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Accuracy, precision, and error in age estimation of Florida manatees using growth layer groups in earbones

Gina L. Lonati, Amber R. Howell, Jeffrey A. Hostetler, Paul Schueller, Martine de Wit, Brandon L. Bassett, Charles J. Deutsch,* and Leslie I. Ward-Geiger

Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute, 100 8th Avenue SE, St. Petersburg, FL 33701, USA (GLL, ARH, JAH, MdW, BLB, LIW-G)
Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute, 7386 NW 71st Street, Gainesville, FL 32653, USA (PS)
Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute, 1105 SW Williston Road, Gainesville, FL 32601, USA (CJD)

* *Correspondent: Chip.Deutsch@myFWC.com*

Ages of Florida manatees (*Trichechus manatus latirostris*) can be estimated by counting annual growth layer groups (GLGs) in the periotic dome portion of the tympanoperiotic complex of their earbones. The Florida Fish and Wildlife Conservation Commission manages an archive of more than 8,700 Florida manatee earbones collected from salvaged carcasses from 1989 to 2017. Our goal was to comprehensively evaluate techniques used to estimate age, given this large sample size and changes to processing protocols and earbone readers over time. We developed new standards for estimating ages from earbones, involving two independent readers to obtain measurements of within- and between-reader precision. To quantify accuracy, precision, and error, 111 earbones from manatees with approximately known ages (first known as calves: "KAC") and 69 earbones from manatees with minimum known ages ("MKA," based on photo-identification sighting histories) were processed, and their ages were estimated. There was greater precision within readers (coefficient of variation, *CV*: 2.4–8.5%) than between readers (*CV*: 13.1–13.3%). The median of age estimates fell within the true age range for 63.1% of KAC cases and was at least the sighting duration for 75.0% of MKA cases. Age estimates were generally unbiased, as indicated by an average raw error ± *SD* of −0.05 ± 3.05 years for the KAC group. The absolute error (i.e., absolute value of raw error) of the KAC data set averaged 1.75 ± 2.50 years. Accuracy decreased and error increased with increasing known age, especially for animals over 15 years old, whose ages were mostly underestimated due to increasing levels of resorption (the process of bone turnover that obscures GLGs). Understanding the degree of uncertainty in age estimates will help us assess the utility of age data in manatee population models. We emphasize the importance of standardizing and routinely reviewing age estimation and processing protocols to ensure that age data remain consistent and reliable.

Key words: accuracy, aging, earbone, error, growth layer group, manatee, precision, validation

Accurately determining age-specific demographics of populations is an important aspect of mammal conservation. Data on individual animal ages can illuminate age distributions, growth rates, age at sexual maturity, age-specific reproductive rates, and the life span of a species—all essential information for understanding population dynamics (Klevezal and Kleinenberg 1969; Morris 1972). One of the most common techniques for estimating ages is counting incremental growth layer groups (GLGs) in continually growing tissues (Perrin and Myrick 1980; reviewed in Klevezal and Kleinenberg 1969). This technique has become ubiquitous in fisheries science, where otoliths can be used to estimate the age of fishes and garner information about stock dynamics (Campana and Thorrold 2001). In mammals, GLGs can occur in teeth, horns, claws, ear plugs, or the periosteal region of skeletal components (Klevezal and Kleinenberg 1969). GLGs provide reliable measurements of absolute age in many mammalian species, including toothed whales (Perrin and Myrick

1980; Myrick et al. 1983; Goren et al. 1987; Hohn et al. 1989; Dellabianca et al. 2011; Neuenhoff et al. 2011), pinnipeds (Laws 1952; Jonsgard 1969; Payne 1978; Childerhouse et al. 2004; Molina-Schiller and Pinedo 2004), dugongs (*Dugong dugon*—Scheffer 1970; Mitchell 1976; Marsh 1980), bears (Stoneberg and Jonkel 1966; Calvert and Ramsay 1998), and large ungulates (Sergeant and Pimlott 1959; McEwan 1962; Geist 1966; Ransom 1966).

Age estimation using GLGs relies on the assumption that animals deposit these layers at certain time intervals as they age. In bone, periosteal GLGs consist of a broad band plus an adhesion line, which often appears darker upon staining (Klevezal 1996). It is thought that the narrow adhesion line forms during periods of reduced growth (in most cases during winter or when there is reduced foraging, migration, hibernation, or environmental stressors), whereas the broader band forms during periods of rapid growth (when food resources are abundant or an animal is allocating more nutrients toward growth—Weinmann and Sicher 1947; Sissons 1949, 1971; Morris 1972; Klevezal 1996). The rate of GLG deposition can vary among species and individuals, as well as with environmental variables, sex, geographic region, life-history events, and various stressors (Scheffer and Peterson 1967; Harwood and Prime 1978; Klevezal and Myrick 1984; Manzanilla 1989; Boyd and Roberts 1993; Klevezal and Stewart 1994; Klevezal 1996; Hanson et al. 2009; Medill et al. 2010; Dellabianca et al. 2011; Knox et al. 2014; Wittmann et al. 2016 and references therein; Hamilton et al. 2017).

The Florida manatee (*Trichechus manatus latirostris*) is a subspecies of the threatened West Indian manatee (*Trichechus manatus*). Florida manatees are fully aquatic, herbivorous marine mammals that mainly inhabit the coastal waters of the southeastern United States. Knowing the ages of Florida manatee carcasses could provide useful insights into population dynamics; age at sexual maturity; potential age-specific susceptibility to red tide, cold stress, and other causes of death; and other aspects of manatee biology. Marmontel et al. (1990) analyzed the structural patterns of a variety of Florida manatee bones (i.e., teeth, mandibles, ribs, humerus, radius, ulna, phalanxes, carpals, pelvic bones, hyoids, mastoids, and various parts of the tympanoperiotic complex) relative to approximate known age and determined that the periotic dome portion of the manatee tympanoperiotic complex (Fig. 1) was the most reliable tissue for determining a manatee's age at death. Marmontel et al. (1996) verified the presence of annual GLGs in the periotic domes (hereafter, "earbones") and found that manatees known to have been less than 2 years old at death exhibited a transition of structure from compact lamellar (CL) bone to the white rim (WR) and lacked adhesion lines. The CL bone was characterized by long, horizontally organized vascular canals, and either gradually or abruptly transitioned into the less organized structure of the WR, which consisted of shorter, irregular vascular canals (Fig. 2). The WR is located just deeper than the GLGs of the earbone edge and was named for its typically lighter appearance (when stained with hematoxylin) compared to other earbone layers (Fig. 2).

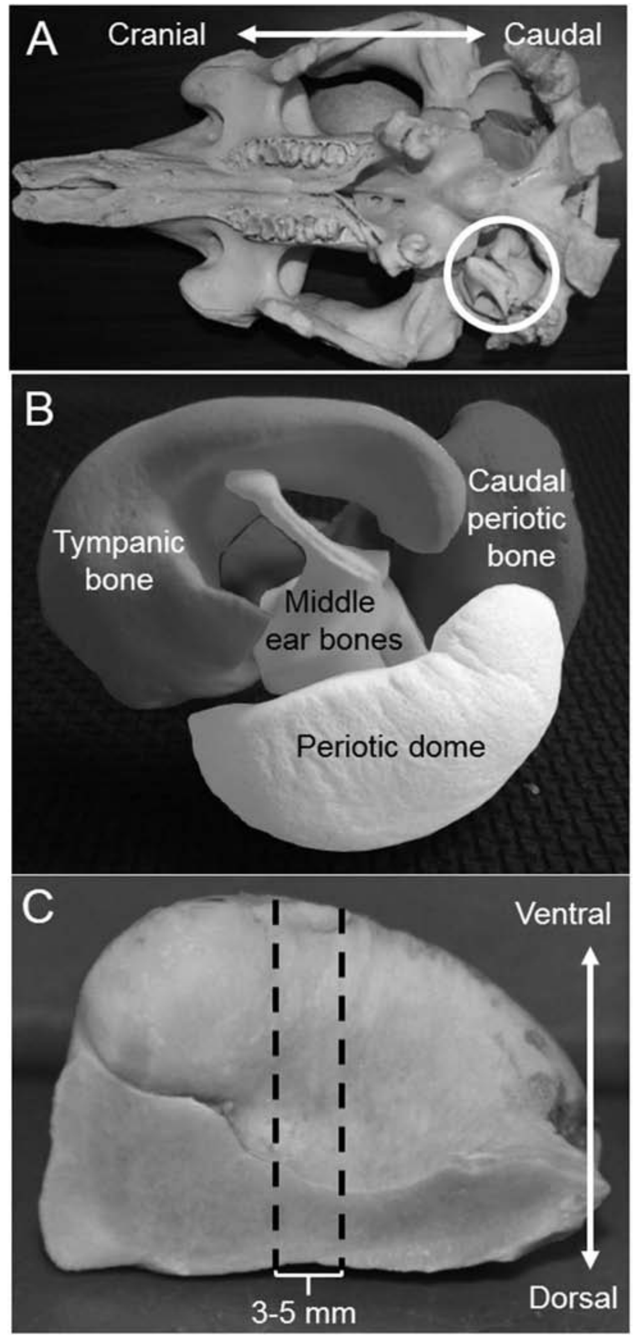

Fig. 1.—Florida manatee (*Trichechus manatus latirostris*) earbone anatomy. (A) Ventral view of the upper skull, with the right tympanoperiotic complex encircled in white. The left earbone has been removed. (B) The cranial-lateral view of an intact, right-sided tympanoperiotic complex removed from the skull. (C) Caudal view of the periotic dome, broken off from the rest of the tympanoperiotic complex. The dashed black lines represent the planes of rough cross-section, which is 3–5 mm thick.

Estimating the age of mammals via GLG counts can be challenging for at least two reasons. First, bones undergo resorption as animals age (Klevezal 1996). Resorption is a natural process of bone turnover that is common in mammals, especially old ones; it can be accelerated by dietary mineral imbalances and reduced calcium-processing efficiency (Hansard et al. 1954;

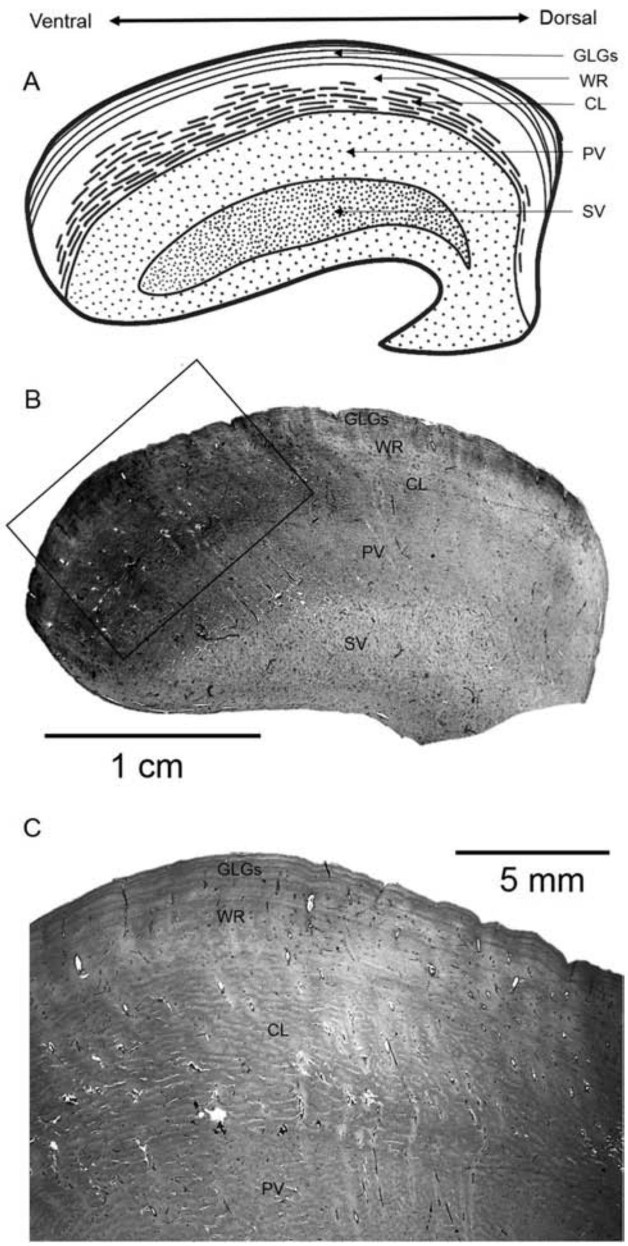

Fig. 2.—Histological bone structures visible upon cross-section of the Florida manatee (*Trichechus manatus latirostris*) periotic dome (earbone). (A) Abstracted diagram of the earbone cross-section, and (B) hematoxylin and eosin–stained earbone cross-section demonstrating structural layers. (C) A 10× magnified view of the structural layers from (B). GLGs = growth layer groups; WR = white rim; CL = compact lamellar bone; SV = secondary vascular bone; PV = primary vascular bone.

Maynard and Loosli 1969; Hancox 1972; Underwood 1981). Resorption can obscure or obliterate bone GLGs, making it difficult to estimate age (Fig. 3). Marmontel et al. (1996) found resorption in the earbones of manatees as young as 4–7 years old, and it started to obscure GLGs in manatees > 15 years old. Females had significantly higher levels of resorption than males, likely due to the additional nutritional demands of reproduction. Therefore, age estimation of older manatees, particularly females, is challenging, and often approximate age ranges must be assigned. Resorption, depending on the extent, can introduce age- or sex-specific error into earbone age estimates (Marmontel et al. 1996). Second, previous studies of age estimation have detected supplementary streaks called accessory lines in various animal tissues. Accessory lines are additional adhesion lines that are not truly annual (Klevezal and Kleinenberg 1969; Kasuya and Brownell 1979; Coy and Garshelis 1992; Stevenson and Campana 1992; Harshyne et al. 1998), and they can result in overestimations of GLGs in tissues. They can be difficult to differentiate from truly annual GLGs and require the analysis of known-age specimens.

The Florida Fish and Wildlife Conservation Commission's (FWC) Marine Mammal Pathobiology Laboratory (St. Petersburg, Florida) curates more than 8,700 earbones collected from dead manatees since the 1980s, building on the initial work of Marmontel and colleagues. Up until now, the FWC has used a one-reader age-estimation protocol to evaluate manatee earbones according to Marmontel et al. (1996). In long-term programs assigning ages to specimens, it is essential to revalidate age-estimation methods to ensure quality control, and to assess accuracy, error, and bias, especially since these metrics may change over the course of the program (Campana 2001). Establishing repeatable, standardized protocols allows for consistency in the age estimation of samples throughout the lifetime of a research program. For statistical purposes, it is also crucial to evaluate within- and between-reader precision, particularly because age estimation can often have a subjective component (Campana 2001).

The goals of this study were to: 1) develop standardized protocols for estimating ages of manatee earbones with a new, two-reader system; 2) assess the accuracy, precision, error, and bias (see definitions in "Materials and Methods") of these age-estimation protocols by comparing estimates to ages of known-age or minimum-known-age manatees; and 3) evaluate the results of age estimation within the context of future manatee research.

Materials and Methods

Specimens.—Two groups of manatees with known ages or sighting histories were selected for age estimation: the known-as-calf (KAC) group, which consisted of manatees that were first identified as calves and therefore of approximate known age, and the minimum-known-age (MKA) group, which consisted of free-ranging manatees with sighting histories of variable durations. Data from 1980 to 2015 were compiled from manatee rescues, research captures, live photo-identification sightings, and carcass recoveries conducted by the FWC, US Geological Survey (USGS), US Fish and Wildlife Service, and Mote Marine Laboratory.

For the KAC analysis, we selected manatees that had been: 1) originally sighted as dependent calves with unique features (e.g., scars) that could be re-identified through photo-identification over time; 2) rescued as dependent or orphaned calves and injected with passive integrated transponder tags upon release that allowed for re-identification upon death;

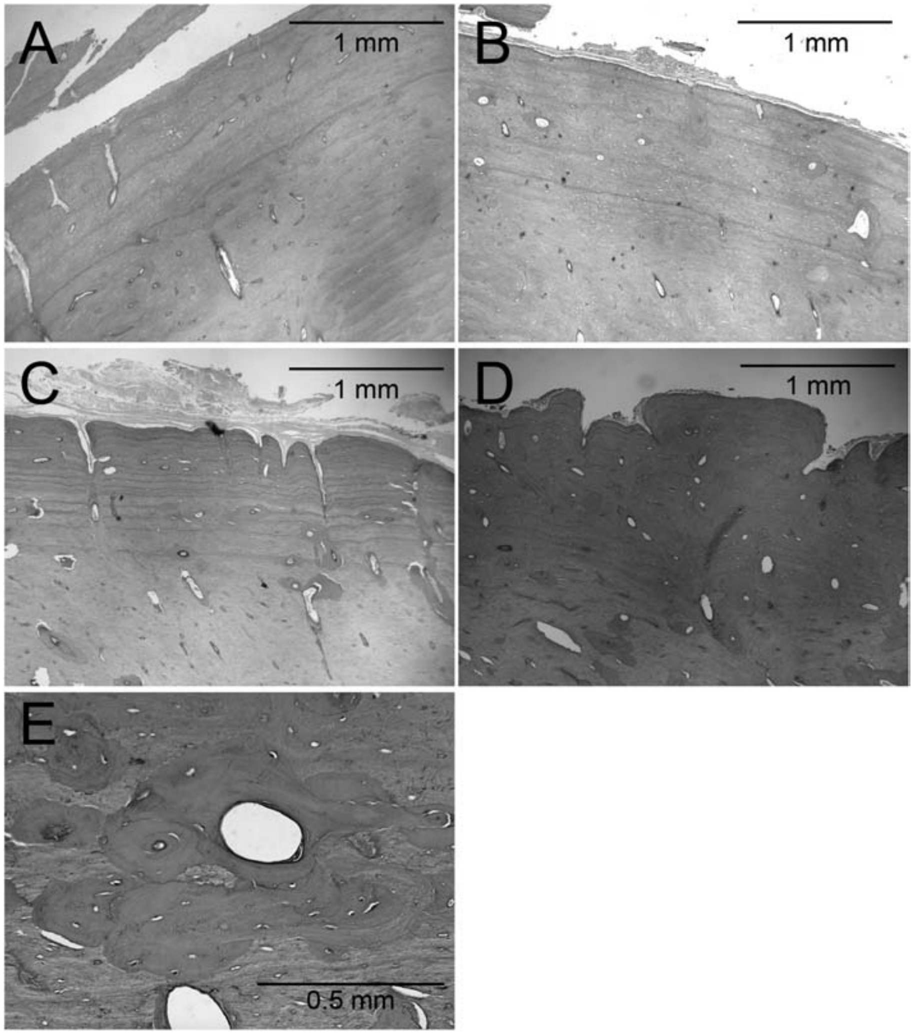

Fig. 3.—Progressive stages of resorption in the Florida manatee (*Trichechus manatus latirostris*) earbone: (A) none, (B) light, (C) moderate, and (D) heavy, and (E) a magnified view of resorbed earbone tissue. Slides were stained with hematoxylin and eosin.

3) captured as dependent calves during routine research and health assessments and injected with passive integrative transponder tags upon release; or 4) born in managed care. Of these cases, 111 earbones were in the FWC's collection. These 111 earbones were processed and ages were estimated according to the methods described below.

A manatee was considered a calf if it was still dependent on its mother or had a straight-line total length (hereafter referred to as "total length") of < 236 cm when first observed. A calf's dependency period typically extends over one or two winters (Hartman 1979; Rathbun et al. 1995), although manatee calves have been documented with their mothers for as long as 3 years (B. Bonde and C. Beck, USGS, Gainesville, Florida, pers. comm., February 2018). Regardless, the photo-identification sighting-history duration (hereafter referred to as "sighting duration") is likely shorter than the true lifetime of the manatee. Thus, each KAC animal was assigned to one of three initial age class categories: 1) perinatal (newborn manatees < 150 cm in total length); 2) born in managed care (manatees whose exact birth dates were known); or 3) calf (nonperinatal manatees < 236 cm in total length) (Table 1). If manatees were known only through photo-identification and no total length measurements were available, they were included in the calf category. These categories were used to refine each individual's true age range before comparison with age estimates (Table 1). We expected accurate age estimates to fall within these ranges.

For the MKA analysis, photo-identified manatees were categorized based on sighting duration into 5-year bins. A staff member not involved with age estimation randomly selected 8–12 manatees (from which an earbone had been collected) from each of the following seven age bins: < 5 (n = 11), > 5–10 (n = 10), > 10–15 (n = 9), > 15–20 (n = 12), > 20–25 (n = 8), > 25–30 (n = 10), and > 30 years (n = 9). Earbones from 69 MKA manatees were processed and their ages were estimated similarly to those from KAC manatees (see below). We did not adjust any of the sighting durations because the majority (n = 66) of specimens were known only through photo-identification and lacked a total length measurement at initial sighting (Table 2). Therefore, the sighting duration represents an absolute minimum for the manatee's true age. We expected that the MKA earbone age estimates would be at least the length of the sighting duration for each animal.

Table 1.—Criteria used to calculate true age ranges for the three initial age class categories in the known-as-calf (KAC) group of Florida manatees (*Trichechus manatus latirostris*). True age ranges were assigned based on the manatee's total length (in cm) at the time of initial sighting. If manatees were known only through photo-identification and did not have a total length at initial sighting, they were placed into the calf (nonperinatal) category.

Initial age class category	*n*	Range of initial total lengths of specimens	Basis for minimum true age	Basis for maximum true age	Justification
Perinatal	14	110–145 cm	Sighting duration	Sighting duration + 3 weeks	Schwarz (2008).
Born in managed care	6	102–160 cm	Known age	Known age	Birth date is known.
Calf (nonperinatal)	91	151–228 cm	Sighting duration + 2 weeks	Sighting duration + 2.5 years	Minimum age accounts for uncertainty in perinatal maximum; maximum age accounts for uncertainty in length of calf stage.

Table 2.—Known-as-calf (KAC) and minimum-known-age (MKA) specimen summary information, including sexes, minimum and maximum total lengths (TL) at death, minimum and maximum amounts of time known alive, and methods used for identifying individual Florida manatees (*Trichechus manatus latirostris*).

Study	*n*	# males	# females	Min TL	Max TL	Min known time	Max known time	Identification methods			
								Photo-ID	Rescued	Wild health assessments	Born in managed care
KAC	111	55	56	110 cm	361 cm	A few hours	35.2 years	33	65	6	7
MKA	69	34	35	274 cm	387 cm	3.4 months	36.6 years	66	2	1	0
Combined	180	89	91	110 cm	387 cm	A few hours	36.6 years	99	67	7	7

Earbone processing.—All earbones were collected from manatee carcasses during standard necropsies. Earbones collected before 2010 were processed according to Marmontel et al. (1996). Earbones collected during and after 2010 were processed with small adaptations, although such small changes through the years have been shown to not significantly affect the precision of age estimation of earbones (Brill et al. 2016). Specifically, periotic domes were separated from the rest of the tympanoperiotic complex (Fig. 1) and stored dry until a 3- to 5-mm rough section was cut from the middle of the dome (Fig. 1C) using a Rock's 6″ Gem Trim high-speed table saw (Polaris Tool & Machine, Wellington, Ohio) with a Dayton 3M292D capacitor motor (Dayton Manufacturing Company, Dayton, Ohio) and an MK303 diamond-edge lapidary blade (MK Diamond Products Inc., Torrance, California). Cutting usually occurred within 2 weeks to 2 months of collecting the earbone. The rough section and the remaining portions of the earbone were fixed in 10% neutral buffered formalin (Thermo Fisher Scientific Inc., Waltham, Massachusetts) for at least 2 weeks. After fixation, the remaining portions were archived in vacuum-sealed plastic bags for long-term storage, while the rough section was kept in formalin until the decalcification process (sometimes up to several years). Before decalcification, the rough sections were rinsed with or soaked in tap water for approximately 1 h. RDO, a rapid decalcifier (Darlco Products, Inc., Oradell, New Jersey), was used to decalcify rough sections for 24–72 h or until the section became soft, slightly translucent, and flexible. The time required for decalcification increased with the thickness of the section and how long it had been stored in formalin. Decalcified rough sections were again rinsed with water for approximately 1 h before returning them to formalin. Within approximately 1 week, the decalcified rough sections were embedded in paraffin with a Tissue-Tek VIP6 tissue processor (Sakura Finetek USA, Inc., Torrance, California) and then thin-sectioned at 4 µm on a rotary microtome (HM325, Thermo Fisher Scientific or RM2235, Leica Biosystems, Buffalo Grove, Illinois). These thin sections were mounted on glass microscope slides. A Mayer's hematoxylin, Richard-Allan hematoxylin, or hematoxylin and eosin stain was used to enhance the contrast of adhesion lines in the thin section.

Age estimation of earbones.—Two readers (Reader1 and Reader2) were trained in-house in the methods developed by Marmontel et al. (1996) and had multiple years of experience estimating ages of manatee earbones at the time of this study. For each specimen, the two readers each conducted two independent readings in the blind (i.e., without knowledge of the previous reading), with at least 24 h between readings to limit the possibility of remembering identification numbers and ages. Each age reading included a best, minimum, and maximum age estimate, which accounted for any double, bifurcating, vanishing, faint, or resorbed lines. In addition, a level of bone resorption (none, light, moderate, or heavy; Fig. 3) was assigned, and the reader's confidence in the estimate was graded on a four-point scale (see Supplementary Data SD1). A third person not involved with age estimation checked for agreement between Reader1's two ages; this process was repeated for Reader2's two ages. If one of the best ages fell outside the minimum–maximum range of the other age reading, then the respective reader performed a third independent reading in the blind. Ages were then compared between Reader1 and Reader2. If any of Reader1's best ages fell outside the minimum–maximum range of Reader2's age estimates (and vice versa), then a collaborative reading was performed. During collaborative age estimation, both readers looked at the earbone simultaneously while considering all previous age readings, photographs, and notes; as with independent readings, this process included a best, minimum, and maximum age estimate, as well as resorption level and grade. In the MKA analysis, if an individual reader performed three age readings and two of the bests fell within each other's minimum–maximum range, then the third age reading that did not agree was ignored during the comparison between Reader1 and Reader2's readings. Known-age data were not compared to age estimates until all age readings, including collaborative readings, had been completed; thus, the animals' histories were unknown to the readers during age estimation.

During each independent age reading, the first step was to look for the presence of the earbone's WR (see Marmontel et al. 1996). The presence of the WR was first investigated at low magnification using either a loupe (8×, Agfa Lupe, Germany) and a light box (Tru-View Logan Electric, Smith-Victor Corporation, Bartlett, Illinois) or a compound microscope at 40× (either Olympus BH-2 or Olympus CK-2, Olympus Corporation, Tokyo, Japan). If the WR was not visible and the edge of the earbone did not appear consolidated (i.e., the bone appeared porous) under 40× magnification (e.g., Figs. 4A and 4B), then the age of the manatee was estimated as 0 (less than 1 year old). If the transition from CL bone to WR was visible, the slide was then examined at 40×, 100×, and 200× under the compound microscope to count GLGs.

The entire edge of the earbone was examined, and lines were counted where contrast was the best, with emphasis on estimating age at the middle of the earbone cross-section (as recommended by Marmontel et al. 1996). Due to limited knowledge about the rate of GLG development in manatee earbones, fractions of a year could not be determined when estimating age. If the edge of the earbone had a band of growth but no additional adhesion line, then the age was estimated based on the last visible adhesion line. Thus, age estimates,

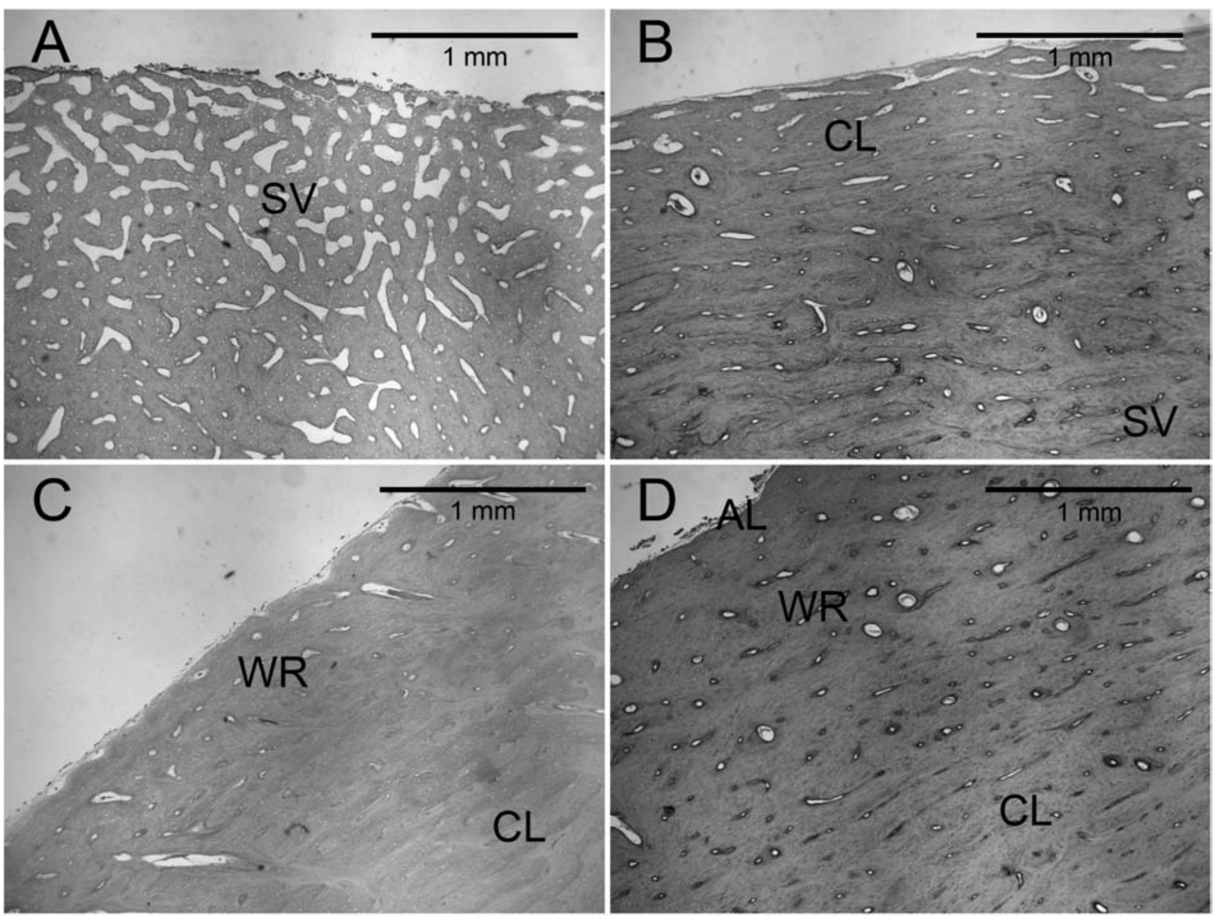

Fig. 4.—Stages of edge development in earbones from Florida manatees (*Trichechus manatus latirostris*) from perinatal to 2 years of age. (A) Earbone from a 110-cm manatee that died during rescue; lacks compact lamellar bone layer and has a porous, nonconsolidated edge = 0 years (perinatal). (B) Earbone from a 167-cm manatee that was rescued and rehabilitated for 2 months before its death; has compact lamellar layer along edge, lacks a white rim, and has a porous, partially consolidated edge = < 1 year. (C) Earbone from a 200-cm manatee that died 4 days after rescue; has compact lamellar layer and thin white rim; the edge is slightly porous and almost fully consolidated, but no adhesion lines are present = 1 year. (D) Earbone from a manatee that was rescued, rehabilitated, and released but found dead 2 years after its rescue date at 255 cm; has a somewhat subtle transition between compact lamellar and white rim layers; the edge is consolidated, and there is one dark adhesion line present = 2 years. All slides were stained with hematoxylin or hematoxylin and eosin. SV = secondary vascular bone; CL = compact lamellar bone; WR = white rim; AL = adhesion line.

true age ranges, and sighting durations were rounded down to the nearest integer for subsequent analyses. Photographs were taken of the earbone cross-section with a SPOT Insight QE camera fitted to the microscope and SPOT 5.0 Advanced imaging software (Diagnostic Instruments Inc., Sterling Heights, Michigan) when possible. These images were later annotated using Windows 7 Professional Paint (version 6.1) to illustrate the best age estimate for that age reading. This practice aided age estimation during collaborative readings.

Statistics.—For all analyses, we used R (version 3.4.1—R Core Team 2017). Within- and between-reader precision of the best estimates were calculated using the coefficient of variation (*CV*—Campana 2001). Precision is a measure of the repeatability of obtaining the best age estimate; lower *CV* percentages represent greater precision.

Accuracy was defined as the percentage of cases in which the given statistic was within the true age range of KAC specimens or was at least the sighting duration for MKA specimens. The six statistics used to calculate accuracy were: 1) the median of all age readings; 2) the mode of all age readings; 3) the mean of all age readings; 4) the collaborative age (when one was performed), otherwise the median of all independent age readings; 5) the collaborative age, otherwise the mode of all independent age readings; and 6) the collaborative age, otherwise the mean of all independent age readings. Based on these analyses, the median of all age estimates (#1) was used for subsequent analyses (see "Results").

Three metrics of error were calculated for the KAC group: mean raw error, mean absolute error, and error range. First, mean raw error is a measure of bias (in our case, under- or overestimating a manatee's age). For the KAC group, mean raw error represented the difference between the median age estimate and the midpoint of the true age range. In the presence of uncertainty about the true age, mean raw error using the midpoint can be biased if the true ages are not evenly distributed within the true age ranges (see Supplementary Data SD2). Second, mean absolute error is another measure of accuracy or fit and is a common alternative to mean squared error (Williams et al. 2002), although the use of the midpoint may result in overestimates of mean absolute error (see Supplementary Data SD2). Mean absolute error was calculated for the KAC group as an average of the absolute value of the median age estimate's raw error. Third, an error range was calculated for each KAC specimen as the difference between the median age estimate and the nearer and farther bounds of the true age range (see Table 1). If the median age estimate was within the true age range, then the minimum error was 0, and the maximum error was the difference from the farther bound. If the median age estimate was equidistant from both bounds of the range, then the lower bound was used to calculate maximum error and avoid biasing the error low. KAC error ranges were calculated using both raw error and absolute error (absolute value of raw error) and then averaged across the data set to produce a mean raw error range and a mean absolute error range.

Only mean raw error was calculated for the MKA group; error could result only from underestimations, because there was no upper bound to the known-age information. MKA mean raw error represented the difference between the median age estimate and the sighting duration. If the median age estimate was equal to or greater than the sighting duration, then error was set to 0. Finally, we compared accuracy and raw error versus the midpoint of the true age range for KAC manatees and versus sighting duration for MKA manatees, in the context of resorption level. LOESS smoother (nonparametric fit) functions were used to fit the nonlinear relationships (Cleveland 2012).

Rankings of resorption (from none to heavy) sometimes varied between age readings and between readers, likely due to the subjective nature of estimating resorption level. For analyses, the modal resorption category of all age readings was used, and ties were settled by selecting the lower (less resorbed) category, to be conservative. We evaluated resorption level in relation to age by performing a chi-square test on younger KAC manatees (minimum true age ≤ 10 years old) versus older KAC (minimum true age > 15 years old) and MKA (sighting duration > 15 years) manatees. These categories were defined based on declines in the accuracy of age estimation at approximately 15 years of age (see "Results"). MKA manatees with sighting durations ≤ 15 years were excluded because manatees could be much older than their sighting duration. We omitted KAC manatees that were 11–15 years old due to uncertainty in known age. Furthermore, since Marmontel et al. (1996) found differences in the levels of resorption between male and female manatees, a chi-square test was performed to determine whether resorption levels were dependent on sex (all ages pooled). Finally, we plotted carcass total length against the median age estimate to obtain an approximate growth curve for males and females separately based on median age estimates. A LOESS smoother function was used to fit the nonlinear relationship between total length and the median age estimate for each sex (Cleveland 2012).

Results

Specimen data.—The 180 earbones analyzed across the two groups included 89 males and 91 females ranging in total length at death from 110 to 387 cm (Table 2). These individuals were known for periods of time ranging from a few hours (e.g., calves that died during rescue) to 37 years (Table 2).

Microscopic earbone characteristics.—The transition from CL bone to the WR was easier to distinguish in younger animals (typically less than 3 years). Earbones from manatees that died less than 1 year after birth were characterized by either porous secondary vascular bone (Fig. 4A) or horizontally organized CL bone at the edge of the earbone section (Fig. 4B). Earbones from calves that were approximately 1 year old exhibited a subtle transition from CL bone to WR as the horizontal vascular canals gradually (or sometimes abruptly) changed into circular, less organized vascular canals, moving outward from the center of the cross-section (Fig. 4C). An animal was not estimated as 2 years old unless it possessed a dark adhesion line at the very edge of the WR (Fig. 4D). The CL–WR transition became more difficult to locate in older animals.

Even so, if GLGs with multiple adhesion lines were present, the location of the transition was approximated based on the location of the first adhesion line and was still counted as the first year of growth. The transition was often best observed at the dorsal and ventral aspects of the cross-section (see Fig. 2).

In earbones from older manatees, adhesion lines tended to bifurcate, merge, disappear, or vary in darkness of stain, requiring readers to evaluate GLGs at multiple locations along the edge of the earbone. However, earbones from some younger manatees also had these confounding adhesion lines, which may have been accessory lines. In these cases, if the line was prominent along the majority of the edge of the earbone cross-section, then it was counted as a GLG. Where resorption obscured lines, the minimum–maximum range was adjusted to reflect any uncertainty.

Light, moderate, or heavy resorption was observed in 85 of the 180 earbones (Fig. 3). The smallest manatee with an earbone that exhibited resorption was a 277-cm-long female (MNW0832) that was first seen as a dependent calf in January 2005 and was found dead in June 2008. Except for MNE1127, which was likely a mislabeled slide (see below), the largest manatee with an earbone that did not exhibit resorption was a 327-cm-long female (MSE1157) that was first seen in 2001 as an adult and was recovered dead in 2011. Manatees with minimum true ages (KAC) or sighting durations (MKA) > 15 years had significantly higher levels of resorption than manatees with known minimum ages (KAC) ≤ 10 years ($\chi^2 = 117.33$, $df = 3$, $P < 0.0001$; Table 3). Specifically, 97% of young specimens (≤ 10 years old) had no or light resorption, while 83% of old specimens (> 15 years old) had moderate or heavy resorption (Table 3). Qualitatively, there was little sex-related difference in resorption within the younger age group, but there was a possible sex-related difference within the older group. Twice as many older females (70%) showed heavy resorption compared to older males (29%) (Table 3). The chi-square test comparing sex and resorption level (all ages pooled) failed to demonstrate that resorption was dependent on sex ($\chi^2 = 4.54$, $df = 3$, $P = 0.21$). Therefore, when evaluating accuracy and error, we separated comparisons among resorption levels from comparisons between sexes.

Age estimates.—The best age estimates from age readings ranged from 0 to 26 years in the KAC group and from 1 to 52 years in the MKA group. The age of one manatee in the MKA group (MNE1127), which was known for nearly 24.5 years, was underestimated by 23 years based on the median of all age estimates. Its earbone lacked resorption, while the manatee's total length at death was 351 cm. It is likely that this slide belonged to a younger manatee that was not included in this study and was mislabeled as MNE1127. Therefore, we omitted this case from all analyses except calculations of precision.

Precision.—For the KAC group, Reader1 and Reader2 had within-reader *CV*s of 2.4% and 7.0%, respectively, and a between-reader *CV* of 13.1%. For the MKA group, they had within-reader *CV*s of 5.9% and 8.5%, respectively, and a between-reader *CV* of 13.3%. Collaborative age estimates were required for 37 (33%) and 51 (74%) of the specimens in the KAC and MKA groups, respectively.

Accuracy.—The median and mode of all best estimates per specimen as well as the collaborative age (if performed, otherwise the median or mode) demonstrated the greatest accuracy (all 63.1%; Table 4) in the KAC group. Using collaborative ages (if performed, otherwise the median, mode, or mean) demonstrated the greatest accuracy (all 76.5%; Table 4) in the MKA group, followed closely by using the median of all best estimates per specimen (75.0%; Table 4). Based on these results and the FWC's desire to phase out collaborative age estimation due to increased workload, we decided to use the median of all best estimates for the remainder of the age analyses for both groups.

Median age estimates for both groups ranged from 0 to 48 years. The percentage of median age estimates that were within the true age range (KAC) or at least the sighting duration (MKA) decreased with increasing true age range midpoint or sighting duration, respectively (Fig. 5A). These percentages also generally decreased with increasing levels of resorption (Fig. 5B). Median age estimates for KAC males and females were within the true age range 53.0% and 73.2% of the time, respectively. Median age estimates for MKA males and females were at least the sighting duration 73.5% and 76.5% of the time, respectively.

Error.—The mean raw error (± *SD*) for the median age estimates of KAC specimens was indistinguishable from 0

Table 3.—Number of Florida manatees (*Trichechus manatus latirostris*) showing different levels of earbone resorption, separated by age group and sex. The young group included manatees with minimum true ages ≤ 10 years for known-as-calf (KAC) specimens only, and the old group included manatees with minimum true ages or sighting durations > 15 years for KAC or minimum-known-age (MKA) specimens, respectively.

Age category	Sex	Resorption level				Total
		None	Light	Moderate	Heavy	
Young (age ≤ 10 years)	Male	44	1	3	0	48
	Female	47	3	0	0	50
	Total	91	4	3	0	98
Old (age > 15 years)	Male	1	5	11	7	24
	Female	0	2	5	16	23
	Total	1	7	16	23	47

Table 4.—Six analyses of the accuracy of age estimation of Florida manatees (*Trichechus manatus latirostris*). Accuracy was defined as the percentage of cases where the given statistic was within the true age range of known-as-calf (KAC) specimens ($n = 111$) and at least the sighting duration of minimum-known-age (MKA) specimens ($n = 69$). Based on these results, the median of all age readings was used for subsequent analyses.

Statistic	% within KAC range	% at least MKA sighting duration
Median	63.1%	75.0%
Mode	63.1%	73.5%
Mean	58.6%	73.5%
Collaborative age, otherwise median	63.1%	76.5%
Collaborative age, otherwise mode	63.1%	76.5%
Collaborative age, otherwise mean	59.5%	76.5%

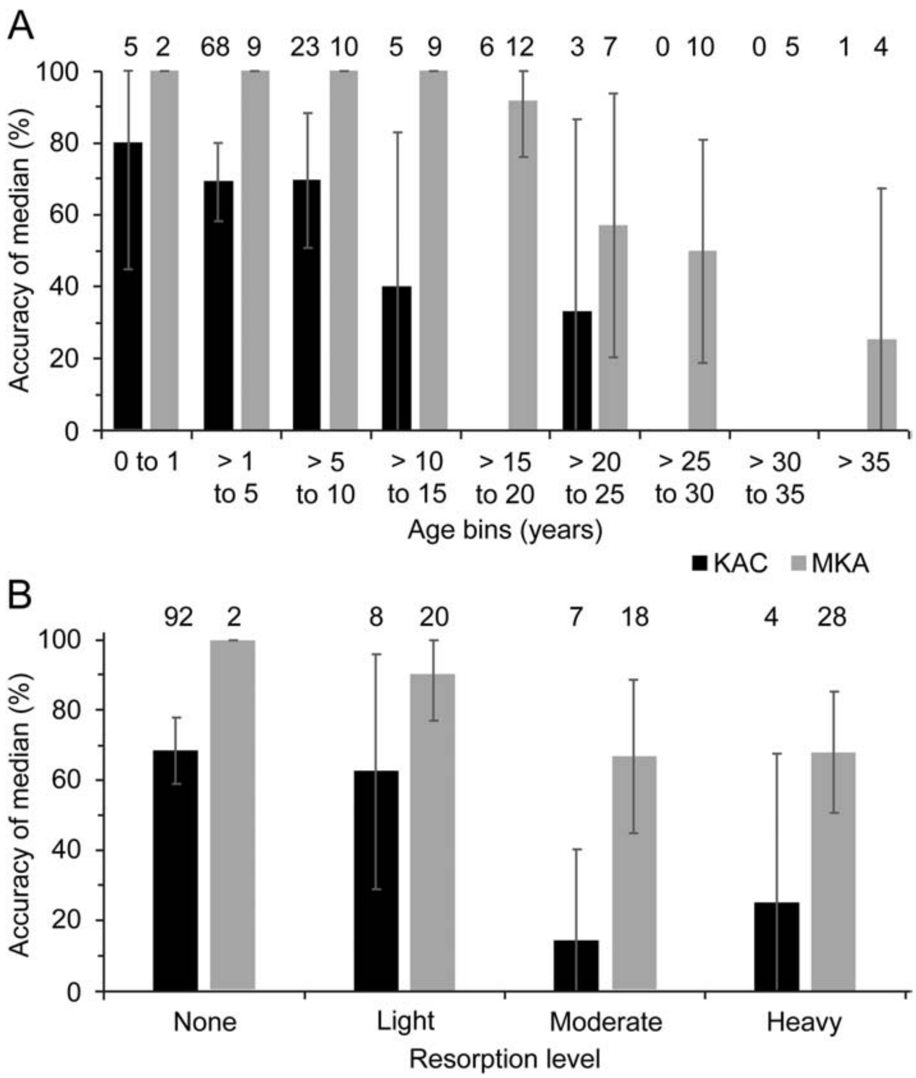

Fig. 5.—Accuracy (% of cases) of the median age estimate for known-as-calf (KAC, black) and minimum-known-age (MKA, gray) Florida manatees (*Trichechus manatus latirostris*) grouped by (A) known-age bin, and (B) earbone resorption level. Accuracy is defined as the percentage of median age estimates that fell within the KAC true age ranges or were at least the MKA sighting durations. Age bins were assigned based on the midpoint of the true age range for KAC manatees or the sighting duration for MKA manatees. Error bars represent 95% binominal confidence intervals, which were based on a normal approximation of the error distribution and truncated to 0 or 100. Numbers above the bars represent the number of specimens in that group.

(midpoint of −0.05 ± 3.05 years), indicating a lack of bias for the data set as a whole (Table 5). The mean absolute error of KAC specimens was 1.75 ± 2.50 years (Table 5). If only inaccurate KAC cases were considered ($n = 41$), the mean absolute error was 3.65 ± 3.31 years (Table 5). The largest underestimation of KAC age was 16–18 years, and the largest overestimation was 7–9 years; these paired numbers account for the error calculated from the nearer and farther bounds of these specimens' true age ranges. The mean raw error for MKA specimens was −1.65 ± 5.20 years (Table 5). When considering only the MKA manatees whose ages had been underestimated ($n = 17$), the mean raw error was −6.62 ± 3.67 years (Table 5). The largest MKA underestimation was at least 13 years (MEC13157). For both groups, raw error became increasingly negative (i.e., age was further underestimated) with increasing levels of resorption or with increasing minimum true age, especially over 15–20 years (Fig. 6).

Total length at death increased with the median age estimate (Fig. 7). The rate of growth slowed considerably at about age 10 for both sexes, based on the LOESS smoother function. After approximately 15 years of age, females continued to grow slightly more than males and attained greater total lengths than males at a given estimated age (Fig. 7).

Discussion

This study comprehensively assessed the accuracy, precision, and error in age estimation of manatee earbones using two readers. Detecting and accurately counting GLGs in earbones is challenging and somewhat subjective due to variations and

Table 5.—Mean raw error, mean absolute error, and error range (in years, ± *SD*) of median age estimates for the known-as-calf (KAC) group, and mean raw error (in years, ± *SD*) of median age estimates for the minimum-known-age (MKA) group of Florida manatees (*Trichechus manatus latirostris*). Values were averaged for the number of cases indicated (*n*). For the KAC group, mean raw error represented the difference between the median age estimate and the midpoint of the true age range. For the MKA group, mean raw error represented the difference between the median age estimate and the sighting duration. Mean absolute error was calculated for the KAC group as an average of the absolute value of the median age estimate's raw error. The mean error ranges represented error calculated from the nearer and farther bounds of each KAC true age range (either using raw or absolute values of error). There were no upper bounds to the MKA group's known-age data, so only underestimated error was possible.

KAC	*n*	Mean raw error from true age midpoint (years)	Mean absolute error from true age midpoint (years)	Mean raw error from true age bounds (years)	Mean absolute error from true age bounds (years)
All cases	111	−0.05 ± 3.05	1.75 ± 2.50	−0.18 to 0.19	1.05 to 2.66
Males	55	−0.08 ± 2.94	1.75 ± 2.35	−0.18 to 0.16	1.16 to 2.60
Females	56	−0.01 ± 3.19	1.74 ± 2.66	−0.17 to 0.22	0.94 to 2.72
Inaccurate cases only	41	−0.23 ± 4.95	3.65 ± 3.31	−0.48 to 0.01	2.84 to 4.45
MKA	*n*	Mean raw error (years)			
All cases	68[a]	−1.65 ± 5.20			
Males	34	−1.84 ± 3.87			
Females	34[a]	−1.47 ± 2.90			
Inaccurate cases only	17	−6.62 ± 3.67			

[a] One female MKA specimen was omitted from the analysis (MNE1127, see "Results" and "Discussion").

occasional anomalies in the bone tissue as well as the resorption that often occurs with increasing age. Our analysis shows that age estimation is relatively unbiased until about 15 years of age, after which GLG counts typically underestimate true age. This finding agrees with the results of Marmontel et al. (1996). The plot of total length at death versus the median age estimate demonstrates that manatee growth may slow after approximately 10 years of age. If earbone GLG deposition rates slow in tandem with somatic growth, it may explain why the ages of manatees > 15 years old are often underestimated. The utility of earbone ages for manatee population analyses will depend on the level of precision required and whether accurate age estimation of older animals is important.

The KAC results revealed that we accurately estimated the age of approximately 63% of manatee earbones. This may seem low, but we were also conservative with defining accuracy. For example, an age estimate that was only 1 year off from the true age was classified as inaccurate for KAC specimens that were either captive-born or perinatal at initial sighting ($n = 20$). The mean absolute error of KAC specimens was only 1.75 years, despite the greater errors associated with estimating ages of older animals. This value of error may even be overestimated (see Supplementary Data SD2), but it is difficult to determine, given that we do not know the exact ages for most KAC specimens. There was more precision within readers than between readers. This is likely why there were a high number of collaborative age readings required for the KAC and MKA groups (49% of all specimens). Campana (2001) reported that the median between-reader *CV* of a subset of 117 studies of age estimation in fish was 7.6%, although *CV* values ranged up to 28% (see figure 5 in Campana 2001). In the present study, the between-reader *CV* was approximately 13%. Based on the *CV* benchmark provided by Campana (2001), we believe that our age-estimation methods are reasonably repeatable and reliable.

Accuracy of age estimation was inversely correlated and absolute error was positively correlated with age. Similarly, age estimation became less reliable with increasing resorption level, which is correlated with age. The MKA group, which included more older animals, showed trends that were similar to the KAC group. These findings are logical, given that resorption progressively obscures GLGs. Also, GLGs compress, split, and merge more frequently in older manatees, making age estimation from these earbones more difficult. The general pattern of underestimating age of older animals has been reported in other studies of mammalian age estimation (Gilbert and Stolt 1970; Coy and Garshelis 1992; Calvert and Ramsay 1998; Childerhouse et al. 2004). Even so, ages of some older manatees (e.g., a 23-year-old KAC manatee) were estimated accurately. While we tried to be conservative with our analyses of accuracy and error, the paucity of truly known-age manatees (i.e., those with known birth dates) makes it difficult to distinguish whether an age estimate is truly accurate or simply within reason based on general sighting information.

Resorption level increased with increasing age. As mammals age, their calcium-processing efficiency declines (Hansard et al. 1954) and their bones become increasingly resorbed to allow vascularization and ion exchange in the outer layers of bone (Morris 1972; Klevezal 1996). Furthermore, chronic dietary mineral and vitamin imbalances can increase rates of bone resorption (Maynard and Loosli 1969; Hancox 1972; Underwood 1981). In contrast to Marmontel et al. (1996), we did not detect a significant difference in resorption levels between males and females, which could not be stratified by age due to limited sample sizes. However, the raw data suggest that the highest levels of resorption occur in older females. Females undergo additional calcium demands during pregnancy and lactation, which are likely to increase resorption in the bones of mature female manatees (Atkinson and West 1970; Rasmussen 1977; Pitkin et al. 1979; Kwiecinski et al. 1987).

Besides increasing levels of resorption with age, there are other potential sources of error when estimating ages of earbones. First, processing error can be introduced during the cutting, decalcifying, embedding, and staining processes and can result in samples that are poorly stained, fragmented, or

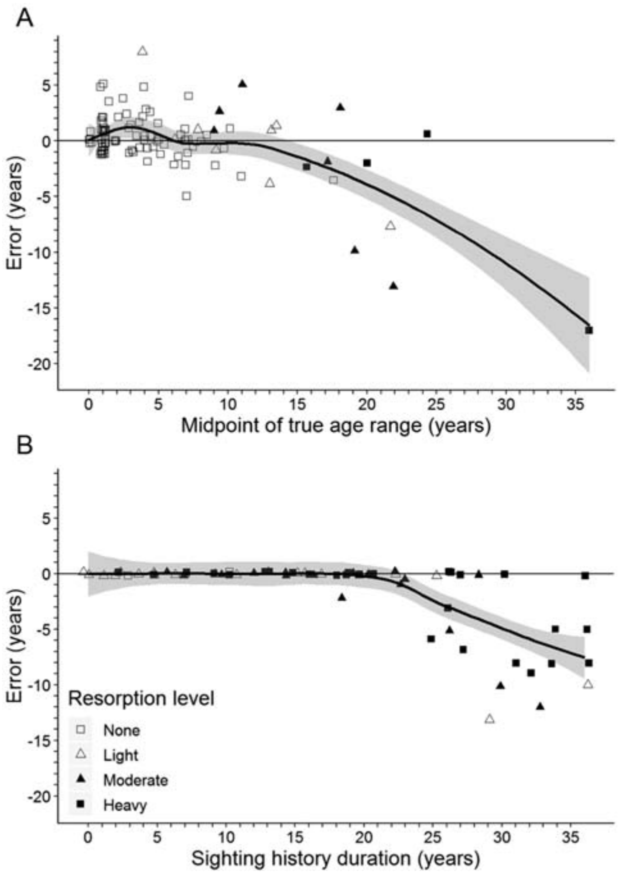

Fig. 6.—Raw error of the median of earbone age estimates for Florida manatees (*Trichechus manatus latirostris*) versus (A) midpoint of the true age range for the known-as-calf (KAC) group, and (B) sighting duration for the minimum-known-age (MKA) group. For the KAC group, mean raw error represented the difference between the median age estimate and the midpoint of the true age range. For the MKA group, mean raw error represented the difference between the median age estimate and the sighting duration. There are no upper bounds on MKA known ages, so only underestimates of age are possible. Data points are differentiated by shapes and shading corresponding to levels of resorption. The gray area surrounding the LOESS smoother line (Cleveland 2012) represents the 95% confidence interval. The horizontal black line represents 0 error, as a reference. A jitter function was used for both graphs to avoid stacking multiple data points.

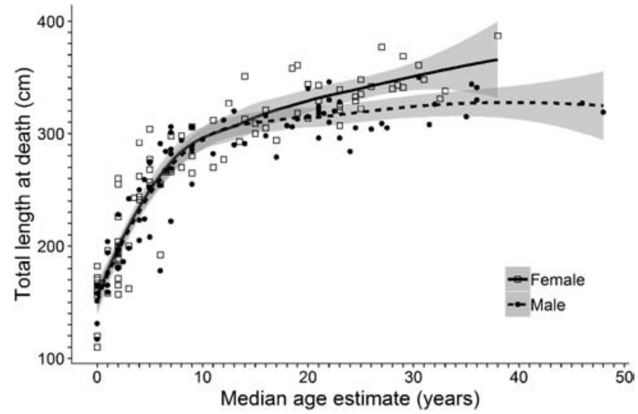

Fig. 7.—Total length (cm) at death versus the median age estimate (years) for female (open squares, solid line) and male (closed circles, dashed line) Florida manatees (*Trichechus manatus latirostris*). The gray area surrounding the LOESS smoother lines (Cleveland 2012) represents the 95% confidence interval. For cases where the head or fluke was decomposed, scavenged, or missing, total length was estimated. Cases were removed (n = 6) from this analysis if the difference between the estimated total length and total length of actual remains was greater than 5 cm, or if this difference was unknown.

mislabeled. Strict labeling protocols should reduce processing error to avoid sample mix-up like the case of MNE1127. If processing error is sufficiently large, mistakes can often be detected with proper checks for logical consistency, as was done with MNE1127. Some of the outliers in Fig. 7 may be the result of processing error, although closer examination of these specimens' histories in relation to photographs of their earbone slides was not convincing enough to exclude them from the study. Second, error in age estimation can occur if GLG patterns are misinterpreted. Error in age estimation can hopefully improve with additional studies like this one and with proper training of earbone readers. Third, errors associated with variable biological processes can occur (reviewed in Read et al. 2018). They can be hard to identify and are likely the most common form of inaccuracy in these analyses. For example, the deposition of additional, nonannual accessory lines could confound earbone age estimates and may have been responsible for some of the overestimates seen in Fig. 7. Accessory lines have been observed in the tissues of other animals, such as fish otoliths during metamorphosis and settling (Stevenson and Campana 1992) and black bear (*Ursus americanus*) teeth during reproductive events (Coy and Garshelis 1992; Harshyne et al. 1998). More research needs to be done to determine possible sources of biological variations in manatee earbones, such as how individual growth rates affect earbone growth and how environmental factors affect GLG deposition.

Growth layers in animal tissues have been correlated with life-history events in some mammals. For example, trace elements and hormones extracted from sequential tissue growth layers have been used to reconstruct diet, stress levels, reproductive events, chemical exposure, and health dynamics over an animal's lifetime (Evans et al. 1995; Edmonds et al. 1997; Ando-Mizobata et al. 2006; Trumble et al. 2013). In other cases, specific line and band patterns have been associated with the attainment of sexual maturity (Harwood and Prime 1978; Klevezal and Stewart 1994; Medill et al. 2010), pregnancy and nursing cycles (Scheffer and Peterson 1967; Klevezal and Myrick 1984; Coy and Garshelis 1992), and dietary shifts due to climatic anomalies (Robinette and Archer 1971; Manzanilla 1989). Marmontel (1995) investigated sexual maturity in manatees by looking for ovarian follicles or uterine scars upon necropsy and relating the findings to earbone ages. However, she did not remark on any earbone GLG patterns that coincided with pregnancy or nursing. This could be a future research endeavor involving manatee earbones.

Teeth GLGs have been used to assess climate–growth relationships in odontocetes (Dellabianca et al. 2011; Hamilton et al. 2017) and fur seals (Boyd and Roberts 1993; Hanson et al. 2009; Knox et al. 2014; Wittmann et al. 2016). Earbone GLGs of manatees, whose health is closely associated with seagrass habitat and coastal water temperature, may also be influenced by extrinsic variables. Some of the manatees included in the KAC group were born in managed care or spent some time in rehabilitation after rescue from the wild. If environmental stressors influence bone growth, then time in managed care may affect GLG formation in manatee earbones and make GLG calibrations using manatees raised in managed care challenging (reviewed in Read et al. 2018). Thus, it is difficult to analyze the effects of intrinsic and extrinsic variables on GLG deposition in manatee earbones, although we acknowledge that doing so would provide useful insight into interpreting GLG patterns more accurately.

Recommendations for age estimation.—As with any program assigning ages to specimens, processing and age-estimation methods must be standardized and routinely checked for quality control to ensure consistent, reliable data collection over time and across readers (Campana 2001). Revalidations should also be conducted when there are changes to any protocol or when new readers are introduced to the program. This may be accomplished by re-estimating ages of a standard training set of earbones or by establishing a set of known-age earbones that can be reprocessed with any new procedures.

In summary, we selected the median of all age estimates from readers as an accurate estimation of ages of manatee earbones. Using only collaborative ages (or else the median or mode) was slightly more accurate in the MKA group and had the same accuracy as the median in the KAC group. However, the FWC has decided to eliminate the collaborative age-estimation step that requires the two readers to meet at the microscope, because the added workload did not substantially improve accuracy. Since the accuracy of all statistics was relatively similar, we suggest that future studies also evaluate the median, mode, and mean to determine which is most appropriate for analyses.

Extracting, processing, storing, and estimating age of manatee earbones can be time-consuming and costly. We recommend that future studies using ages of manatees consider the target demographic (younger versus older manatees) and the level of accuracy and precision required for robust interpretations before investing in a program that estimates ages of earbones. A matrix population model derived from the Florida manatee Core Biological Model, which is an age- and sex-structured population viability analysis (Runge et al. 2017), suggests that approximately 68% of the manatee population and 79% of manatee carcasses are < 15 years old (see Supplementary Data SD3). Therefore, earbones could be used effectively for investigations of age at sexual maturation, growth dynamics in young manatees, and susceptibility of these younger cohorts to diseases and other threats (e.g., boat strike, cold stress, or red tide).

The Core Biological Model uses total length to estimate age class for certain analyses, with calves < 236 cm, subadults between 236 and 265 cm, and adults > 265 cm (Runge et al. 2017). Although total length can be obtained faster and more easily than earbone age, calculating age via growth curve analyses can be variable and uncertain (Schwarz and Runge 2009). Molecular and genetic techniques are increasingly being investigated for estimating ages of wild mammals, including those in the marine environment (Olsen et al. 2014; Polanowski et al. 2014; Jarman et al. 2015; Pal and Tyler 2016; Paoli-Iseppi et al. 2017; Read et al. 2018). An advantage of these techniques over GLG counts is that samples can be taken from both living and dead animals, to get a broader picture of the population. However, genetic ages do not necessarily correspond with chronological ages, estimates can be very imprecise, and these methods require a great deal of ground-truthing and calibration before they are effective at estimating age. Given that the FWC has a long-term genetics program, using genetic techniques for estimating age may be an avenue for manatee research in the future.

Age data provide useful information for understanding animal demographics, reproductive rates, and health. However, interpreting age data in the context of these research questions requires robust estimates of accuracy, precision, and error, which, in light of the present study, are now available for age-estimation methods using manatee earbones. Given our results, age data for manatees could be used most effectively to understand the biology of the younger portion of the population. More research should be done to understand the physiological processes that govern GLG deposition in general. This could help account for some of the error associated with age estimation of manatees, particularly older ones.

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Supplementary Data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—The four-point grading scale used to estimate a reader's confidence in his or her best, minimum, and maximum age estimates for each age reading.

Supplementary Data SD2.—Simulation of effects of using midpoint of range on error.

Supplementary Data SD3.—Proportions of manatee population by age.

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