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The natural armors of fish: A comparison of the lamination pattern and structure of scales

Sandra Murcia[a], Ellen Lavoie[b], Tim Linley[c], Arun Devaraj[c], E. Alex Ossa[d], D. Arola[a,*]

[a] *Department of Materials Science and Engineering, University of Washington, Seattle, WA, USA*
[b] *Molecular Analysis Facility (MAF), MolES, University of Washington, Seattle, WA, USA*
[c] *Physical and Computational Sciences Directorate, Pacific Northwest National Laboratory, Richland, WA, USA*
[d] *Production Engineering Department, School of Engineering, Universidad Eafit, Medellín, Colombia*

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ABSTRACT

Fish scales exhibit a unique balance of flexibility, strength and toughness, which is essential to provide protection without encumbering locomotion. Although the mechanical behavior and structure of this natural armor are of recent interest, a comparison of these qualities from scales of different fish species has not been reported. In this investigation the armor of fish with different locomotion, size and protection needs were analyzed. Scales from the *Arapaima gigas*, the tarpon (*Megalops atlanticus*) and the carp (*Cyprinus carpio*) were compared in terms of the stacking sequence of individual plies and their microstructure. The scales were also compared with respect to anatomical position to distinguish site-specific functional differences. Results show that the lamination sequence of plies for the carp and tarpon exhibit a Bouligand structure with relative rotation of 75° between consecutive plies. The arapaima scales exhibit a cross-ply structure, with 90° rotation between adjacent plies. In addition, results indicate that the volume fraction of reinforcement, the number of plies and the variations in thickness with anatomical position are unique amongst the three fish. These characteristics should be considered in evaluations focused on the mechanical behavior.

1. Introduction

Engineered composites of ceramics, metals and polymers are designed to achieve a combination of specific stiffness and strength that are not available from the individual constituents alone. Interestingly, the biological structural materials in nature are composites, and have developed over millions of years of evolution. These structural materials generally follow a common pattern in combining organic (soft) and inorganic (hard) materials, which are organized according to a hierarchical architecture to achieve incredible combinations of strength and toughness (Currey, 1999; Barthelat, 2007, 2013; Meyers et al., 2008; Ortiz and Boyce, 2008). Despite the consistency in the constituents amongst most of Nature's composites, by virtue of variations in their microstructure they achieve a range of properties that rivals that of engineered materials.

One area of research on natural structural materials is focused on the mechanical behavior of dermal armors like the ones found in the seahorse (Porter et al., 2013, 2015), turtles (Chen et al., 2015), alligator (Sun and Chen, 2013) and fish (Ikoma et al., 2003; Bruet et al., 2008; Torres et al., 2008; Lin et al., 2011; Chen et al., 2012; Zhu et al., 2012; Allison et al., 2013). In the case of fish with elasmoid scales, their armor is flexible and enables locomotion without sacrificing the protective capacity of less evolved armors. Elasmoid scales are constructed from a combination of type I collagen fibers and apatite (Lin et al., 2011; Garrano et al., 2012; Zhu et al., 2012), which are the two constituents that are widely encountered in natural mineralized systems. Unlike mineralized hard tissues like bone, dentin and enamel, which possess mineral content exceeding 60% by weight, elasmoid scales exhibit a much lower degree of mineralization and strong spatial gradients that appear to be unique amongst species (Zylberberg and Nicolas, 1982; Murcia et al., 2016).

Elasmoid scales can be divided into three different layers, which correspond to the relative degree of mineralization (Fig. 1a). The outermost layer or “limiting layer” (LL) is a mineral matrix with randomly dispersed thin collagen fibers (30–50 nm in diameter) (Zylberberg and Nicolas, 1982; Zylerberg, 1985; Sire and Huysseune, 2003). It is the highest mineralized region in the scale and acts as the first barrier against penetration. The elasmodine, or basal plate, is a laminate consisting of discrete plies of unidirectionally aligned collagen fibers with a diameter of between 100 and 160 nm (Zylberberg and Nicolas, 1982; Garrano et al., 2012) The elasmodine can be further divided into the external and internal layers according to the mineral

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[*] Correspondence to: Department of Materials Science and Engineering, University of Washington, Roberts Hall, Box 352120, Seattle, WA 98195, USA.
E-mail address: darola@uw.edu (D. Arola).

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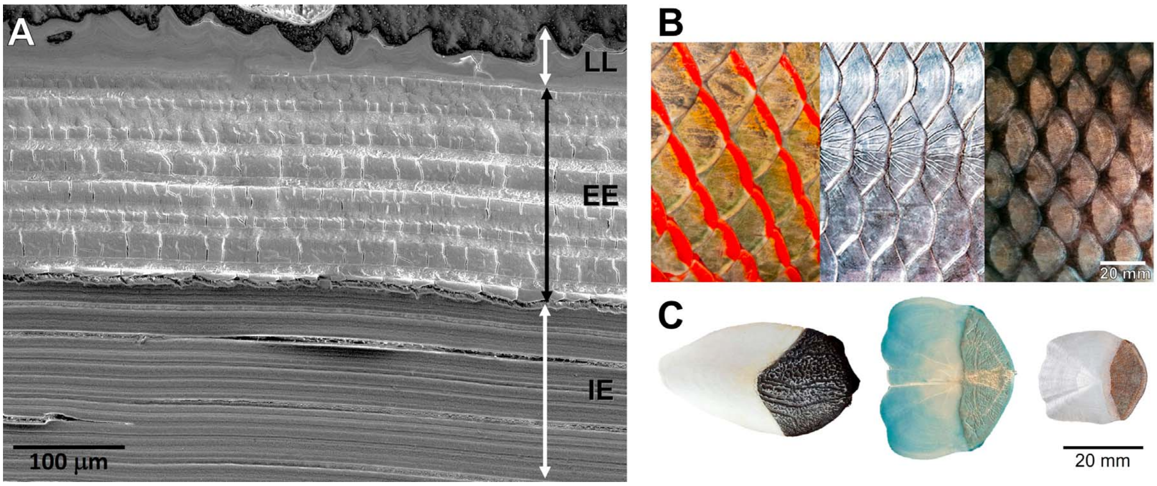

Fig. 1. A comparison of the scales for the three Teoleost. A) A cross sectional SEM image from the scale of the Carp denoting the three distinct regions including the limiting layer (LL), external elasmodine (EE) and internal elasmodine (IE). B) Scale patterns for the *A. gigas*, tarpon and carp (left to right). C) A comparison of single scales from the arapaima, tarpon and carp (left to right). As evident, the overlap between scales, their geometry and size are different for each fish in spite of having similar scale patterns.

content. The collagen matrix of the external elasmodine (EE) is reinforced by platelets of apatite similar to those in bone (McNally et al., 2012; Schwarcz et al., 2014). The EE is also critical to the puncture resistance of fish scales, which dissipates energy by brittle fracture once the LL has undergone gross failure, resulting in delamination of the scale (Dastjerdi and Barthelat, 2015). Thereafter, the collagen fibers of the internal elasmodine (IE) undergo stretching, rotation and delamination (Zimmermann et al., 2013; Yang et al., 2015). Due to its composition and the host of contributing mechanisms, the IE undergoes substantial deformation and contributes to the large strain to failure (≥15%) of the scales (Ikoma et al., 2003; Garrano et al., 2012; Zhu et al., 2012; Yang et al., 2014; Murcia et al., 2016).

The mechanical behavior of fish scales has been the primary focus of most investigations in this area. Studies have been reported on the puncture resistance (Zhu et al., 2012, 2013), hardness (Lin et al., 2011), tensile strength (Ikoma et al., 2003; Lin et al., 2011; Garrano et al., 2012; Zhu et al., 2012; Zimmermann et al., 2013; Torres et al., 2014; Yang et al., 2014; Gil-Duran et al., 2016) and resistance to fracture (Yang et al., 2013; Dastjerdi and Barthelat, 2015; Murcia et al., 2015). While some have identified that the scales exhibit mechanical anisotropy, the reported degree of anisotropy is not consistent (Table 1). For example, in an evaluation of scales from the *Morone saxatilis* or striped bass, Zhu et al. (2012) found that the maximum strength (~60 MPa) was perpendicular to the longitudinal axis of the fish (Zhu et al., 2012). In contrast, the maximum strength of scales from the *Arapaima gigas* (~30 MPa) (Yang et al., 2014) and *Megalops atlanticus* (~20 MPa) (Gil-Duran et al., 2016) is reportedly parallel to the longitudinal axis of the fish. Moreover, in scales from *Cyprinus carpio,* the anisotropy was found to depend on anatomical position, with scales near the head exhibiting higher resistance to fracture in the longitudinal direction than in other orientations and locations (Murcia et al., 2015).

Differences in the reported mechanical behavior and anisotropy of scales amongst previous studies could be attributed to many factors. The most likely contribution is the unique microstructure of the scales from each fish, and their functional requirements in relation to their habitat and principal predators. But in comparison to the efforts focused on mechanical behavior, the microstructure of these natural composites and important differences between fish have not been explored in detail. Therefore, the objective of this study was to evaluate and compare scales from three different teleost fish and develop a quantitative description of the microstructure that addresses important details contributing to their mechanical behavior.

2. Material and methods

Scales were obtained from the bodies of three different fish including the *Cyprinus carpio* (i.e. freshwater carp), the *Megalops atlanticus* (i.e. tarpon), and the *Arapaima gigas* (i.e. Pirarucú). The carp represents the control for our study. Being raised in captivity, this fish currently has no natural predators and special needs for locomotion or protection. The *Megalops atlanticus* (Atlantic tarpon) inhabits both fresh and salt waters of the Atlantic. With a top speed of near 70 kph it is one of the ten fastest fish of the world. Speed is important for evading predators and feeding. Lastly, the *A. gigas* is one of the largest freshwater fish in the world and inhabits the Amazon River Basin in South America. In previous reports in this field the *A. gigas* has been identified as "arapaima", and to maintain consistency it will be regarded as such here. The scales of the arapaima are credited with protecting this fish from attacks of the piranha (Meyers et al., 2012) and have enabled it to survive for centuries.

All three of these fish are teleost and possess elasmoid scales. Carp scales were extracted from farmed fish marketed as East Asian carp. In comparison, the tarpon and the Arapaima were wild fish captured in the Caribbean Sea and the Amazon River, respectively. For the studied fish, no additional information was available for record. One of the differences between these three teleost is their overall size at maturity (Ibáñez and O'Higgins, 2011; Levin et al., 2012; Baudouin et al., 2015).

Table 1
Summary of mechanical and structural properties previously reported for elasmoid scales.

Fish	Direction of maximum strength	Tensile strength (MPa)	Ply orientation	Source
Pagrus major	–	~90	Orthogonal	Ikoma et al., 2003
Cyprinus carpio	–	~30 (head scales)	–	Garrano et al., 2012
Morone saxatilis	perpendicular	~60	Orthogonal	Zhu et al., 2012
Megalops atlanticus	parallel	~20	Boulligand (60°)	Gil-Duran et al., 2016
Arapaima gigas	parallel	~30	Boulligand (angles between adjacent lamellae vary)	Yang et al., 2014

According to their weight (3.4, 5.5 and 20 kg, respectively) and length (53, 75 and 157 cm, respectively) the fish ranged between 3 to 6 years of age, indicating that each had reached maturity. In order to compare the scales objectively, it was necessary to have a similar reference system beyond size and weight. During the process of growth, a specific number of scales emerge from the epidermis covering the body of the fish. Afterwards, the existing scales increase their size during the maturation of the fish (Levin et al., 2012; Metz et al., 2012). Thus, the scale count along the lateral line was recorded and served as a reference in the process of extracting the scales. There were 38, 45 and 55 scales on the lateral line for the carp, tarpon and Arapaima, respectively.

Scales were extracted from the body of each fish within three characteristic regions, including adjacent to the head, mid-length and near the tail (Fig. 2a). To be consistent, these regions were defined at distances from the gill plate of 15%, 40% and 80% of the total number of scales along the lateral line. In recognition of possible variations in the scale structure, four scales were obtained in each region as shown in Fig. 2b; scales 1 and 3 are along the lateral line of the fish, and scales 2 and 4 were above and below the lateral line, respectively. After extraction, the scales were stored in Hanks Balanced Salt Solution (HBSS) at room temperature and were fixed within a week of harvesting the fish for further evaluation.

The comparison of scales from the three fish included an evaluation of the ply orientations, the lamination stacking sequence and the overall microstructure. As such, the scales were divided into two equal parts along the centerline. Half of the scale was used for conducting the ply rotation measurement and the remaining half was used to evaluate the cross-section, including the overall thickness, number of plies, thickness of the individual sections (i.e. limiting layer and elasmodine), and the depth of mineralization from the limiting layer. In recognition of potential variations in thickness of the scales (Zhu et al., 2013), cross sectional samples were cut where the thickness is most uniform as indicated in Fig. 2c. The scales were fixed by submerging them in 2% glutaraldehyde buffered with 0.1 M sodium cacodylate with pH=7.2 for 4 h. They were then rinsed in 0.1 M sodium cacodylate buffer followed by post-fixation in 1% osmium tetroxide buffered with 0.1 M sodium cacodylate for 2 h. After rinsing, the scales were dehydrated through an ascending ethanol series from 50 to 95%, followed by 100% of acetone and instant dehydration in 2,2- dimethoxypropane (DMP) for 5 h. Instant dehydration and rinsing with 100% acetone was followed by infiltration of the scales in Mollenhauer resin. To expose the microstructure, the cross-sectioned samples were trimmed and faced with a microtome using a glass knife (Leica EM UC6 Ultramicrotome, Vienna, Austria). To evaluate the ply orientations and rotations, the natural curvature of the scales after dehydration resulted in a bias angle that allowed the measurement of all the layers within a single viewing plane. The samples for fiber orientation measurement were polished with SiC abrasive paper from mesh numbers of #800 to #4000. Final polishing was performed with a liquid suspension of 3 µm diamond, followed by a liquid suspension of 0.04 µm Al_2O_3.

Samples were sputtered with Au/Pd and the microstructure of scales within each region of evaluation was examined with a scanning electron microscopy (SEM) in secondary electron imaging mode (JEOL, model JSM- 6010PLUS/LA, Peabody, MA). Final measurements of the ply rotation angles and distribution of ply thickness were obtained by post-processing of the SEM images using cellSens (Olympus, Tokyo, Japan). All angles were measured clockwise (cw) from the longitudinal axis of the fish, which was defined as the reference (Fig. 2c).

3. Results

A cross-section view of a representative scale from the tarpon is shown in Fig. 3a. The limiting layer and the individual plies of the elasmodine are clearly evident. There is also a gradient in the degree of mineralization that is apparent beneath the LL, which is reflected by the change in contrast in the micrograph with depth and divides the elasmodine into two distinct regions. A more detailed view of the lamination pattern of the elasmodine for representative scales from each fish is shown in Fig. 3b. According to a comparison of the micrographs in this figure, there are two important findings to highlight. Firstly, the average ply thickness in the three scales is not equivalent. For instance, the average ply thickness in the Arapaima is approximately 30 µm and is nearly five times that of the carp. Secondly, the carp and tarpon scales appear to share a similar arrangement of adjacent plies, resulting in a stacking sequence that repeats every five plies. In comparison, the Arapaima scales have a stacking sequence that repeats every two plies.

A quantitative comparison of important microstructural characteristics of the scales is presented in Figs. 4 and 5. The overall total thickness of the scales is shown in Fig. 4a and was found to be significantly different between the fish analyzed. Although the arapaima scales showed the greatest thickness overall, they did not exhibit the greatest thickness in all three regions of the body as the thickness changed with anatomical position. For the carp and the arapaima, the scales obtained from the head and middle regions were significantly thicker than the tail scales. In contrast, the head scales for the tarpon were significantly thinner than scales from the middle and the tail.

The overall thickness of the scale is an important feature but the depth of mineralization is the unique characteristic that makes the elasmodine special. As shown in Fig. 3a, the macrostructure of the scales can be divided into three different regions (or layers) according to the differences in composition and mineral content. The thickness of the LL is shown in Fig. 4b. It can be seen that the LL thickness of scales from the carp and tarpon is similar, and that it does not undergo large changes in thickness with anatomical position. The LL of the arapaima is the greatest of the three fish, and its thickness in the head and middle scales is greater than that of scales from the tail. Note that the LL of the

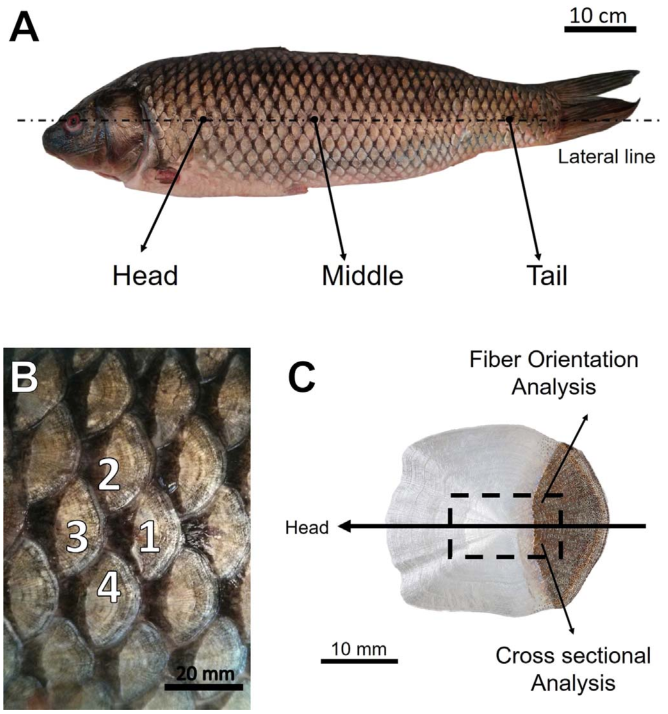

Fig. 2. Details of the scale samples evaluated, including preparation and analysis. A) The three anatomical positions of the extracted scales (head, middle and tail). In each location, four adjacent scales were extracted (B, head region). Scale numbers 1 and 3 are on the lateral line of the fish. C) An extracted scale with description of the locations used for analyzing the fiber orientation and cross section. Note that the 0° fiber orientation corresponds to the longitudinal axis of the fish and all angles are defined according to clockwise rotations from this axis.

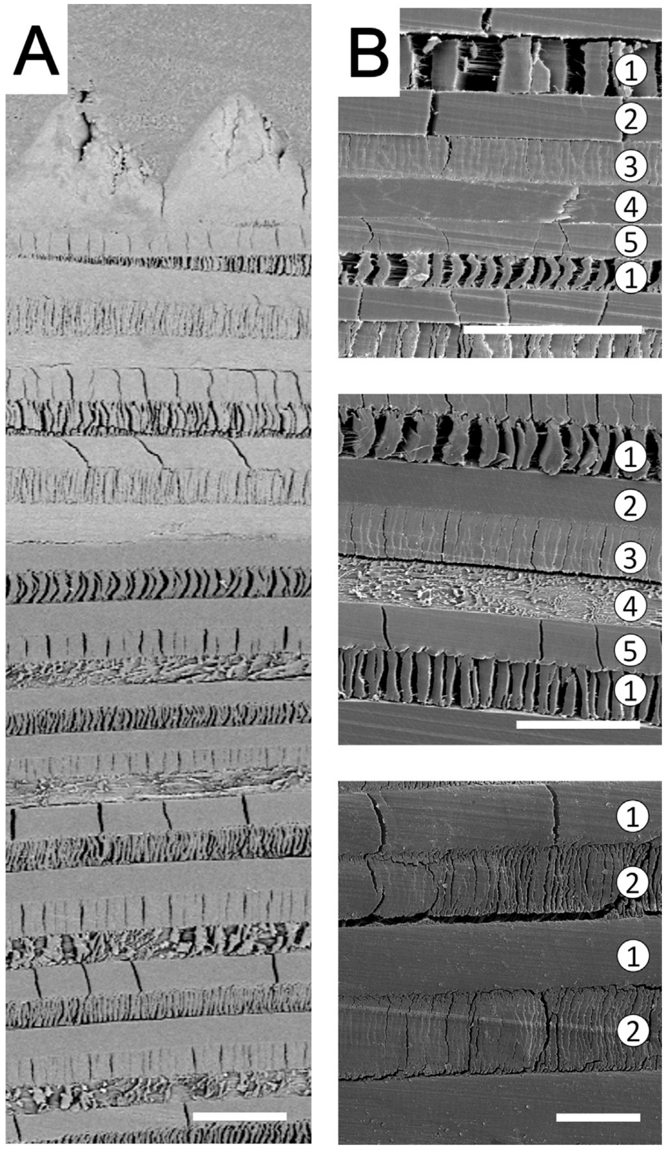

Fig. 3. Evaluation of the collagen fiber ply orientations of the scales after fixation and dehydration. A) Backscattered SEM image from a tarpon scale from the middle region. As evident from the decrease in brightness from top to bottom, there is a reduction in relative mineral content from the limiting layer to the internal elasmodine. B) Stacking sequence of the plies for the carp, tarpon and arapaima (from top to bottom). The carp and tarpon share similar stacking patterns with an apparent replication every five plies, suggesting a Bouligand-type or a double twisted plywood structure. In contrast, for the arapaima scale the stacking pattern exhibits a replication every other ply. Scale bar=50 μm.

arapaima scales is between 2 to 6 times thicker than that of the other two fish in all three regions of evaluation.

In a previous evaluation regarding the fracture resistance of scales from the carp it was found that the ratio of the external elasmodine to the total elasmodine thickness (EE ratio) was important (Murcia et al., 2015). The EE ratio represents the relative number of plies in the elasmodine that are more highly reinforced by apatite. In Fig. 4c the EE ratio is shown for each fish as a function of the anatomical position. As evident in this figure, the arapaima scales possess an EE ratio that is significantly greater than that of both the carp and tarpon. Note that scales from the head of the carp and arapaima have a much higher percentage of mineralized plies than scales of the middle and tail regions. In contrast, for the tarpon the greatest number of mineralized plies was found in scales of the tail, although the difference was not significant from the other anatomical regions sampled.

In engineered fiber reinforced composites the number of individual plies and their thickness are important to the mechanical behavior of the laminate (Jones, 1998). Consequently, these qualities of the fish scales were evaluated and are shown in Fig. 5. The total number of collagen fibril plies in scales of each fish is shown in Fig. 5a. Interestingly, although scales of the carp and tarpon were much thinner than those of the arapaima (Fig. 4a), they both possessed a larger number of plies (Fig. 5a). A comparison of the ply thicknesses within the external and internal elasmodine layers is shown in Figs. 5b and c, respectively. For both of these regions the average ply thickness was relatively similar over the entire body of each fish. Akin to the results shown in Fig. 4a, the arapaima scales have the thickest plies overall within both the EE and IE. In addition, there was a significant difference in the ply thickness between the three fish studied. As evident from Figs. 5b and c, the plies of the arapaima scales are roughly 2 and 4 times thicker than those of the tarpon and carp scales, respectively. There are additional important features evident from comparisons of Fig. 5b and c. For example, the EE plies are generally thicker than those of the IE, except for scales of the head and middle regions of the arapaima and the scales of the tail in the tarpon, where there were no significant differences. The arapaima and carp had increases of nearly 50% and 150% in the EE ply thickness, respectively.

Based on a comparison of the cross-sectioned scales (Fig. 3b) it was apparent that there are differences in the ply stacking sequence of the elasmodine for the three fish. The ply rotation angles were determined from a top view of the scales and sequential polishing. One of the major concerns to address was whether fiber orientations remained constant through the whole scale. To this end, measurements were performed over a large area of the scale with high and low magnification, and revealed that the fibers were primarily straight and parallel over the entire ply. In a previous report on the scales from striped bass a cross-ply evaluation was performed on the elasmodine after removal of the mineralized layers (Zhu et al., 2012). If plies of the external elasmodine were revealed by a pull out method, that could result in disruption and artifacts. To avoid this concern, all of the layers where exposed through the thickness of the scales by the polishing approach. Results from this analysis can be seen in Fig. 6. Specifically, micrographs of exposed plies for carp and tarpon scales are shown in Figs. 6a and b, respectively. For the scales of these two fish it was found that the fiber orientation of adjacent plies was rotated an average of 75° with respect to one another, which results in 5 increments of rotation to achieve a complete revolution. This Bouligand type structure forms a helix through the thickness of the scale. In comparison, adjacent plies in the arapaima scales exhibited a rotation of approximately 90°, which is equivalent to a cross-ply orthogonal laminate.

Fig. 7 shows the primary ply orientations in the tarpon scales and relative angle of rotation in terms of the cumulative frequency distribution. The ply orientations are shown in Fig. 7a for the three regions of evaluation. This figure enables a visual comparison of the ply distribution with respect to the longitudinal axis of the fish (0°). Each polar plot presents results for the four scales evaluated in these regions. Interestingly, the ply orientations were not aligned with the longitudinal axis of the fish. Surprisingly, scales of the head and tail regions do not appear to exhibit a preferential orientation. In contrast, scales from the middle region exhibited a preferential alignment with respect to the longitudinal axis of the fish. The average ply rotation angles for scales of the three regions are shown for all three fish in Fig. 7b. Although the tarpon scales did not share a common preferential orientation in the three regions of evaluation, the average ply rotation between adjacent plies was approximately the same. The average rotation angle for the scales of the carp and tarpon is 75° and in all three regions of the body. For the arapaima, the average ply rotation was approximately 90°.

A comparison of the cumulative frequency distribution of the ply orientations for scales of the three fish is shown in Fig. 8. These results were obtained for the head scales and serve as an illustration that is

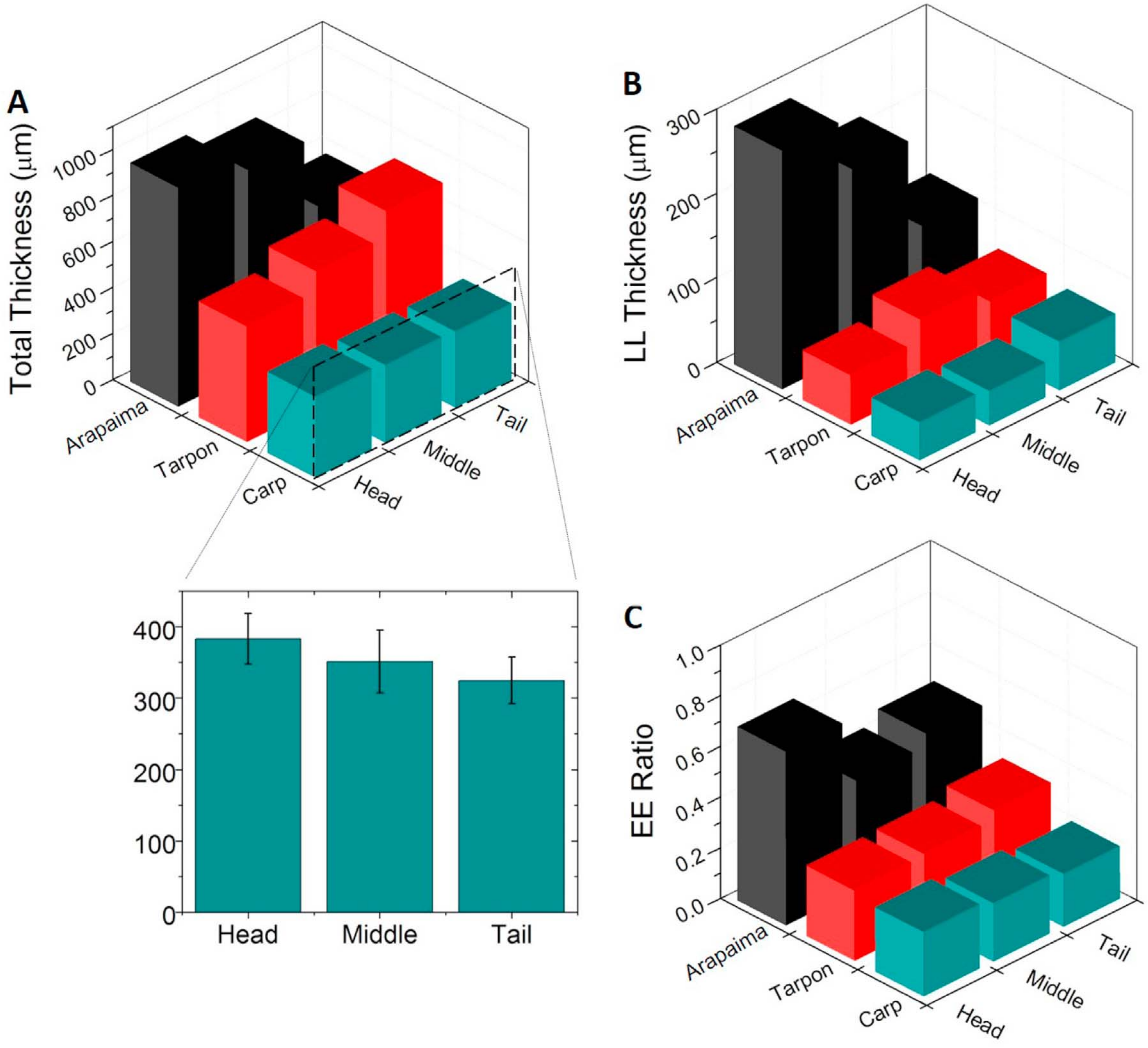

Fig. 4. A comparison of the microstructural characteristics of the carp, tarpon and arapaima scales in the three anatomical positions. A) Total thickness, B) Limiting layer thickness and C) External elasmodine ratio, which represents the ratio of the EE thickness to the total elasmodine thickness. All measurements where conducted after fixation and dehydration of the scales.

representative of the other anatomical locations. The ply orientation distributions in Fig. 7b show that the carp and tarpon share the same rotation angle between adjacent plies. However, the distributions in Fig. 8 reveal that the plies of scales from the three regions have different preferred alignment with the longitudinal axis of the fish. Scales from the head and tail regions of the carp had a preferential offset angle from the longitudinal axis of the fish of 30° and 40°, respectively. In comparison, the tarpon scales did not exhibit a preferred alignment of the ply orientations for these same locations. In the case of the arapaima, the plies of scales from the head were aligned primarily with the 0°/90° orientation. However, in the remaining two locations, no preferred alignment of the plies was evident.

4. Discussion

Over recent years the quest to develop tougher and more durable materials has led to explorations involving natural dermal armors. Elasmoid fish scales are an attractive candidate within this group of materials, particularly for applications that require both flexibility and resistance to penetration. Early efforts focused on modeling and mimicking the mechanical response and structure have treated elasmoid scales as uniform plates imbricated in a soft substrate (Browning et al., 2013; Liu et al., 2016). This assumption, although valid for an initial approach, does not reflect the complexity of the laminate structure. In a recent study, (Funk et al., 2015) fabricated a biomimetic fish skin material where the importance of the overlapping of the scales and the mechanical properties of the constitutive materials to the overall mechanical behavior of these dermal armors was shown. Numerical (Browning et al., 2013; Martini and Barthelat, 2016) and analytical (Vernerey and Barthelat, 2010; Rudykh and Boyce, 2014) models have been used to analyze the flexural response of fish skin. However, no study has been reported that explores the mechanical behavior of elasmoid scales where they are regarded as a composite material. For fish scales to inspire future generations of structural materials, a better understanding of the relationships between scale structure, mechanical behavior and their contribution to performance is essential. In this investigation elasmoid scales from three different teleost fish were evaluated, and a comparison of their microstructure was performed to identify important differences. Overall, results of the evaluation showed that the scales of the three fish exhibited unique characteristics, and that some of the differences between fish are dependent on the anatomical location.

In addition to the unique size and shape of the scales, one of the most notable differences amongst the three fish was the change in scale thickness across the body. Scales of the carp and arapaima decreased in thickness from the head to tail, whereas those of the tarpon increased in thickness in this direction (Fig. 4a). Garrano et al. (2012) noted that variations in the mechanical properties of carp scales over the body

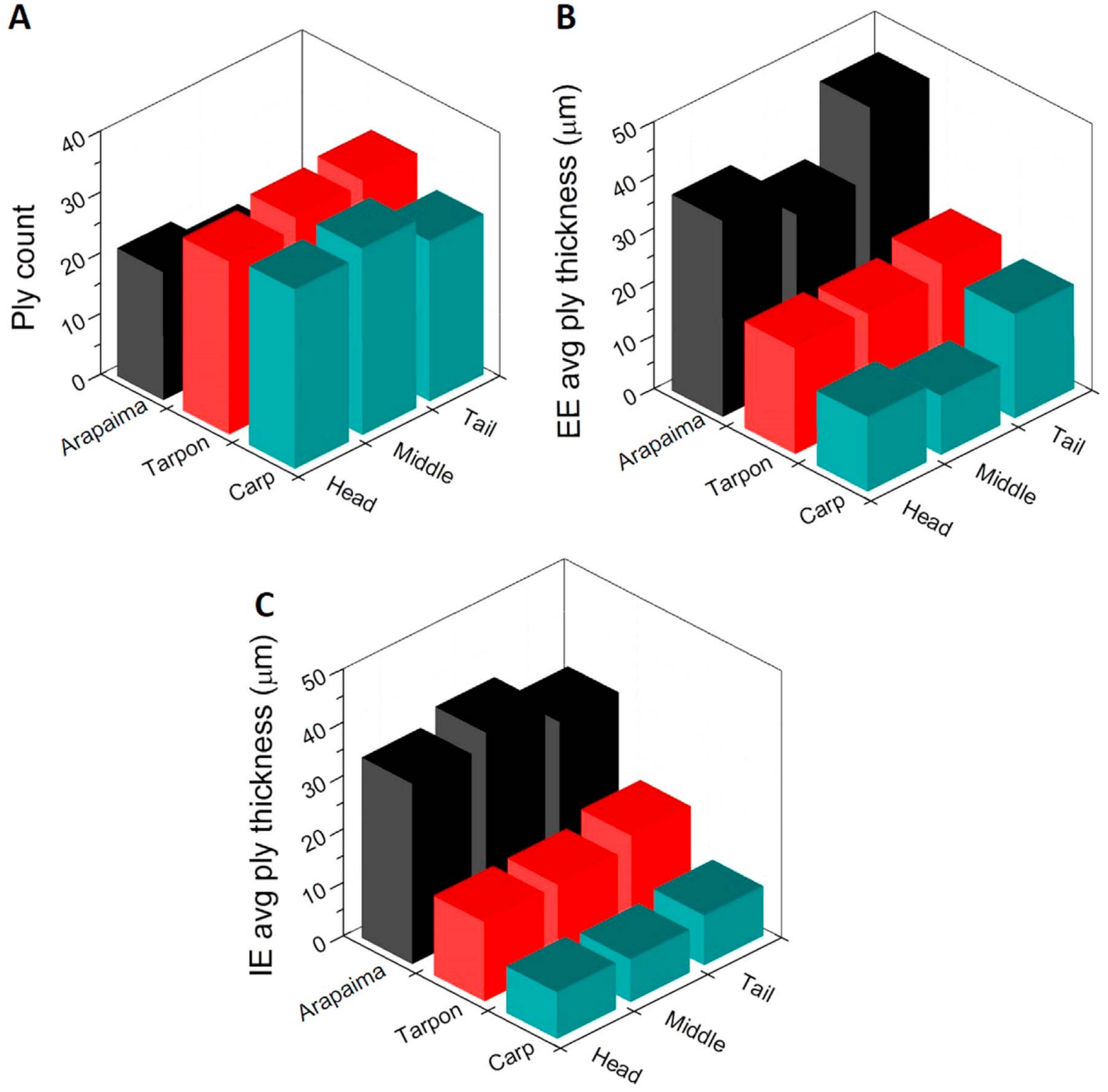

Fig. 5. Qualities of the collagen fibril plies of the elasmodine for the carp, tarpon and arapaima fish. A) Number of plies in the elasmodine, B) Average ply thickness of the external elasmodine and C) Average ply thickness in the internal elasmodine. All measurements were conducted after fixation and dehydration of the scales.

were related to the region-specific functional and protective needs. For instance, the strength and elastic modulus of the scales were greatest in the head and middle regions, which are areas with vital organs. The scale thickness was also greater in these regions, which would increase the level of protection achieved. A later study concerning the fracture resistance of carp scales suggested that the spatial variations in thickness could be due to the squamation sequence (Murcia et al., 2015).

Squamation refers to the generation of scales from the dermis and influences the relative period of time that the scales have undergone external growth. This process starts in different anatomical regions and follows a designated pattern over the body. A mechanical stimuli is apparently responsible for the preferential development of scales in specific anatomical regions (Sire and Akimenko, 2004). Once started, the growth process of the scales involves an increase in projected area and thickness to cover the body of the fish (Bereiter-Hahn and Zylberberg, 1993; Zhu et al., 2012). Indeed, there is a direct correlation between these two variables (Fig. 9), which is essentially independent of fish species.

The most common squamation sequence is from anterior to posterior, which occurs in the carp and arapaima. There are cases where the process starts from the posterior and progresses to the anterior, including the zebra fish (Sire and Akimenko, 2004) and the tarpon (Tsukamoto and Okiyama, 1997). Indeed, the thickness of the tarpon scales follows that trend (Fig. 4a). One concern with this interpretation is that the entire squamation process (from head to tail) extends over roughly 40 to 80 days depending on the fish (Park and Lee, 1988; Tsukamoto and Okiyama, 1997). With the expected age of the three fish evaluated ranging over 3 to 6 years, it is difficult to attribute the entire anatomical variations in scale thickness to the range in number of days of squamation. Nevertheless, ontogenetic effects early in life can be carried forward for extended periods of time, sometimes permanently (de Vrieze et al., 2011). Clearly the factors contributing to spatial variations in scale thickness and its importance to the performance of dermal armors will require further study.

Differences in scale thickness between the three fish are reflected in all three layers, but are most prominent in the LL (Fig. 4b). Zhu et al. (2012) described the LL as the first barrier to penetration, highlighting that it dissipated contact energy by brittle fracture and mitigated the stresses transmitted to the elasmodine by redistributing them over a large area. Due to the relatively slow speed of the arapaima and the aggressiveness of its predators (including crocodiles and piranha), a

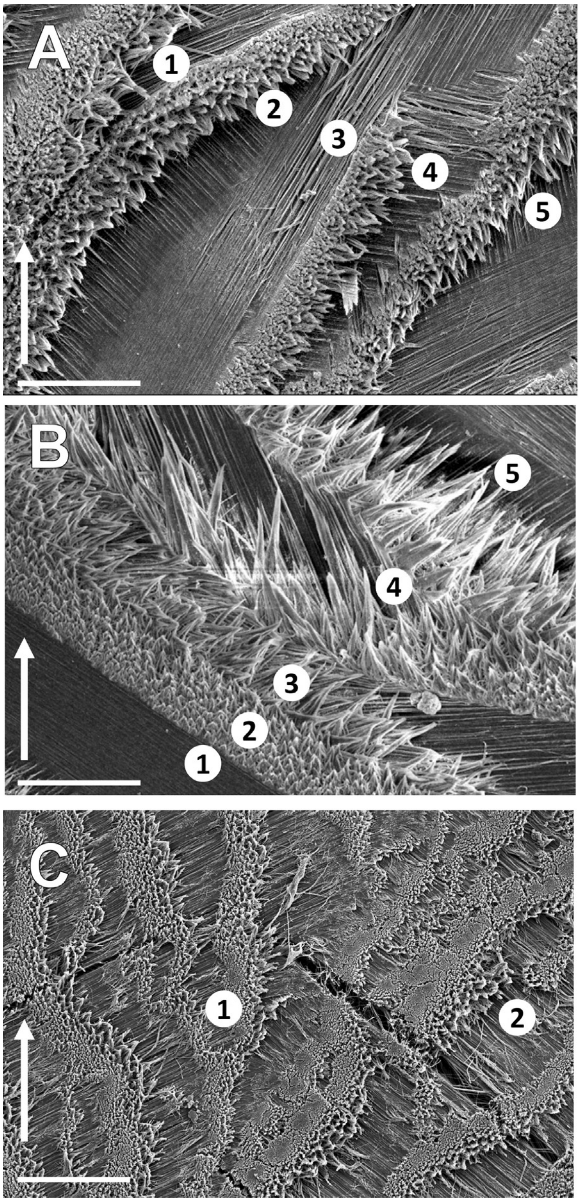

Fig. 6. Fiber orientations of the individual plies. A) Carp. B) Tarpon, C) Arapaima. The arrow in the SEM images represents the direction of the lateral line of the fish and points towards the head of the fish. Scale bar=50 µm.

larger LL thickness could be expected to achieve higher puncture resistance. Nevertheless, there was also a marked difference in the LL thickness in the arapaima with anatomical position, which was not reflected in this highly mineralized layer of the other fish.

One possible contribution to the unique spatial variations of the LL for the three fish is their body movements. Locomotion patterns in fish are classified according to which parts of the body are involved in propulsion and the degree of movement (Helfman et al., 2009). About a dozen general types of fish locomotion are recognized, but two are of interest here. Akin to the subcarangiform species, the arapaima undulates its body into less than one full wavelength, but more than a half wavelength. In comparison, both the carp and tarpon are carangiform species and undulate their bodies into shallow waves less than one-half wavelength. They achieve faster locomotion by involving only posterior segments of the body in wave generation (Hart and Reynolds, 2008). One major disadvantage in the subcarangiform is that the attack angle of the tail changes constantly as the tail sweeps back and forth, producing less thrust at low angles and creating more drag at greater angles (Helfman et al., 2009). Perhaps the pronounced decrease in the LL thickness of arapaima scales from head to tail is related to the larger movement of the mid and tail sections during swimming. A lower LL thickness in the posterior region would reduce the stiffness of the scales and facilitate this type of body motion. That would diminish the stored energy in the scales of these regions and its potential contribution to the energy needed for locomotion (Vernerey and Barthelat, 2014).

The elasmodine of the three fish possesses a laminated structure, and both the thickness and orientations of the plies were quantified. There was a significant increase in ply thickness from the internal to the external elasmodine in the scales of all three fish. Scales of the arapaima had the greatest average ply thickness overall (Figs. 5b and c). Furthermore, the EE ratio was substantially greater in scales of the arapaima than the other two fish (Fig. 4c). These characteristics are attributed to the degree of mineralization of the collagen plies and are important to the scale properties. For instance, in a comparison of reported mechanical properties for scales of the carp (Garrano et al., 2012), arapaima (Yang et al., 2014) and tarpon (Gil-Duran et al., 2016), the maximum tensile strength is relatively similar for the three fish (~25 MPa). Scales of the carp and tarpon possess similar elastic modulus (0.4 vs 0.3 GPa, respectively), but less than half the value of scales from the arapaima (0.86 GPa). The elastic moduli measurements are very consistent with the trends in EE ratio (Fig. 4c); the carp and tarpon scales have EE ratios that are approximately only half that of the arapaima. The elastic modulus is clearly influenced by the number of more highly mineralized plies. The distribution in EE ratio for the arapaima (Fig. 4c) suggests that scales of the head region should exhibit substantially larger modulus than the middle and tail regions. Yet, an analysis of spatial variations in arapaima scales has not been reported.

Zylberberg and Nicolas (1982) studied the mineralization of scales and found that it primarily occurs in two stages. The LL is mineralized first and occurs by cellular processes, starting with the interfibrillar nucleation of crystals that cluster and form needle-shape crystals until a highly mineralized matrix is formed. Crystals in the LL are arranged in a radiating manner (Sire et al., 1997; Lin et al., 2011) with pattern of mineral deposition that is regarded as spheritic mineralization (Meuniee, 1984). The mineralization of the elasmodine follows the LL and is dependent on the fiber organization and contributing mechanisms. The mineralization front progresses from the LL within the elasmodine and its extent is reflected by the EE ratio (Fig. 4c). The depth of mineralization is a consequence of the distribution of the elasmocytes (mineralizing cells similar to the osteocytes) into the elasmodine, as well as species-specific differences in mineralization (Meuniee, 1984; Sire and Huysseune, 2003). The elasmodine of the arapaima and tarpon have elasmocytes that extend along the collagen fibers. That results in a more uniform progression of the mineralizing front (Meuniee, 1984). Elasmocytes are present throughout the whole scale thickness in the arapaima, whereas in the tarpon they are only located in the first few layers. Thus, the elasmodine of the arapaima scales appears to be mineralized more extensively due to the distribution of the elasmocytes throughout the thickness.

In cyprinidae or carp, additional thin collagen (TC) fibers (≤30 nm) can control the depth of mineral deposition (Zylerberg, 1985). The TC fibers are oriented perpendicular to the scale surface and connects the dermis of the fish with the interior of the scale from the LL to the elasmodine (Sire and Huysseune, 2003). With the presence of TC fibers, minerals are first found at the mineralization front and later the crystallites are located alongside the collagen fibrils but they do not penetrate into the intrafibrillary matrix (Zylerberg, 1985). TC fibers are mineralized more rapidly than the rest of the fibers due to the presence of mucosubstances around them creating a mineralization front that resembles inverted trees. Hence, the inferior EE ratio of the carp can be attributed to the difference in mechanisms of mineralization and limited penetration depth of the TC fibers in the elasmodine.

A comparison of the ply orientations within the elasmodine showed

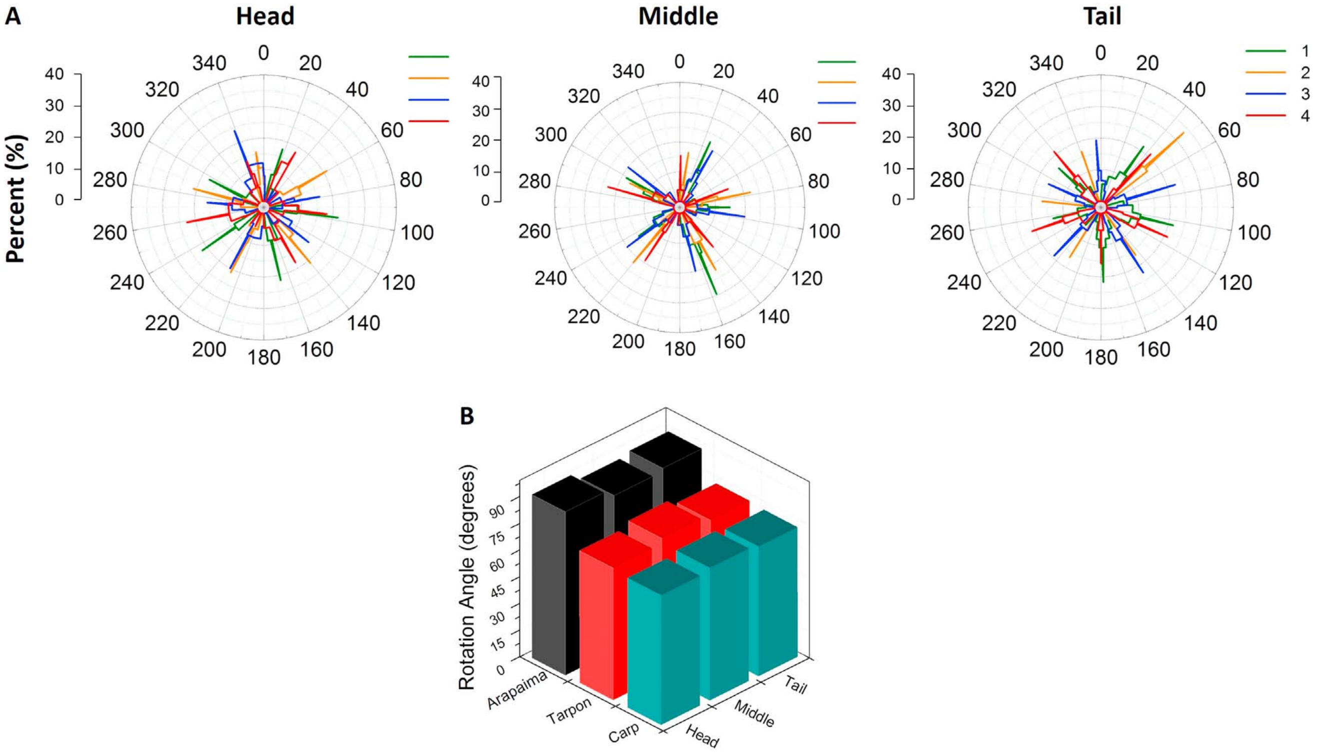

Fig. 7. Primary ply orientations in scales of the tarpon. A) A comparison of the cumulative frequency distribution of the ply orientations for the head, middle and tail regions. Note that the 0° orientation is defined parallel to the lateral line of the fish. B) Average rotation angles between consecutive collagen fiber plies for the three fish and within the three regions of evaluation.

that there were unique characteristics of the lamination sequence amongst the three fish. Mandl (1839) was the first to describe how fish scales are stratified by layers of aligned collagen fibers whose directions change from one layer to the next. In general, the lamination pattern of fish scales is usually described as either orthogonal (Ikoma et al., 2003; Zhu et al., 2012), twisted (Meuniee, 1984), or double twisted plywood (Zylberberg et al., 1988; Bigi et al., 2001). The orthogonal plywood structure has alternating layers of aligned fibrils that are oriented roughly orthogonal to one another. In contrast, the twisted plywood collagen fibrils rotate progressively through successive layers, forming a Bouligand type structure. The double twisted plywood structure is described as a modified orthogonal plywood, where every second layer is rotated by a small angle, so that the orthogonal system is just slightly twisted (Bigi et al., 2001). Earlier studies on the arapaima (Hofer, 1889) and carp (Meuniee, 1984) described the laminate system as more or less orthogonal, but an accurate measurement of the fiber rotations was not given. More recent studies have characterized the laminate system of the arapaima as either an orthogonal (Meyers et al., 2012) or a twisted Bouligand type with a rotation close to 70° (Lin et al., 2011; Zimmermann et al., 2013; Yang et al., 2014). These different interpretations appear to arise from the measurement approach and fiber rotation resulting from prior deformation incurred by testing.

In the present study, direct measurement of the fiber rotation was performed on scales without prior deformation and after chemical fixation, thereby eliminating uncertainty. Our results show that regardless of location, the arapaima scales have a double twisted plywood structure as subsequent plies are roughly orthogonal to one another; there is a small rotation in every couple of layers that can change the fiber orientation just slightly (≤10°). After removal of the offset angle from the lateral line, the average rotation between adjacent plies of the arapaima is 90° (Fig. 10). These results agree with those of (Meuniee, 1984). Both the carp and tarpon scales clearly exhibited a twisted plywood structure with an average rotation angle of 75° (Fig. 10). That distribution results in a stacking sequence that repeats every 5 plies as indicated in Fig. 6.

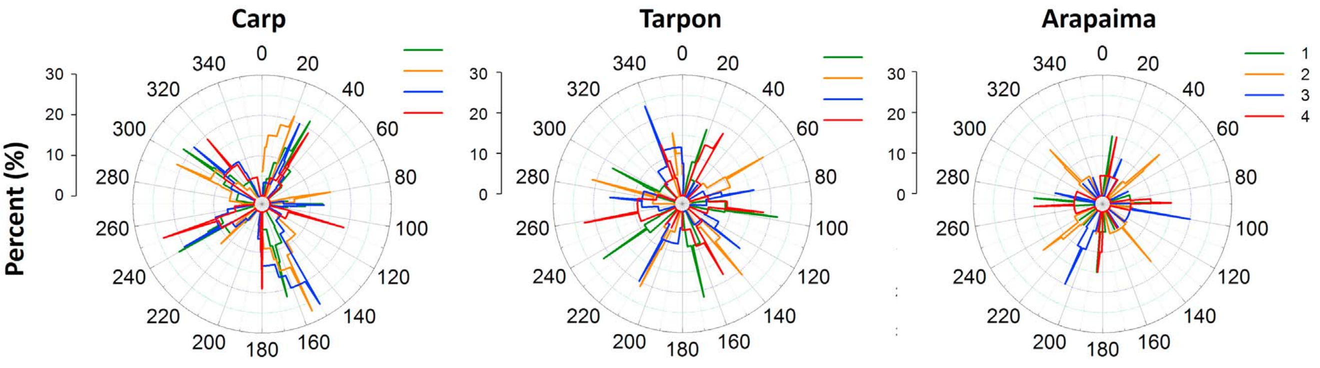

Fig. 8. A comparison of the primary ply orientations of scales for the three fish. Each polar plot describes the cumulative frequency distribution of ply orientations for the head region. The 0° orientation is defined parallel to the lateral line of the fish. Note that the carp and tarpon scales have similar ply orientation distributions, while the arapaima scales have a rotation between plies of ~90°. Additionally, the carp scales show greater consistency in the alignment of the collagen fibers in consecutive plies with respect to the other fish.

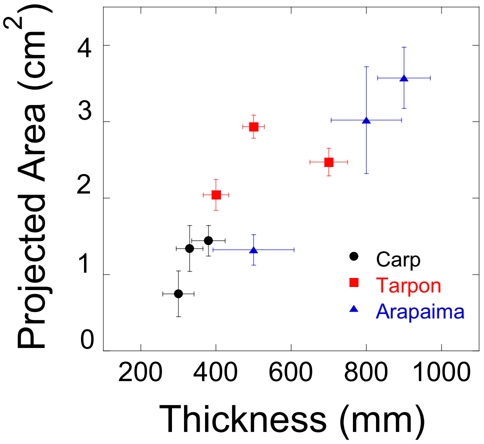

Fig. 9. Correlation between the projected area of the scales with its respective measured thickness. As evident, scales with larger projected area are thicker as a consequence of the radial growth and top to bottom deposition of collagen plies during their development.

The complex distribution of collagen fiber plies of the elasmodine stimulates questions regarding what controls the development and organization of these structures. Meuniee (1984) concluded that rather than a sequential assembly process that results automatically, the fiber organization could be a result of “long distance order” that develops from simultaneous crystallographic and biological control processes. From a mechanics perspective, the unique ply thickness and orientations of the three fish could be attributed to the body shape and stress states generated during locomotion. Subcarangiform ancient fish with body shape similar to the arapaima, including the *Latimeria chalumnae* and several species of *Dipnoi* (lungfish), also have elasmodine with double twisted plywood structure (Meuniee, 1984). Less primitive species with small scales like *Poecilia reticulate* (guppy) have an orthogonal plywood structure (Zylberberg et al., 1988). In comparison, evolved carangiform families similar to the carp and tarpon including *Hemichromis bimaculatus* (African jewelfish), *Leporinus friderici* and *Carassius auratus* (gold fish), have a twisted plywood structure with angle of rotation that varies between 25° and 35°. The ply rotation frequency in the scales of these smaller fish is much greater than found for the carp and tarpon, which could imply that the rotation angle is at least partly related to the fish growth rate.

Although the collagen fibril orientations and ply stacking sequence are expected to influence the mechanical properties of the scales, these qualities are seldom considered in investigations of the structural behavior. Zimmermann et al. (2013) commented that the collagen matrix undergoes rotation (along with other mechanisms) under tension and is dependent on fiber direction. Plies with fiber offset angles between 15 and 30° from the tensile axis rotate towards the tensile direction and deform in tension, whereas fibers at an angle > 60° rotate away from the tensile axis. That increases the importance of an offset between the ply directions and the lateral axis of the fish, as well as with respect to the specimen orientations used in evaluating the mechanical behavior. Results for the ply stacking sequence analysis showed that the plies could have an initial offset angle with respect to the lateral axis of the fish (Figs. 7 and 8) that contributes to the relative fiber orientations and mechanical properties of specimens obtained with respect to the principal coordinates of the body. In addition, while the offset angle may be consistent amongst scales of an anatomical region, it is not necessarily consistent over the entire length of the fish. For example, scales from the head region of the tarpon had an offset angle of approximately −20°, whereas that angle changes to +20° and then +40° in the middle and tail regions (Fig. 7a). The consistency of this offset angle is greater for the middle region than in scales from the head and tail regions. For the tarpon evaluated, scales of the middle

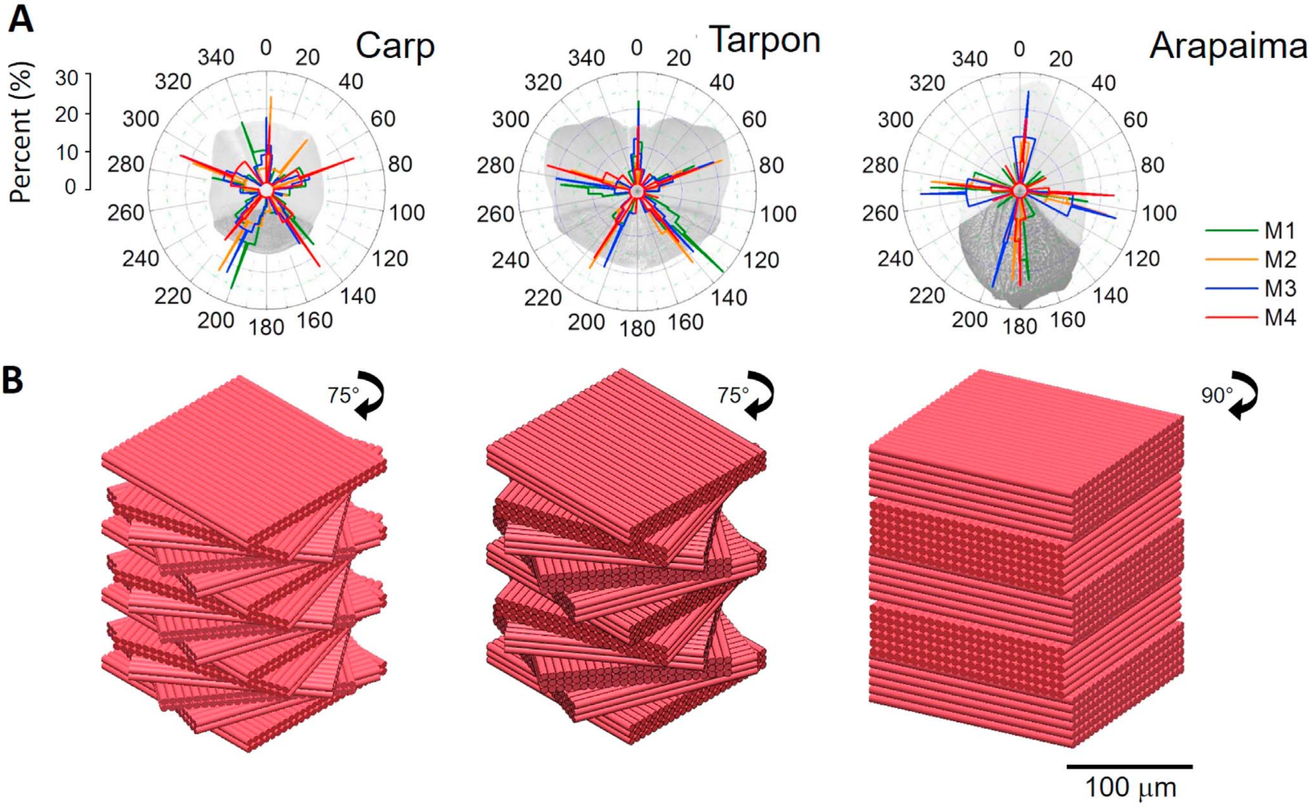

Fig. 10. Rotation angles between consecutive plies. A) Polar plots for the cumulative frequency distribution of scales from the middle region of each fish. Each plot represents the ply rotations from four scales after aligning the plies to the same initial offset. B) Schematic representation of the stacking sequence and average ply thickness for the carp, tarpon and arapaima (left to right). Note the differences in the lamination sequence and thickness. Fibril diameters are not to scale.

region would appear more likely to exhibit anisotropic behavior than those of the head and tail regions due to the consistency in offset angle.

In order to fully understand the anisotropy reported in previous evaluations on the mechanical behavior of scales it is necessary to know the ply orientations as well as the offset angle. Unfortunately, that information has not been included in previous studies of scales. And based on the regional variations in these parameters (Fig. 7a) it is also important to know the spatial variations in these qualities. For instance, due to the double twisted plywood structure of the arapaima scales, similar strength would be expected parallel and transverse to the primary ply directions (Fig. 10a). However, Yang et al. (2014) found that specimens with longitudinal orientation were stronger than those with transverse orientation. For the arapaima scales evaluated in the present study, the head scales had an offset that preferentially aligns with the 0 and 90° directions as shown in Fig. 8. The consistency in orientations was greater when the offset angles were aligned (Fig. 10). But these values are not necessarily indicative of the offset angles and consequent ply orientations in all locations, enabling differences in strength in the axis defined by Yang et al. (2014). Therefore, to understand sources of anisotropy in the mechanical behavior of the scales it is necessary to know the preferential orientations of the fibers and offset angles.

Results from this investigation provide new understanding concerning the microstructure and laminations patterns of fish scales, as well as the potential contribution of these qualities to their mechanical behavior. Despite the importance of these findings, there are concerns and limitations to consider. There were biological variables that were not controlled, like growth conditions, environment and age. In addition, the thickness of the LL or the EE ratio could vary depending on the age of the scale irrespective of the fish if the scales were regenerated due to prior events. Therefore, further study involving environmental control of the fish growth would be desirable. Perhaps the largest limitation is that scales from only a single fish were evaluated from each species, which included four samples per anatomical region. Although the investigation was designed to compare results of structure and lamination patterns between the three fish, it is not clear how consistent these qualities are amongst fish of the same species. There are variations in the mechanical behavior of scales from different fish of the same species (Murcia et al., 2015), which appears to be a reflection of the variations in structure. Furthermore, while the rotation angles are expected to be consistent amongst fish of the same species, it is not known if the offset angles are as well. Hence, a full characterization of the structure is recommended in evaluations of mechanical properties of scales from each fish, even for fish of the same species. The spatial variations in microstructure of the scales from the three fish highlight the importance of recording the anatomical position in future studies on fish scales. Important differences in the LL thickness, EE ratio, fiber rotation and preferential orientation of the scales with respect to the longitudinal axis appear to be important parameters to the performance of natural armored systems.

5. Conclusion

A comparison of the lamination pattern and microstructure of scales from the *Cyprinus carpio* (carp), *Megalops atlanticus* (tarpon), and the *Arapaima gigas* was conducted. Important differences were found in the scales between the three fish and with respect to anatomical position. For example the thickness of scales varied with location with a distribution that was unique amongst the three fish. The differences in thickness were associated with the degree of mineralization reflected in the dimensions of the limiting layer (LL) and external elasmodine (EE). For the carp the LL thickness is essentially uniform over the fish length, whereas in the arapaima and tarpon the LL decreased and increased from head to tail, respectively. Furthermore, the thickness of mineralized plies in the EE is substantially greater than that of the non-mineralized collagen plies, and scales of the arapaima exhibit an EE ratio nearly twice that exhibited by the carp and tarpon scales. Thus, the arapaima scales have a larger proportion of their total thickness occupied by more highly mineralized plies, which appears essential for achieving puncture resistance.

An evaluation of the lamination sequence of the elasmodine showed that the scales of the three fish possess spatially dependent characteristics. The carp and the tarpon have a twisted plywood structure with average ply rotation between adjacent plies of 75°, resulting in a repetition of the stacking sequence every 5 plies. In comparison, the arapaima was characterized as a double twisted structure with average rotation of 90° and slight rotation ($<10°$) offset between adjacent plies. Furthermore, the scales of each fish exhibited a unique preferential offset angle of the ply orientations with respect to the lateral line of the fish. This characteristic appears to be important to the direction dependence in the mechanical behavior of fish scales and in quantifying the degree of anisotropy. The preferred rotation and offset also appeared location-dependent. As such, experimental evaluations concerning the mechanical properties of fish scales should account for the potential importance of anatomical position.

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