Micro-anatomical structure of the first spine of the dorsal fin of Atlantic bluefin tuna, *Thunnus thynnus* (Osteichthyes: Scombridae)

Nicoletta Santamaria[a], Giambattista Bello[a], Letizia Passantino[a], Mariasevera Di Comite[b], Rosa Zupa[a], Chrysovalentinos Pousis[a], Robert Vassallo-Agius[c], Vincenzo Cicirelli[a], Gualtiero Basilone[d], Salvatore Mangano[d], Aldo Corriero[a*]

[a]Department of Emergency and Organ Transplantation, Section of Veterinary Clinics and Animal Production, University of Bari Aldo Moro, Valenzano (BA), Italy

[b]Department of Basic Medical Sciences, Neurosciences and Sensory Organs, University of Bari Aldo Moro, Bari, Italy

[c]Malta Aquaculture Research Centre, Marsaxlokk, Malta

[d]Institute for Marine Coastal Environment, National Research Council, Capo Granitola (TP), Italy

Abbreviations: ABFT, Atlantic bluefin tuna; FS, first spine of the first dorsal fin.

***** Corresponding author at: Department of Emergency and Organ Transplantation, Section of Veterinary Clinics and Animal Production, University of Bari Aldo Moro, Valenzano (BA), Italy.

Email address: aldo.corriero@uniba.it (A. Corriero)

Abstract

The first spine of the first dorsal fin (FS) of the Atlantic bluefin tuna (ABFT), *Thunnus thynnus*, is customarily used in age determination research because its transverse sections display well-defined growth marks. In this paper the FS structure was studied to explain its known dramatic age- and season-related morphological modifications, which are evidently caused by bone remodeling. Cross sections of samples from six adult ABFT were in part decalcified to be stained with histological, histochemical and immunohistochemical methods and in part embedded in metyl-metacrylate to be either observed under a linear polarized light or microradiographed. FS showed an external compact bone zone and an inner trabecular bone zone. The compact bone zone consisted of an outer non-osteonic primary bone layer (C_1) and an inner osteonic bone layer (C_2). C_1 was in turn characterized by alternate translucent and opaque bands. Evidence of spine bone remodeling was shown by the presence of osteoclasts and osteoblasts as well as by tartrate-resistant acid phosphatase (TRAP)-positive bands at the boundary between old and newly formed bone. The examination of plain, i.e. not-fixed and not-decalcified, FS from 28 ABFT showed that the average thickness of C_1 remained fairly constant during fish growth, whereas C_2 increased significantly, indicating that the periosteal primary bone apposition is counterbalanced by the parallel bone remodeling occurring inside the compact bone zone. The present study revealed the structure of the ABFT FS and the pattern of its bone remodeling. Both of them underlay phenomena, never examined in detail before, such as the appearance followed by the progressive disappearance of growth bands.

Key words: Atlantic bluefin tuna; dorsal fin spine; fish bone; bone remodeling.

1. Introduction

Teleost fishes are provided with a variable number of median (one or more dorsal, one caudal and one anal) and paired (pectoral and pelvic) fins (Lauder, 2006). These are supported by dermal bone rays (lepidotrichia), which are composed of either small articulated bony segments (soft rays or, simply, rays) or a single bony rod (spiny rays or, simply, spines) (Tortonese, 1975; Drucker and Lauder, 2001; Kalish-Achrai et al., 2017). The structure of skeletal elements in fishes varies according to the species and is constituted by different kinds of bone tissues: cellular/acellular; non-lamellar/lamellar; compact/trabecular (Kölliker, 1859; Amprino and Godina, 1956; Moss, 1961, 1965; Meunier, 1987; Sire et al., 1990; Witten and Huysseune, 2009; Cohen et al., 2012; Shahar and Dean, 2013; Kalish-Achrai et al., 2017). Incidentally, acellular bone, i.e. bone without osteocytes, should be better named anosteocytic bone since it includes other bone cell types, i.e. osteoblasts and osteoclasts (Weiss and Watabe, 1979; Shahar and Dean, 2013). In teleost fishes the occurrence of either osteocytic or anosteocytic bone is a species-specific feature: in some species only one bone type occurs, in some others both types co-occur (Amprino and Godina, 1956; Moss, 1963). The Atlantic bluefin tuna, *Thunnus thynnus* (Linnaeus, 1758), (ABFT) is provided with median (two dorsal and one anal) and paired (pectoral and pelvic) fins, as well as with several unpaired finlets (Fisher et al., 1987). The first (cranial) dorsal fin is supported by 12-15 spines, the second one is supported by a spine followed by 11-13 soft rays (Tortonese, 1975; Fisher et al., 1987). The first spine of the first dorsal fin (FS) – an elongated rod tapering to a free tip, articulated to the radial bone by means of a condyle – is the most suitable hard structure for age determination studies and is in fact the one customarily used for this purpose because its transverse sections display well-defined growth marks; in addition, it is comparatively easy to collect (Cort, 1991; Megalofonou and De Metrio, 2000; Corriero et al., 2005; Santamaria et al., 2009; Berkovich et al., 2013; Luque et al., 2014). The presence of growth marks is due to the progressive apposition of bone tissue at different rates according to the season, which becomes apparent as an ordered series of alternate opaque and

translucent rings (Cort, 1991; Megalofonou and De Metrio, 2000; Santamaria et al., 2009). In the ABFT, concomitantly with bone apposition on the outer surface, a physiological progressive resorption of bone tissue occurs in the innermost part of the spine (the so-called core or nucleus) (Cort, 1991; Megalofonou and De Metrio, 2000; Santamaria et al., 2009; Luque et al., 2014). Santamaria et al. (2015) assessed the pattern of spine bone resorption in both wild and captive-reared (i.e. individuals caught alive from the wild and confined in captivity for fattening) ABFT from the Mediterranean Sea. They concluded that the fraction of compact bone, as measured in a spine cross section surface, progressively decreases with age; moreover, they found that bone resorption is dramatically enhanced in captive-reared ABFT individuals with respect to wild animals. The understanding of the FS structure and its bone apposition/resorption pattern has important bearings in applied matters, such as the age-reading technique (Luque et al., 2014) and the well-being of farmed individuals (Santamaria et al., 2015; Campobasso et al., 2017). Furthermore, the histological features of this skeletal element deserve to be studied for their own sake in order to provide solutions to several unanswered questions, e.g. which types of bone tissue are specifically present in *Thunnus* and allied genera and, in general, in perciform fishes , and the corresponding relevance to their evolutionary history. Notwithstanding the above, no histological study on FS is reported in the vast literature on this species.

The present research was prompted by the awareness, gained during a previous investigation on the FS (Santamaria et al., 2015), that virtually nothing was known about the microscopic structure of the spine bone and its remodeling. Purpose of the present study was to describe the structure of the FS of the ABFT, identify its different types of bone tissue, and provide a background to elucidate its bone remodeling processes.

2. Materials and Methods

No fish was experimentally reared or sacrificed for the present study. All the samples were taken from freshly dead animals, killed for commercial purposes.

To study the FS structure, six adult ABFT (fork length, *FL*, 136 to 225 cm), reared in captivity (MFF Ltd tuna farm, Malta) for about five months after their capture in the wild, were sampled during commercial slaughtering operations, on 11 November 2014. From the region above the condyle of each spine, 1 mm-thick serial cross sections were with an ISOMET® saw (Buehler, Lake Bluff, Illinois, USA) and fixed overnight at 4°C in 4% paraformaldehyde and afterwards rinsed in phosphate buffer saline (PBS) 0.01 M pH 7.4.

Some sections were decalcified for eight months at 4°C in 10% buffered EDTA (Sigma Aldrich, Milan, Italy) pH 7.4, dehydrated in ethanol and embedded in paraffin wax. Five-µm sections were de-parafinized in xylene and stained with: hematoxylin-eosin; periodic acid-Schiff (PAS) reaction followed by hematoxylin counterstaining; 1% toluidine blue in 0.1 M citric acid, 0.1 M disodium phosphate pH 6; Masson-Goldner trichrome (Bio Optica, Milan, Italy).

The identification of osteoclasts and osteoblasts was carried out on de-parafinized sections by the enzyme-histochemical demonstration of tartrate-resistant acid phosphatase (TRAP) (Witten et al., 2001) and the immunohistochemical detection of osteonectin, respectively. For osteonectin immunodetection, rabbit antibodies produced against a recombinant human osteonectin sequence (Lot number C105955; Sigma-Aldrich, Milan, Italy) were used. This antibody was chosen among those commercially available because of the very high similarity between the amino-acid sequence of the human recombinant protein used by the antibody producer for immunization and the osteonectin sequence of fish species available in GenBank. The immunohistochemical staining was performed according to the producer protocol (https://www.sigmaaldrich.com/technical-documents/protocols/biology/immunohistochemistry-procedure.html; accessed 04 January 2018) with some modifications. Deparaffinized sections were re-hydrated through graded ethanol solutions and pre-treated for 30 min with 0.3% H_2O_2 in methanol to inhibit endogenous peroxidase activity. The sections underwent an antigen retrieval procedure by boiling in citrate buffer (0.01 M, pH 6.0;

4x5 min cycles) in a microwave oven on high power (750 watts). The sections were incubated for 30 min in normal horse serum (NHS; Vector, Burlingame, CA, U.S.A.) diluted 1:50 in phosphate buffered saline (PBS; 0.01M phosphate buffer at pH 7.4, containing 0.15M NaCl) to block non-specific binding sites for immunoglobulins. The sections were then incubated for 30 min at room temperature in a moist chamber with anti-osteonectin antibodies diluted 1:200 in PBS containing 1% bovine serum albumin. After rinsing for 10 min in PBS, the immunohistochemical visualization was carried out using the Vectastain Universal Elite Kit (Vector, Burlingame, CA, U.S.A.). Peroxidase activity was visualized by incubating for 10 min with Vector DAB Peroxidase Substrate Kit (Vector, Burlingame, CA, U.S.A), which produces a brown precipitate. To confirm the specificity of the immunoreaction, a control-staining procedure was performed by replacement of the primary antibody with NHS and/or PBS.

Some other sections were dehydrated in ethanol and embedded in metyl-metacrylate (Sigma-Aldrich, Milan, Italy). These samples were sectioned by a Leica SP1600 microtome saw (Leica, Wetzlar, Germany) provided with a water cooling system to both prevent overheating and remove dust, and ground to a final thickness of 50 μm. They were observed under a linear polarized light microscope (Photomicroscope Ultraphot - Zeiss, Oberkochen, Germany) as well as microradiographed at 8 kV and 14 mA by a micro radiographer (Ital Structures - Riva del Garda, TN, Italy) with high resolution film (Kodak, Cinisello Balsamo, MI, Italy).

The progress of the FS bone layer thickness during fish growth was analyzed using unfixed, undecalcified spines from 28 wild ABFT (71.5 to 163.0 cm *FL*). These spines, belonging to a collection kept at the University of Bari, were sectioned and processed according to Santamaria et al. (2009, 2015). In each spine section, the thickness of the compact bone layers were measured along five representative radial directions (Fig. 1) and averaged in order to obtain an index for the thickness of those layers. The correlations between compact bone layer thickness and *FL* were examined.

All measurements were performed on spine section images by an interactive function (i.e. measurements of operator selected distances between layer borders by a specific software function), by means of the image analysis software Quantimet 500 W (Leica, Wetzlar, Germany) with a digital camera DC 300 (Leica, Wetzlar, Germany) connected to a binocular lens microscope Wild M3C (Leitz, Heerbrugg, Switzerland).

3. Results

Cross sections of undecalcified FS samples showed that the spine was composed of an external compact bone zone and an inner trabecular bone zone. The former was characterized by alternating translucent and opaque bands (growth marks), whereas the latter contained many irregularly shaped apparent cavities delimited by anastomosing bone trabeculae (Fig. 1).

In fixed, decalcified sections, the FS appeared to be surrounded by a periosteal membrane composed of an external fibrous layer and an inner highly cellularized layer, containing active osteoblasts (see further) (Fig. 2a). Periosteal cells entered radially-directed bone canals that permeated the compact bone zone throughout its thickness. Bone canals, identifiable in both the histological sections (Fig. 2a, b, c) and the microradiographs (Fig. 2e, f), contained cells and blood vessels (Fig. 2). They were larger at the spine periphery (diameter = 9.8 μm ± 0.1 μm) and slightly tapered towards the inner side of the spine compact bone zone (diameter = 7.2 ± 0.1 μm). The mean distance between two adjacent bone canals decreased from the spine periphery (64.7 ± 1.5 μm) towards the trabecular bone zone (45.2 ± 0.8 μm). The compact bone zone (Fig. 2a, b, c, e, f) was composed by an outer layer (C_1), mostly made of non-lamellar bone (homogeneously dark under polarized light), and an inner layer (C_2), rich in osteons with variable in diameter central canals. Most osteons were clearly delimited by a cement line and some of them distinctly extended over previously deposited osteons, which indicates that they were secondary osteons (Fig. 2e, f). The

organic matrix of both C_1 and C_2 was PAS positive (Fig. 2a), orthochromatically stained with toluidine blue (Fig. 2b) and acidophilic (Fig. 2c).

The immunohistochemical staining with anti-SPARC antibodies labelled the cytoplasm of cuboidal/columnar cells of the inner periosteum constituting an almost continuous layer adhering to the irregular surface of the FS (Fig. 3a) as well as some flattened cells lining the internal surface of the bone canals (Fig. 3b). Tartrate-resistant acid phosphatase (TRAP) positive, mononucleated osteoclasts, were observed within the bone canals (Fig. 3c). The enzyme activity of TRAP released in the bone matrix, sometimes persisted after the active osteoclasts disappearance as a TRAP-positive red-violet band between old and newly formed bone tissues (Fig. 3d, e), which was evidence of the occurrence of bone remodeling.

The spine core consisted of lamellar bone trabeculae (Fig. 4), which contained rare osteons and delimited large cavities, occupied by adipocytes.

No differences were found in the above described overall spine microstructure in ABFT within the range of examined size.

The thickness indexes of C_1 and C_2 (IC_1 and IC_2, respectively) were best correlated to FL by the following regression equations, respectively: $FL = 801.9\ IC_1^{-0.040}$ and $FL = 0.3\ IC_2^{1.716}$ (Fig. 5). The regression equation of IC_1 on FL showed that IC_1 did not increase significantly as the animal grew (its slope, b, was not significantly different from 0; $P >> 0.05$). On the other hand, the increase of IC_2 with fish size was notable and its slope significantly differed from 0 ($P < 0.001$). Obviously, $IC_1 + IC_2$, representing the overall thickness of both outer and inner compact bone layers, also increased with fish size and was significantly different from 0 ($P < 0.001$). The dispersion of both IC_1 and IC_2 values increased with the animal size, which shows that the spine bone deposition/remodeling processes are rather uniform in young individuals but become more variable in older animals.

4. Discussion

The ABFT is a large pelagic fish, capable of trans-oceanic migrations, that shows unique locomotive performances (Carey and Teal, 1966; Carey and Lawson, 1973). Its fins play different roles during locomotion. As shown in other acanthopterygian fishes, the spiny dorsal fin, which is quickly erected during high-speed turns, plays an important role as a stabilizer by resisting fluid forces that might promote roll movements of the body (Lauder, 2006; Helfman et al., 2009).
The ABFT is a long-lived fish: according to the von Bertalanffy growth equation, it may reach 50 years of age (Santamaria et al., 2009). Its growth is continuous throughout life and may attain notable body length and weight, i.e. 315 cm *FL* (Hamre et al., 1971) and 685 kg (Sarà, 1969). Correspondingly with body growth, its skeletal structures, including fin spines, grow throughout its lifetime, so that both spine diameter and spine cross section surface are significantly correlated to fish size (Santamaria et al., 2009 and 2015, respectively).
The spine cross sections display, under transmitted light, an ordered series of alternate opaque and translucent marks, which correspond to a faster spring-summer and a slower autumn-winter growth, respectively (Cort, 1991; Megalofonou and De Metrio, 2000; Corriero et al., 2005: Santamaria et al., 2009). In the chinook salmon *Oncorhyncus tshawytsch*, the optical differences between translucent and opaque marks were assumed to be related to different calcium concentrations, higher in the translucent ones (Ferreira et al.,1999). In the present study, alternate translucent and opaque bands were observed only in the sections of plain (i.e. not decalcified) spines, under both light microscopy and microradiography, but not in the decalcified sections. This clearly corroborates that the alternating translucent and opaque bands only depend on the mineral component of the bone tissue, which component occurs in greater amount in the former than in the latter bands.

A wide array of bone tissues has been reported in different teleost fishes. The available literature on this subject is broad and somehow contradictory. For instance, osteocytic bone has been deemed

233 an ancestral trait, whereas advanced teleosts have been reported to display anosteocytic bone
234 (Parenti, 1986, 2008). However, osteocytes have been described in a number of phylogenetically
235 advanced teleosts (Kölliker, 1859; Meunier and Sire, 1981; Zylberberg et al., 1992; Hughes et al.,
236 1994). The presence of osteocytic bone in one of the most evolved tuna species is in agreement with
237 old observations on other scombroids (Kölliker, 1859) and corroborates that the cellular bone may
238 represent a derived state within percomorphs.
239 The present histological examinations of decalcified sections showed that the FS consists of
240 three different bone tissues: an external layer of non-osteonic bone, an intermediate layer of
241 compact lamellar bone with osteons, and an inner zone of lamellar trabecular bone. Kalish-Achrai et
242 al. (2017) showed that the dorsal fin spines of farmed blue tilapia *Oreochromis aureus* and common
243 carp *Cyprinus carpio* have a central canal containing blood vessels and adipocytes instead of the
244 inner trabecular bone observed in ABFT. This difference likely depends on the fact that the dorsal
245 fin spines of the ABFT are subjected to much more intense strain because of its long-distance
246 seasonal migrations and fast swimming features (Santamaria et al., 2009, 2015).
247 All the decalcified spine sections used in the present study were from adult individuals (their *FL*
248 was larger than the reported size at first sexual maturity; Corriero et al., 2005). Hence the
249 occurrence of active osteoblasts in the inner periosteal layer shows that the periosteal bone
250 apposition persists after puberty and seemingly throughout the lifetime. Thank to this apposition the
251 spine diameter progressively increases throughout lifetime as observed in age determination studies
252 (Cort, 1991; Megalofonou and De Metrio, 2000; Santamaria et al., 2009; Luque et al., 2014).
253 As newly formed primary non-osteonic bone is apposed at the FS surface, older primary bone is
254 reabsorbed from its inner side at a roughly similar rate, so that the overall primary bone thickness
255 does not increase as fish grows (IC_1 does not change significantly as fish size increases). Primary
256 bone is reabsorbed by mononucleated osteoclasts, visible in the bone canals. In turn, secondary
257 osteonic bone is apposed inside by osteoblasts, also present in bone canals, and its width
258 progressively increases, as shown by the IC_2 trend with respect to fish growth in length.

In this paper, we showed the occurrence of secondary osteons in the ABFT first dorsal spine bone. These osteons are delimited by a cement line and some of them overlap with each other. According to the established literature on bone structure (Amprino and Godina, 1955; Moss, 1961, 1965; Mori and Burr, 1993; Witten and Huysseune, 2009; Currey and Shahar, 2013; Currey et al., 2017), these phenomena are a clear evidence of a remodeled bone. Hence, the osteons in the ABFT dorsal spine bone displaying such features are to be interpreted as secondary osteons. In this respect, Atkins et al. (2014) stated that bone remodeling is strictly associated to bone micro-damages, mainly caused by mechanical stress; they ostensibly based such assumption on experimental evidences provided by Mori and Burr (1993). Indeed, the latter authors reported that bone remodeling occurs preferentially, hence not exclusively, in fatigue-damaged regions. Moreover, Currey et al. (2017) overtly recognized that "the current paradigm of bone remodeling – that it is a response to damage in the bone material caused by strain resulting from everyday loading – may not be right", contrary to the Atkins et al.'s (2014) statement. By the present research, we showed that, in the process of active bone remodeling, secondary osteons were deposited in the ABFT dorsal fin spine of both wild and captive individuals that did not suffer any particular mechanical stress. The occurrence of bone remodeling in the FS was further corroborated by the TRAP-positive bands situated at the boundary between primary and secondary bone; see Witten et al. (2001) about the interpretation of TRAP-positive bands. Therefore, we believe that the spine bone remodeling is just a physiological process that follows a seasonal pattern and continuously reorganize the spine internal structure (see Santamaria et al., 2015, about the seasonal changes in the spine internal structure).

The cancellous bone of the ABFT FS is characterized by trabeculae delimiting large cavities almost entirely filled with connective tissue rich in large adipocytes, as reported in bone tissues of other fishes (Meunier, 2002), including peculiar tuna scales (Wainwright et al., 2018). The FS trabecular bone expands during ageing (Santamaria et al., 2015) and, as a consequence, both the extent of bone cavities and the amount of adipocytes and lipids therein accumulated increase. The

accumulation of large quantities of lipids in ABFT spines may contribute to keeping bone mass low and may also participate in the energy storage system. It is known that ABFT need to accumulate large energy reserves in order to support the development of large amount of yolk-rich eggs during the reproductive season (Mourente et al., 2001), hence it is possible that fin spines play also a role in the mechanism of accumulation and mobilization of energetic reserves. Furthermore, when reared in captivity, ABFT showed a significant increase of spine bone resorption, with respect to wild individuals, which caused a further expansion of bone cavities (Santamaria et al., 2015). This phenomenon may be related either to metabolic alterations due to captivity-induced stress or to the high-fat diet provided to captive-reared ABFT in order to increase their body mass and muscle fat content (Mylonas et al., 2010). From the applied fisheries standpoint, the present results show that the ABFT dorsal fin spine growth bands occur only in the primary compact bone and that the disappearance in time of older growth bands, which incidentally makes fish age estimation difficult, is caused by the bone remodeling processes rather than the purported "spine core vascularization" (Cort, 1991; Megalofonou and De Metrio, 2000; Santamaria et al., 2009; Luque et al., 2014).

To conclude, the present study provides a description of the micro-anatomical characteristics of the first spine of the first dorsal fin of the ABFT. The types of bone tissue involved in the spine development and remodeling were identified and their organization within the spine was described. Lastly, this study contributes to a deeper understanding of well-known phenomena – such as the presence and the progressive disappearance of growth bands in fin spines and the resorption of bone in the spine core – which were never examined in detail before.

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416

Figure Captions

Fig. 1. Cross section of the first spine of the cranial dorsal fin of the Atlantic bluefin tuna showing the five radial directions for the measurements of compact bone layers' thickness (C_1, non-osteonic compact bone layer; C_2, osteonic compact bone layer; *T*, trabecular bone). Arrowheads indicate the inner edges of C_1 and C_2. Translucent (arrows) and opaque (asterisks) bands represent periodic events (growth bands or annuli).

Fig. 2. Micrographs of Atlantic bluefin tuna spine cross sections. a) Spine surrounded by a periosteal membrane made of an external fibrous layer (*) and an internal highly cellularized layer (arrow). Scattered osteocytes within their lacunae are visible as dark spots. PAS-hematoxylin staining. Magnification bar = 50 μm. Arrowheads: bone canaliculi. b) Spine section showing a peripheral zone composed of compact bone devoid of osteons (C_1) and an inner zone with osteons (C_2). Toluidine blue staining. Magnification bar = 200 μm. Arrowheads: bone canals; arrows: osteons. c) Spine section showing compact bone with matrix at different mineralization degree, osteocyte lacunae (arrowheads) and bone canals (arrow). Masson-Goldner trichrome staining. Magnification bar = 100 μm. d) Particular of the inner side of the compact bone zone showing a secondary osteon. Hematoxylin-eosin staining. Magnification bar = 25 μm. Arrows: osteocytes; asterisk: cement line. e) Microradiograph of a spine section showing the external compact and internal trabecular bone layers. Bone trabeculae are less mineralized (higher radiolucency) than the adjacent compact bone. Many bone canals as well as thin radiopaque bands (corresponding to light microscopy translucent bands) are visible. Osteon density increases from the spine periphery towards the spine core. Magnification bar = 300 μm. Arrowheads: bone canals; arrows: osteons; double arrows: growth bands (annuli). f) Microradiograph of the compact bone zone of a spine section crowded with secondary osteons. Osteonic bone is less mineralized than surrounding bone;

442 osteons are surrounded by cement lines. A dashed line encircles a group of overlapping osteons.
443 Arrowheads: bone canals; arrows: osteons. Magnification bar = 150 μm.

444 Fig. 3. Micrographs of Atlantic bluefin tuna spine cross sections. a) Osteoblasts of the inner
445 periosteum layer immunostained with anti-osteonectin antibodies. Magnification bar = 50 μm. b)
446 Anti-SPARC positive flat osteoblasts in a bone canal (arrowhead). Magnification bar = 50 μm. c)
447 Presence of flat mononucleated osteoclasts (red) in bone canals revealed by TRAP demonstration.
448 Magnification bar = 50 μm. d) and e) Red-violet band revealing the persistence of TRAP enzyme
449 activity between old bone layer (white asterisk) and newly formed bone layer (black asterisk) ,
450 which indicates the presence of bone remodeling. Magnification bars = 100 μm in d) and 25 μm in
451 e).

452 Fig. 4. Micrographs of the inner trabecular bone zone of Atlantic bluefin tuna spine cross sections.
453 a) Bone trabeculae delimiting cavities filled with adipocytes. PAS-hematoxylin staining.
454 Magnification bar = 100 μm. b) Bone trabeculae showing bone matrix at different mineralization
455 degree. Bone canals and an osteon (arrow) are visible. Masson-Goldner trichrome staining.
456 Magnification bar = 50 μm. c) Bone trabeculae under transmitted polarized light showing both dark
457 and light lamellae. Magnification bar = 100 μm.
458
459 Fig. 5. Regression curves of thickness indices of compact bone on fork length (*FL*). a) Index for
460 compact non-osteonic layer (IC_1). b) Index for compact osteonic layer (IC_2). c) overall index (IC_1 +
461 IC_2). The regression equations with related correlation coefficients (r) and significance levels (P_r)
462 are reported in the graphs.