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AN EVALUATION OF USING ENHANCED DIGITAL MICROSCOPY TO ESTIMATE AGES OF SHORT-BEAKED COMMON DOLPHINS *(Delphinus delphis)*

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U.S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
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Abstract

Precision and bias in age estimates of short-beaked common dolphin (*Delphinus delphis*) teeth were compared between two readers and two viewing platforms: a compound light microscope and a digital image analyzer. Coefficients of variation for both readers ranged from 11.5% to 11.7% on the microscope and from 9.0 % to 14.4% on the image analyzer. For both readers, precision of age estimates was not significantly different between viewing platforms, and precision was not correlated with age (i.e. number of growth layer groups (GLGs) counted) for either reader on either viewing platform. For specimens with ten GLGs or more, age estimates by Reader 1 were not significantly different between viewing platforms, whereas Reader 2 made lower estimates on the image analyzer compared to the microscope. This negative bias in estimating age for older animals by Reader 2 is likely due to a change in technique when using the image analyzer, which can likely be remedied by improving the training protocol for using the image analyzer. Use of the digital image analyzer is promising for age estimation primarily because precision was comparable to traditional light microscopy. However, the system provides additional benefits by enabling reference GLGs to be marked, and as a storage medium resistant to fading.

Introduction

Age is a fundamental parameter for describing the life history of a species. It provides the basis for quantifying reproductive potential of a population and estimating individual growth rates from birth to adulthood and schedules of birth and survival rates for population modeling. Thus, it is essential that the method of estimating this parameter maximize precision and accuracy in order to obtain the best age estimate possible. Age has been estimated in many species of delphinids by counting growth layers in the teeth. Incremental growth in the dentine and cementum of the tooth begins after birth and accumulated layers defined by regularly spaced major lines are referred to as growth layer groups (GLGs). In small delphinids, the concept that GLGs correspond to an annual rate of accumulation (Perrin and Myrick 1980) is generally accepted because several calibration studies (Gurevich et al. 1980; Myrick et al. 1984; Hohn et al. 1989; Myrick and Cornell 1990) support the interpretation. In one calibration study, using tetracycline labeled teeth, Gurevich et al. (1980) determined that one GLG is laid down annually in the teeth of short-beaked common dolphins, *D. delphis*, our selected study species. In this study we explore the use of a new method which has proven successful in aging fish otoliths (Neal 1987; Laidig and Pearson 1992; Caillet et al. 1996) and examines its associated precision and biases in aging *D. delphis* teeth.

Traditionally, age estimates are obtained in delphinids by counting GLGs in stained thin sections of teeth mounted on slides and viewed through a compound light microscope. Typically, several readers read each specimen multiple times to make a best estimate of age (Myrick et al. 1983; Hohn and Hammond 1985). However,

stained thin sections run the risk of fading with time and past studies cannot be reviewed or repeated. This has occurred in the past and been remedied with alternative sealing media (Lockyer 1995). However, it is currently not known how long current stains will last. A method for archiving prepared tooth sections is needed so that teeth may be referenced far into the future. In addition to this need, the prospect of improving the clarity of GLGs (and therefore precision and accuracy) and saving reader GLG designations with their associated teeth led to the exploration of using enhanced digital images obtained from the microscope for estimating ages. Digital imaging equipment has been used to measure widths of incomplete GLGs in Pacific white-sided dolphins (*Lagenorhynchus obliquidens*) (Ferrero and Walker 1996) but not as a platform to estimate age from prepared tooth sections of marine mammal teeth.

In order to determine whether this method might be a viable alternative to traditional microscopy, this study focused on whether (a) precision in age estimations could be maintained or improved on the image analyzer, (b) age estimates differed between viewing platforms, and (c) discrepancies in age differences between readers could be resolved by reviewing saved GLG demarcations. Teeth from *D. delphis* incidentally caught in gillnets (Chivers et al. 1997) were used to investigate these questions.

Methods

Preparation and Age Determination

Teeth were obtained from 36 *D. delphis* incidentally killed in the California gillnet fishery between 1994 and 1997. Following the protocol of Myrick et al.

(1983), teeth were decalcified, cut with a freezing microtome into 25μm thick longitudinal serial sections, and stained with hematoxylin. Decalcification times ranged from one to 16 hours, with longer times needed for larger, older animals that had accumulated more dentine and cementum. Sections were mounted on gelatin-coated slides, and cover slip margins were sealed with DPX mounting medium (Lockyer 1995). Ages were determined by examining GLGs in the dentine (Myrick et al. 1983; Hohn et al. 1989), using both a compound light microscope and enhanced video microscope images. Two readers aged each tooth three times, with at least a week between readings, on each viewing platform. GLG estimates were made without reference to specimen information, such as total body length, reproductive status, or previous GLG counts. The mean GLG count of a reader's three age estimations was used to compare precision and ages between readers. The mean GLG count of both readers' three readings is referred to as the total pooled mean age estimate for each specimen.

Video microscope images

Tooth images were captured using a precision megapixel digital camera (DVC-1310C) and then viewed and enhanced using Image Pro-Plus software (version 4.5). Multiple partial images of each tooth, viewed with the 100x objective, were captured and spliced to produce a single image of the entire tooth. In older animals, it was often necessary to save an additional image centered on the pulp cavity at 400x. Images were then enhanced by increasing brightness and contrast and applying sharpen and Hi Gauss filters. To maintain consistency, both readers viewed the same enhanced image. During each aging session, the boundaries of each GLG were

marked and the corresponding width measurement of each GLG was saved with the specimen's age file. These marked GLG boundaries were not viewed during subsequent aging sessions.

Intra-Reader Variation

For each reader and viewing platform, the coefficient of variation (CV = $\frac{SD}{\overline{X}} \times 100$) and index of precision ($D = CV/\sqrt{n}$) were calculated for each tooth and the mean of these values were used for comparisons (Chang 1982). Because these measures of precision were effectively the same (demonstrated the same trends), D is reported only for comparison to other studies and CV was used in analyses of reader precision and bias. To determine whether precision varied with increasing GLGs, a Spearman rank correlation test was conducted on CV and GLGs.

Inter-Reader Variation

In addition to *t*-tests and analyses of variance (ANOVA), CVs, age-frequency tables, and age-bias plots were used to compare matched pairs of GLG determinations. Campana et al. (1995) suggested these additional comparison methods as a way of detecting non-linear biases (i.e., bias that differs with age), which *t*-tests and ANOVA generally cannot detect. The mean ages reported by the two readers were compared for each viewing platform using a paired *t*-test. Due to heteroscedascity in CV data, the non-parametric alternative to the two-way ANOVA, the Scheirer-Ray-Hare test (Sokal and Rohlf 1995), was used to compare CV across readers for two GLG groups. The GLG groups were based on a departure of paired age estimations from the 1:1 line at approximately ten GLGs in age-bias plots. This

observation and its associated implications will be discussed further in following sections. The following GLG groupings were used in this and subsequent analyses: (a) 0 – 9 ("young animals) and (b) 10 or more GLGs ("older" animals).

Viewing-Platform Variation

For each reader, a paired *t*-test was used to compare CV between viewing platforms, age-bias plots were used to visually compare ages between viewing platforms, and a one-way ANOVA was used to compare differences in GLG counts between viewing platforms across GLG groups. For each specimen, assignment to GLG group was based on the total pooled mean age estimate.

Results

Microscope

Intra-reader variation

The mean CV and D for Reader 1 were 11.70% and 6.75%, respectively. For Reader 2, mean CV was 11.53% and D was 6.66%. A Spearman rank correlation test for each reader indicates that there is no relationship between CV and number of GLGs (Table 1).

Inter-reader variation

On the microscope, GLG counts for Reader 1 ranged from 0 to 24 and from 0 to 23 for Reader 2 (Table 2). Mean age estimates for each specimen were not significantly different between readers (*t*-test: $t_{35} = 0.109$, $P = 0.914$). However, the age-bias plot illustrates a subtle bias between readers (Figure 1a). Compared to Reader 1, a small negative bias for GLG counts by Reader 2 is present for GLGs zero to nine (*t*-test: $t_{17} = 2.14$, $P = 0.047$), whereas a linear bias is not evident for GLGs

Table 1. Results of Spearman rank correlation test of CV and GLG counts.

	r	P-value
Reader 1		
Microscope	-0.321	0.057
Image Analyzer	-0.053	0.759
Reader 2		
Microscope	0.033	0.850
Image Analyzer	0.060	0.728

Table 2. Frequency of age estimates in GLGs made by Reader 1 and Reader 2 using the microscope. Gray cells illustrate where frequency of age estimates would be located if there were complete concordance in age estimates between readers.

	Ages estimated by reader 2																									
Ages estimated by reader 1		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	Total
	0	4																									4
	1		4	1																							5
	2			3																							3
	3																										0
	4			1																							1
	5							1																			1
	6						1																				1
	7								1																		1
	8							1																			1
	9									1																	1
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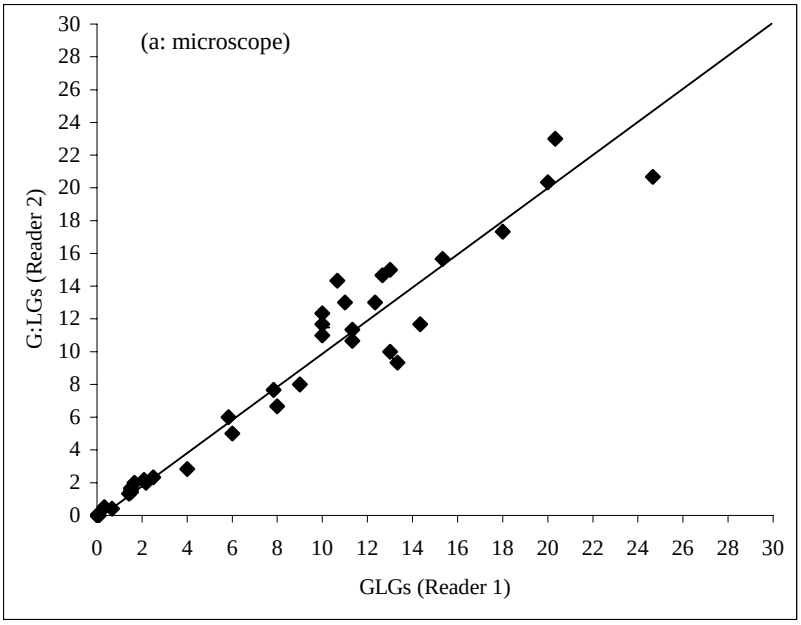

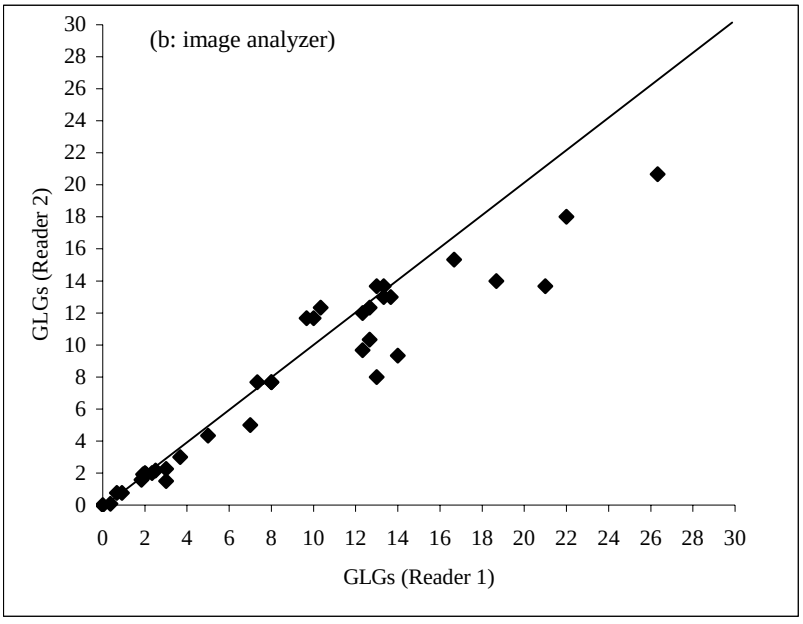

Figure 1. Age-bias plots for Reader 1 and Reader 2 by viewing platforms: (a) microscope and (b) image analyzer. The 1:1 line is included for reference to illustrate how the plot would look if there were complete concordance in age estimates between readers.

greater than or equal to 10 (*t*-test: $t_{17} = 0.374$, $P = 0.713$). However, the cloud of data points off the 1:1 line for GLGs 10 to 14 indicates variability in ages between readers for this group of specimens. For specimens where GLG estimations did not agree, 43% agreed to within one GLG and 71% agreed to within two GLGs. Mean CV was not significantly different across readers or age groups ($0.25 < P < 0.50$). Table 3 presents the Scheirer-Ray-Hare summary. Pooling the ages from both readers resulted in a mean CV of 21.3%.

Image Analyzer

Intra-reader variation

Mean CV and D for Reader 1 were 9.00% and 5.20%, respectively. For Reader 2, mean CV was 14.37% and D was 8.30%. Spearman rank correlation tests for each reader indicate that there is no relationship between CV and number of GLGs (Table 1).

Inter-reader variation

On the image analyzer, mean GLG counts ranged from 0 to 26 for Reader 1 and from 0 to 20 for Reader 2 (Table 4). Mean GLG estimates were found to be significantly different between readers (t-test: $t_{35} = 3.11$, $P = 0.004$). For specimens where GLG estimations did not agree, 37% agreed to within one GLG and 63% agreed to within two GLGs. Age-bias plots indicate that compared to Reader 1, GLG counts by Reader 2 were negatively biased overall, with the exception of a positive bias for three specimens between nine and 10 GLGs (*t*-test: $t_2 = 17.0$, $P = 0.003$) (Figure 1b). Mean CV was not significantly different between readers or age groups ($0.90 < P < 0.95$). Table 5 presents the Scheirer-Ray-Hare summary.

Table 3. Scheirer-Ray-Hare summary of CV comparisons across readers and age groups (0-9, 10+) for the light microscope.

Source	Sum-of-squares	df	H	P
Reader	2.512	1	0.001	0.975
GLG Group	979.548	1	0.539	0.25 < P < 0.50
Reader*GLG Group	2345.058	1	1.291	0.25 < P < 0.50
Error	125628.194	68		

Table 4. Frequency of age estimates in GLGs made by Reader 1 and Reader 2 using the image analyzer. Gray cells illustrate where frequency of age estimates would be located if there were complete concordance in age estimates between readers.

	Ages estimated by reader 2																											
Ages estimated by reader 1		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	Total
	0	4																											4
	1		2																										2
	2		1	3																									4
	3		1	1	1																								3
	4																												0
	5					1																							1
	6																												0
	7						1		1																				2
	8								2																				2
	9												1																1
	10												1	1															2
	11																												0
	12										1	1		2															4
	13									1					4														5
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	25																												0
	26																					1							1
	Total	4	4	4	1	1	1	0	3	1	2	1	2	3	5	1	1	0	0	1	0	1	0	0	0	0	0	0	36

Table 5. Scheirer-Ray-Hare summary of CV comparisons across readers and age groups (0-9, 10+) for the image analyzer.

Source	Sum-of-squares	df	H	P
Reader	525.420	1	0.328	$0.50 < P < 0.75$
GLG Group	2744.170	1	1.713	$0.10 < P < 0.25$
Reader*GLG Group	7.670	1	0.005	$0.90 < P < 0.95$
Error	110448.486	68		

Pooling the data from both readers resulted in a mean CV of 16.2.

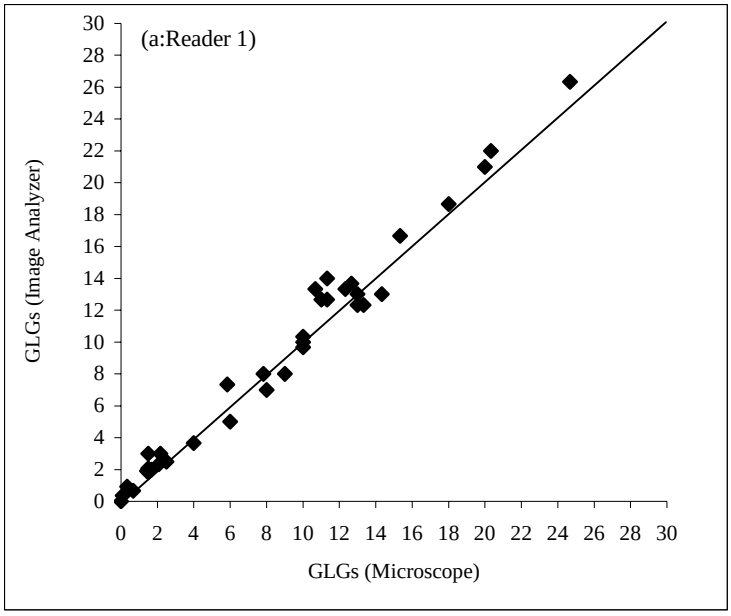
Viewing platform comparison

Mean CVs were not significantly different for Reader 1 (*t*-test: $t_{35} = 1.33$, P = 0.193) or Reader 2 (*t*-test: $t_{35} = 0.548$, P = 0.587) on the image analyzer compared to the microscope. The age-bias plot (Figure 2) indicates a departure in GLG comparability between viewing platforms, for Reader 2, at approximately 10 GLGs. For specimens with 10 GLGs or more, age estimates by Reader 1 were not significantly different (ANOVA: $F_{1,34} = 3.277$, P = 0.079), whereas Reader 2 estimated lower on the image analyzer compared to the microscope (ANOVA: $F_{1,34} = 5.256$, P = 0.028). Table 6 presents the ANOVA summary tables for both readers.

Age-bias plots illustrate differences in reader behavior between viewing platforms (Figure 1). Biases in GLG counts between readers exhibited the same trend for both viewing platforms until approximately 10 GLGs. Up to this point, Reader 2 GLG estimations were negatively biased compared to those of Reader 1. After 10 GLGs, this negative bias continued and increased on the image analyzer whereas on the microscope a clear bias was not evident for these older animals.

Discussion

Although no significant difference in CV across platforms was observed, the readers compared in this study exhibited opposite trends in precision between platforms. Reader 1 had higher precision (lower CV and D) when using the image analyzer, whereas Reader 2 had higher precision when using the microscope (Table 7). This difference in reader behavior may reflect individual comfort level using the different viewing platforms to count GLGs. Reader 1 had limited aging experience on the microscope, and had developed the use and protocol for using the image



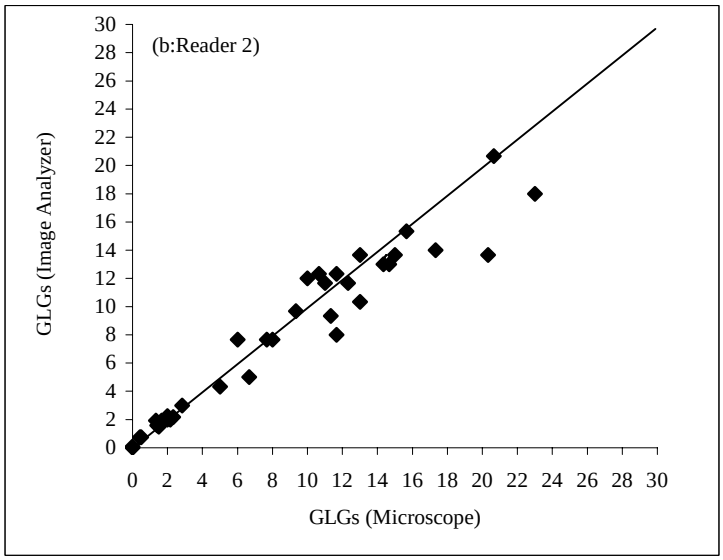


Figure 2. Age-bias plots for two different viewing platforms by reader: (a) Reader 1 and (b) Reader 2. The 1:1 line is included for reference to illustrate how the plot would look if there were complete concordance in age estimates between platforms.

Table 6. ANOVA summary for differences in GLG counts between platforms by age class groups: 0-9 and 10+ for Reader 1 and Reader 2.

Reader 1					
Source	Sum-of-squares	df	Mean-square	F-ratio	P
Age Group	3.053	1	3.053	3.277	0.079
Error	31.670	34	0.932		
Reader 2					
Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Age Group	15.232	1	15.232	5.257	0.028
Error	98.516	34	2.898		

Table 7. Calculated estimates of precision for individual readers using different viewing platforms for this study and precision values for ages obtained using the microscope in other odontocete age studies .

	Reader 1		Reader 2		Reilly et al.		Evans et al.	
	CV (%)	D (%)	CV (%)	D (%)	CV (%)	D (%)	CV (%)	D (%)
Microscope	11.70	6.75	11.53	6.66	6.90 - 11.28	4.55 - 6.59	10.6	4.8
Image Analyzer	9.00	5.20	14.37	8.30				

analyzer system, and therefore was more at ease using the computerized system. However, Reader 2 had several years experience aging teeth on the microscope and had little experience with the computerized system and was therefore less at ease with this system. The CVs and Ds obtained on each reader's "stronger" viewing platform were similar to values reported by other odontocete aging studies (Reilly et al. 1983; Evans et al. 2002). However, CVs for ages estimated on each reader's "weaker" viewing platform were higher than published values (Table 7). Although these differences were not statistically detectable, they suggest that readers should gain experience to increase their comfort level using a new viewing platform before readings from a new platform are used in an age-related study.

Precision of GLG counts did not vary with number of GLGs, reflecting what has been found for sperm whales (Evans et al. 2002) and contrasting with the decrease in precision with increasing GLGs found in pantropical spotted dolphins (Reilly et al. 1983). Conflicting results of precision variability with age have been found in pinnipeds as well (Lawson et al. 1992; Bernt et al. 1996), suggesting that the ability to accurately estimate all ages repeatedly differs by species (i.e., older spotted dolphins are likely more difficult to age than sperm whales, primarily because of the size of the tooth). *D. delphis* teeth are similar in size to spotted dolphin teeth and by analogy, aging older *D. delphis* may be expected to be comparable to aging spotted dolphins. However, the consistency of precision across age groups found in this study implies that GLGs in older *D. delphis* teeth might be less compacted or distorted and thus have a more consistent layering pattern than other species. The teeth used in this study may also have more clearly defined layers due to the temperate habitat where

the specimens were collected. Variation in diet and growth, typical of seasonal habitats may lead to more distinct layering in the teeth (Klevezal 1980).

The high pooled mean CV for both readers on both viewing platforms is similar to that reported by Reilly et al. (1983), and is most likely due to differences in interpretation of the layering patterns of later GLGs between readers. Future work with older known-age specimens to determine the correct interpretation of layering patterns could reduce reader interpretation error that is likely contributing to the large pooled mean CVs. Unfortunately, the availability of older captive *D. delphis* teeth is low, and long-term studies of wild populations are nearly impossible due to the pelagic nature of this species.

Matched paired *t*-tests and age-bias plots (Figure 1) indicated that GLG counts were more comparable between readers on the microscope than on the image analyzer. However, a slight negative bias in Reader 2 GLG counts (relative to Reader 1) for younger specimens is apparent on both viewing platforms. Using image analyzer files, GLG demarcations between readers were compared on younger specimens to provide insight into potential reasons for this trend. One explanation may be that Reader 1 counted accessory lines as growth layers (thus estimating more GLGs) in the younger animals because they are more pronounced in the characteristically wider GLGs typical of younger specimens.

Tooth image files were also examined for the three specimens contributing to the positive age-bias of Reader 2 compared to Reader 1 on the image analyzer (Figure 1b). Pearling, unusual shapes, or shredded areas of the pulp cavity were present in these teeth, which may have made it more difficult for the less experienced person

(Reader 1) to interpret these inner GLGs, leading to an underestimation of age (Figure 3). Four teeth in addition to those mentioned above contributed to the cloud of data points above the 1:1 line in middle-aged animals on the microscope (Figure 1a). In these teeth, it is likely that what Reader 1 considered accessory lines (and therefore did not count), Reader 2 counted as GLGs. Reader 2 often used layers in the cementum to help verify GLG counts in the dentine, whereas Reader 1 did not. However, Reader 2 could not use this verification method on the image analyzer because high magnification images of cementum were not taken. This is likely why the image analyzer has fewer specimens contributing to the positive bias in middle aged animals.

The marked negative bias of reader 2 GLG counts among older animals on the image analyzer (Figure 1b) illustrates the differences in reader behavior between platforms. Age-bias plots (Figure 2) show that Reader 1 had a slight positive bias for older animals on the image analyzer compared to the microscope and Reader 2 counted fewer GLGs in older animals on the image analyzer. These trends are likely due to Reader 2 routinely using a higher magnification on the microscope to count these inner GLGs, whereas Reader 1 rarely did this because of limited experience tracking GLG lines when switching to higher objectives. Interestingly, Reader 1 began routinely using a higher magnification to count inner GLGs on the image analyzer, because images on two different objectives could be viewed simultaneously, and therefore the difficulty in tracking GLGs was eliminated for this reader.

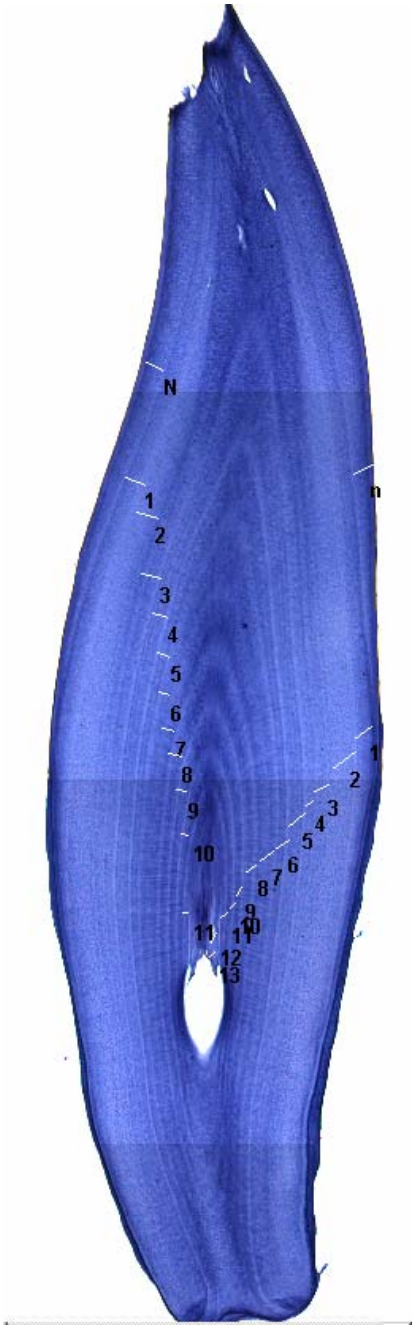


Figure 3. GLG demarcations of Reader 1 (left) and Reader 2 (right) for specimen JYB0021. Note increased number of GLGs towards pulp cavity (center) by Reader 2.

The increased use by Reader 1 and the decreased use by Reader 2 of the higher objective on the image analyzer likely resulted in the negative bias of Reader 2 GLG counts compared to those of Reader 1 (Figure 1b). This is supported by examination of marked GLG image analyzer files for older specimens; Reader 1 observed and marked more GLGs near the pulp cavity than Reader 2 (Figure 4), and fewer discrepancies between readers were noted in the identification of GLGs nearer to the outer edge of the tooth. These opposing trends in age-bias on the image analyzer for the two readers (Figure 2) essentially magnify the difference in GLG counts between readers in older specimens (Figure 1b).

Potential implications of aging biases

Our study demonstrates that not only may biases in aging exist between aging platforms, but that there are also several factors that may bias ages independent of the platform being used. Accessory lines, tooth section condition, and GLG compaction can all influence how GLGs are interpreted. Age frequency distributions generated from aging data could potentially be skewed depending on the degree and direction of a particular bias. A skewed age distribution could then bias estimates of age at attainment of sexual maturity as well as longevity. Unfortunately, the degree and direction of potential biases relative to the "true" age of a specimen is generally unknown and may be unknowable.

Conclusions

Comparable precision to traditional light microscopy, ability to reference GLG demarcations to resolve reader differences in age, and use as a storage medium resistant to fading make digital microscope images in combination with image

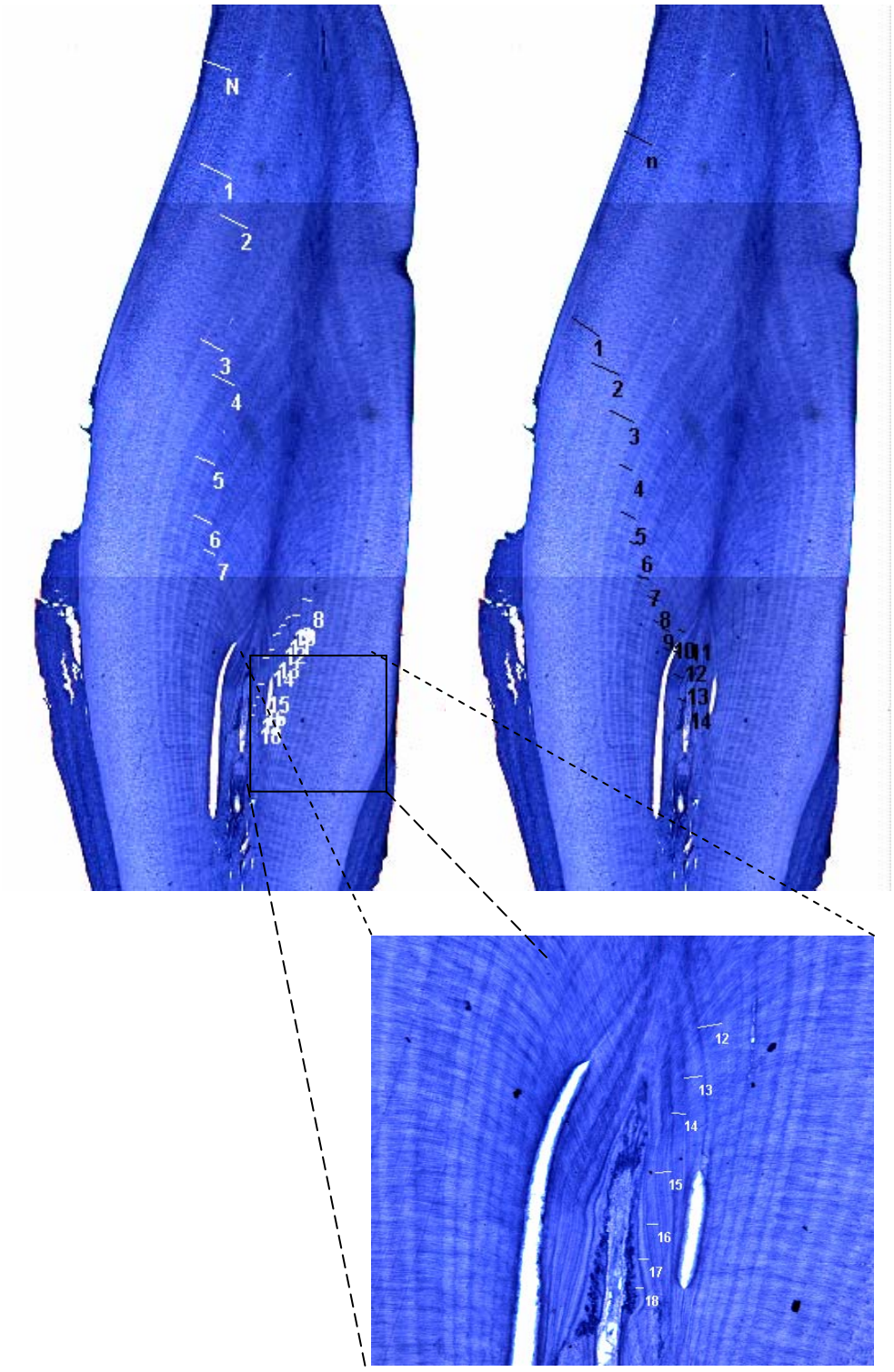


Figure 4. GLG demarcations of specimen SHB003 by Reader 1 (left side with lower inset of higher magnification) and Reader 2 (right side). Note greater number of GLGs towards pulp cavity (center) for Reader 1.

analysis software a promising tool for GLG estimation in small delphinid teeth. However, before such a system is set as a standard procedure for age related studies, an additional analysis similar to this should be performed after readers have gained sufficient experience with the system and a standard protocol is in place for using higher objectives and cementum layers to aid in GLG estimation of older animals. If GLG counts in the cementum are needed to verify GLG counts in the dentine, high magnification images of the cementum should be taken. If reader behavior were comparable after such protocols were implemented and experience was gained, image analysis would be the preferred method for aging delphinid teeth.

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