



Aging-related histomorphometric changes in cortical bone tissue of bottlenose dolphins (*Tursiops truncatus*)

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Received: 10 January 2020 / Revised: 10 March 2020 / Accepted: 23 March 2020
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Abstract

Morphological characteristics of bones have shaped as a result of adaptation to life in the aquatic environment and whale diving habits. This study deals with the postnatal bone development and microscopic characteristics of bones of bottlenose dolphins (*Tursiops truncatus*). Bone sections of the right humerus and the fifth right rib of 106 dolphins aged less than 1–21 years were used to determine the density of the primary, secondary and remodelling osteons, the magnitude of the area occupied by the osteons, and the number of osteon lamellae. Bone sections were analysed using a compound microscope with standard and polarized light. Our study showed the primary osteons of bones to occupy a smaller area than the secondary ones. The secondary osteon area in the humerus is significantly larger than that in the rib. All rib osteons are significantly denser than those of the humerus. In both bones, the number of osteon lamellae is higher in secondary than in primary osteons. The number of secondary and remodelling osteons and the areas occupied by them increase with age, as opposed to primary osteons in which a decrease was noted. The number of lamellae in primary osteons decreases with age, while in secondary osteons their number increases. Within the study frame, morphometric osteon values of the bottlenose dolphin were determined, to the best of our knowledge, for the first time ever as concerns the aquatic mammals.

Keywords Bottlenose dolphin · Cortical bone tissue · Histomorphometry · Humerus · Rib

Introduction

Morphological characteristics of bones have shaped as a result of adaptation to life in an aquatic environment and whale diving habits (Wall 1983). The transition from land to aquatic lifestyle has caused remarkable changes in the bone microstructure (Wiffen et al. 1995; Uhen 2007). As a result, cetacean bones are of lesser density as compared to those of terrestrial mammals (de Buffrénil and Mazin 1990), which

allows the cetaceans to exercise dynamic buoyancy control (de Buffrénil et al. 1986; Gray et al. 2007). At the microscopic level, compact cortical bone thickness in cetaceans is lesser than that of terrestrial mammals; however, marrow cavities of the cancellous bone are enlarged (Ricqlès & de Buffrénil 2001). On the contrary, sea cows or dugongs that feed in shallow waters have thick-walled and solid bones to achieve negative buoyancy (Currey 2003).

Mammalian bone tissues constantly change during life to adapt to the requirements of the organism; at first, a developmental modelling takes place, turning into mature remodelling at some point (Samuelson 2007). Primary osteons of the compact bone exist within the lamellar bone and are created during the transformation from woven to lamellar bone (Currey 2002). Along the line of this process, primary osteons remodel into secondary ones. Secondary osteons can be distinguished from the primary ones based on several morphological characteristics, as follows: they are delimited by a cement line created when bone resorption ceases and a new bone is laid down; on top of that, they intersect circumferential lamellae, resulting in interstitial lamellae. Furthermore, primary osteons tend to be smaller than the secondary

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ones (Currey 2002; Hillier and Bell 2007). Unlike the woven bone tissue, the matrix of the lamellar bone tissue consists of an ordinary crisscross texture causing a typical birefringence under polarized light (Malluche and Faugere 1986).

It is well known that the osteon area of the human cortical bones decreases with age, while the number of osteons per unit area increases (Kerley 1965; McCalden et al. 1993; Wachter et al. 2002). Additionally, higher strain and larger weight result in a smaller osteon area of the cortical bone (van Oers et al. 2008; Britz et al. 2009). The microstructure of a cortical bone also depends on the function of the bone (Pearson and Lieberman 2004; Pfeiffer et al. 2006), which is particularly influenced by the locomotion environment, e.g. land versus water. In land mammals and humans, the rib cortical bone has a persistently active modular tissue that keeps up with growth, as the thorax expands. On the contrary, the tissue of the femur and humerus needs to respond to a wider range of mechanical loading requirements, from disuse to extreme loading (Pfeiffer et al. 2006). When it comes to whale ribs, apart from taking part in breathing, they also protect vital organs from pressure exerted during deep-diving (Rommel and Reynolds 2002). On the other hand, whale humerus, as a part of the fluke, is not exposed to strong forces because it plays a role in reducing drag while moving at high speeds, generating greater lift and improving body attitude control (Reidenberg 2007). Changes in the secondary osteon area and density in correlation with gender and age were studied in the human rib (Mulhern 2000; Pfeiffer et al. 2006). Additionally, a study of the rhesus monkey rib offers data on the secondary osteon area magnitude and the number of such osteons but correlated only to age, not gender (Przybeck 1985).

Most of the research published on cetacean bones has focused on bone microstructure in terms of tissue density (Wall 1983; de Buffrénil and Schoevaeret 1988; Gray et al. 2007; Dumont et al. 2013; Houssaye et al. 2015, 2016; Rolvien et al. 2017). However, there is a lack of precise description of the structure of the cetacean cortical bone that would explain its built at the microscopic level and changing during postnatal life. Hence, the purpose of our study was to describe the postnatal microscopic characteristics of the developing cortical bone of bottlenose dolphins. The presented morphological and morphometric descriptions of osteons of dolphins of different ages are the first detailed description of the bottlenose dolphin (and whale in general) cortical bone microstructure ever reported.

Materials and methods

For the purpose of this study, samples of 81 right humeri and 91 fifth right ribs retrieved from 106 bottlenose dolphins (*Tursiops truncatus*) aged less than 1–21 years were

analysed. The bottlenose dolphins were found dead in the Croatian part of the Adriatic Sea between 1990 and 2011. They were examined post-mortem with the permission of the Croatian authorities. The skeletons were archived, together with the necropsy reports, at the Department of Anatomy, Histology and Embryology of the Faculty of Veterinary Medicine University of Zagreb. Pectoral flippers were stored after soft tissue removal with a sharp knife. The tissue between the bones was left intact to preserve the structure and form of the flipper in whole. Pectoral flippers were fixed for 48 h in 4% formaldehyde water solution, bleached for 48 h in 4% hydrogen peroxide water solution and then air-dried. The rest of the skeleton was preserved by virtue of cooking and drying.

The gender of each dolphin was determined by virtue of macroscopic examination of the genitalia during the necropsy. Age estimation was based on the counts of dentinal growth layer groups (GLGs) found in teeth sections (Hohn et al. 1989; Slooten 1991). This method operates under the assumption that one GLG found in the dentine corresponds to a calendar year. For the purpose of this research, in total 109 dolphins were divided into five age groups: the 1st group—dolphins less than a year of age ($n = 14$, 7 males and 7 females), the 2nd group: 1–3-year-old dolphins ($n = 12$, 7 males and 5 females), the 3rd group: 4–7-year-old dolphins ($n = 19$, 10 males and 9 females), the 4th group: 8–14-year-old dolphins ($n = 22$, 11 males and 11 females), and the 5th group: dolphins older than 14 ($n = 39$, 20 males and 19 females). Age groups were established based on the growth curve published for bottlenose dolphins inhabiting the Adriatic Sea (Đuras et al. 2016).

The right humerus and the 5th rib were selected for sampling because, insofar, these bones have been the most frequent subject of histomorphometric analysis. Additionally, these bones have different biomechanical loading histories and remodelling rates (Goliath et al. 2016). The right humerus was cut at the humeral neck (*collum humeri*), which lies distal to the humeral head (*caput humeri*) (Fig. 1). This area is built of thick cortical tissue and is a discernible landmark of the bottlenose dolphin humerus (Felts and Spurrell 1965).

Humerus sampling involved cutting with an Ultimate 500 saw (NSK, Japan) at the level of the humeral neck (Fig. 1a), yielding a triangular, 0.5-cm-thick transversal sample (Fig. 1b). Using the same tool, two parallel transversal sections were made through the ventral end of the rib groove (*sulcus costae*) of the 5th right rib (Fig. 1c), yielding a roughly 0.5-cm-thick bone segment (Fig. 1d). Greasy bone segments were defatted in tetrachloroethylene for 48 h before gluing onto slides using a dual-component glue. The bone segments were ground with sandpaper on Struers LaboPol-1 to a thickness suitable for light microscopy.

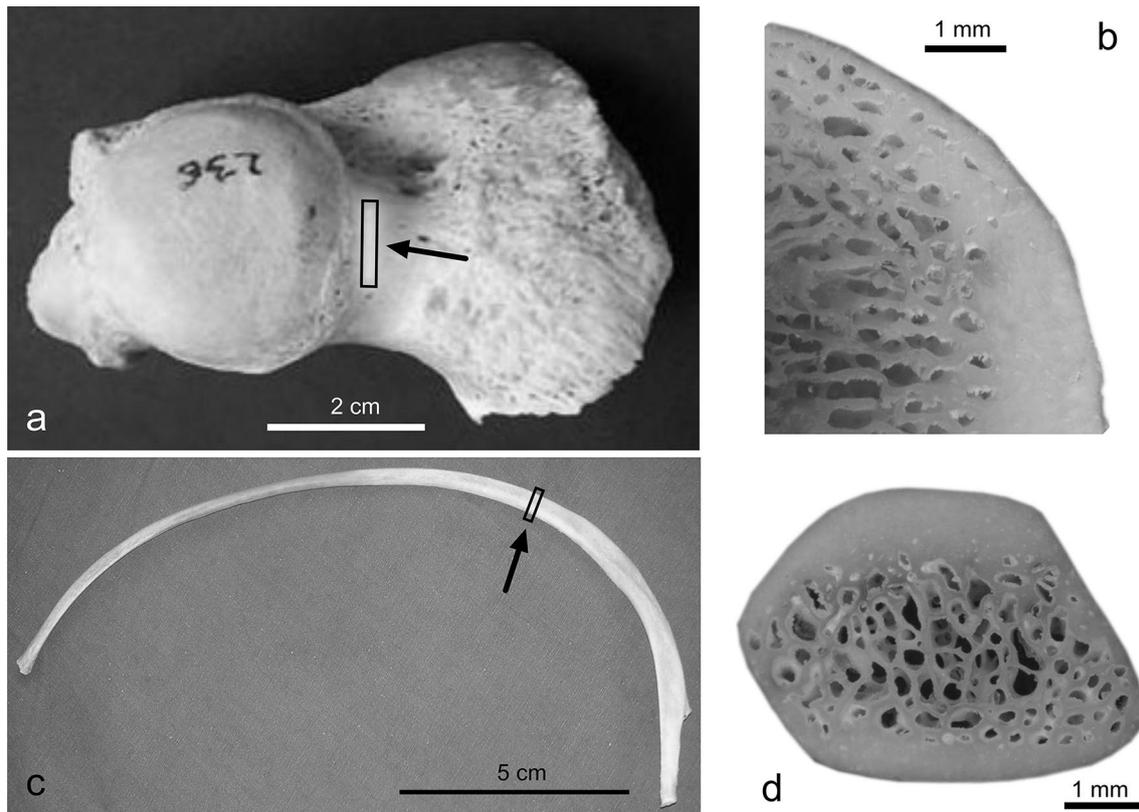


Fig. 1 Sampling location and sample morphology. **a** Right humerus with marked sampling location; **(b)** transversal surface of a right humerus sample; **(c)** right 5th rib with marked sampling location; **(d)** transversal surface of a rib sample

Bone sections were analysed and photographed under $100\times$ magnification using a Zeiss Axio Imager M2 (Zeiss, Germany). Five fields in the compact bone tissue of each sample were randomly selected to best represent the meso-osteal area of the cortical bone. The fields were $890\times 670\ \mu\text{m}$ large. The number of primary, secondary and remodelling osteons was established in each field and expressed as the osteon density (number of osteons/ mm^2). Polarized light was used to distinguish primary from secondary osteons. Additionally, osteon diameter was measured, and the osteon area calculated using the Zeiss AxioVision Software (Zeiss, Germany). The lamella number was counted in five (primary and secondary) osteons per sample. In remodelling osteons, no exact lamellae number was determined due to their different stage of remodelling.

Statistical analysis was performed using the StatSoft, Inc. (2007) STATISTICA (data analysis software system), Version 8.0, www.statsoft.com. Descriptive statistics is provided for qualitative (mean, standard deviation and percentage) and quantitative data (range, median and percentage). Statistical comparison was performed using general linear models (GLM). The effects of gender, age and bone type, as well as the interaction between gender, age and bone type, were tested. The Turkey post hoc HSD test for unequal n

was performed to depict and test inter-group differences. The latter were considered statistically significant should the P be ≤ 0.05 .

All data generated or analysed during this study are included in this published article.

Results

Qualitative analysis of the cetacean bone tissue in the humerus and the rib

The cortical tissue of the humerus and the rib of the 1st age group (< 1 year old) bottlenose dolphins (Figs. 2 and 3, 1st line) consisted of the woven bone and a few longitudinal canals. These canals were identified as forerunners of the primary osteons embedded into the woven bone tissue. Each canal was small in diameter and surrounded by 5–7 lamellae. In the rib, a thin layer of woven bone tissue was observed beneath the periosteal surface. Just under this peripheral layer, a well-expressed compact bone tissue was observed.

In the 2nd age group (1–3-year-old dolphins) (Figs. 2 and 3, 2nd line), compact tissue of the humerus and the rib mainly contained longitudinal vascular canals. A relatively

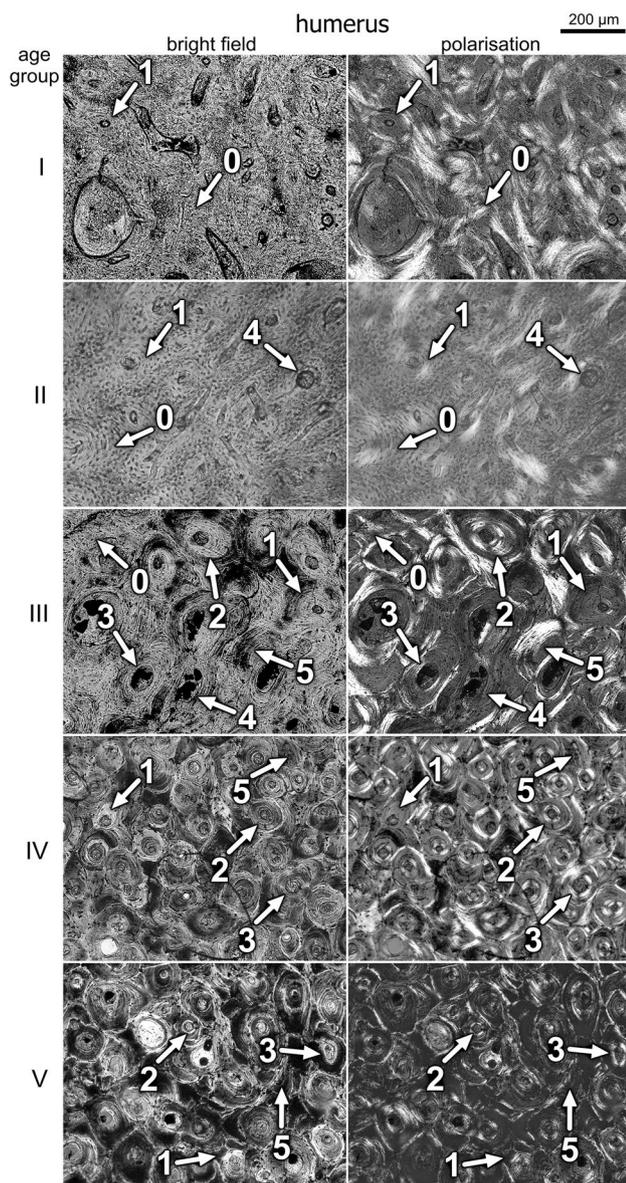


Fig. 2 Development of the bottlenose dolphin cortical bone tissue in humerus with aging. 0- woven bone tissue; 1- primary osteon; 2- secondary osteon; 3- remodelling osteon; 4- resorptive cavity; 5- intermediate lamellae

large amount of woven bone tissue was observed in the humerus between the primary osteons, while in the rib the amount of woven bone tissue around the primary osteons was very small. The primary osteons in the compact bone tissue of both bones were surrounded by 5–7 lamellae. About 10% of the primary osteons in the rib had resorptive cavities that indicate bone remodelling inception. In the humerus, no remodelling osteons were observed.

Compact tissue of the humerus and the ribs of the 3rd age group (4–7-year-old dolphins) (Fig. 2 and 3, 3rd line) had many elliptical to oval primary osteons with 6–8 lamellae.

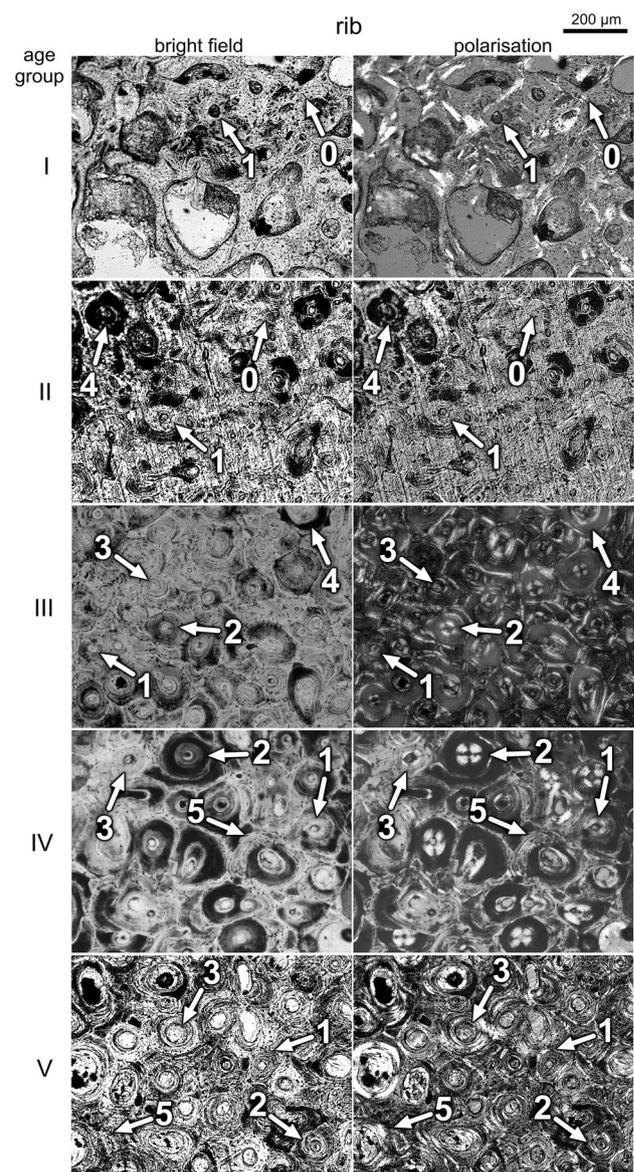


Fig. 3 Development of the bottlenose dolphin cortical bone tissue in the rib with aging. 0- woven bone tissue; 1- primary osteon; 2- secondary osteon; 3- remodelling osteon; 4- resorptive cavity; 5- intermediate lamellae

A bigger amount of woven tissue surrounded the primary osteons in the humerus as compared to the ribs. Deeper layers of the compact tissue of the humerus and the ribs contained resorptive cavities surrounded by woven bone tissue and elliptical to oval secondary osteons.

In the 4th age group (8–14-year-old dolphins) (Fig. 2 and 3, 4th line), compact bone of the humerus and the ribs was built of tightly packed elliptical to oval osteons. Still, a few primary and many secondary osteons were observed. Interstitial lamellae that remained after bone remodelling were noted between the two. Compact tissue of the humerus had

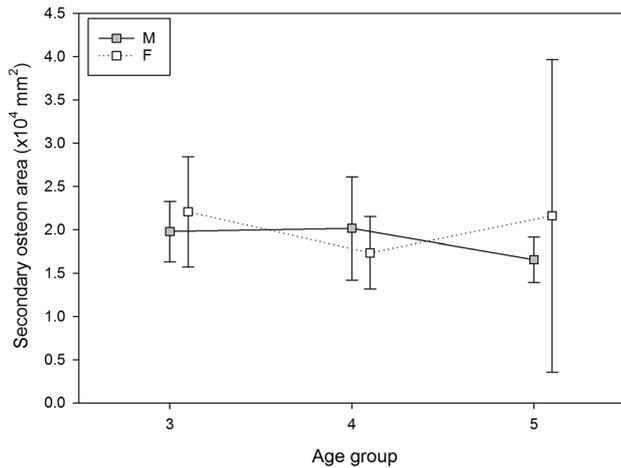


Fig. 4 Secondary osteon area (mean \pm 1SD) in male and female humerus of bottlenose dolphins falling into the 3rd, the 4th and the 5th age groups

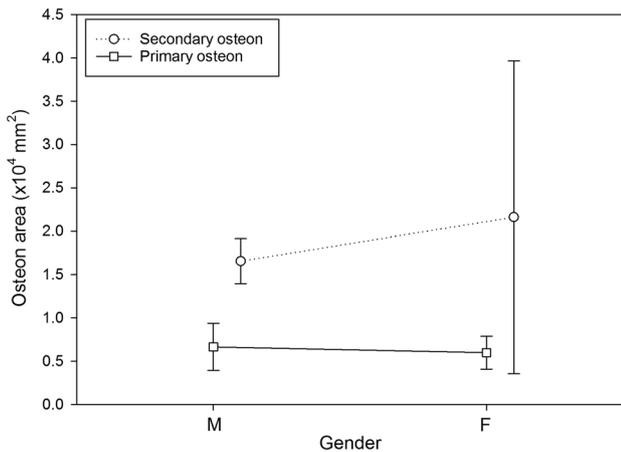


Fig. 5 Primary and secondary osteon areas (mean \pm 1SD) of male and female humerus of the 5th age group bottlenose dolphins

large resorptive cavities surrounded by woven bone tissue transforming from the compact into the cancellous tissue. In the compact tissue of the ribs, no woven bone tissue was to be seen.

In the 5th age group (> 14-year-old dolphins) (Figs. 2 and 3, 5th line), compact tissue of the humerus and the ribs was built of densely stacked elliptical to oval secondary osteons. The density of the latter appeared even greater in the rib. A larger number of remodelling osteons was observed between the primary and the secondary osteons. Intermediate lamellae were present in-between, too. Deeper layers of the compact tissue had resorptive cavities.

No gender-associated differences in the microscopic pattern of the humerus and the rib bone tissue were observed in any of the bottlenose dolphin age groups.

Quantitative analysis of the cetacean bone tissue of the humerus and the rib

Average magnitude of the osteon area, osteon density and osteon lamellae number in the bottlenose dolphin humerus and the rib are shown in Table 1. In general, in both the humerus and the rib primary osteons occupy more than twice smaller area than secondary ones. The secondary osteon area in the humerus is significantly larger than in the rib. The osteon density is higher in secondary than in primary osteons. In all osteon types, the osteon density is significantly higher in the rib than in the humerus. The number of osteon lamellae is higher in the secondary than in the primary osteon of both bones. Furthermore, the number of secondary and remodelling osteons increased with age, while the number of primary osteons that appear earlier in life, decreased. The secondary osteon area increased with age, as opposed to the primary osteon area that decreased with aging. The number of lamellae in the primary osteons

Table 1 Average magnitude of the osteon area, osteon density and osteon lamellae number in bottlenose dolphin humerus and rib

	Osteon type	Humerus		Rib		<i>P</i>
		Mean \pm SD	<i>n</i>	Mean \pm SD	<i>n</i>	
Osteon area ($\times 10^4 \mu\text{m}^2$)	Primary	0.65 \pm 0.27	27/81(33%)	0.76 \pm 0.37	63/91(69%)	0.172
	Secondary	1.89 \pm 1.06	57/81(70%)	1.61 \pm 0.39	80/91(88%)	0.029*
Osteon density (osteon number per mm^2)	Primary	7.77 \pm 7.06	53/81(65%)	9.43 \pm 11.65	68/91(75%)	0.000*
	Secondary	9.06 \pm 5.81	80/81(99%)	14.20 \pm 7.32	82/91(89%)	0.000*
	Remodeling	5.45 \pm 2.48	78/81(96%)	6.39 \pm 2.31	86/91(95%)	0.000*
	Osteon type	Median [min–max]	<i>n</i>	Median [min–max]	<i>n</i>	<i>p</i>
Lamella number	Primary	6 [4–8]	26/81(32%)	6 [4–10]	59/91(65%)	0.925
	Secondary	9 [3–5]	52/81(64%)	9 [7–11]	79/91(87%)	0.505

SD standard deviation, *n* number of samples with the osteon type analysed/total number of samples (%), *n.d.* not determine

* $P \leq 0.05$

decreased with age, as opposed to the secondary osteons in which it increased.

Osteon area associated with bone type, age and gender

In the female humerus (Table 2), primary osteons of the 1st, the 2nd and the 3rd age group dolphins and secondary osteons of the 2nd age group dolphins were not clearly defined, so that accurate measurements of the osteon area are not provided. For the same reason, data on primary osteon area of the male humerus of the 1st age group dolphins and secondary osteon area of the 2nd age group dolphins are lacking. In the female humerus, age-related changing of the primary osteon area could not be determined due to the lack of data. In the male humerus, primary osteon area decreased with age, but this decrease was insignificant (Table 2). In male dolphins falling into the 5th age group, primary osteon area was greater than in females of the same age, but this difference was insignificant ($P=0.507$). In the female humerus, secondary osteon area decreased from the 3rd to the 4th age group and increased again in the 5th age group. On the contrary, in the male humerus secondary osteon area increased up to the 4th age group and then decreased in the 5th age group; however, in an insignificant manner. Secondary osteon area in the humerus of both genders showed no significant difference ($P=0.488$) (Fig. 4). Primary and secondary osteon areas were mutually compared in the male and female humerus of the 5th age group dolphins. The analysis showed secondary osteons to occupy a significantly larger area than the primary ones (Fig. 5).

In the rib, primary osteon area increased in both genders from the 1st up to the 2nd age group. That area was

significantly larger in the 2nd age group dolphins of both genders as compared to other age groups. After that age, primary osteon area in the ribs decreased; however, the difference seen in dolphins falling into the age groups 3, 4 and 5 was not significant (Fig. 6). Secondary osteon area in the rib of dolphins of both genders did not show significant differences associated with age. In both genders, that area increased up to the 3rd age group and then slowly decreased in males (Fig. 7).

The analysis of the secondary osteon area in the humerus and the rib of both male and female 3rd, 4th and 5th age group bottlenose dolphins showed the secondary osteon area to be larger in the humerus than in the rib (in both genders and all age groups). Additionally, secondary osteon area of

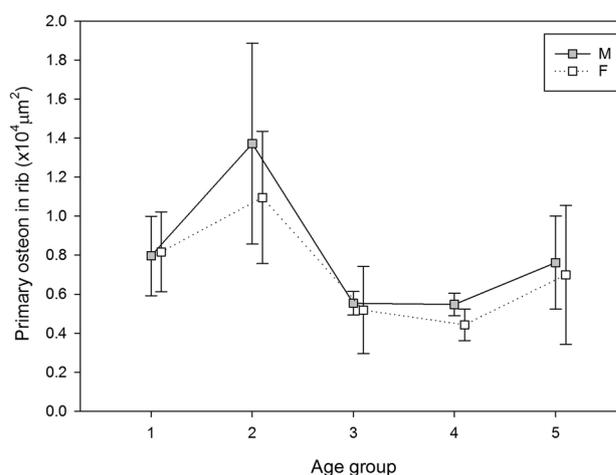


Fig. 6 Primary osteon area (mean \pm 1SD) in the bottlenose dolphin rib associated with age and gender

Table 2 Primary and secondary osteon areas ($\times 10^4 \mu\text{m}^2$) of the bottlenose dolphin humerus and rib relative of gender and age

Humerus								
Age group	Primary osteon (Mean \pm SD)				Secondary osteon (Mean \pm SD)			
	Male	<i>n</i>	Female	<i>n</i>	Male	<i>n</i>	Female	<i>n</i>
1st	n.d	0/7(0%)	n.d	0/5(0%)	1.15 \pm n.d	1/7(14%)	1.50 \pm 1.31	2/5
2nd	1.44 \pm n.d	1/7(14%)	n.d	0/4(0%)	n.d	0/7(0%)	n.d	0/4(0%)
3rd	0.63 \pm 1.17	2/4(50%)	n.d	0/4(0%)	1.98 \pm 0.35	3/4(75%)	2.21 \pm 0.64	2/4(50%)
4th	0.67 \pm n.d	1/10(10%)	0.37 \pm n.d	1/9(11%)	2.1 \pm 0.59	9/10(90%)	1.73 \pm 0.42	9/9(100%)
5th	0.67 \pm 0.27	10/14(71%)	0.60 \pm 0.19	12/17(71%)	1.65 \pm 0.26	14/14(100%)	2.16 \pm 1.80	17/17(100%)
Rib								
1st	0.80 \pm 0.20	4/5(80%)	0.82 \pm 0.21	5/6(83%)	1.11 \pm 0.16	3/5(60%)	1.40 \pm 0.73	5/6(83%)
2 nd	1.37 \pm 0.52	6/6(100%)	1.10 \pm 0.34	4/5(80%)	1.63 \pm n.d	1/6(17%)	1.39 \pm 1.13	3/5(60%)
3rd	0.55 \pm 0.06	3/10(30%)	0.52 \pm 0.22	5/7(71%)	1.78 \pm 0.28	10/10(100%)	1.58 \pm 0.35	7/7(100%)
4th	0.55 \pm 0.06	6/11(55%)	0.44 \pm 0.08	4/11(36%)	1.68 \pm 0.29	11/11(100%)	1.65 \pm 0.42	11/11(100%)
5th	0.76 \pm 0.24	9/11(82%)	0.70 \pm 0.36	9/19(47%)	1.60 \pm 0.25	10/11(91%)	1.65 \pm 0.28	19/19(100%)

SD standard deviation, *n* number of samples with the osteon type analysed/total number of samples (%), n.d. not determined

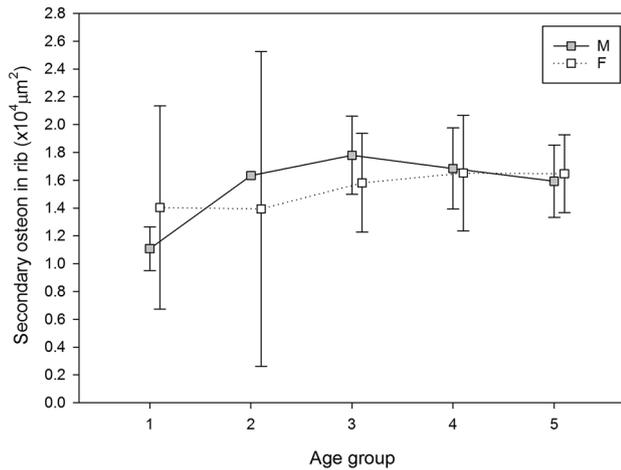


Fig. 7 Secondary osteon area (mean \pm 1SD) in the bottlenose dolphin rib associated with age and gender

the humerus of females falling into the 5th age group was significantly larger ($P=0.000$) than in the rib of males of the same age (Fig. 8).

Osteon density associated with bone type, age and gender

Primary osteon density significantly decreased ($P=0.000$) with age in both the humerus and the rib of dolphins of both genders (Table 3, Fig. 9 and 10). The average values descriptive of dolphins of the 1st and the 5th age group were nearly the same in both genders. Secondary osteon

density found in the 1st and the 2nd age group was nearly equal in both studied bones and increased with age in both bones and both genders. A significant increase ($P=0.000$) was observed in the ribs of the 3rd age group; however, the density decreased in the ribs of the 5th age group dolphins (Figs. 9 and 10). Primary osteon density ($P=0.000$) was significantly higher in the ribs of the 1st and the 2nd age group dolphins as compared to that of the humerus (Fig. 11). With aging, that difference slowly decreased, so that in dolphins falling into the 5th age group, the density almost equalised. On the other hand, when comparing secondary osteon density in the humerus to that in the rib, no significant change was observed for the 1st and the 2nd age group dolphins (Fig. 12). A significantly higher secondary osteon density was observed in the rib of the 3rd age group dolphins as compared to the humerus. In the humerus and the rib, the density of remodelling osteons was significantly lower than that of primary and secondary osteons ($P=0.000$). The density of remodelling osteons increased slower with age than the density of secondary osteons (Fig. 9 and 10). However, a significant influence of gender and age on the density of remodelling osteons of both bones was witnessed in the 5th age group dolphins (Table 3, Fig. 13). In the 5th age group, a significant increase in the density of remodelling osteons was recorded in females. In animals of the 1st to the 3rd age group, the density of these osteons was just about the same in both genders. On the contrary, a significantly higher density of remodelling osteons was observed in males of the 4th age group, whereupon that density decreased in the 5th age group (Fig. 13).

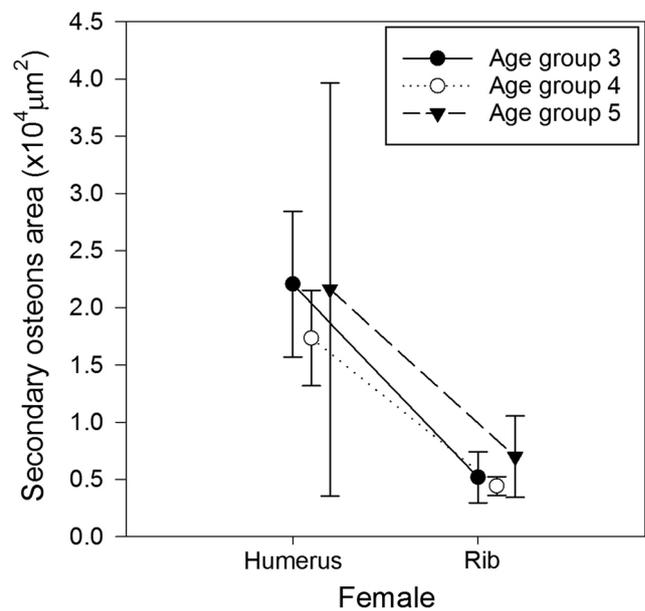
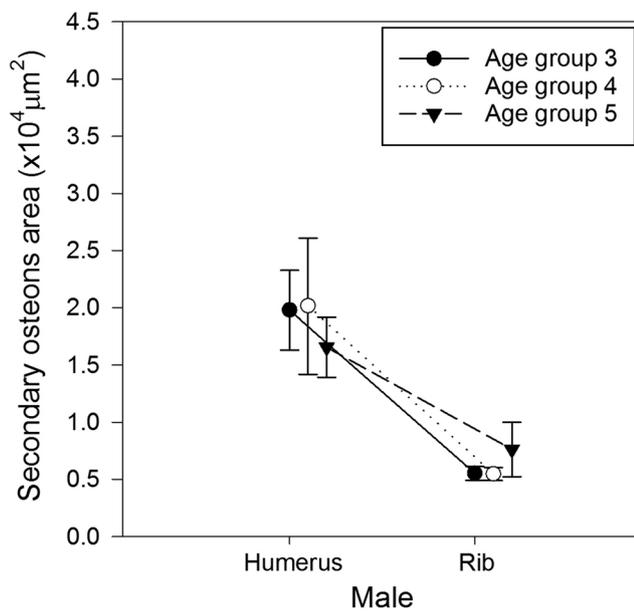
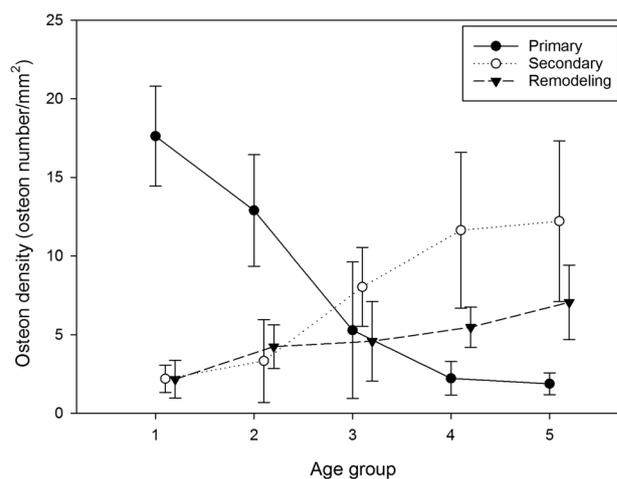
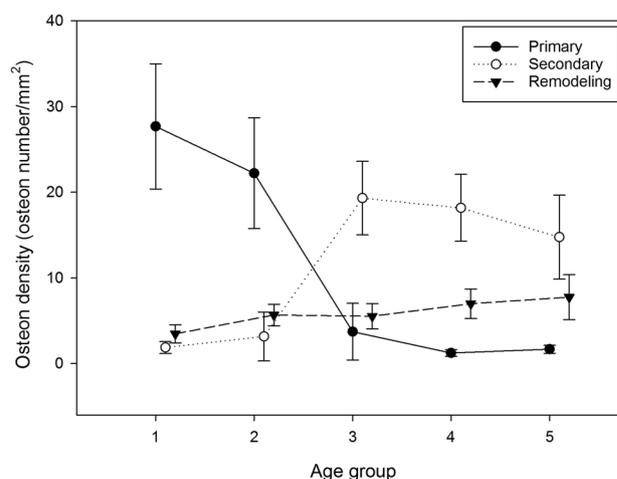


Fig. 8 Differences in the secondary osteon area (mean \pm 1SD) of the humerus and the rib associated with age and gender of bottlenose dolphins

Table 3 Primary, secondary and remodelling osteon densities (osteon number/mm²) of the bottlenose dolphin humerus and the rib relative of gender and age

Age group	Primary osteon (mean ± SD)						Secondary osteon (mean ± SD)						Osteon in remodeling (Mean ± SD)					
	Male		Female		n		Male		Female		n		Male		Female		n	
	Mean	SD	Mean	SD	n	%	Mean	SD	n	%	Mean	SD	n	%	Mean	SD	n	%
Humerus																		
1st	17.88 ± 4.15	6/7 (86%)	17.30 ± 1.87	5/5 (100%)	5/5	100%	2.03 ± 0.92	6/6 (100%)	2.38 ± 0.87	5/5	100%	2.54 ± 1.26	6/7 (86%)	1.40 ± 0.69	3/5 (60%)			
2nd	11.58 ± 2.29	7/7 (100%)	15.18 ± 4.51	4/4 (100%)	4/4	100%	3.76 ± 3.28	7/7 (100%)	2.56 ± 0.77	4/4	100%	4.64 ± 1.60	7/7 (100%)	3.53 ± 0.45	4/4 (100%)			
3rd	3.20 ± 1.44	3/4 (75%)	8.40 ± 6.22	2/4 (50%)	2/4	50%	8.55 ± 3.36	4/4 (100%)	7.51 ± 1.62	4/4	100%	4.25 ± 1.47	4/4 (100%)	4.90 ± 3.54	4/4 (100%)			
4th	2.83 ± 0.23	2/10 (20%)	1.00 ± n.d.	1/9 (11%)	1/9	11%	10.09 ± 4.68	10/10 (100%)	13.35 ± 4.92	9/9	100%	5.30 ± 1.35	10/10 (100%)	5.66 ± 1.24	9/9 (100%)			
5th	1.83 ± 0.60	11/14 (79%)	1.92 ± 0.79	12/17 (71%)	10/10	100%	10.10 ± 4.15	14/14 (100%)	13.95 ± 5.25	17/17	100%	5.49 ± 1.96	14/14 (100%)	8.35 ± 1.88	17/17 (100%)			
Rib																		
1st	29.17 ± 3.09	5/5 (100%)	26.43 ± 9.74	6/6 (100%)	6/6	100%	2.10 ± 1.07	4/5 (80%)	1.72 ± 0.31	6/6	100%	3.66 ± 1.38	5/5 (100%)	3.23 ± 0.57	4/6 (67%)			
2nd	20.71 ± 6.86	6/6 (100%)	24.0 ± 6.2	5/5 (100%)	5/5	100%	1.50 ± 0.71	2/6 (33%)	3.99 ± 3.27	4/5 (80%)	5.61 ± 1.65	6/6 (100%)	5.74 ± 0.69	5/5 (100%)				
3rd	1.50 ± 1.00	4/10 (40%)	5.20 ± 3.53	6/7 (86%)	6/7	86%	19.91 ± 4.29	10/10 (100%)	18.46 ± 4.50	7/7 (100%)	5.38 ± 1.44	10/10 (100%)	5.72 ± 1.6	7/7 (100%)				
4th	1.22 ± 0.37	8/11 (73%)	1.25 ± 0.50	4/11 (36%)	4/11	36%	17.64 ± 4.14	11/11 (100%)	18.71 ± 3.78	11/11	100%	7.25 ± 1.82	11/11 (100%)	6.70 ± 1.67	11/11 (100%)			
5th	1.72 ± 0.47	8/11 (73%)	1.66 ± 0.47	16/19 (84%)	16/19	84%	11.60 ± 4.55	8/11 (73%)	16.07 ± 4.53	19/19	100%	5.10 ± 1.64	8/11 (73%)	8.86 ± 2.09	19/19 (100%)			

**Fig. 9** Age-related changes of osteon density (mean ± 1SD) in bottlenose dolphin humerus**Fig. 10** Age-related changes of osteon density (mean ± 1SD) in bottlenose dolphin rib

The number of osteon lamellae

Primary osteon of bottlenose dolphin humerus and the rib had six lamellae on average, while the average number in a secondary osteon of both bones was nine (Table 1). Changes in lamellae number in both the humerus and the rib relative of dolphin age and gender are shown in Table 4. Osteon lamellae in the humerus of the 1st, the 2nd, the 3rd and the 4th age group dolphins were not clearly defined, so that the number of lamellae in primary and secondary humeral osteon was compared only in animals of the 5th age group. The average lamellae number contained by a primary osteon of the humerus of the 5th age group dolphins was significant higher ($P = 0.00$) in males than in females. Humeral primary osteons contained

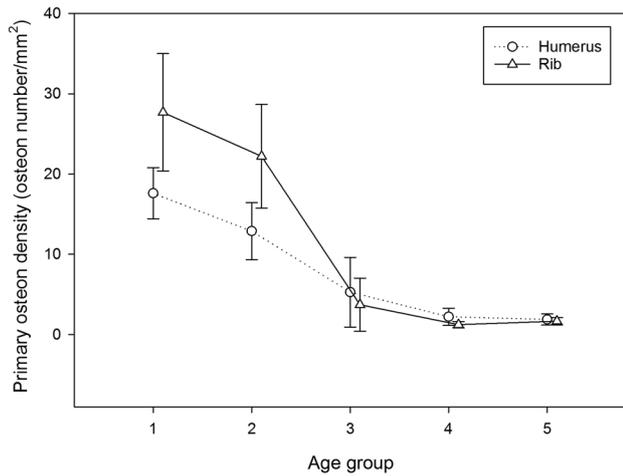


Fig. 11 Age-related changes of the primary osteon density (mean \pm 1SD) in the humerus and the rib of bottlenose dolphins

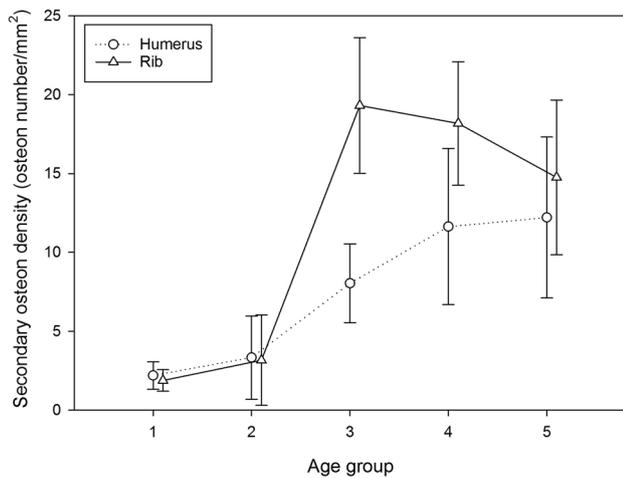


Fig. 12 Age-related changes of the secondary osteon density (mean \pm 1SD) in the humerus and the rib of bottlenose dolphins

a significantly lower number of lamellae than the secondary ones ($P = 0.00$) (Fig. 14).

The average number of lamellae in the primary and the secondary rib osteon was six and nine, respectively. The difference in the lamellae number between the two was proven significant ($p = 0.00$) (Fig. 15). The number of lamellae in the primary rib osteon of both genders increased from the 1st age group up to the 2nd age group, and then decreased in the 3rd and 4th age group. The lamellae number increased in males of the 5th age group; however, only a slight increase was observed in females (Table 4, Fig. 15). In both genders, the number of lamellae contained by the secondary rib osteon increased up to the 4th age group, and then decreased in the 5th age group (Table 4, Fig. 16). Differences in the average lamellae number contained by the primary osteons,

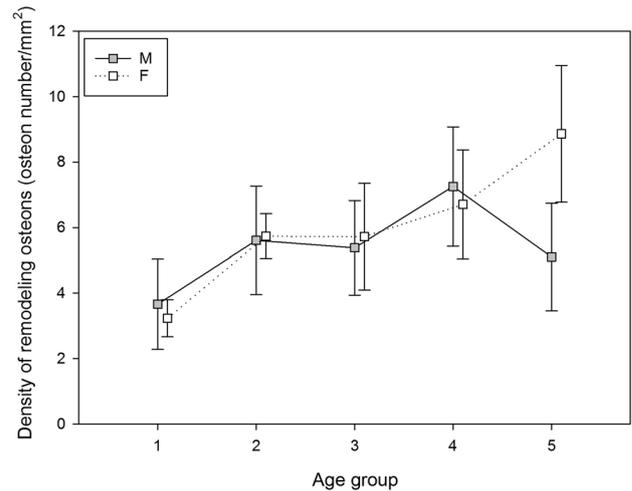


Fig. 13 Differences in density (mean \pm 1SD) of remodelling osteons relative of bottlenose dolphin age and gender

seen between genders and age groups, were statistically significant ($P = 0.04$), but the differences witnessed between the primary and the secondary osteons were not ($P = 0.204$).

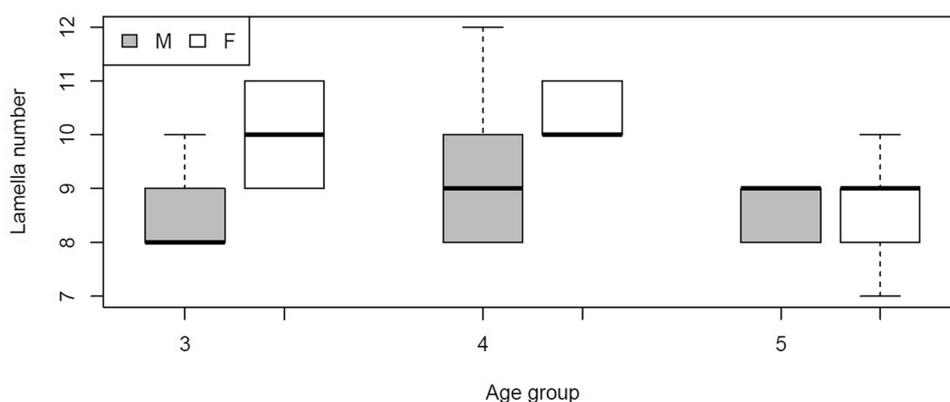
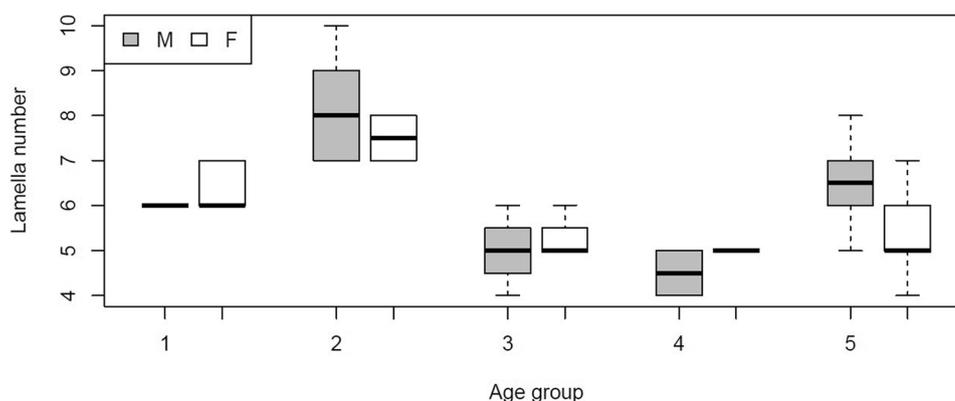
Discussion

This study aimed to establish qualitative and quantitative changes in bone microstructure of the humerus and the rib of bottlenose dolphins of both genders and different ages. Qualitative analysis did not show any gender-related differences in the above; however, quantitative differences in the studied bones of females and males of different ages were observed. Morphometric osteon values were determined, as well; to the best of our knowledge, these are the first data of the kind provided for any aquatic mammal ever.

The humerus and rib are bones frequently used for the analysis of the bone microstructure in terms of compact versus spongy bone tissue, orientation and thickness of trabeculae and size of intratrabecular spaces. The cetacean rib is built of loose spongiosa surrounded by a layer of rather compact cortex whose thickness varies among taxa. The cetacean humerus has a spongiosa surrounded by a thin cortex homogeneously or heterogeneously surrounding the bone (Houssaye et al. 2016). Similar microstructure of the humerus and rib was observed in extinct whales called the archaeocetes (Houssaye et al. 2015). The variation of the bone microstructure that has been determined among different skeletal elements should be considered in terms of functional requirements and phylogenetic heritage (Houssaye et al. 2016). Exceptional swimming and diving behaviour of whales results in extreme tension and compression on the vertebral body due to the work of

Table 4 The lamellae number in primary and secondary osteons of bottlenose dolphin humerus and rib relative of gender and age (n-number of samples with the osteon type analysed/total number of samples (%); n.d.-not determined)

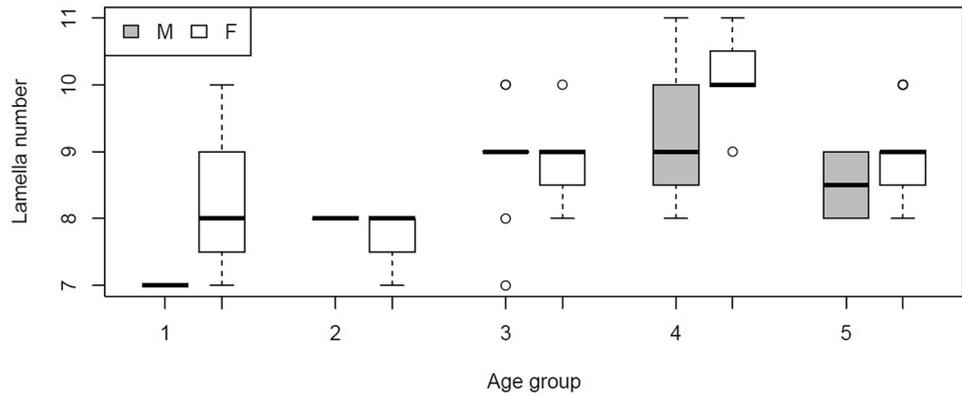
Humerus								
Age group	Primary osteon median [min–max]				Secondary osteon median [min–max]			
	Male	n	Female	n	Male	n	Female	n
1st	n.d	0/7 (0%)	n.d	0/5 (0%)	6 [6–6]	1/7 (14%)	n.d	0/5 (0%)
2nd	n.d	0/7 (0%)	n.d	0/4 (0%)	n.d	0/7 (0%)	n.d	0/4 (0%)
3rd	5 [5–5]	2/4 (50%)	n.d	0/4 (0%)	8 [8–10]	3/4 (75%)	10 [9–11]	2/4 (50%)
4th	4 [4–4]	1/10 (10%)	5 [5–5]	1/9 (11%)	9.5 [8–12]	8/10 (80%)	10 [9–11]	9/9 (100%)
5th	7 [6–8]	10/14 (71%)	5 [4–7]	12/17 (71%)	9 [8–9]	13/14 (93%)	9 [7–10]	17/17 (100%)
Rib								
1st	6 [6–6]	4/5 (80%)	6 [6–7]	5/6 (83%)	7 [7–7]	3/5 (60%)	8 [7–10]	4/6 (67%)
2nd	8 [7–10]	6/6 (100%)	7.5 [7–8]	4/5 (80%)	8 [8–8]	1/6 (17%)	8 [7–8]	3/5 (60%)
3rd	5 [4–6]	3/10 (30%)	5 [5–6]	4/7 (57%)	9 [7–10]	10/10 (100%)	9 [8–10]	7/7 (100%)
4th	4.5 [4–5]	6/11 (55%)	5 [5–5]	1/11 (9%)	9 [8–11]	11/11 (100%)	10 [9–11]	11/11 (100%)
5th	7 [5–8]	10/11 (91%)	5.5 [5–7]	16/19 (84%)	8.5 [8–9]	10/11 (91%)	9 [8–10]	19/19 (100%)

Fig. 14 The number of osteon lamellae in the secondary osteon of bottlenose dolphin humerus (descriptive of dolphins of the 3rd, the 4th and the 5th age groups)**Fig. 15** The number of osteon lamellae in the primary osteons of bottlenose dolphin rib, displayed based on the age groups

propulsive muscles. Thus, the vertebral bone microstructure has been intensively studied (Dumont et al. 2013; Rolvien et al. 2017) and showed that increased trabecular length is of special importance for the vertebral strength in cetaceans (Dumont et al. 2013). Moreover, in deep-diving species such as the sperm whale (*Physeter macrocephalus*),

patterns similar to diving-associated bone pathologies were described (Rolvien et al. 2017). Bone tissue constantly changes throughout a mammal's lifetime. Fibrous, woven bone tissue with numerous longitudinal bone canals prevails after birth (Locke 2004). The canals in the fibrous tissue are later surrounded by lamellae and form primary osteons. In

Fig. 16 The number of osteon lamellae in the secondary osteons of bottlenose dolphin rib, displayed based on the age groups



pigs, ruminants (Mori et al. 2005) and horses (Mori et al. 2003), the plexiform bone occurs in the long bones during maturing. However, the plexiform bone does not appear in the ribs of deer, dogs and pigs (Morris 2007). Within this study frame, plexiform bone tissue failed to be observed in the humerus and the rib of bottlenose dolphins of any age. In the youngest bottlenose dolphins, the bones were built of woven tissue, which was replaced by lamellar bone tissue as described in other mammals, too. Primary osteons are gradually remodelled into secondary ones; therefore, an adult compact bone of the bottlenose dolphin is built of three main elements: secondary osteons, remodelling osteons and intermediate lamellae that represent remodelling leftovers located between the osteons, as also described in pigs, cows and sheep (Martiniaková et al. 2007).

In different animal species, as well as in humans, the shape of the secondary osteons is cylindrical to oval (Martin 1998; Zeda et al. 2008). In general, the shape of the secondary osteons of the studied bottlenose dolphin bones was also cylindrical to oval. Likewise, it was observed that osteon circularity increases with age, as noticed by Goliath et al. (2016) in humans. This is opposite to the findings of Michell (2016), who claimed the shapes of osteons of aquatic mammals (genus *Phocanella*) to be far more irregular than those of terrestrial mammals. It has to be noted, though, that our findings on osteon circularity are subjective.

Our study specifies, for the first time ever, the average primary osteon density in the bottlenose dolphin humerus ($7.77 \pm 7.06/\text{mm}^2$) and rib ($9.43 \pm 11.65/\text{mm}^2$). The primary osteon density is significantly higher in the rib than in the humerus. To the best of our knowledge, no other study has reported about the primary osteon density in animals insofar; data on age-related primary osteon density were published for the human humerus only (Pitfield et al. 2017).

Bearing in mind that the humerus and the rib have different functions in the bottlenose dolphin skeleton, we presumed that certain differences in the magnitude and density of the secondary osteon area shall be encountered. Our findings are in line with those of Morris (2007), who studied

the density and the magnitude of the secondary osteon area in the humerus and the rib of dogs, pigs and deer. Morris (2007) recorded that the rib has a smaller secondary osteon area and a higher secondary osteon density than the humerus. Smaller secondary osteon area in the rib as compared to the femur was observed by Horni (2002) in humans, and by Mulhern and Ubelaker (2009) in chimpanzees (*Pan troglodytes*). In our study, a similar pattern was witnessed in the bottlenose dolphin, as well, namely secondary osteons in the bottlenose dolphin rib are denser and smaller (secondary osteon area $1.61 \pm 0.39 \times 10^4 \mu\text{m}^2$; secondary osteon density $14.20 \pm 7.32/\text{mm}^2$) than in the humerus (secondary osteon area $1.89 \pm 1.06 \times 10^4 \mu\text{m}^2$; secondary osteon density $9.06 \pm 5.81/\text{mm}^2$). We presume that water as the living environment does not affect osteon density and the magnitude of osteon area in the rib versus a limb bone (humerus), given that the values established in the bottlenose dolphin show a pattern similar to that of terrestrial mammals. Skedros et al. (2013) explained that ribs, contrary to limb bones, are metabolically more active and sensitive to hormonal changes, and receive frequent low-strain loading; hence, bigger osteons are geometrically more advantageous when it comes to resisting to bending and compression loads (Felder et al. 2017). In addition, van Oers et al. (2008) stated that smaller osteons are located in higher strain areas. That could explain our finding in the bottlenose dolphin rib that suffers strong forces during deep dives, as compared to the humerus, which plays a role in the body attitude during swimming. Consequently, we observed a higher density of remodelling osteons in the bottlenose dolphins' rib ($6.39/\text{mm}^2$) than in their humerus ($5.45/\text{mm}^2$). That can be explained by more intensive remodelling of a bone that has to endure higher strains (Currey 2003).

In general, the density of primary osteons decreases with age in most mammalian species (Kerley 1965; Hillier and Bell 2007). So far, just Pitfield et al. (2017) published results about the primary osteon density in the humerus of humans of different ages. They observed the same pattern: in humans aged up to 1.9 years, the density of primary osteons was

$5.47 \pm 1.31/\text{mm}^2$, while in 13–18-year-old adolescents the density in question was $0.70 \pm 1.06/\text{mm}^2$. Our results also corroborate that the density of the primary osteons significantly decreases with age ($P=0.000$). We determined the average primary osteon area in the bottlenose dolphin rib to measure $0.76 \pm 0.37 \times 10^4 \mu\text{m}^2$, while its density equals to $9.43 \pm 11.65/\text{mm}^2$. To the best of our knowledge, these are the first data on the magnitude and density of the primary osteon area of any mammalian species—terrestrial or aquatic—ever published. We also observed that the primary osteon area in the bottlenose dolphin rib decreases with age in a gender-irrespective fashion, which is in line with findings concerning other mammals (Hillier and Bell 2007).

Secondary osteons of mammalian species are generally better described than primary ones. Nganvongpanit et al. (2017) studied the secondary osteon area in dogs (Golden Retriever). They studied several types of bones, among other the humerus of puppies and adults. Their results show that puppies have a significantly smaller secondary osteon area ($0.71 \times 10^4 \mu\text{m}^2$) than adults ($1.64 \times 10^4 \mu\text{m}^2$). Based on our results, the magnitude of the secondary osteon area of the bottlenose dolphin humerus varies across age and gender groups. In dogs, the humerus and the femur represent weight-bearing bones (higher strains—smaller osteons). According to Britz et al. (2009), mobility-related behaviour has an impact on secondary osteons. In light of the above, it can be expected that an aquatic medium, which reduces body weight, would have an impact on the magnitude of the secondary osteon area of the bottlenose dolphin humerus. Changes in the secondary osteon area and density in correlation to gender and age were studied in the human rib by Mulhern (2000) and Pfeiffer et al. (2006). Additionally, a study of the rhesus monkey (*Macaca mulatta*) rib offers data on the secondary osteon area and the number of such osteons correlated only to age (Przybeck 1985), but not gender. Mulhern (2000) recorded that the secondary osteon area in the human rib does not follow a linear trend. After initial growing, when the female secondary osteon area becomes bigger than that of males, the area in question starts to decrease in both males and females, that of males thereby becoming bigger as compared to the female one. At the same time, the density of the secondary osteons follows the osteon area streamline. In young animals, the density of the area is low; in later life, it grows until the middle age and then decreases again. The same pattern was observed in the bottlenose dolphin, in which, in the early stage of life, the secondary osteon area in a female rib is a bit larger than that of a male rib. Older males have a larger secondary osteon area. In adult dolphins, the secondary osteon area is similar in size in both genders. As in humans, the osteon density follows the same pattern. Przybeck (1985) studied the secondary osteon area in the rib of rhesus monkeys of different ages and observed the same pattern as in humans and bottlenose

dolphins; however, the gender was not taken into account. Pfeiffer et al. (2006) studied the ribs of humans aged 30 to 50+. This group of authors did not specify the density of the secondary osteons in the ribs, but only the magnitude of the area occupied by them. They noticed that the osteon area decreases with age, the same as reported in the studies by Mulhern (2000) and Przybeck (1985), and by our study.

Based on our results and the morphometric values published insofar, it could be assumed that no differences between the magnitude and the density of the secondary osteon area can be found between terrestrial and aquatic mammals. Differences in the bones are probably caused by some other factors. Like Skedros (2013) said, “It is not well known how the relative importance of these different osteon functions might be at work in specific bone types, where metabolic functions might predominate over mechanical functions (e.g., ribs vs. weight-bearing bones, respectively)”.

The remodelling osteons were studied in young animals (rats, monkeys and dogs) and humans (Enlow and Brown 1958). Many authors stated that the number of such osteons increases with age (Kerley 1965; Ortner 1974; Mulhern and Ubelaker 2003), which is in concordance with our findings in bottlenose dolphins. That could be explained by the fact that in that age the mature bone tissue, which tends to recover by virtue of remodelling, is formed (Currey 2003).

Lamellae number does not increase linearly as was the case with the osteon area. In the bottlenose dolphin humerus, the lamellae number in primary osteons of the 5th age group dolphins is significantly higher in males (7) than in females (5). It is an interesting observation considering that the primary osteon area of both genders is almost equal in size (male $0.67 \pm 0.27 \times 10^4 \mu\text{m}^2$, female $0.60 \pm 0.19 \times 10^4 \mu\text{m}^2$). However, the lamellae number contained by the secondary humeral osteons did not significantly change relative of age and gender. In the rib, a significant change in the lamellae number contained by the primary osteon was observed relative of gender and age (the median lamellae number in males of the 5th age group 5–8, that in female peers 5–7). As in the humerus, the rib area occupied by the primary osteons in the male and female 5th age group dolphins is almost equal in size ($0.76 \pm 0.24 \times 10^4 \mu\text{m}^2$; $0.70 \pm 0.36 \times 10^4 \mu\text{m}^2$). In the rib of animals of both genders, the lamellae number contained by the secondary osteons increases (but not significantly) with age. In the accessible literature, the lamellae number was defined just in the secondary osteons of terrestrial mammals. The lamellae number found in raccoons (*Nyctereutes procyonoides*) was 3–5, in badger (*Meles meles*) 5–8 (Hidaka et al. 1998), in cows (*Bos taurus*) 5–7 (Zeda et al. 2008), in horses (*Equus caballus*) up to 10, in pigs (*Sus scrofa*) 10–12 (secondary osteon lamellae number) (Castrogiovanni et al. 2011), and in humans 16–20 (Martin 1998).

In this study, we determined the primary osteon area magnitude and density in an aquatic mammal rib and humerus for

the first time ever. Also, for the first time ever, the magnitude and the density of the secondary osteon area, the density of the remodelling osteons and the lamellae number in primary and secondary osteons of the bottlenose dolphin during aging was determined. It was presumed that aquatic media affect rib and humeral cortical bone structure during life. However, it does not appear that water, as a living medium, influences the secondary osteon density in the humerus and the rib, but may have an impact on the magnitude of the humeral secondary osteon area of bottlenose dolphins. Our study also showed that the growth pattern of the humeral and rib cortical bone does not differ between terrestrial and aquatic mammals, not even between genders of the same species. The same pattern of bone maturing and differences between osteon area and density were observed in young and adult animals in both studied bones. Therefore, the findings of Britz et al. (2009) and Skedros et al. (2013), who claim that age, gender and weight affect osteon geometry and that different bone osteon functions, having specific purposes in specific bone types in which metabolic functions may predominate over mechanical ones, can be applied to sea mammals, as well. Our study has several weaknesses, as follows: the quality of microscopic slides of young animals' humeri was too poor to perform certain measurements. Consequently, some of the obtained data did not suffice for statistical analysis. Also, some of the measurements would be more precise if a whole transversal section of the humerus was sampled. These flaws aside, this study reports the first data on aging-related histomorphometric changes in cortical bone tissue of the bottlenose dolphin.

Acknowledgements Funding of this study was provided by the Gesellschaft zur Rettung der Delphine, Munich, Germany, and the Croatian Veterinary Institute, Zagreb, Croatia. Many thanks to our colleagues Relja Beck, VMD, PhD, affiliated with the Croatian Veterinary Institute, Zagreb, for his help during microscopy, and to the retired Professor of the Faculty of Veterinary Medicine University of Zagreb Zvonimir Kozarić for the idea and useful suggestions.

Author contributions Conceptualization and methodology: MĐ; formal analysis, investigation and original text preparation: MZ; formal analysis: NM-S; resources and visualisation: TG and MM; review and editing: MĐ and SK.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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