation.
- Morphometrics: a method that is fast, consistent and cheap, *e.g.,* body length, but is not precise either for young animals, which may grow at variable rates, or older animals, with substantial overlap in age at length. Over-all growth may be too sensitive to energy intake to precisely measure age.

Validation:
Validation is essential to aging methods but has rarely been done in marine mammals. Validation can be effected through

- Known age, known-history animals. Photo-ID may help in tracking such free-living animals.
- Captive animals born in captivity or of known age when captured. GLG might not be as well defined as in wild animals
- Biomarkers, ideally administered on the animal's birthday and at set intervals, *e.g.,* lead acetate or tetracycline antibiotics that leave a time-mark in hard tissues.
- Bomb radiocarbon isotope fallout (a natural biomarker), based on ^{14}C from nuclear experiments in the 1950s and 1960s. The method requires samples from before and after 1958. This is high technology and needs expensive equipment.
- Artifacts found in animals. These may include tags of natural or artificial origin, *e.g.,* "Discovery" tags, harpoon heads found in whales. It lacks precision due to the time span in which the technology was used and the life span of the animal before being 'tagged.'

General conclusions:

- Hard tissues, *e.g.,* teeth and bones, are good materials for aging but are species-specific.

- More than one method may be accurate for a species but time and funding constraints often preclude the use of more than one.
- There is a need for standardisation among research labs for both method and reading GLG.
- All methods should be validated.
- Combining complementary methods might be required for 'tricky' species.
- Often the ages estimated are only minimum ages.

Monodontids

Beluga and narwhal are problematic species with respect to aging. To date, there are still discrepancies among methods and further work on validation is required. However, advances have been made for narwhal with the application of AAR techniques to eye lenses, although teeth (embedded or tusks) are not generally useable except in males. For belugas, teeth are potentially the most useful method of aging. There is a need for standardisation of GLG counting, and especially for stating the number of GLG in addition to the age estimated. There is not yet full agreement on a deposition rate of GLG for beluga, although one per year is now favoured. Once GLG deposition rate is certain, all previous age data should be revised. Until agreement on GLG deposition rate is reached, management should be cautious about age parameters.

Focusing on belugas, a summary of methods for estimating age is provided in Table 1 where suitability, cost, source reference, and other factors are noted.

Discussion

Read was thanked for her comprehensive review. Steve Campana queried whether new imaging technology can reveal previously invisible structures in teeth of marine mammals. Imaging technology is relatively accessible. However, it is difficult to standardise the enhancements to the point where one does not see structures which do not relate to age. The possibility of enhancement by chelation by EDTA was raised. Care should be taken to interpret age artifacts which may be either opportunistic or experimental. It is important that animals tagged or marked at birth be followed throughout life for validating age methods.

Table 1. Different methods of age estimation in belugas with an appraisal of relative cost and efficacy. Green cells indicate preferred methods; yellow indicates some merit and areas for possible development; and red indicates little merit for routine aging.

BELUGA WHALE		Relative cost	Time required	Precision	Accuracy (where tested)	Pros	Cons	Reference
Teeth	**Untreated-dentine**	low	Short	medium	high	Clearer than cemental GLG. Does not close	Severe wear of the tip; some stocks show less distinct GLG	Perrin and Myrick 1980
	Untreated-cementum	low	Short	medium	medium		Cemental GLG form too closely to read; often the cemental GLG count is less than the dentinal GLG	Lockyer *et al.* 1999, Stewart *et al.* 2006
	Stained-dentine	medium	Long	high				
	Stained-cementum	medium	Long	high	high			
	Acid etching	low	Medium	low	low	GLG visible	Did not improve readability	Perrin and Myrick 1980, Pierce and Kajimura 1980
	Scanning Electron Microscope	high	Medium	high	low		GLG visible but not countable	Goren *et al.*1987
	Microradiography	medium	Short	low	n/a			
Mandible	**Untreated**	low	Short	medium	medium			
	Stained	medium	Long	high	medium			
Others	**Bone density**	medium	Medium	high	n/a			

BELUGA WHALE		Relative cost	Time required	Precision	Accuracy (where tested)	Pros	Cons	Reference
	Genetic telomere	high	Long	medium	n/a			
Validation	**Aspartic acid racemization**	high	Long	high	n/a			
	Fatty acid signatures	high	Long	high	n/a			
	Relative age from ovarian *corpora*	low	Short	low	low		Accessory *corpora lutea*	Suydam 2009
	Known-age / history (incl. wild)	low	Long	high	medium/high	photo-id project has started		Hohn and Lockyer 1999, Lockyer *et al.* 2007
	Tetracycline	low	Long	high	medium			Brodie 1982, Hohn and Lockyer 1999, Lockyer *et al.* 2007
	Captive	low	Long	high	high			Brodie1982, Hohn and Lockyer 1999
	Bomb Radiocarbon	high	Long	high	high		Requires animals pre- and post 1958	Stewart *et al.* 2006
	Artifacts	low	Short	medium	n/a			George and Bockstoce 2008

Presentation 2: Direct aging in dolphins, including belugas – Aleta Hohn

ABSTRACT: *Early studies suggested the possibility that teeth in dolphins (and pinnipeds) contained growth layers that served as an indicator of age. Since that time, most of the emphasis of direct age estimation in dolphins and porpoises has focused on validation of deposition rates, improved methods of preparing teeth for optimal resolution of growth layers, and standardising protocols for counting growth layers. A workshop in 1978 discussed the complexity of growth layer patterns and the term "growth layer group" (GLG) was agreed to best represent that annual layers included smaller incremental growth layers. These incremental layers have been referred to in various ways, with particularly distinct incremental layers appearing as accessory layers that confuse readings of annual GLG or as marker lines that represent life-history events. Despite remaining questions, with the exception of long-terms studies of known individuals, counts of GLG still serve as the best means of estimating age in dolphins.*

Hohn discussed the advantages of using teeth for age estimation and some caveats. Tooth structure reflects the animal's physiology at the time of deposition, which is a bonus for life history and stock information. The anatomy of teeth, with specific examples, was presented. The disadvantages of using teeth included sub-annual incremental growth laminae, which confuse age estimation, and crown wear, which erodes layers. It is critical that the orientation of the section is in the midline from crown to root apex for dentinal counts. In some species GLG become compressed with age, so that layers are missed towards the root apex in old animals. There can be variations in tooth ultrastructure within species according to stock and region. The use of teeth for aging is relatively easy and inexpensive leading many "non-experts" to use it without training, producing erroneous, non-standard ages. Accurate age estimation is important, as it is important to be aware that there are biases associated with the techniques (Hohn and Fernandez 1999).

Discussion

The possibility that dentinal-cemental layers might not be consistent was raised, but this seems to be a species-specific question. The cemental GLG counts can help in cases where the young dentinal layers are missing, depending on the species and how the cementum is formed. With reference to harbour porpoise, the cement can be used to greatly determine the first GLG.

With respect to potential differences between captive-held and free-living animals, it was stated that there are no differences in tooth GLG in *Tursiops* in captivity or in the wild.

Presentation 3: Investigating the deposition of growth layer groups in dentine tissue of captive common dolphins - Sinéad Murphy, Matthew Perrott, Jill McVee, Wendi Roe and Karen Stockin

ABSTRACT: *Knowledge of age structure and longevity (maximum age) are essential for modelling marine mammal population dynamics. Estimation of age in common dolphins* (Delphinus sp.) *is primarily based on counting Growth Layer Groups (GLG) in thin sections of decalcified and stained hard dental tissues. The incremental deposition rate was validated for* Delphinus sp. *30 years ago through the use of tetracycline, an antibiotic that was employed as a fluorescent vital marker in teeth of captive dolphins. Although an annual GLG deposition rate was identified, it is not known if the pulp cavity becomes occluded in older individuals or if GLG continue to be deposited in dentine tissue. To date, the oldest wild common dolphin has been aged at 30 years. To investigate the deposition of GLG in dentine, tooth samples were obtained during the necropsies of two New Zealand common dolphins that were held in captivity for 31 and 34 years. Individuals were captured together in Hawkes Bay, and classified as juveniles based on physical appearance. Teeth were processed in two aging laboratories, using four different bone decalcifiers, two sectioning techniques incorporating the use of both a freezing microtome (-20˚C) and paraffin wax microtome, and two different stains. Time required for decalcification was determined by manual assessment of pliability, calcium oxalate precipitation end point tests or radiography. A maximum age was estimated for one of the dolphins, in line with that proposed based on estimated age at capture and period in captivity. However, a hypomineralized area was observed in the dentine tissue close to the pulp cavity of the second individual, preventing estimation of maximum age. The presence and structure of this anomaly is explored further within the study.*

A general introduction was given on common dolphin from different regions of the world with estimates of longevity from teeth up to *ca.* 30 years. However, this presentation focused on the species around New Zealand, where two common dolphins, *Delphinus sp.*, captured young, were held in captivity for more than three decades before death. Shona died in 2006 at 206 cm length, after 31.3 years in captivity, suggesting an age of 4 years from body length at time of capture (Kastelein *et al.* 2000); Kelly died in 2008 at 204.5 cm length after being held captive for 33.75 years, suggesting an age of 3 years from length at time of capture (Kastelein *et al.* 2000). Two preparation methods were applied to the teeth although both fixed the teeth in 10% neutral buffered formalin initially. Wax embedding, sectioning at 5 micron, and haematoxylin staining were employed in St Andrews University, Scotland, and frozen sectioning at 18-25 micron with toluidine blue staining

at Massey University, NZ. Before sectioning, different decalcification methods were tried and compared, using whole teeth from each animal:

St Andrews:
- RDO - up to 3 days
- Formical-4 - more than 6 months

Massey:
- 10% Formic Acid - up to 6 weeks
- 10% EDTA - up to 6 weeks

Different endpoint tests (radiology or ammonium oxalate) were found for all chemicals. In some instances, the period for decalcification extended into months, and the chemicals were not completely effective. RDO was the most effective. Estimated ages for Shona were up to 27-36 years, while for Kelly were up to 19 years, far less than anticipated due to areas of hypomineralization around the pulp cavity. Both captive dolphins had lighter skulls than wild dolphins of a similar skull length found stranded along the New Zealand coastline.

Discussion
There was a suggestion that the teeth be pared down so that only the central part of thickness of about 2-3 mm is decalcified. This method would facilitate and hasten the decalcification process because of better permeation of the decalcifying agent.

Presentation 4: Age estimation in seals - Fiona L. Read

ABSTRACT: *Accurate age estimates provide valuable information about the age structure, age at sexual maturity, and longevity of a population and are fundamental for understanding and interpreting the dynamics of a population. Age estimation in seals is particularly important due to the large numbers harvested in management systems and their large fluctuations in population size resulting from viral epidemics, e.g., the harbour seal* (Phoca vitulina) *phocine distemper epidemics in northern Europe in 1988 and 2002. Traditionally in seals age is determined by counting growth layer groups GLG in the dentine and/or cementum of teeth and, less frequently, in claws. In recent years, more novel approaches of age estimation have also been attempted with varying degrees of success, e.g., telomere length and aspartic acid racemization. The following presentation will review the methods used to establish age estimates in seals, the pros and cons of each method, the best tooth for age estimation and how the methods have been validated and calibrated, e.g., known age animals and multi-reader experiments. The presentation will conclude with how our present knowledge for obtaining age*

estimates in seals can be applied to age estimation of monodontids (narwhals and belugas).

Direct methods of aging include tagging, freeze-branding and photo-ID. For indirect methods, claws and teeth are used. The presentation covered the following species: grey seals (*Halichoerus grypus*), harbour seals (*Phoca vitulina*), harp seals (*Phoca groenlandica*), and ringed seals (*Pusa hispida*), although other species were mentioned (Table 2). The best tooth for aging is the canine, although others have been used, and tooth selection largely depends on whether the animal is alive or dead. Mineralization anomalies such as pulp stones were discussed. Their presence complicates the counting of GLG. Other methods tried for aging were mentioned, *e.g.*, AAR, genetic telomere length, radiography and X-ray of bone density and teeth. However, teeth remain the best method for seals, although AAR and telomere length are promising.

Table 2. Tooth preparation and age estimation techniques in seal species.

Seal Species	Section	Untreated / Stained	Stain	Cementum / Dentine	Validated	Reference
Baikal	Longitudinal	Stained	Haema-toxylin	Cementum	-	Amano *et al.* 2000
Bearded	Transverse	Untreated	-	Cementum	With claws	Benjamins en 1973
Grey	Longitudinal	Untreated	-	Cementum	Known-age	Hewer 1964; Mansfield 1991
Harbour	Longitudinal	Stained	Toluidi ne Blue	Cementum	Known-age	Dietz *et al.* 1991; Lockyer *et al.* 2010
Harp	Transverse	Untreated	-	Dentine	Known-age	Bowen *et al.* 1983; Frie *et al.* 2011
Monk	Longitudinal / Transverse	Untreated	-	Cementum	-	Murphy *et al.* 2012
Ringed	Longitudinal	Stained	Haema-toxylin	Cementum	-	Stewart *et al.* 1996

Discussion

The possibility of scaling the weight of teeth with body size/age to obtain an approximate age was discussed.

Presentation 5: A brief review of age estimation in sirenians, focusing on dugong tusks - Christina Lockyer

ABSTRACT: *The different evolutionary origins of sirenians, with links to elephants, means that teeth in this Order cannot generally be used for age estimation. The specialised molars in manatees erupt at different times and wear down and move forward so that they are replaced (Marsh 1980) – as in elephants. Dugongs however have molars and premolars, which wear down, and a pair of incisors. These incisors erupt in males and continue growing throughout life to become tusks. Internally their structure shows a regular annual incremental GLG pattern (Mitchell 1976, 1978). Longevity can exceed 60 years. Tusks generally do not erupt in females, so other techniques must be employed for aging, e.g., dry eye lens weight. In manatees, ear bones can be used for aging, but this topic is not discussed. The similarities between internal GLG patterning in dugong tusk and both sperm whale and beluga whale teeth are reported.*

Lockyer emphasised the longevity of sirenians and the very similar GLG patterning in teeth of monodontids. In some respects the dugong tusk in males has similarities to the tusks of male narwhals.

Presentation 6: Prospects for genetic age estimation of cetaceans - Morten T. Olsen, Martine Bérubé, Jooke Robbins and Per J.Palsbøll

ABSTRACT: *Although the proliferation of tools available to cetologists has increased our understanding of whale ecology and evolution, there are questions of a temporal nature that will remain unanswered until a reliable and accurate method of age estimation is developed for free-ranging cetaceans. Telomeres are DNA sequences situated at the end of chromosomes and tend to shorten with age, suggesting that telomeres may be used as a marker for age estimation. Here we report on the relationship between telomere length and age in the humpback whale* (Megaptera novaeangliae). *We used four different qPCR methods to estimate the rate of telomere shortening both across samples and* in vivo *in individual humpback whales for which multiple skin samples were available. The overall correlation between telomere length and age was weak, and highly variable among individuals of similar age, suggesting that telomere length measured by the qPCR method is an imprecise predictor of chronological age in humpback whales. We discuss the potential factors responsible for the observed patterns as well as the prospects for age estimation of cetaceans by use of the above and alternative methods for telomeres length measurement, such as TRF analysis and the novel dot blot method.*

Olsen's presentation indicated genetic material had been isolated from skin biopsies of 56 humpback whales using quantitative PCR analysis. A ratio between T/S was made using a known reference gene (Cawthon 2002, 2009*)*. Telomere length was not precise for determining the absolute age, however, it might be used for characterizing the age classes of a population. Presently there are inherently large experimental errors. The method was nevertheless promising for relative age and for use in aging live animals. Telomeres can express biological age and are thus reflect external factors that may affect health and growth such as oxidative stress, *e.g.*, pollution, metabolism and diving hypoxia, reproduction and general health, which may affect the length of telomeres.

Comparing methods, TRF (telomere restriction fragmentation) has been found to be a precise and more accurate, while relatively expensive, technique for aging. The Dot Blot method (Kimura and Aviv 2011) requires less refined DNA and is relatively quick and inexpensive, and is useful for standard applications.

Species-specific calibration is required for aging. For humpback whales, additional older known-age animals are required for calibration, and a cross-lab calibration is needed. The rate of telomere shortening is generally low although there is large individual variation. At best, telomeres may be proxies of life-history trade-offs.

Discussion
The study was accepted as preliminary but with interesting results. Although it is perhaps unlikely that this method may be useable for precise aging in any marine mammal, the technique has promise for defining broad age classes – useful in live populations, and may reflect life history of individuals.

Presentation 7: Age estimation from teeth in large odontocetes - Christina Lockyer

ABSTRACT: *This presentation introduces the use of teeth from large whales to estimate age. The concept of counting Growth Layer Groups (GLG) that form throughout life is discussed with reference to its validity, and the assumption of an annual incremental rate of GLG. The species used as examples include sperm whales, killer whales, bottlenose whales, and beaked whales. The method of halving the tooth from crown through root for sperm whales* (Physeter macrocephalus)*, and etching the smooth cut surface with 10% formic acid to throw the GLG into relief is satisfactory. However, methods of thin sectioning at 100-200 micron, as well as decalcification, thin-sectioning at 25 micron and subsequent staining, are discussed for smaller species such as beaked whales, e.g.,* Mesoplodon sp. *In sperm and killer*

whales, problems of wear at the crown of the tooth lead to under-estimation of age in old animals. The presentation concludes with comments on the relevance of the methods of tooth preparation to beluga, and finds similarities in sperm whale teeth GLG patterns as well as crown wear and possible longevity to belugas

Methods described included acid-etching half-teeth with 10% formic acid or other agents (Gambell and Grzegorzewska 1967, Evans *et al*. 2002), thin sectioning, and decalcification methods with stained thin sectioning. For large teeth such as sperm whales, the best method is the simple acid-etching of half teeth. Special problems arise with teeth from some beaked whales *(Ziphiidae)* in which teeth are curved and difficult to cut/section. In conclusion, the size of the tooth dictates the method, but untreated sections work for small sperm whales, killer whales, beaked whales, and bottlenose whale (*Hyperoodon ampullatus*), teeth. The estimation of total age can be problematic when the crown is worn down and GLG are missing. The relevance to monodontids includes superficial similarities between beluga and sperm whale teeth; the untreated section method is good for both and crown wear is often severe and leads to underestimation of age. It is helpful to have body size data to compare with GLG age.

Validation of age has been feasible for known-history killer whales, some also with tetracycline antibiotic time-marking of teeth, but requires subsequent retrieval of teeth (Myrick *et al*. 1988). ^{14}C incorporated from atomic bomb fallout may be a useful validation marker in all species born pre-1958.

Presentation 8: Age estimation in mysticetes with a focus on ear plugs - Christina Lockyer

ABSTRACT: *The different methods that have been employed to age Mysticetes are briefly noted with comment on their applicability. The focus is on ear plugs as being the best method in Mysticetes. The ear plug (paired) which is of epidermal origin and is found in the external auditory meatus, grows continuously throughout life and thus holds a complete record of age. The incremental rate of deposition of GLG is annual and the ways this has been validated are reported. The ear plug anatomy, its collection, and the method of preparation for GLG counting are described as are the internal GLG pattern that changes with age to permit estimation of age at sexual maturity at the transition phase. The ear plug is generally used successfully in fin, sei and minke whales, and also can be used in blue, Bryde and humpback whales. The total age and the transition phase enable life history parameters to be determined retrospectively. A comparison is made with beluga tooth GLG patterning, noting the occurrence of accessory lines which*

are primarily a juvenile feature in Mysticetes, and the compacting of GLG in old beluga teeth.

Age estimation has been done using ear bones, jaw bones and tissues of epidermal origin (ear plugs and baleen plates) which contain GLG. Other methods relying on physical or chemical analysis include eye lens weight and opacity, AAR of eye lens, and isotopes in baleen plates. However, these latter require calibration using more precise methods that have GLG as a reference point.

The ear plug anatomy (Purves 1955) was described as was the method of extraction from carcasses. Ear plugs have been used for age estimation in several species of baleen whales (Ichihara 1964, Ohsumi 1964). The easiest approach for extraction is from the back of the skull when severed from the vertebral column so exposing the occipital condyles. However, this approach may require major flensing. Once the tympanic bullae are located, the ear plugs can be found and extruded via the external auditory meatus. The ear plug is suitable for age estimation in balaenopterid whales, especially blue whales (*Balaenoptera musculus*), fin whales, humpback whales, sei whales (*Balaenoptera borealis*), and Bryde's whales (*Balaenoptera brydei*). Minke whales also have useable ear plugs but there are challenges in aging those from certain populations, *e.g.*, North Atlantic, due to fragility and also poor GLG definition. Also, minke whale GLG are generally not as clear as in other balaenopterid species, and readability of the GLG can be variable (Kato 1984, Kato *et al.* 1991). Ear plugs from balaenid whales are problematic and ear plugs have been used in gray whales (*Eschrichtius robustus*) (Rice and Wolman 1971).

The ear plug requires fixation and preservation in neutral buffered 10% formalin. The paired ear plugs are "shaved" down lengthways to the core centre using an old-fashioned straight hand razor, exposing the neonatal line and GLG above the "glove finger." Once exposed, the GLG can be counted using low-power magnification. A complete record of age is recorded as well as life-history stages from GLG growth pattern changes, *e.g.*, age at sexual maturity (transition phase) and even physical maturity (Lockyer 1972, 1974, 1984), providing parameters that can be used at a population level. Roe (1968) provided validation of annual GLG deposition in fin whales.

The relevance to monodontids is indirect. Ear plugs are very different structures from teeth and are not generally applicable in odontocetes. However, ear plugs and teeth have continued growth and potential for total age records; both have accessory lines that may confuse age estimation. The transition phase in ear plugs might be something to look for in teeth GLG patterns.

Presentation 9: Feasibility study on the incorporation of the gelatinized collection method and the freeze-section technique of the ear plug in age estimation in common minke whales - Hikari Maeda, Tadafumi Kawamoto and Hidehiro Kato

ABSTRACT: *Because of its soft structure, ear plugs of common minke whales* (Balaenoptera acutorostrata) *are easily damaged during their collection from the external auditory meatus, especially among younger animals. In addition, there are still problems existing for ear plugs with unclear lamination on the bisected surface of the core. The present study tried to solve these two problems in age estimation of the common minke whales, by examining the feasibility of new techniques incorporating the gelatinized collection method and a histological approach by the freeze-sectioning of the core in ear plugs.*

For the first problem, we have tried a new ear plug collection method as follows; i) filling the space in the external auditory meatus with gelatin, ii) hardening the gelatin encasing the ear plug and any fragments by spraying a cooling gas, iii) removing the gelatinized ear plug from the meatus. Using a total of 214 trials on the minke whales at the scientific permit survey platform (JARPN II coastal program) in 2007 to 2009, it was revealed that embedding ear plugs with gelatin material minimized the proportion of breakages at the neonatal region, especially among ear plugs in younger animals. This obviously leads to an increased proportion of readable ear plugs and identifies high utility of the present gelatinized collection method. For the second problem, so as to have clearer core surface images of growth layers, we examined histological sections (thickness 5-10μm) sliced by the Kawamoto specialized frozen sectioning techniques (Kawamoto 2003), with staining by three different agents: Sudan III, Haematoxylin – Eosin, and Alizarin red. Through a total of 8 experiments, the histological section with Alizarin red gave the clearest growth laminations where we easily identified both dark and pale laminations, suggesting a close relation to the seasonal changes in intake of calcium through feeding. The present frozen section is also useful for investigating further detailed structure of ear plugs.

This presentation concluded that using the gelatinized technique for collection of ear plugs with the injection of liquid gelatin around the ear plug and the solidification, improves ease of extraction and, by maintaining the integrity of the ear plug, also improves the subsequent readability of GLG. The histological experiments indicated that the frozen sectioning technique and staining with Alizarin red helps clarify the GLG.

Discussion
The point was raised that probably the gelatin extraction technique could be used on the bowhead whale in which the ear plug is generally very soft and disintegrates easily. In general, the gelatin technique seems to be improving the extraction of ear plugs.

Kato noted that the correlation of GLG between ear plug and tympanic bullae in minke whales was good and that such a correlation might be sought for GLG in teeth and tympanic bullae in odontocetes, *e.g.,* belugas, especially for predicting real age when wear was present in the tooth crown. The ear bones are common to all whales.

Presentation 10: Age estimation with age validation from eye lens of fin whales and harbour porpoises - Nynne Hjort-Nielsen

ABSTRACT: *The aspartic acid racemization (AAR) method is based on the fact that the amino acids in nearly all living tissue, consists solely of L-isomers, but once the life process has ceased, the L-isomer amino acids undergo racemization to its D-isomer. This racemization occurs at a constant rate and it is thus theoretically possible to calculate the time that has elapsed once the racemization rate (k) and the ratio of D and L at birth ((D/L)0) are known. However, k is highly temperature-dependent and it is thus of great importance to keep this in mind when handling the samples. The AAR method was originally developed for dating marine sediments (Bada* et al. *1970) and fossils (Bada 1972; Bada and Protsch 1973) but later it has been applied in forensic science on human tooth enamel and dentine (for a review, see Meissner and Ritz-Timme 2010) and on human eye lens nuclei (Masters* et al. *1977, 1978). Studies of known-age humans (Ohtani* et al. *1995) and zoo animals (Eva Garde pers. comm.) found conclusive agreement between AAR age estimates and actual ages.*

This study estimated the age of 121 fin whales (Balaenoptera physalus) *and 83 harbour porpoises* (Phocoena phocoena) *by the AAR method and by counting the growth layer groups (GLG) in teeth (harbour porpoises) and ear plug (fin whales) respectively. The aspartic racemization rate (k_{Asp}) for fin whales was established from 15 foetuses classified to age, based on body length, and 15 adult whales age estimated by counting the GLG in the ear plugs. The k_{Asp} for harbour porpoises was derived from thirteen 1+ year old porpoises age-estimated by counting GLG in the teeth and four neonate porpoises classified to age based on length. The k_{Asp} values were determined by regression of GLG against aspartic acid D/L ratios. For the fin whales k_{Asp} of 1.10 x 10-3 year-1 (SE ± 0.00005) and a D/L ratio at birth ((D/L)0) of 0.028 (SE ± 0.0012) were determined. For the harbour porpoises a k_{Asp} of*

3.10 x 10-3 year-1 (SE ± 0.0004) and a (D/L)0 value of 0.023 (SE ± 0.0018) were determined. The fin whale k_{Asp} is in agreement with rates for other baleen whales, whereas the rate for harbour porpoise is considerably higher. Correlation between age estimates from AAR and GLG counts (individuals not included in the estimation of k_{Asp}) indicated that AAR might be a suitable method for determining age in marine mammals.

The theory and history of the AAR method were presented. Details of the method used in marine mammals were described, specifically for fin whales (n = 121) and harbour porpoises (n = 83) for which a known method of age estimation (GLG in ear plugs and teeth respectively) was available for calibration. The art of extracting the nucleus from the eye lens, which is surrounded by layers like an onion, is in rolling the lens until the nucleus is exposed and then peeling off the outer layers. Sources of error in the analysis can come from contamination and also cataracts in the lens. Calibration of the AAR age was by GLG in ear plugs for fin whales and GLG in teeth for porpoises. There were large variations in D/L ratios in young animals and a high racemization rate, k_{Asp}, in harbour porpoises for which this was the first study.

One surprising finding was an AAR estimated age of 120 years for an old stranded fin whale off coastal Denmark in 2010. This is the oldest estimated age for this species hitherto.

Validation of the AAR ages still requires reference to known-age animals.

Presentation 11: Comparison of aging techniques, estimation of racemization rates and validation - Eva Garde

Presentation 11a: Background, the harp seal study and the known age animals study

ABSTRACT: *This talk will focus on the aspartic acid racemization (AAR) technique and the AAR results from two different studies. One study (Garde* et al. *2010) compares age estimates of harp seals* (Pagophilus groenlandicus) *obtained by 3 different methods, the traditional technique of counting growth layer groups (GLG) in teeth and 2 novel approaches, aspartic acid racemization (AAR) in eye lens nuclei and telomere sequence analyses as a proxy for telomere length. The other (Garde* et al. *2012) uses animals of known age or ages estimated by another aging method to determine species-specific racemization rates and to examine the effect of body temperature on the rate of racemization. Both studies address the question of the AAR technique as a valid method for age estimation of mammals.*

Lower jaws (containing the teeth), eyes, and skin samples were collected from harp seals in the southeastern Barents Sea for the purpose of comparing age estimates obtained by 3 different methods, the traditional technique of counting growth layer groups (GLG) in teeth and 2 novel approaches, AAR in eye lens nuclei and telomere sequence analyses as a proxy for telomere length. A significant correlation between age estimates obtained using GLG and AAR was found, whereas no correlation was found between GLG and telomere length. An AAR rate (k_{Asp}) of 0.00130/year ± 0.00005 SE and a D-enantiomer to L-enantiomer ratio at birth (D/L_0 value) of 0.01933 ± 0.00048 SE were estimated by regression of D/L ratios against GLG ages from 25 animals (12 selected teeth that had high readability and 13 known-aged animals). AAR could prove to be useful, particularly for aging older animals in species such as harp seals where difficulties in counting GLG tend to increase with age. Age estimation by telomere length did not show any correlation with GLG ages and is not recommended for aging harp seals.

The AAR technique has been applied for age estimation of humans and other animals over the past three decades. In this study, eyeballs from mammals (n =124; 25 species) of known age or age estimated by another aging method were used to determine species-specific racemization rates and to examine the effect of body temperature on the rate of racemization. Strong correlations (range: r = 0.93-0.99) were found by regression of D/L ratios against known/estimated ages for 7 mammal species. Racemization rates (as $2k_{Asp}$ values) were well correlated (r = 0.91) with average core temperatures (°C), and a linear relationship was found between rate and temperature.

The presentation demonstrated that the AAR method is valid for several mammal species for which age is known, showing a strong correlation of D/L ratio with actual age. A total of 124 animals from 3 groups of species were examined. In pygmy goats the known age was similar to the AAR age. Racemization rates were different among species and racemization rates correlated well with core temperatures. When applied specifically to harp seals (n=113), there was also a strong correlation between tooth GLG and D/L ratio. AAR and GLG ages were similar but the AAR method appeared more accurate for old animals. The eye lens in narwhal was soft and clear in young animals but became hard and yellow in old animals. Some fine-tuning and further calibration are still needed for harp seals. However, there was no correlation with telomere length, which was deemed as an unsuitable method of aging in harp seals.

Presentation 11b. Narwhal age from eye lens and age validation

ABSTRACT: *This talk will focus on the AAR technique in age estimation of narwhals* (Monodon monoceros). *I will present the results from two studies.*

One (Garde et al. *2012) estimates a species-specific racemization rate for narwhals by regressing aspartic acid D/L ratios in eye lens nuclei against growth layer groups in tusks. The other (Garde* et al. *2015) is a large-scale study of age estimation in narwhals using the AAR technique, followed by construction of age distributions and estimation of life history parameters. The obtained parameters were subsequently used in a population dynamic analysis.*

Ages of marine mammals have traditionally been estimated by counting of dentinal growth layers in teeth. This method is, however, difficult to use on narwhals because of their special tooth structures. Alternative methods are therefore needed. The AAR technique has been used in age estimation studies of cetaceans, including narwhals (Garde et al. *2007). The purpose of this study was to estimate a species-specific racemization rate for narwhals by regressing aspartic acid D/L ratios in eye lens nuclei against growth layer groups in tusks. Two racemization rates were estimated: one by linear regression (r^2 = 0.98) based on the assumption that age was known without error, and one based on a bootstrap study, taking into account the uncertainty in the age estimation (r^2 between 0.88 and 0.98). The two estimated $2k_{Asp}$ values were identical to two significant digits. The $2k_{Asp}$ value from the bootstrap study was found to be 0.00229 ± 0.000089 SE, which corresponds to a racemization rate of 0.00114-year ± 0.000044 SE. The intercept of 0.0580 ± 0.00185 SE corresponds to twice the $(D/L)_0$ value, which is then 0.0290± 0.00093 SE. We recommend that this species-specific racemization rate and $(D/L)_0$ value be used in future AAR aging studies of narwhals.*

Eyes, reproductive organs and body length measures were collected from 280 narwhals in East and West Greenland in 1993, 2004, and 2007 – 2010. The purpose was a large-scale study of age estimation using the AAR technique, followed by construction of age distributions and estimation of life history parameters. The obtained parameters were subsequently used in a population dynamic analysis. Age estimates were based on the racemization of L-aspartic acid to D-aspartic acid in the nucleus of the eye lens. The ratio of D- and L-enantiomers was measured using high-performance liquid chromatography (HPLC). The age equation used was determined from data from Garde et al. *(2015). Asymptotic body length was estimated to be 405 cm for females and 462 cm for males from East Greenland, and 399 cm for females and 456 cm for males from West Greenland. Age at sexual maturity based on data from reproductive organs was estimated to be 8 years for females and 17 years for males. Pregnancy rates for East and West Greenland were 0.42 and 0.38, respectively. Maximum lifespan expectancy for narwhals was found to be ~100 years of age. A population projection matrix was parameterized with narwhal data on age structure and fertility rates. Under the assumption of stable age structure it is calculated that*

narwhals in East Greenland have a potential annual growth rate of 3.8% while narwhals in West Greenland have a potential growth at about 2.6%.

Narwhal tusks were sectioned using a jigsaw so that the cut surface of a half tusk was prepared. The surface was acid-etched with acetic acid by immersing the tusks in specially built tanks for many hours. The etched tusks were subsequently rinsed in water and then dried so that the GLG were thrown into relief and could be counted. The surface was also rubbed over with soft pencil lead to highlight the GLG. Deposition rate of GLG in narwhal is not known but inter-GLG spacing is very thick (ca 3 mm) and thus likely represents annual growth. A species-specific racemization rate was estimated for narwhals by using the tusk age as a calibration.

Discussion
This method showed great promise for a species where age is largely unknown. The possibility of using embedded tusks in young animals was suggested, as these are relatively easy and inexpensive to acquire. It was recommended that comparisons be made in future studies of narwhal population dynamics, age distribution and life-history parameters between samples from east and west Greenland using reproductive status and AAR techniques.

Presentation 12: Aging beluga (white) whales from measurements of specific fatty acids present in their outer-blubber biopsy tissues - David P. Herman, Roderick C. Hobbs, Barbara A. Mahoney and **Gina M. Ylitalo**

ABSTRACT: *Age estimation of individual cetaceans and estimation of the age distribution of entire whale populations is fundamental to assessments of status and long-term viability. Until recently, there was no reliable benign method to determine the specific ages of live animals for remote populations where long-term longitudinal sighting studies were not practical. In two recent studies involving populations of eastern North Pacific (ENP) killer whales and humpback whales from both the ENP and western North Atlantic, we described a new method by which age could be estimated with good precision from measurements of specific endogenous fatty acids (FAs) and FA ratios present in the outer blubber layers obtained by remote dart biopsy techniques. Although the precisions ($\pm\sigma$) of the FA-age models derived for these populations of whales were somewhat variable (ranging between $\pm$3.1 and $\pm$5.3 years), the results indicated that it should be possible to estimate the age of an individual whale from any population of these two species with better than decadal resolution using this approach. In this presentation, we provide some new preliminary data suggesting that it should be possible to age individual Cook Inlet beluga (white) whales following this approach based on FA results obtained from a combination of capture and release*

(biopsy) and stranded (necropsy) samples acquired between 2001 and 2007. Unlike the two previous studies in which exact or minimum known-ages were known and thus served as calibration standards to derive empirical FA-age models, ages of the Cook Inlet belugas described in this study were initially estimated from the von Bertalanffy allometric relationship between body length and teeth growth layer groups (hence age, assuming 1 GLG/year) derived for this population of belugas in the 1990s from a large number of stranded animals, (Vos 2003). Whereas body lengths may only be used to derive crude age estimates for juvenile and sub-adult whales not yet having achieved maximum physical maturity (size), the proposed FA ratio – age model described in this presentation seemingly should enable the ages of physically mature adult whales of both sexes to be estimated following this approach.

The paper reviewed studies employing this method using specific endogenous fatty acids (FAs) and FA ratios (Herman *et al.* 2008, 2009). The method is robust and generally viable for age estimation, and is a non-lethal method that can use biopsies of blubber. Focusing on northeastern Pacific killer whales, despite some differences in blubber FAs in residents and transients, an empirical killer whale age-FA model was developed using this technique, which can predict ages with a precision of ±3.9 year.

In humpback whale FA studies (endogenous and dietary in origin), where there has been a comparison with Photo-ID aging (southeast Alaska vs Gulf of Maine), a robust model could predict ages within ±5.3 year regardless of stock, sex and dietary preference. A generic species model is not optimal for precise age. The humpback whale studies highlighted the need to develop individual stock-based models. This may in part be due to different dietary habits affecting the FA composition. However, the underlying biological mechanism is not well understood. The outliers appear mainly to be very young and suckling animals. When the two stocks were analysed separately, a greater precision of $\pm$3.1 year for Gulf of Maine and $\pm$4.5 year for southeast Alaska was obtained.

When FA techniques were applied to Cook Inlet belugas, body lengths and ages from teeth (Vos 2003) were compared with FA ratio-derived ages for 11 males and 11 females, using outer blubber. Analysis was based on the ratio of a single pair of blubber FA: C16:1n9/iso-C16:0. Preliminary results indicated that such a model can be used to predict ages within ±5.8 years for juvenile/sub-adult belugas and appears to be independent of sex. Results appear to be contiguous thus enabling the ages of physically mature adult belugas to also be estimated. It is anticipated that age prediction uncertainties will be substantially reduced when biopsy samples from animals of exact known age are acquired and their blubber FA compositions fit to a linear

combination of two FA ratios, similar to the killer whale and humpback whale models. However, as in humpbacks, there is no clear understanding of the underlying biological mechanisms responsible for the beluga age/FA relationship.

Discussion
One issue with using FAs, especially from remote biopsy sampling, is that small differences in the sampling of blubber might be significant. The sample would be affected by the angle of penetration of the biopsy tip or the selection of blubber analysed. The beluga work is in progress and, although the age precision is not as high as for killer whales, the technique is promising and should be investigated further. In time, the technique may be applicable to narwhal if there is an independent method for aging available.

Presentation 13: Growth and maturity of belugas in Cumberland Sound compared to those raised in captivity - Paul Brodie, K. Ramirez and M. Haulena

ABSTRACT: *The beluga* (Delphinapterus leucas) *is one of the few odontocetes to adapt, year-round, to a polar environment, one of the most challenging marine habitats in the world, with shallow estuaries, high turbidity, shifting pack-ice and extreme tidal ranges. Adaptation is attributed in part, to year-round herd integrity and synchrony, occupying a sequence of restricted seasonal habitats and calving sites, which are reflected in tooth laminae. Newborn and the first four year-classes are recognisable by comparing length, body colour and morphology. Assessment of body colour is highly subjective in the field and provides a crude index of maturity. Field research, 1966-1969, led to the conclusion that females are sexually mature at 5.75 years and males are at 8.75 years, gestation is 15-16 months, and the reproductive cycle is 3 years, with a lifespan of 30-35 years. The 2- year nursing period results in rapid growth of the calf, coincident with a training period to acquire social, feeding and crucial navigational skills. The population in Cumberland Sound had been reduced through exploitation, thus it is unlikely that the present numbers are food limited, reflecting maximum rate of increase for a wild stock. We examine similar growth indices for captive belugas, some captured as calves, as well as first and second generations born in captivity, to compare known-age animals. Growth to onset of sexual maturity of males and females is similar to findings for the Cumberland Sound population, which was based on two growth layer groups per year in the teeth, or GLG/2. We analyse studies where previous oral doses of tetracycline, as well as bomb ^{14}C were used to argue for single annual GLG. Dedicated field studies, using appropriate dosage of intramuscular tetracycline, provide evidence for GLG/2. The ^{14}C study appears to have been compromised by preparation technique, and burdens sampled in the 1990s*

are probably of maternal origin, transferred during foetal growth and lactation. Direct observations and cross-referenced parameters fail to substantiate GLG/1, which requires halving the somatic growth rate, thus doubling the age of sexual and physical maturity as well as lifespan, resulting in a 40% reduction in the intrinsic rate of natural increase.

This presentation gathers together diverse information regarding age, reproductive history and growth rate for the Cumberland Sound belugas during the pre-1970 period, and a comparison with biological parameters from known-history captive belugas primarily from Churchill, western Hudson Bay, the source of most captive beluga. Based on younger, known-age belugas from Churchill, the conclusions are that the deposition rate of tooth GLG is two per year. The ages at capture of two belugas in Hohn and Lockyer (1999) and later included in Lockyer *et al.* (2007) were assessed as wrongly estimated and GLG counts based on realistic ages at capture (Robeck *et al.* 2005) were more consistent with GLG/2 for

- Churchill (male) at total age 12.7 years (4.9 years wild + 7.83 years captive) = an expected 25.4 GLG, the average count of five readers being 27.8 (SD 3.63, range 24-32), while for
- SW-DL-7903 (female) at total age 10.75 years (2.75 years wild+ 8 years captive) = an expected 21.5 GLG, the average count of five readers being 18.20 (SD 2.17, range 16-21) – neither consistent with GLG/1 nor GLG/2. (However, with reference to Lockyer *et al.* (2007), this animal had a tetracycline mark which clearly established a GLG/1 rate at least in the adult phase – see Fig. 5 later under presentation 26. by Hohn and Lockyer.)

When appropriate ages at capture, and estimated GLG loss, were applied to the other captive belugas examined in Lockyer *et al.* (2007), they were all (sample of 10) assessed as not conforming with GLG/1. Examples were:

- Aurora, a 246 cm female, possibly as old as 3.2 years, plus 15.2 years captivity = 18.3 years, thus 36.6 GLG. The average count in Lockyer *et al.* (2007) Table 4, is 35 (SD 3.32, range 27-35).
- No-See-Um, a 257 cm male was a maximum of 3.2 years at capture in August, plus 21.7 years captivity = 24.9 years, thus 49.8 GLG. The maximum count in Lockyer *et al.* (2007) Table 4, is 46+, the "+" indicating lack of neonatal line and possible tooth wear. The average count was 42.8 (SD 4.66, range 35-46).

Discussion

There was much discussion, particularly with respect to the apparently circular arguments pertaining to GLG deposition rates, used in the

presentation. The full arguments on interpretation of all GLG counts are discussed in Lockyer *et al.* (2007), and different scenarios were tested based on both minimum and maximum GLG counts estimated, which clearly diminishes the strength of an argument for GLG/2 based on using the average count alone.

While body length can be taken as an indicator of age, especially in juvenile animals, growth rates and parameters vary among both stocks and individuals. It is important to compare like with like. With reference to the phasing of colour from grey to white, Brodie (1971) stated: "Whitening of female beluga in Cumberland Sound occurs after 6 years, and of males after 7 years. The white colour can be used in the field to establish at least a minimum age and to indicate that the animal is near physically mature size…" Light grey beluga females of 6+ years of age have been observed to be pregnant both in Cumberland Sound and in Hudson Bay. Body colour which can change from grey to white in adult belugas, is not a knife-edge transition, and there are many documented cases of so-called "juveniles" of grey colouration that have produced a few young before becoming white (see presentation 20. by Michaud below; also Stewart, pers. comm., who reports dissecting foetuses from grey female belugas). Colour can, therefore, not be used reliably to assess maturity. In terms of allometric life-history, it seemed odd that belugas alone would live only half as long as pilot whales (*Globicephala sp.*), for example, which are about the same size, and be the only species of mammal that has a different tooth GLG deposition rate.

Convincing evidence for the one GLG per year hypothesis requires appropriate numbers of productive females within a population, either pregnant and/or lactating, whose ages can be verified at 30-60 years. Moby, a female, appears to be one of the oldest known history belugas, dying after 30 years in captivity. According to Lockyer *et al.* (2007), among 5 readers, at least 30-43 GLG were observed in the tooth dentine which was worn at the crown. According to length, she was a juvenile on capture.

On balance the workshop members supported the current interpretation of annual GLG deposition rate. There were now many other studies (see Michaud presentation 20) that confirmed an annual deposition rate.

One detail from the presentation that the workshop found promising was a simple technique, whereby GLG lost to erosion could be estimated. As additional GLG are added to the pre-natal tooth, the total angle at the dentine interface with the root tissue expands in increments of 1-2 degrees, beginning at 25-30 degrees, 40-50 degrees after approximately 12 GLG, 70-80 degrees after approximately 18 GLG; ultimately to 150-170 degrees (Fig. 2). Examining this matter in detail, perhaps through a special study specific to

the population, could help identify cases where crown wear results in lost GLG and help to estimate the number of GLG worn away.

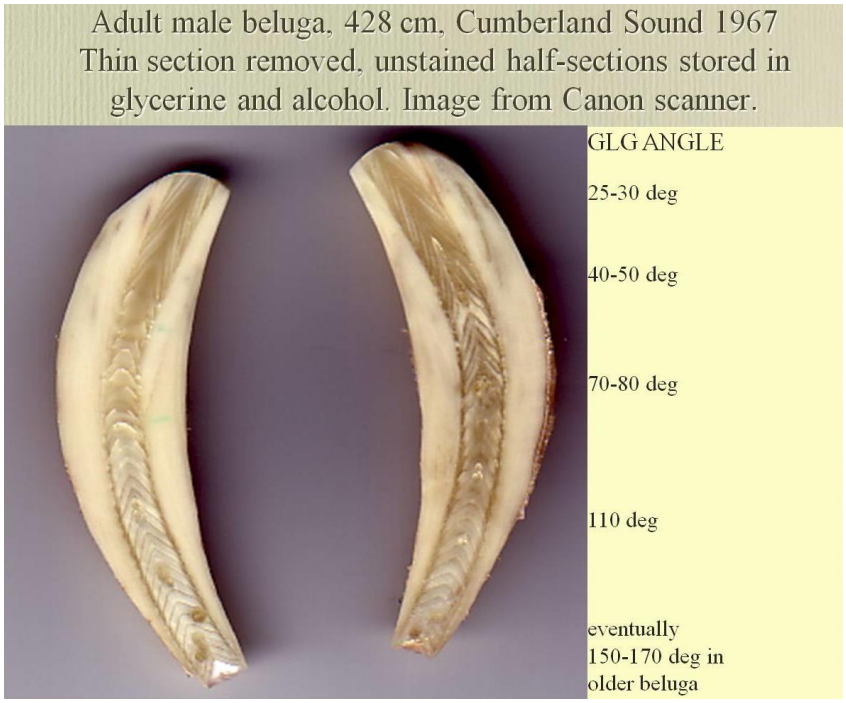

Fig. 2. Beluga tooth sections showing the change in total angle of the GLG at the pulp cavity with age. Picture by courtesy of Paul Brodie, Balaena Dynamics Ltd, Halifax, Canada.

Presentation 14: Use of micro-computed tomography for dental studies - Carolina Loch, Donald Schwass, Jules A. Kieser and R. Ewan Fordyce

ABSTRACT: *Teeth are important elements in studies of modern and fossil cetaceans, providing information on feeding habits, estimations of age, and phylogenetic relationships. The growth layer groups (GLG) recorded in dentine have demonstrated application for aging studies, but also have the potential to elucidate life history phenomena such as metabolic or physiologic events. Micro-Computed Tomography (Micro-CT) is a non-invasive and non-destructive technique that allows 3-dimensional study of mineralized tissues and their physical properties. It has mostly been used for qualitative dental studies in humans. Teeth from extant dolphins* (Globicephala *sp. and* Sotalia guianensis) *and an unnamed Oligocene fossil dolphin (OU 22108) were scanned in a Skyscan 1172 Micro-CT desktop system. X-rays were generated at 100 kV and 100 µA for extant samples and at 80kV and 124 µA for the fossil tooth. Aluminum and copper filters, 0.5 mm thick, were used in the beam. Reconstructed images were finely resolved for the fossil, showing the enamel, internal layers of dentine, and the pulp cavity. The enamel layer was well defined in both extant species throughout the images, but the dentinal*

layers were less resolved. We are refining the use of Micro-CT for dental studies in cetaceans, to allow resolution of internal structure and potential application in non-destructive aging techniques. Imaging software should elucidate grayscale values observed in the dentinal region of extant specimens and their relation to GLG. Future Micro-CT analysis will involve paired scans of teeth alongside resin-hydroxyapatite calibration standards of known densities to quantify mineral density of dental tissues in odontocetes.

This technique, Micro-CT, is a non-destructive alternative for looking into hard structures such as bones and teeth of fossil and living animals to investigate internal structures. This may be very helpful for examining teeth that are difficult to cut / section because of shape or fragility. The examples shown demonstrated internal layering, and thus may have potential use in age estimation from tooth GLG. The technique is promising albeit still being developed. A significant limitation on application is that the actual size of the teeth that can be scanned is limited because of the dimensions of the investigative chamber.

Presentation 15: Applications of Aspartic Acid Racemization for Aging Bowhead Whales - Craig George

ABSTRACT: *The Aspartic Acid Racemization (AAR) technique has been applied to estimating the age of several mammals. For bowhead whales, the technique has been applied in several publications on cetacean age, growth, animal health, and management advice (Bada 1972, Bada* et al. *1970, 1980, 1983, Rosa* et al. *2004, Rosa* et al. *2014, George* et al. *2011, Garde* et al. *2007).*

While age estimates for bowheads have high SEs there has been no indication of bias (George et al. *1999, Rosa* et al. *2014). For sub-adult bowheads (<15 years), the CV of the AAR estimates exceeds 100% and should be applied cautiously. For young animals, the "baleen aging" technique (stable carbon cycles in baleen) is recommended as it is more accurate.*

With regard to the question of accuracy and bias, the age estimates for bowheads have been verified independently by several different approaches. These are reviewed below:

1. *The "baleen aging" technique suggested age at sexual maturity (ASM) for bowheads in their early 20 years (Lubetkin* et al. *2008 , Schell and Saupe 1993) corroborating the AAR estimates of* ca *25 years. (Rosa* et al. in press*).*
2. *Growth rate data from photogrammetry estimated ASM to be late 20 years (Koski* et al. *2004). In their approach, they estimated growth*

rates using inter-year photographs and calculated the number of years to reach 13-14; i.e., the well-documented length at sexual maturity.

3. *Recovery of stone weapons in harvested bowhead whales indirectly confirms that bowhead whales live in excess of 100 years, also corroborating the AAR maximum longevity estimates.*
4. *The recovery of a Yankee whaling projectile patented in 1879 in a recently harvested bowhead whale also corroborated the longevity estimates (George and Bockstoce 2008).*
5. *Population dynamics models for bowhead whales suggest that low values for ASM are unlikely and favour over 20 years (Givens* et al. *1995).*

In view of the above, the AAR technique appears useful for long-lived cetaceans and possibly other vertebrates; however, its applications to species with shorter life spans (<30 years) must be approached cautiously.

Samples of eyes of bowhead whales hunted in the traditional hunt were collected for analysis using AAR (George *et al.* 1999). For females, ovarian *corpora* were used as an age proxy for calibration. Investigating carbon isotopes in baleen plates of younger animals indicated an age at sexual maturation of 17-20 years from the regular annual oscillations associated with feeding migration (Schell *et al*. 1989a, b). These data, together with the knowledge of potential longevity from artifacts, and relative age from body length, could be used to calibrate age derived from the AAR method. Improvement of the AAR method (Wetzel and Reynolds 2011) and testing of the consistency of k_{Asp} with calibration using known rates for humans and fin whales has allowed more reliable estimation of age. Results of investigating racemization rate k_{Asp} indicated a possible age at sexual maturation of >20 years in females and a longevity of *ca* 120 years in males.

In summary, the AAR technique was found useful for long-lived species such as bowhead whales, for which other techniques are unavailable. There is no evidence of bias but there is a high variance, especially for young animals. Baleen plates are recommended for age estimation in young animals, *i.e.*, <20 years. To get reliable results using AAR, repeated measures/samples are required, together with a good lab procedure and corroborative age estimates.

Discussion

Details were provided on age estimation using the ovarian *corpora* counts. The estimates of age at sexual maturity must necessarily be added to *corpora* age to obtain total age and are not totally independent from AAR ages. There is thus some circular dependency of the data. However, the AAR technique demonstrates reliably that bowheads are long-lived. In this respect, the

apparent old ages estimated using AAR for narwhal (Garde presentation 13.2 above) must be considered feasible.

Presentation 16: Value of long-term studies of humpback whales for determining population parameters and ground-truthing new age estimation methods - Christine M. Gabriele

ABSTRACT: *Photo-identification of individual animals has become an important source of information on humpback whale behaviour and population parameters via long-term studies occurring at several sites worldwide. Through photo-identification, researchers have determined age at first calving, reproductive rates and calf mortality for this species. The sheer length of sighting histories in long-term studies is also shedding light on the lifespan of humpbacks, in that many whales have sighting histories approaching 40 years, although this species was earlier thought to have a 30-year lifespan. Tissue samples from known-age humpback whales are also contributing to development of techniques for determining the age of unidentified individuals (i.e. stranded animals or those without a sighting history) from blubber fatty acids, chromosome telomeres, and eye lens aspartic acid racemization.*

This method of direct observation of free-living animals demonstrates the value of long-term monitoring for assessing age and life history. Since 1974, 626 animals have been identified using marks on tail flukes and supplemental marks on dorsal fins. Recently, validation of ear plug GLG deposition rate (one per year) was possible for a known-history female # 68 from Glacier Bay, Alaska, which was first sighted in 1975 as an adult with a calf and washed up dead 25+ year later after a ship strike in July 2001. Her estimated age from the ear plugs was 44 years with a likely age at sexual maturation (from the transition phase) of 7 years (Gabriele *et al.* 2010). From sighting histories, it is possible to get a minimum age estimation and age at first calving. Biopsy analysis has enabled comparative age studies to be undertaken on this population, including telomere length analysis (Olsen presentation 8 above) and endogenous FA ratios (Ylitalo presentation 14 above), which have also correlated with an annual GLG deposition rate in known-age animals. The AAR technique is now being applied to eye lenses from recovered dead animals.

Discussion

The Glacier Bay photo-ID sightings database has been used to validate several other aging techniques such as FA ratios, eye lens extraction for AAR, and genetic telomere length analysis. The previous presentation on photo-ID in belugas also indicates the potential of this kind of study, although it is labour intensive and will probably never cover all members of a population.

Humpbacks are coastal and migratory but generally return to their mother's feeding range, so that resighting is feasible. The oldest known-age whale so far recorded is 37 years (first sighted in 1974). The importance of collection of ear plugs from stranded known animals was stressed.

Presentation 17: Validation of growth layer deposition rates from known history and photo-ID (dolphins) - Aleta A. Hohn

ABSTRACT: *Long-term field studies have provided the opportunity to know the age or the approximate age of free-ranging studies. These studies are valuable for validating growth layers because the alternative generally is use of captive animals, for which it is possible that captivity, itself, has affected growth layer deposition patterns or rates. The best study, to date, that has provided and continues to provide teeth from free-ranging animals occurs in the Sarasota Bay region of Florida (Hohn* et al. *1989). From that study, teeth have been extracted from live animals during temporary catching and holding of animals. Additional teeth have been available when known dolphins died. In some cases, teeth available after death represent a second opportunity to examine a tooth from the same individual. These studies will be limited due to the nature of conducting such studies, but what has been learned is invaluable.*

The Sarasota Bay Photo-ID project on *Tursiops truncatus* has now run for at least 5 generations of dolphins and has provided a mass of life-history data for known individuals. The oldest known-age female, Nicklo, is 61-year-old and produced her last known calf at age 48 years, but several females of age >40 years have produced up to 8 calves during the monitoring programme. Although this study is a special case in that animals have been regularly captured and released to monitor individual health, growth, and development, aspects may be applicable to belugas. By extracting teeth from live animals, it has been possible to validate aging methods by comparing actual known age to the numbers of tooth GLG counted blind without reference to any data. Again, the value of long-term studies was underlined.

Presentation 18: Bio markers and tetracycline antibiotic time marking - Aleta A. Hohn

ABSTRACT: *Examining teeth from known-age animals does not, in itself, calibrate growth-layer deposition. That is, a count of growth layer groups (GLG) in teeth that corresponds to a known or approximately-known age could match but that does not allow for the actual GLG boundaries to be known. Actual calibration would be required to be certain when an annual layering pattern starts and ends. A means to obtain this information is using a bio-marker. The most common biomarker for cetaceans has been*

oxytetracycline. This compound is incorporated into actively mineralized teeth. When those teeth are sectioned (not decalcified) and viewed under reflected UV light, the incorporated tetracycline fluoresces. This technique has been used across a spectrum of mammals (terrestrial and marine).

The most commonly used bio-marker for marking teeth is tetracycline which can be administered orally or intra-muscularly. The former method is perhaps better as the drug, which may be required in a relatively large quantity, is absorbed and circulated in the body quickly without potential damage to muscle tissue. The dosage must be calculated according to the body weight and can be administered once or in lower dosages over a few days. A typical dosage is between 10-50 mg/kg body weight and the intensity of the mark appears to increase with dosage. Circumstances will dictate which is practicable. The tetracycline binds with calcium during new growth of the tissue. The method is reliable for time-marking (Myrick *et al.* 1984), but it is important to recognise that the drug can also be transferred via milk during lactation and that, rarely, undocumented marks in teeth may be the result of food or prey ingested. Other problems that may affect the correct interpretation in teeth are the effect of captivity on GLG deposition and autofluorescence, an edge effect, including that due to cracks in the tooth. When teeth so-marked are extracted for age estimation, it is important not to fix in formalin or decalcify as these processes leach out the chemical. Exposure to light will also degrade the mark in teeth.

Presentation 19: Bomb dating and age validation: conclusive results in a fuzzy world - Steve Campana

ABSTRACT: *Atmospheric testing of atomic bombs in the late 1950s resulted in an abrupt increase in atmospheric radiocarbon which was soon incorporated into all organisms that were growing at the time. Thus the period is analogous to a large-scale chemical tagging experiment, wherein all body hard parts formed before 1958 contain relatively little ^{14}C and all those formed after 1968 contain elevated levels. For fish and aquatic organisms born between 1958 and 1968, bomb radiocarbon in growth increments can be used to confirm the accuracy of more traditional aging approaches with an accuracy of $\pm$ 1-3 years. This approach has proven to be effective in validating the age of fish, bivalves, sharks and belugas, and would be expected to be effective in many other organisms.*

The method is based on identifying and quantifying nuclear fallout elements from atomic testing, namely ^{14}C. The increase in concentration of radiocarbon first started in 1958 in surface marine waters around the world. The ^{14}C concentration curve reached a peak and plateau in the late 1960s, and is now slowly declining although still strong. Hard tissues bearing GLG were micro-milled for internal sampling of individual GLG. About 2 mg of material is

sufficient for testing. The analysis is expensive – *ca* 1,000 – 1,500 USD per sample, but only 5 samples are needed for pre-1958 born animals. During the presentation, examples of the technique were given for halibut (*Hippoglossus sp.*) and yellowtail flounder (*Limanda ferruginea*), otoliths. There is a difference between freshwater and marine environments and an offset for surface or deep animals. Other examples included porbeagle shark (*Lamna nasus*), mako shark (*Isurus sp.*), and dogfish (*Squalus acanthias*), for which radiocarbon was used to calibrate ages.

The method is suitable for all hard tissues, including beluga teeth and narwhal tusks, and for investigating individual GLG. In the study of beluga teeth (Stewart *et al.* 2006), the radiocarbon method was robust enough to validate age from GLG. A comparison of results making assumptions of deposition rate of one or two GLG per year had a strong offset (Fig. 3). Concluding, bomb radiocarbon is an excellent age validation method for long-lived animals; the age of individual animals can be validated; and marine mammals, especially monodontids, are good study subjects.

Discussion

Earlier, Paul Brodie (presentation 15), had raised criticism to the findings of the bomb radiocarbon method. Brodie mentioned that the study by Stewart *et al.* (2006) using bomb radiocarbon had been examined by B. Buchholz, senior research physicist, Center for AMS Livermore, California, who provided the following assessment:

> *"My problems with the paper are the incomplete methods and corrections they used to remove large amounts of dead carbon from the embedded samples. None of the samples embedded in epoxy are suitable for these measurement. The corrections seem arbitrary, and can be used to obtain whatever answer you want. You can make GLG/1 fit the late 1950s rise with a suitable correction. Ignoring all data after 1982 is not justified. If the corrections are accurate, they should work for the entire curve, not just a segment. Hence the data have significant problems." (Pers. comm. to P. Brodie; see also Brodie* et al. *2013.)*

Brodie also commented that an additional complication is that the radiocarbon burdens in the belugas may not have originated during their lifetime and were actually transferred during gestation and two years of intensive nursing. Campana responded that there is no difference in ^{14}C signature for animals feeding directly or lactating. The ^{14}C signal comes through milk or diet to calves, but the signal is the same. Even if gross resorption took place in the mother's skeleton, this would not have an appreciable effect on ^{14}C content

and transfer. Thus maternal transfer to calves should not be a confounding factor in the deposition rate controversy.

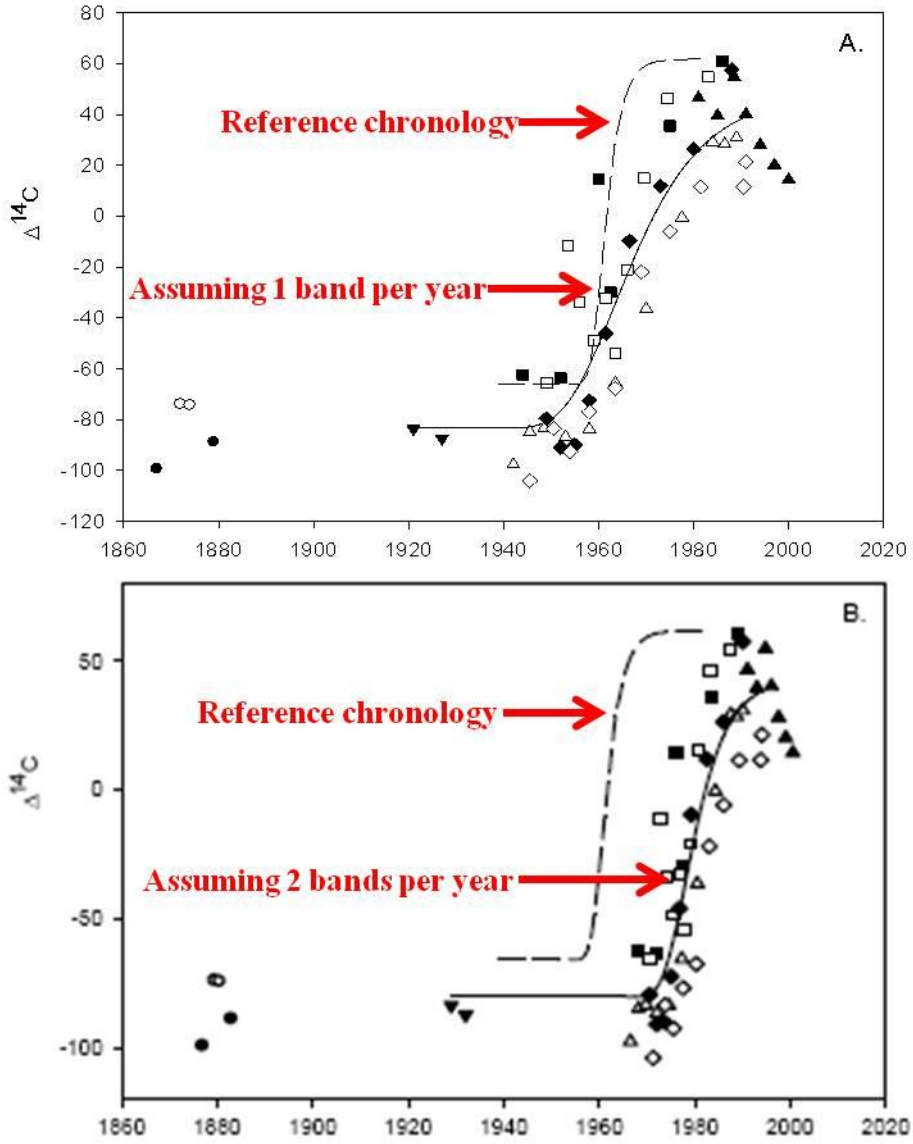

Fig. 3. The radiocarbon ^{14}C signatures for belugas pre- and post-atomic bomb fallout. The reference graph is shown in both A and B. The graphs overlap only for the assumption of one GLG per year in A. (After Stewart *et al.* 2006).

Presentation 20: Age validation through known history captive studies in belugas – Aleta A. Hohn and **Christina Lockyer**

ABSTRACT: *This presentation is a recap of the now published work on examination of known-history captive beluga teeth (Lockyer* et al. *2007). A sample of teeth from 10 beluga specimens was examined for total age. Data on sex, capture date, length at capture, history of tetracycline antibiotic medication, general health, and date of death were available. The results of agreed GLG counts for the sample teeth were compared to the life history, including time in captivity (ranging 4 – 30 years), of each animal under two hypotheses: one and two GLG deposited annually. Resulting counts were more consistent with the hypothesis of one GLG per year. In five of the seven animals for which the neonatal line was present, given the length of the animal at capture and time in captivity before death, under the assumption of two GLG per year, the animals were younger than would be possible. The number of GLG between the tetracycline mark and death also corresponded to a deposition rate of one GLG per year. We believe the evidence supports that beluga whales deposit GLG at the same rate (one GLG per year) as other cetaceans for which this has been calibrated. Additional support for this conclusion is drawn from reference to other techniques that indicate an annual deposition rate.*

The tooth samples were from 10 animals all captured near Churchill, Manitoba, Canada. Details of tooth preparation were presented for thin untreated sections and thin stained sections. The untreated sections were examined under magnification using reflected UV light which makes tetracycline marks fluoresce. The belugas had been captive for 4-30 years and, although none was of known age, all were captured when very young. Problems of crown wear in some animals meant that only minimum age could be estimated but many had an intact neonatal line. Although the evidence was not clear for some animals, from actual time in captivity and GLG age, at least 3 supported an annual GLG deposition rate, while a further 5 neither supported nor refuted an annual GLG deposition. There were however, another 2 specimens that supported an annual rate from tetracycline marking (see Fig. 4). On balance, the annual GLG deposition was accepted.

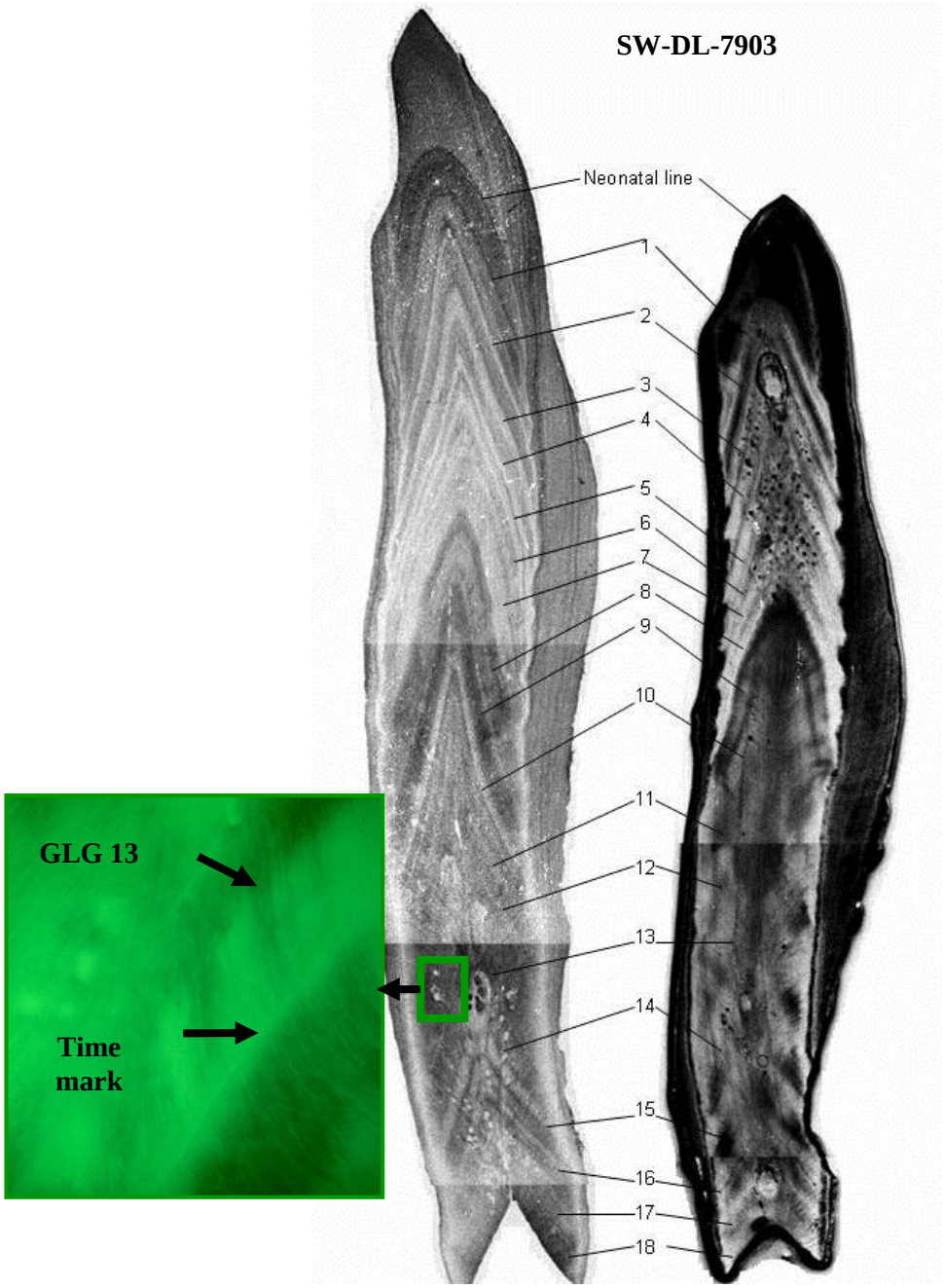

Fig. 4. To the left is the decalcified and stained section of the tooth of SW-DL-7903, and to the right is the untreated section from the same tooth. There are 18 GLG marked up in the dentine, and the neonatal line is intact. This animal was in captivity almost 8 years. Presence of a fluorescent time mark in the dentine around GLG 14 originates from a tetracycline treatment 4 years and 2 months before death. The conclusion is that a one GLG per year deposition rate is validated for this animal.

Discussion

Brodie queried the authors' conclusions and offered an alternative conclusion for possible evidence of two GLG deposition rate instead, based on his experience with size and growth of free-living animals. He drew attention to specific examples used by Hohn and Lockyer in his earlier presentation (15.), and he believed that all the animals presented could be interpreted this way based on body lengths at capture. Suydam raised the issue of potential problems in growth, especially tooth growth, with the change from wild to captive status. For other species, *e.g.*, *Tursiops*, there has not been evidence of a change in tooth structure due to captivity. Lockyer noted that geographical variations in ultrastructure occur in teeth (Lockyer 1999) and that probably climatic events also leave traces in the teeth (Manzanilla 1989). Possibly some animals taken into captivity also show such events in tooth ultrastructure. The events of capture (when often an animal fails to eat for a while), ill health, and reproductive events (births) can change the GLG pattern in short-finned pilot whales (*Globicephala microcephalus*; Lockyer 1993) but these do not eliminate any GLG. Hohn noted that in dusky dolphins (*Lagenorhynchus obscurus*) the difference is in mineral density of GLG deposited during El Niño years. However, the changes were not deposition rate or thickness of the GLG. Clearly response to captivity is likely species-specific.

Stewart argued that there seemed to be no physiological rationale for 2 GLG per year in comparison with other species in which one per year is normal. Brodie persisted that the length at birth of two of the animals mentioned by Lockyer, plus the time in captivity, matches the two GLG per year hypothesis. For example, one beluga (Moby, captive 30 years, and age > 43 years) should be consistent with two GLG per year if extra GLG are added for crown wear and the age at first capture was as a calf. Lockyer concluded that the juvenile phase in the teeth is the controversial item where most GLG identification problems exist and effort should be put there into assessing deposition rates and patterns and how to identify a GLG in the juvenile period.

Presentation 21: Accuracy, precision and quality control in the age estimation of aquatic animals - Steve Campana

ABSTRACT: *Many calcified structures produce periodic growth increments useful for age estimation at the annual scale. However, age estimation is invariably accompanied by various sources of error, some of which can have a serious effect on age-structured calculations. This review highlights the best available methods for insuring aging accuracy and quantifying aging precision, whether in support of large-scale production aging or a small-scale research project. Through use of quality control monitoring, aging*

errors can be readily detected and quantified; reference collections are the key to both quality control and reduction of costs.

Ageing is a very important aspect of living resource management. Some examples of precisely wrong ages in different species were presented, which were only identified when known ages became available. Age validation methods are essential and age corroboration using different methods provides support for the ultimate age. In producing a successful aging programme a method must first be developed. This must be followed by validation of the method. Preparation of a reference collection, ideally of known-age hard parts or, at least, hard parts aged by international experts is necessary. The reference collection is best for monitoring aging consistency through time, in the training of new age readers, and for testing consistency among readers.

Quality control must also exist with regular monitoring and preparation of age bias graphs for readers and CVs. The best measures of precision were coefficient of variation (CV), average percent error (APE) and index of dispersion (D); the least reliable was percent agreement among readers (Campana 2001).

In conclusion, accuracy is not equal to consistency and age validation methods are not all created equal. Chemical mark-recapture and bomb radiocarbon are the most reliable age validation methods. A reference collection is the key to effective quality control.

Discussion
There was much discussion about the desirability of reference collections and also the usefulness of internationally available digital and accessible images. This would facilitate standardisation and training in methods among diverse labs and workers, and obviate the need to exchange actual material when CITES permitting was problematic.

Ad hoc **Presentations**
The workshop was enriched by several *ad hoc* presentations from researchers in the audience. Their summaries follow.

Presentation 22: Ear bones for aging manatees - Amber Howell

This presentation reviewed the age estimation in manatees. The method entails thin sectioning of periotic bone, which shows GLG (Marmontel *et al.* 1996). The sections are stained and examined in transmitted light. Fig. 5 shows GLG in a stained ear bone section of a 10 year old manatee.

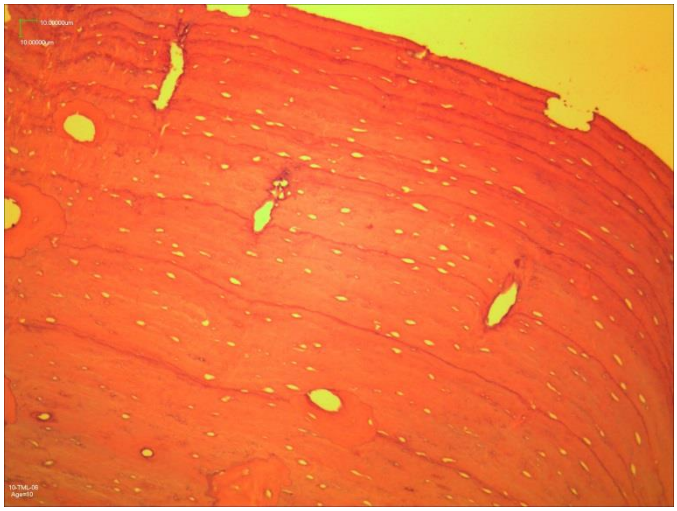

Fig. 5. Stained section of ear bone of a 10 year old manatee. Courtesy of Katherine Brill.

There is some degree of blurring of the layering above 12 GLG, and despite possible resorption, the oldest recorded age using this method is nearly 60 GLG (O'Shea *et al.* 1995). There are difficulties in interpreting GLG because of double laminae and merging of laminae. However, these problems are common to other methods of aging using layered hard tissues.

Presentation 23: Informal progress report on *trace element profiles* in beluga teeth - Cory Matthews

In this interim report of an ongoing pilot study, Matthews reported on stable isotope ratios for diet and ecological determination of beluga (*Delphinapterus leucas*), killer (*Orcinus orca*) and bowhead whales (*Balaena mysticetus*). The teeth of belugas were micro-milled in the light and dark bands of the dentinal GLG and analysed for nitrogen ($^{15}N/^{14}N$) and carbon ($^{13}C/^{12}C$) isotope ratios, which varied among GLG. Dark bands seem enriched in both ^{15}N and ^{13}C. It may be feasible to cross-date at the population level using these chemical signals in teeth GLG. In addition, it appears possible to assess the weaning age using ^{15}N in early GLG in killer whales, and this may be similar in belugas.

Trace elements measured using laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS) show regular oscillations in the GLG, but this is not consistent for all teeth. However, if changes in element concentration can be linked to environment, such as oscillations caused by seasonal movements between summer and winter distributions, the method could be used to calibrate tooth structure. The method might be useful to look

at the structure of the teeth to learn more about life history at both the population and the individual levels.

Discussion
It was pointed out that as of yet replicates of the same animals have not been made, so that the results must be treated with caution. It was suggested that the element ^{18}O could also be useful. Continuation of this work was encouraged.

Presentation 24: Individual identification and life history of the St. Lawrence beluga - Robert Michaud

Michaud offered this update on ongoing work started in 1989 (Michaud 1989, 1993, http://bed2.gremm.org/eng/pag.php?PagRef=Nws&NwsId=4569). To date there have been about 60 surveys per year in which belugas were photographed for reliable marks, identified and classified into 3 classes of re-sightability (RI). Common marks are nicks on the dorsal ridge and small scars including round indentations that were likely bullet wounds. The beluga population is about 1,000 animals, and a photo-identification catalogue holds files of 341 individuals ($RI \geq 1$) identified from both sides, 265 first identified as white and 76 first identified as grey animals. Biopsies were taken during surveys and these used for sexing and genetic profiling. A genetic profile is known for 95 males and 38 females. Males have more scars than females. Between 1983 and 2011, over 350 beluga carcasses were recovered for detailed necropsy and identification. Teeth for aging and reproductive material were collected for determining life-history parameters. Histories of individuals first identified alive and recovered dead provided information on first age at parturition, longevity, calving interval, and a validation for tooth GLG deposition rate at one per year.

- Atomic – DL006, female, was first sighted in 1986, presumably born in 1984, first seen with a calf in 1992, She subsequently had calves in 1994 and 1996, (while she was still light grey), 2000 (when she turned white), 2002, and 2004. She was last sighted in 2007.
- Ligne ligne – DL225, male, first sighted as big grey in 1991, turned light grey in 1993, white in 1997 and died in 2007 at 21 years old assuming 1 GLG per year deposition in the teeth. Assuming 2 GLG a year, DL225 would have been white at its birth!
- DL172 – female, first sighted in 1980 as a large white died in 2008 at 46 years old assuming 1 GLG per year deposition in the teeth. Assuming 2 GLG per year, DL172 would have been seen as a white whale before its birth!

Discussion
This study, apart from monitoring the distribution and social movements of the individuals, has been able to make valuable observations on age, growth and reproductive history. The recovery of biological samples after death provides a means to verify age from teeth and calving history based on reproductive tissues. The limitation of this work has been that there are fewer marked animals now since the end of the hunt (1979) when struck but escaped animals bore scars. In addition, it is not yet possible to quickly access beach-cast carcasses to recover fresh eyes for possible AAR analysis. The workshop welcomed this long-term work which potentially may provide a wealth of life history data and a means for absolute age validation using tooth GLG.

Presentation 25: Artifacts - Craig George

This presentation traced the start of the investigations that were triggered by the discovery of a historic slate end-blade in the mattak of a bowhead whale caught recently in Alaska. Since then there has been a recovery of many stone weapons indicating a possible age of up to 117 years, and also Former Yankee whaling bomb lance fragments (George and Bockstoce 2008), indicating a possible age of up to 129 years. However, with respect to the stone weapons, some may still be employed today in some areas, so that the certainty that these were placed historically is in doubt. Nevertheless, such artifacts indicate a relative age of the animals in which they are found and indicate great longevity in bowhead whales. In summary, recording artifacts is a useful technique for long-lived species where other techniques are unavailable.

Discussion
There was an extended discussion about interpreting the finding of an artifact. An artifact such as a Yankee harpoon head clearly could not be placed in a whale before it was invented but it could be placed any time after it was invented. The presence of a harpoon that was introduced in 1879 tells us that the whale was struck after 1879 but it could have been struck in 1979 if the artifact had been held and deployed later in time. One needs to be aware of such possible biases. The possibility of recovery of historic artifacts from monodontids is a possibility, but longevity is far less than in bowheads.

Presentation 26: The sensitivity of age structured population dynamics models to bias and precision in ages - Roderick C. Hobbs

Published and sourced data on age parameters for belugas were presented as the basis of the talk. The sources of errors were listed as:

1. Ambiguous aging material
    a. Poor or interrupted growth layer groups (GLG) in teeth
        i. Estimation bias and variance increase with age

        ii. GLG formation may be correlated temporally by environmental events
    b. Worn or broken structures (crown, root, etc.) in teeth
        i. Minimum age only. If wear rate is > 1 GLG per year, minimum age declines with age
2. Interpretation of material
    a. Deposition rate of GLG in teeth
        i. Was thought to be 2 per year now 1 per year.
        ii. Do older animals continue to add GLG's?
    b. Racemization rate (AAR method)
        i. Initial value may vary among individuals
        ii. May vary by population and species.

In investigating age-structured models, the following were assumed for belugas:

1. Leslie Matrix
    a. Maximum age (80 years)
    b. Age at first reproduction (11 years)
    c. Survival rate (0.95/year)
    d. Birth rate (0.3/year)
2. 100 individuals were drawn from a stable age distribution for the analyses.

For the analyses, the following were derived sequentially:

1. Age at first reproduction and survival rate estimated from age data.
2. Bias and variation drawn for each individual to simulate aging error.
3. Age at first reproduction and survival rate estimated from simulated aging data.
4. Intrinsic growth rates estimated for each data set for comparison.

In conclusion, errors in estimation of parameters can arise from errors in aging with both negative and positive bias and varying degrees of variance. In turn, these translate to errors in estimation of growth rates. Efforts should be made to quantify bias and precision.

Presentation 27: Summary of the main findings of the workshop with specific reference to monodontids – Christina Lockyer

The breadth and depth of the presentations made it clear that most issues concerning monodontid age estimation are not unique. Many researchers investigating many taxa have considered a diversity of methods and tissues to establish biological records of age. Aside from the biological materials, they have pondered accuracy and precision of the counts or metric, as well as their interpretation.

Relative age can be estimated using biological or chemical changes if the rate of change is known. Attempts to use **telomere length** to estimate age (Olsen: Presentation 6) show telomere lengths provide a measure of individual body fitness and condition rather than age, as environment, migration, health, and reproduction affect telomere length. The method has potential but is still under investigation; problems include locating long-lived known-age humpback whales for calibration. A review of **AAR** techniques on fin whale and harbour porpoise (Hjort-Nielsen: Presentation 10) warned that the presence of cataracts in the eye lens could seriously bias the age estimation and give falsely old ages. Longer-lived animals may be better candidates for the AAR technique, although neither fin whales nor porpoises had a good correlation of ear plug GLG and tooth GLG with AAR age respectively. There is an underestimation of age by AAR in harp seals, as in most animals (Garde: Presentation 11a) although in narwhal (Garde: Presentation 11b) tusk GLG correlated well with AAR age. The AAR technique using eye lens in bowhead whale (George: Presentation 15) showed good correlation with other age estimation methods, *e.g.*, known-age (from photo-ID), baleen plates, and ovarian *corpora* counts. Recent modifications to the hydrolysis technique and heating process at the Mote Marine Lab, Florida, had allowed refinement of the k_{Asp} rate (L_D/L_L) ratio which was found constant over time. Age models using endogenous **fatty acid** (FA) ratios have been successfully derived for killer whales and humpback whales (Ylitalo: Presentation 12), and preliminary results using a single fatty acid ratio for Cook Inlet belugas correlated with age from tooth GLG for physically immature animals. The maximum age from fatty acid ratio was *ca* 76 years using C16:ln9/iso-C16:0. Future work plans to get two FA ratios is expected to provide more precision in age. It may be possible to use **bone density** as a proxy for age in beluga and narwhal flippers (Read: Presentation 1). The method would need to be calibrated with reference to beluga tooth GLG and AAR ages in narwhal. The recording of historic hunting **artifacts** recovered in bowhead whales (George: Presentation 25) in Alaska has presented an opportunistic and remarkable insight into longevity of this species which exceeds 100 years. Other evidence of age from AAR aging technique would support this.

One technique shows promise for bridging relative ages from bone density and counting changes in density. Micro-CT scanning of teeth (Loch: Presentation 14) demonstrated great potential for investigating internal structure of teeth and other hard tissue specimens that are difficult or impossible to section, as there is no destruction of the specimen and it can be viewed in 3-dimensionally. It is also suitable for tympanic bones. The resolution from the technique was 5-50 micron. The main limitation is the small size of the experimental chamber, the height of which is 7-8 cm.

Counts of presumed annual markers can provide a more accurate (absolute) estimate of age than other tissues which show gradual changes with age. Among taxa, hard structures that show regular episodic growth are the most commonly used tissues to investigate for records of age. These can include bones, otoliths, claws, and ear plugs (Read: Presentations 1 and 4) although teeth are most widely used. **Ear plugs** in baleen whales provide a permanent record of total age from GLG, as long as there is no damage (Lockyer: Presentation 8). Apart from longevity, life-history parameters of age at sexual maturity and possibly physical maturity can be identified from the GLG patterns. Such patterns might exist in some teeth and should be investigated. Ear plug extraction from carcasses of minke whales (Maeda: Presentation 9) is facilitated by a new method involving injection of molten gelatin into the surrounding ear canal. Upon cooling, the gelatin supports the fragile ear plug structure, so increasing the possibility of extracting whole and undamaged ear plugs. This method should be tried in bowhead whales in which ear plugs are soft and fragile. The histological study of frozen thin sections of ear plug stained with Alizarin Red was successful in clarifying GLG in minke whale ear plugs.

Manatee periotic **bones** are suitable for age estimation in manatees (Howell: Presentation 22) where a thin section is cut from the middle of the periotic bone rostral lobe, decalcified, sectioned again to 5 micron, stained with Haematoxylin and Eosin, and examined with transmitted light under a microscope. Although some bone resorption occurs after age 15 years, maximum ages up to 59 years have been recorded.

Teeth are commonly used to age carnivorous mammals, including marine mammals. The seal age estimation review (Read: Presentations 1 and 4) indicated that canines are the optimal choice for aging, but that other teeth can be selected, especially in live animals. Techniques for preparing teeth vary. All are directed to obtaining the most complete record of clear lines. The dolphin age estimation review (Hohn: Presentation 2) noted the importance of quality tooth section preparations that included correct orientation providing a central section through crown and root when dentine was examined. The discussions following the dolphin tooth histology and preparation presentation (Murphy: Presentation 3) recommended that teeth prepared for decalcification should be wafered and sectioned thick initially at *ca* 2.5 mm to facilitate permeation of the decalcifying agent. A review of aging in sirenians (Lockyer: Presentation 5) indicated that dugong tusks had many internal similarities with beluga teeth and also perhaps narwhal tusks. GLG deposition rate in dugongs is annual. The most suitable method of age estimation in large odontocetes (Lockyer: Presentation 7) is using acid-etched half teeth in a crown to root-apex orientation, *e.g.*, in sperm whales, although thin untreated sections (*ca* 150-200 micron) are successful for smaller

odontocetes, *e.g.*, killer whales and some beaked whales. Although the former method is unsuitable for belugas, the latter method is suitable. GLG patterns in sperm whales and belugas are very similar.

In beluga, counts of GLG in dentine as seen in medial longitudinal sections of teeth is the standard method and completely consistent with methods used in other taxa. Discussion on the use of cemental GLG for estimating age, which was not so usual for cetaceans, might, in the case of belugas where cement is thick, be used to help estimate age when the dentine is worn down at the crown.

The most direct age estimation technique is the 'birth certificate' method whereby known and recognisable individuals are followed though time. This approach is not applicable to many species but is important in providing calibration animals for other techniques. **Long-term photo-ID** monitoring and surveys of the Gulf of St Lawrence belugas (Michaud: Presentation 24) resulted in a mass of reliable data on life history, age, reproduction, growth, and colour change. Necropsies on recovered known-age and known-history animals have provided teeth for verifying age. A photo-ID study of Alaskan humpback whales (Gabriele: Presentation 16) has also demonstrated the value of long-term monitoring of individuals. Calving intervals and reproductive history are known for several animals and many have been monitored since birth. Validation of an annual GLG deposition rate in humpback whale ear plugs was possible because of the recovery of samples and data from a stranded known-history female in Glacier Bay, Alaska. A long-term monitoring study of bottlenose dolphins in Sarasota Bay (Hohn presentation 17), involving capture and release, has enabled 5 generations to be monitored for life history. Extraction of teeth from live animals has permitted validation of the tooth GLG age technique for known-age animals, and knowledge of life-history parameters.

Once GLG are identified and counted, the next universal issue in age estimation is assessing the repeatability of the counts and validating their relationship to time. The first is precision; the second is accuracy and it is not the same as precision. Quality control is essential for both (Campana: Presentation 21). For quality control, there must be regular monitoring of an aging programme. The best measures of age precision are coefficient of variation (CV), average percent error (APE), and index of dispersion (D); the least reliable is percent agreement among readers which is usually the most commonly used. A permanent reference collection of aging materials, *e.g.*, known-age beluga teeth, is the key to effective quality control. An investigation of precision and bias in aging, focused on belugas with reference to tooth age data (Hobbs: Presentation 26). In conclusion, errors in estimation of parameters can arise from errors in aging with both negative and positive

bias and varying degrees of variance. In turn these translate to errors in estimation of growth rates. Efforts should be made to quantify bias and precision.

One of the most persistent debates pertaining to age estimation in the beluga, has been about the accurate translation of GLG counts into time units (years). The measurement of radiocarbon, ^{14}C, in laminated hard structures of animals (Campana: Presentation 19) has been a precise and successful method for validating age in many species, including belugas where GLG deposition rate was found to be unquestionably annual. Necropsies on recovered known-age and -history animals have provided teeth for verifying age. Several examples support a GLG deposition rate of one per year in beluga teeth (Michaud: Presentation 24). Investigation of the age from teeth of known-history captive belugas, together with data on tetracycline time-marking of teeth (Hohn and Lockyer: Presentation 20), generally supported an annual deposition rate of GLG. However, GLG definition was unclear in some specimens, particularly in the juvenile phase. The use of tetracycline drugs for time-marking of hard tissues (Hohn: Presentation 18) has been proven to be a valuable method of validating age in teeth GLG. Oral administration to both captive and free-living (Sarasota Bay study) animals has enabled precise information on GLG deposition rate and is recommended as the bio-marker of choice.

Claims in support of 2 GLG per year deposition rate based on examination of growth and reproduction in Cumberland Sound belugas (Brodie: Presentation 13) were criticized on a number of counts. The information did not agree with other evidence presented at the workshop where 1 GLG per year deposition rates were verified by using radiocarbon techniques and photo-ID studies of known-age and -history belugas for which teeth were available.

Future research was identified in several areas to fine-tune our understanding. One potential technique for estimating total age from worn beluga teeth by using the angle of the boundary layers relative to the pulp cavity edge appeared promising and should be pursued. Of a broader nature is the potential to understand the ecological correlates to line formation. Laser ablation (ICPMS) for trace elements showing periodic oscillations in beluga tooth GLG (Matthews: Presentation 23) may be promising, and stable isotope ratios focusing on ^{13}C and ^{15}N. The point of weaning can be identified from the N depletion up to this point. Chemical oscillations in the teeth may be linked to ecology and movements associated with feeding and migration, although these may not be annual and thus cannot be used as an age proxy presently. The method offered great potential, and should be investigated further, especially looking at O_2.

RECOMMENDATIONS OF THE WORKSHOP

The participants agreed upon the following recommendations for further studies on monodontids:

1. Inter-method comparisons of alternative aging methods using wild, known-age animals (*e.g.* Sable Island grey seals, St Lawrence belugas).
2. Augmenting the number of samples of known-age captive beluga from which teeth can be collected and comprehensive sampling of other materials useful for age estimation.
3. Examination of the immature phase of growth in teeth in beluga with reference to captive animals to determine GLG patterns.
4. Establishment of reference collections (hard parts) and consideration of a digital image exchange for calibration and training among labs.
5. Establishment of quality control routines.
6. Periodic exchanges among labs and inter-laboratory calibration for all aging techniques.
7. Comparison of tooth preparation methodologies among labs.
8. Estimation of crown wear from the angle of the boundary layers relative to the pulp cavity edge in beluga teeth, perhaps leading to estimation of the number of GLG that have disappeared.
9. Chemical time-marking for age calibration of hard parts.
10. Bomb radiocarbon validation of hard parts and eye lenses.
11. Comparison of GLG structure among stocks (wild and captive).
12. Compare GLG in teeth to growth layers in ear bones from belugas to determine if ear bones might have value for aging belugas and also narwhals.

CONCLUSIONS OF THE WORKSHOP ON AGEING METHODS APPLICABLE TO MONODONTIDS

The workshop members agreed on the methods which are or may be applicable to monodontids, presented in Table 3. The methods are graded according to relative accuracy, feasibility, validity and assumptions made. New methods not yet applied to monodontids are also listed. The limitations of each technique are also given. Some methods, depending on the type of tissue required for analysis, may be applicable both to living and dead animals.

Overall, tooth GLG are judged to be the best and most precise method. Presently, tooth GLG are only useable in belugas, but AAR is promising in narwhals. More work needs to be undertaken on embedded tusks of young narwhals to help establish the AAR rate. The AAR method should also be applied to beluga eye lenses to provide a correlation with beluga tooth GLG.

Such a study might provide more reliability on the narwhal AAR work presently done.

Other than known-age animals, the method that provides the most accurate ages and can be used for calibration is that of bomb radiocarbon. However, the main limitation is that at least some of the teeth or hard tissues must come from animals that were born before the fallout commenced, *i.e.* pre-1958.

Currently, the workshop members agreed that an annual deposition rate of tooth GLG was to be the accepted standard. New evidence from known-age and -history belugas in the Gulf of St Lawrence, combining photo-ID, tooth GLG and known age, also supported this tenet. Any doubts regarding interpretation of GLG would be taken up in detail at the forthcoming workshop at the NOAA lab in Beaufort, North Carolina, where aging experts would examine beluga tooth ultrastructure and define a standard protocol for tooth preparation and GLG counting method.

PUBLICATION OF THE PROCEEDINGS FROM THE WORKSHOP

Acquarone presented the *NAMMCO Scientific Publications* Series to participants of the workshop, explaining that papers submitted to the workshop would be welcomed as submissions to a volume addressing *Age estimation in marine mammals with a focus on monodontids*. Presenters at the workshop and potential other authors would be contacted in early 2012 regarding an invitation to contribute a paper to the volume. NAMCO had already approved the proposed volume which is now published online as Volume 10 in the *NAMMCO Scientific Publication*s Series at http://septentrio.uit.no/index.php/NAMMCOSP/issue/view/236. The editors comprise the members of the Steering Committee for this workshop in addition to the technical editor, Mario Acquarone.

Table 3. Age estimation methods that are or may be applicable to monodontids with an appraisal.

Methods/ Techniques of Aging	Absolute / Relative age	New to monodontids ?	Validated method?	Correl-ational support?	Precision for age at sexual maturation	Alive or dead source	Assumptions	Comments
Eye lens Aspartic Acid Racemization (AAR) with matching tooth-based age estimates	Relative	No -narwhals, yes - belugas	Not in marine mammals	Yes	Not sufficient	Dead	Lens metabolically stable. Racemization rate is constant.	Find surrogate species for testing. Encourage animal facilities to collect samples from known-age animals. Sample storage needs are specific.
Erupted tusks in narwhal	Absolute	No	No	No	Yes if accurate age	Dead	Interpret annual deposition correctly	Relatively difficult to obtain large specimen.
Embedded tusk studies in narwhal	Absolute	Hay (1980), Barner Neve (1995)	No	Pending	Unknown	Dead		Good for the first decades of life. Compare embedded to erupted tooth.
Trace element studies (in teeth and hard tissues)	Relative; unless there are periodic cycles	Yes (other models)	No	Unknown	Unknown	Dead mainly	Trace elements in the diet are cyclically incorporated in the teeth..	Could corroborate direct reading.Work in progress, encourage continuation.
Bone density of flippers	Relative	Yes	No	No	Unknown	Dead mainly	Requires that age and length are correlated	Samples available from some locations.
Telomeres	Relative	Yes	No	?	Probably low	Alive mainly		Requires high quality DNA.
Photo-ID and known age studies in free living animals	Absolute	No	Yes	Not applicable	Yes	Alive	Existing marks do not change over time or can identify changes. Calves accurately identified.	Frequent and continuous sampling is essential. Can be an ideal validation method.
Bomb radiocarbon	Absolute	No for beluga, Yes for narwhal	Yes	Not applicable	Yes	Dead	Some of the animals have to have been born before 1958	On teeth, the core of the eye-lens (and in the ear plug).

Teeth (GLG in dentine/cementum)	Absolute	No	Yes for beluga	Not applicable	Yes	Dead mainly	Interpreting annual deposition correctly	
Ear bones (tympanic bullae)	Absolute	Yes	Not applicable		Yes - potentially	Dead	Interpreting annual deposition correctly. There is no significant resorption.	
MicroCT scanning	Absolute	Yes			Probably	Dead	Mineralized differences can be found in modern teeth.	
Artifacts (found in hunted animals, *e.g.*, harpoon heads, bullets)	Relative	No	No	?	?	Dead	Artifact used close to manufacture date. Correct identification of that date.	Rare, opportunistic. Dead animals only.
Scar accumulation on the body	Relative	Yes	No		No	Both	Can determine scarring rate and permanency.	Crude ages.
Fatty Acid (FA) analysis	Relative	In progress for beluga (2011)	No		No	Dead or biopsy samples	FA are changing consistently from year to year and with age.	Requires analyses specific to the population / stock. Investigate sensitivity to ambient temperature.
Stable isotope ratios	Relative	In progress for beluga (2011)	No	To be done	To be done	Dead mainly	Preliminary studies of ^{13}C and ^{15}N are promising. .	Could corroborate direct reading. Work in progress, encourage continuation.

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Appendix 1

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Appendix 2

SCHEDULE FOR THE
AGE ESTIMATION WORKSHOP

26-27 November 2011 -- Venue Room #19, Tampa Convention Center, Tampa, Florida

PROGRAMME

Terms of Reference (TOR)

1. Review current methods of age estimation in marine mammals with a focus on monodontids.
2. Recommend the method(s) most suitable for monodontids; and trials of any new techniques that are as yet untried in monodontids.
3. Compile papers submitted to the workshop and relevant to age estimation in monodontids in a publication volume entitled "Age estimation in marine mammals" of the NAMMCO Scientific Publication Series.

Day 1: 26 November

08:30 hr	**Registration**	
Opening, Welcome and Introduction		
09:00 hr	Background and the basis for the workshop and its aims (TOR)	Christina Lockyer
Review – Chair: Christina Lockyer		
09:15 hr	Age estimation methods applicable in mammals with special emphasis on marine mammals and especially monodontids	Fiona Read
09:55 hr	**Discussion and questions – led by Chair of session**	
10:15 hr	**Refreshments (30 min)**	
Direct methods of aging - Chair: Aleta Hohn		
10:45 hr	Aging in dolphins including belugas	Aleta Hohn
11:00 hr	Investigating the deposition of growth layer groups in dentine tissue of captive common dolphins *Delphinus sp.*	Sinead Murphy
11:20 hr	Age estimation in seals	Fiona Read
11:35 hr	Age estimation from teeth in large odontocetes	Christina Lockyer
11:50 hr	A brief review of age estimation in sirenians focusing on dugong tusks	Christina Lockyer
12:00 hr	**Discussion - led by Chair of session**	
12:15 hr	**Lunch (1 hr 15 min)**	
13:30 hr	Age estimation in mysticetes with a focus on ear plugs	Christina Lockyer
13:50hr	Feasibility study on the incorporation of the gelatinized collection method and the freeze-section technique of the ear plug in age estimation in common minke whales	Hikari Maeda
14:10 hr	**Discussion – led by Chair of session**	
Indirect methods of aging – Chair: Rob Stewart		
14:30 hr	Porpoise / fin whale age from eye lens and age validation	Nynne Hjort-Nielsen
14:45 hr	Background, the harp seal study and the known age animals study	Eva Garde
15:00 hr	Narwhal age from eye lens and age validation	Eva Garde
15:25 hr	Aging beluga (white) whales from measurements of specific fatty acids present in their outer-blubber biopsy tissues	Gina Ylitalo
15:45 hr	**Refreshments (30 min)**	
16.15 hr	Prospects for genetic age estimation of cetaceans	Morten Tang Olsen
16.35 hr	Growth and maturity of belugas (*Delphinapterus leucas*) in Cumberland Sound, Canada, compared to those raised in captivity	Paul Brodie

17:05 hr	**Discussion led by Chair of session**	
17:25 hr	Summing up for Day 1	Christina Lockyer
17:30 hr	**BREAK FOR DAY 1**	

DAY 2: 27 November

New techniques – Chair: Rod Hobbs

08:30 hr	Use of micro-computed tomography for dental studies	Carolina Loch
08:50 hr	Ear bones used for aging of manatees	Amber Howell
09:10 hr	Informal progress report on *Trace element profiles in beluga teeth*	Cory Matthews
09:20 hr	An overview on aspartic acid aging-strengths and weaknesses	Craig George
09:40 hr	Individual identification and life history of the St. Lawrence beluga	Robert Michaud
10:00 hr	**Discussion - led by Chair of session**	

Validation techniques – Chair: Rod Hobbs

10:15 hr	Long-term studies with respect to humpbacks photo-ID, age and reproduction	Chris Gabriele
10:25 hr	Known history and photo-ID (dolphins)	Aleta Hohn
10:35 hr	Bio-markers and tetracycline antibiotic time-marking	Aleta Hohn
10:50 hr	**Refreshments (10 min)**	
11:00 hr	Bomb dating and age validation: conclusive results in a fuzzy world	Steve Campana
11:20 hr	Artifacts (e.g. historic whaling weapon recovery from carcasses)	Craig George
11:35 hr	Age validation through known history captive studies in belugas	Christina Lockyer
11:50 hr	**Discussion - led by Chair of session**	

Application of age data – Chair: Christina Lockyer

12:00 hr	Sensitivity of age structured population dynamics models to bias and precision in ages	Rod Hobbs
12:20 hr	Accuracy, precision and quality control in the age estimation of aquatic animals	Steve Campana
12:40 hr	**Discussion - led by Chair of session**	
13:00 hr	**Lunch (1 hr 30 min)**	

Concluding the workshop - Chair: Christina Lockyer

14:30 hr	Summing up of Days 1 and 2	Christina Lockyer
14:45 hr	Draft recommendations: • *Accepted methods for monodontids* • *Unsuitable methods for monodontids* • *New methods for trial with monodontids* • *Validation methods for monodontids* • *Conclusions on validation of GLG in teeth* • *Conclusions on quality control and any actions arising (e.g. standardisation)*	
15:30 hr	**Refreshments (30 min)**	
16:00 hr	Draft recommendations for the report – on screen Deadline for completion and circulation of the workshop report.	Mario Acquarone
16:30 hr	Contributions to the ***NAMMCO Scientific Publications***: who will contribute, deadlines for submissions, and editors. Author guidelines and planned publication date.	Mario Acquarone, Christina Lockyer
17:00 hr	**CONCLUSION OF WORKSHOP**	