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Distribution of chitin and collagen in the structural organization of the freshwater jellyfish *Limnocnida indica* Annandale, 1912 (Hydrozoa: Limnomedusae: Olindiidae)

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Abstract

In this paper, a structural analysis of the rarely encountered hydrozoan medusa *Limnocnida indica* Annandale is discussed. This diploblastic, radially symmetrical organism has a body plan typical of hydrozoan medusae. In this study more emphasis was given to observation of the supporting components of its anatomical structures. Detailed observation on the distribution of supportive components including chitin, collagen, and noncellular fibers within the tissue organization has been carried out with the application of different types of staining methods. The neuromuscular arrangement at the rim of the umbrella and basal part of the gastrovascular cavity and the distribution of the neurons in the sub epithelial region of ectoderm have been analyzed both by staining and by scanning electron microscopy studies. Supporting blocks of chitin at the base of tentacles, along with statocysts, and ring-like deposition of chitin and collagen on the tentacles are documented. Distribution of collagen around radial canals in the mesoglea was found to be unique to this species. The mesoglea seems to be a storehouse for the macromolecules chitin and collagen.

Key words: Body plan, chitin blocks, collagen fibers, diploblastic, neuromuscular, subepithelial nerve cells, unique collagen

Introduction

Cnidarians are a diverse group of diploblastic, radially symmetrical animals with a simple body plan. As examples, within the cnidarian classes, Anthozoans lack a medusa stage, while hydrozoans and others undergo both polyp and medusa stages. Among hydrozoans, freshwater jellyfishes are interesting because of their unpredictable appearance. Six species of the hydrozoan genus *Limnocnida* Günther (Limnomedusae) have been described. Of these, three species *L. tanganjicae* Böhm, 1883; *L. victoriae* Günther, 1907; and *L. congoensis* Buillon, 1957 are found in Africa. The three other species *L. indica* Annandale, 1912; *L. biharensis* Ahmad et al. (1987); and *L. nepalensis* Dumont, 1976 are present on the Indian subcontinent (Jankowski et al., 2008). Another freshwater species of the same order, *Craspedacusta sowerbii*, Lankester, 1880 is also distributed in a few lakes of India (Riyas and Biju kumar, 2017). The taxonomic diversity and identification of these species is mainly based on six key features—body size, total number of tentacles, maximum length of tentacles, statocyst tentacle ratio, number of oral arms, and presence or absence of scale-like membranes (Jankowski et al., 2008) (but not including the components of the structural organization within the body plan as there is no assessment of their taxonomic role, if any). Whether these structures have the same organization or not in all species of freshwater jellyfishes is still unknown.

In this work, we attempted to demonstrate how the macromolecular components chitin, collagen, and extracellular fibers have contributed to the organization of structures in the species *L. indica.* These findings may be used as comparative standards while studying the structural organization in other freshwater jellyfishes.

The freshwater jellyfish *Limnocnida indica* is a rarely encountered aquatic coelenterate known to be distributed only in India. To present, it has been found only in the Western Ghats (Biological Hotspot) and in river systems originating from the Western Ghats. Birsal (1994) documented a list of studies on this species. Srinivasan and Barton (2021) put forth a detailed account of the current status of *L. indica*, but it has not yet been declared as an endemic species to the Western Ghats. Compared to other coelenterates such as hydra, the structural organization of this species is less studied (Ghaskadbi, 2020), maybe because of its opportunistic availability in nature. Structural analysis of any organism contributes to the significant knowledge of comparative studies between congenerics or other taxonomic groups (Srinivasan and Barton, 2021). Ordinarily, whole organism staining is used to study the internal organization of small transparent or translucent animals. Staining gives a contrast between the structures that can then be more easily visualized under light microscopy. Furthermore, extracellular tissue fibers, muscle fibers, and molecules deposited on the cell and neuronal tissues can also be more easily observed.

During the period of one hundred twenty years from the first identification of *L. indica* to the present study, only about fifteen observational reports could be found and these have mainly dealt with its distribution and occurrence patterns (Annandale, 1912; Gravely and Agharkar, 1912; Agharkar et al., 1913; Hora, 1926; Rao, 1931, 1932, 1933; Darling, 1935; Ramakryshna et al., 1950; Jones, 1951; Krishnamurthy, 1951; Iyengar and Venkatesh, 1955; Birsal, 1994; Sharma and Chakraborty, 2000; Manna et al., 2005; Srinivasan and Barton, 2021). Previous authors have concentrated mostly on the size of the medusa and number of tentacles, radial canals, and gastrovascular structure to explain or discriminate the species of medusa. Hence, this study on the structural components of the freshwater jellyfish *L. indica* was undertaken and the observations recorded are presented in this work.

Species of freshwater Limnomedusae present a defined body plan (Hroudova et al., 2012) with diploblastic tissue layers with mesoglea sandwiched between the tissue layers, radial symmetry, and a gastrovascular cavity. The mouth, a common inlet and outlet for food material is surrounded by the manubrium and directly opens into gastrovascular cavity. Tentacles and statocysts at the base of tentacles are arranged around the rim of the bell. As we initiated a complete structure analysis of the medusa, we had several queries to address. One of these was to find out about the presence of any additional components that give support to the structures in the body of this organism, and if present, to determine how they are organized. Another intriguing factor examined in this study was locomotion. While swimming near to the surface, *L. indica* medusae move the primary (long) tentacles sideward as well as frontward and backward, while the secondary tentacles (short) show lateral movements. While descending in the water, the medusae have the primary tentacles held upright in a stiffened state, whereas the secondary tentacles remain in the original condition without upward stretch. Although this type of tentacle movement was seen and photo recorded by several previous authors, the movements perhaps escaped their attention (Srinivasan and Barton, 2021) as they have not been the subject of further analysis. In this regard, we attempted to investigate whether the structural composition is similar or different for the two tentacle types. Other structures that drew our interest were the four radial canals extended as cross bridges between the gastrovascular cavity and rim of the bell; these were examined to find out whether they have any additional support to be held intact in the mesoglea. All the queries raised were categorically analyzed to present the inferences drawn. All these analyses were carried out in asexual forms of medusae as we did not encounter gonad-bearing sexual forms during this study.

Material and Methods

Twenty-one free swimming medusae of *Limnocnida indica* were collected from the stagnant backwater of Linganamakki Reservoir built across the Sharavathi River in the Western Ghats, India. Linganamakki Reservoir is located at latitude 14.10139°, longitude 74.88056°. The medusae were caught using a planktonic net during the first week of January 2023 between approximately 6:30 to 7:30 am while sailing across the backwater in a boat. The specimens were brought to the laboratory and preserved in 70% alcohol. Medusae were initially photo recorded using a Sony digital or iPad Air4 camera. The stained specimens were photographed using a Carl Zeiss Discovery.V20 stereoscope or a Cresta microscope with a Micron optik industrial digital camera USB 2.0 USA mounting system. All micrographs were taken with a 4× objective. The images were magnified 10× or 20× on the computer screen and were suitably calibrated to the micron scale. A minimum of two individuals was used for staining with each stain solution. The staining solutions used in this study were procured from Nice Chemicals (Pvt.) Limited, Kochi, India.

Silver Nitrate Staining ($AgNo_3$)

A general stain used to trace neuronal structures; it stains neurons and dendrons. Method of Fritzsch and

Zakon (1988), with a suitable alteration in length of time, was adapted for silver staining. Specimens preserved in 70% alcohol were taken out and washed in distilled water, transferred to 1% silver nitrate solution, then left for 24 hours. Stained samples were removed from the coloring solution, washed in distilled water, and mounted on a slide for observation and photographic analysis.

Methylene Blue Staining (MBS)

Applied to stain the neuromuscular rings of medusa, it gives a light blue color to neuromuscular junctions. The procedures established by Cores (1952) were followed, with slight modification for staining of jellyfish. A medusa stored in 70% alcohol was washed and the whole animal was kept in methylene blue solution for 20–30 minutes. The medusa was drawn out of the stain solution, washed in distilled water, and the results were recorded.

Lactophenol Cotton Blue (LCB) Staining

A stain used to demonstrate the presence of chitin as a supportive macromolecule. The protocol given by Thomas et al. (1991) was followed to stain the medusa. The specimen was washed with distilled water to clear the alcohol, then kept in the stain for 20 minutes till the body parts appeared dark blue. To crosscheck if the material that stained blue was chitin, it was treated with strong acetic acid, the tissue was washed, then stained with lactophenol cotton blue. The denatured chitin did not stain blue (because the strong acid denatures chitin).

Haematoxylin, Eosin, and Saffron (HE and S) Staining

The combination of haematoxylin and Eosin is the most widely used stain to observe muscles and connective tissues either in the whole organism or tissues. Saffron stain is exclusively used to stain collagens. Following the instructions given by Ceccopieri et al. (2021), a saffron stain alcohol solution was prepared using natural saffron (*Crocus sativus*) procured from a reliable marketing agency. All the three stains solutions, haematoxylin (Harris), eosin, and saffron, were mixed in equal proportion, the medusa was cleared of the alcohol, and was kept in the stain for 15–20 minutes. The medusa was taken out to clear off excess stain on the surface by dipping in distilled water. Parallel to this, the medusa was also stained separate in combined hematoxylin and eosin for comparison. The results were photo recorded.

Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) studies were done on a Carl Zeiss electron microscope EVO LS 15 following the procedures adapted from Kavya et al. (2018). Thick cross sections of the preserved jellyfish were cut accurately using a blade. The sections were mounted onto aluminum holder stubs using double sticky carbon tape coated with Au/Pd and placed on a grid. The positions of the sections were noted and the images were photo recorded.

Observation of tentacle movement

In its natural habitat, it was observed that the jellyfish had different strokes of its long and short tentacles while swimming. A few live animals were brought to the laboratory for further observation and were kept in a large transparent glass container filled with water collected from its habitat. The jellyfish were observed as active between 7:00 and 7:30 and the stroke of each type of tentacles was observed and recorded.

Results

The general morphology of the *L. indica* medusa is shown in Figure 1A–D. The body of the medusa has a transparent inverted saucer shape with distinct oral or sub-umbrellar and aboral or ex-umbrellar surfaces. The body size of medusae ranged between 10.9 mm to 11.1 mm in diameter. The tentacles are arranged around the rim of the bell as seen in Figure 1A. The number of tentacles was >300 in each of the individuals counted with a maximum of 384. Primary tentacles were longer and measured around 10–11 mm. The number of primary tentacles ranged from 68–79. The secondary tentacles were very small and measured about 1–2 mm in length. Both primary and secondary tentacles had a large number of nematocytes arranged as circular rings along the length of the tentacle. The oral end has a circular mouth at the center surrounded by the ridge-like manubrium. The mouth extended into a circular gastrovascular canal with a whitish translucent structure. Four radial canals were seen radiating from the circumference of the gastrovascular cavity towards the edge of the bell (Fig. 1B).

Observations of specific structures and tissues were facilitated with the use of the staining and microscopy (Figs. 2–6). The medusa had supportive muscular rings, one upper at the aboral end just below the ring canal and the lower at the edge of the bell. These muscular rings had neuronal junctions that could be clearly seen as blue-colored rings stained by methylene blue (Fig. 1C), as observed under the light microscope. In addition to two muscular rings, MBS also stained blue the primary and secondary tentacles along their length, the gastrovascular ring canal, and manubrium ridges, but the velum and umbrellar surface were negative for this stain.

AgNo3 stained the cellular junctions, sub-epithelial nerve cells, nerve endings (Fig. 6), and a circular ring of tissue above the muscular ring of the rim dark brown. The silver impregnation distinctly defined the structure, position, and arrangement of statocysts enclosing a statolith around the rim of the bell below the tentacles base (Fig. 2A). $AgNo_3$ impregnation was darker at the base of the nematocysts and all along the tentacles. Both tentacle types showed a similar staining pattern with this solution (Fig. 3D).

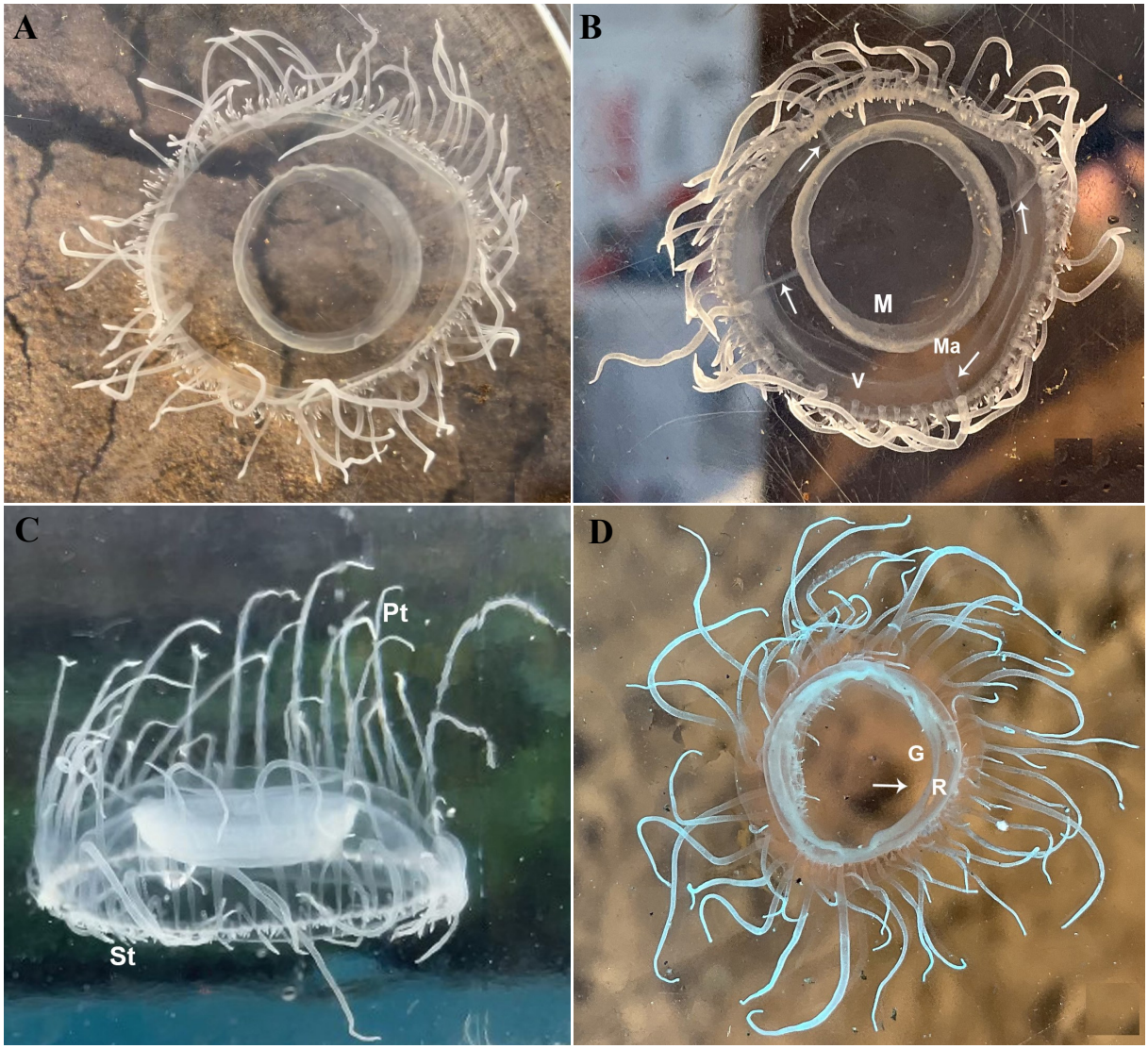

Figure 1: *Limnocnida indica* whole animal captured with digital camera. (A) Aboral view of the medusa. (B) Oral side of medusa. M—mouth, Ma— manubrium ridge. Arrows indicate radial canals. (C) Descending jellyfish with primary tentacles held upright. (D) Methylene blue-stained jellyfish showing neuromuscular rings. G—gastrovascular ring, R—neuromuscular ring at the rim.

LCB stain applied to the whole specimen revealed that the rim of the bell with the tissue junction of both surfaces stained deep blue, whereas the velum, manubrium, ridge of the mouth, ex-umbrellar surface did not take the stain (Fig. 2B). The base of the tentacles originating from the block of tissue, along with the statocysts also, appeared dark blue (Fig. 2B). The dark blue-stained blocks had a gap in between, but the associated muscle ring that had given rise to the tentacles was continuous. The surface of tentacles and the base of nematocyst rings presented deep blue staining (Fig. 2B). The mesoglea nearer to the rim of the muscle ring had dense blue-stained granules.

The statocysts were found below the tentacle base that is surrounded by the blocks of chitin positive for LCB. Each statocyst found below each block (Fig. 2B) had a central statolith surrounded by a membrane-bound vacuity; the statolith and bound membrane were both positive for LCB. The number of statocysts varied from 140–160 among different individuals and were arranged in a circle below the muscular ring.

HE and S-stained tentacles appeared red along their length, and the nematocyst bases appeared dark red (Fig. 3A) and were arranged in the form of several rings along the length of both primary and secondary tentacles. The extracellular matrix around the radial canals stained as reddish dark mesh, a color distinct from haematoxylin staining. Within this mesh-like structure unique red, orange, blue (purple), green, and yellow dot-like structures were scattered (Fig. 4A-C). The velum stained dark red with parallel-running fibers (Fig. 3E), as did the muscular ring. H and E stain without saffron produced a different result (Fig. 3C); this did not reveal the presence of a mesh-like matrix covering around the radial canal. The tentacles and muscular ring around the rim showed a reddish-pink color with this stain, in contrast to the deep red color produced with HE and S.

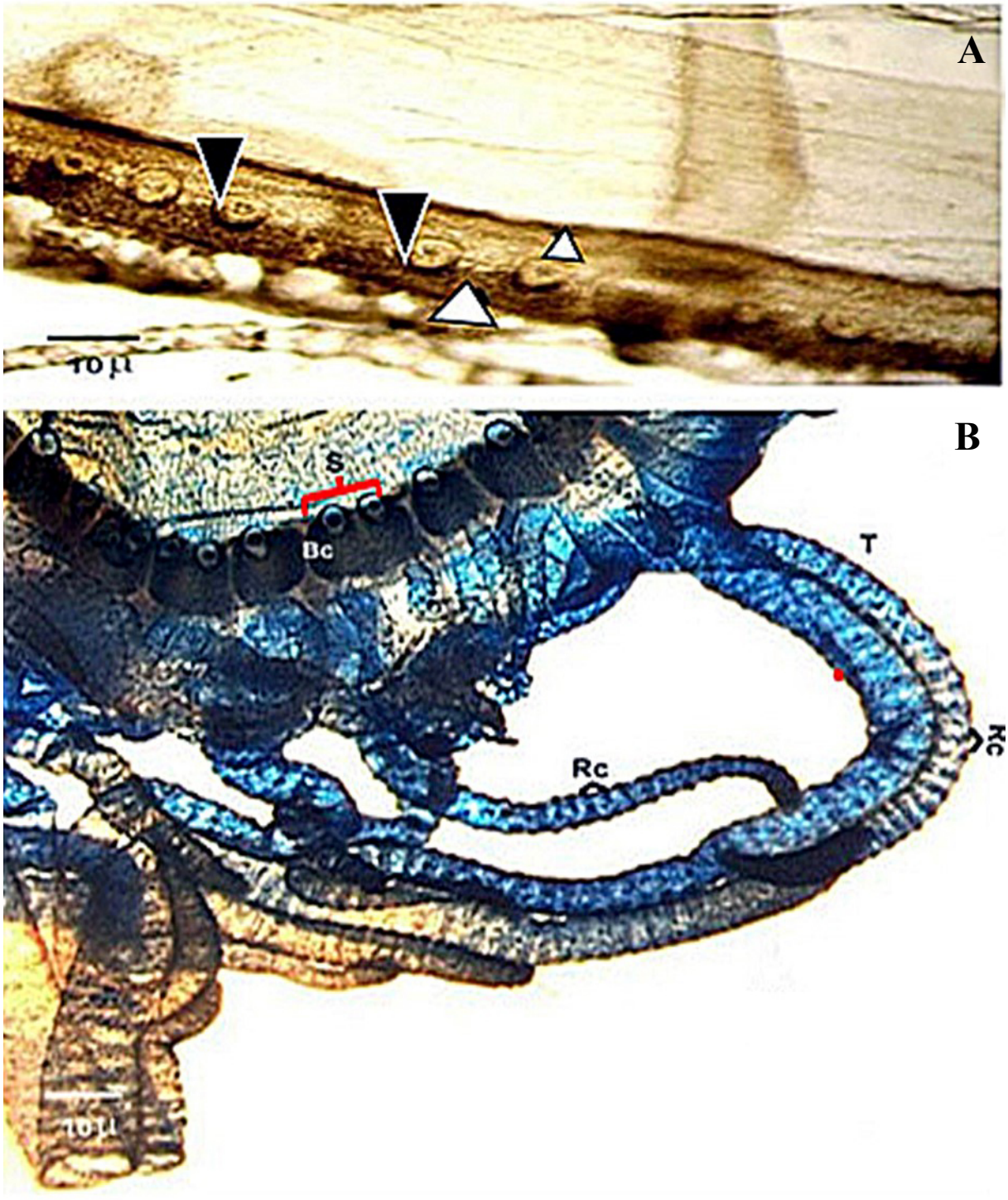

Figure 2: Light microscopy images of *L. indica* tissues with staining. (A) Silver-stained edge of the rim statocysts (black arrowheads) and neuronal rings associated with muscles (white arrowheads). (B) Lactophenol-stained rim. Bc—chitin blocks, S—statocyst, T—tentacle, Rc—chitin rings.

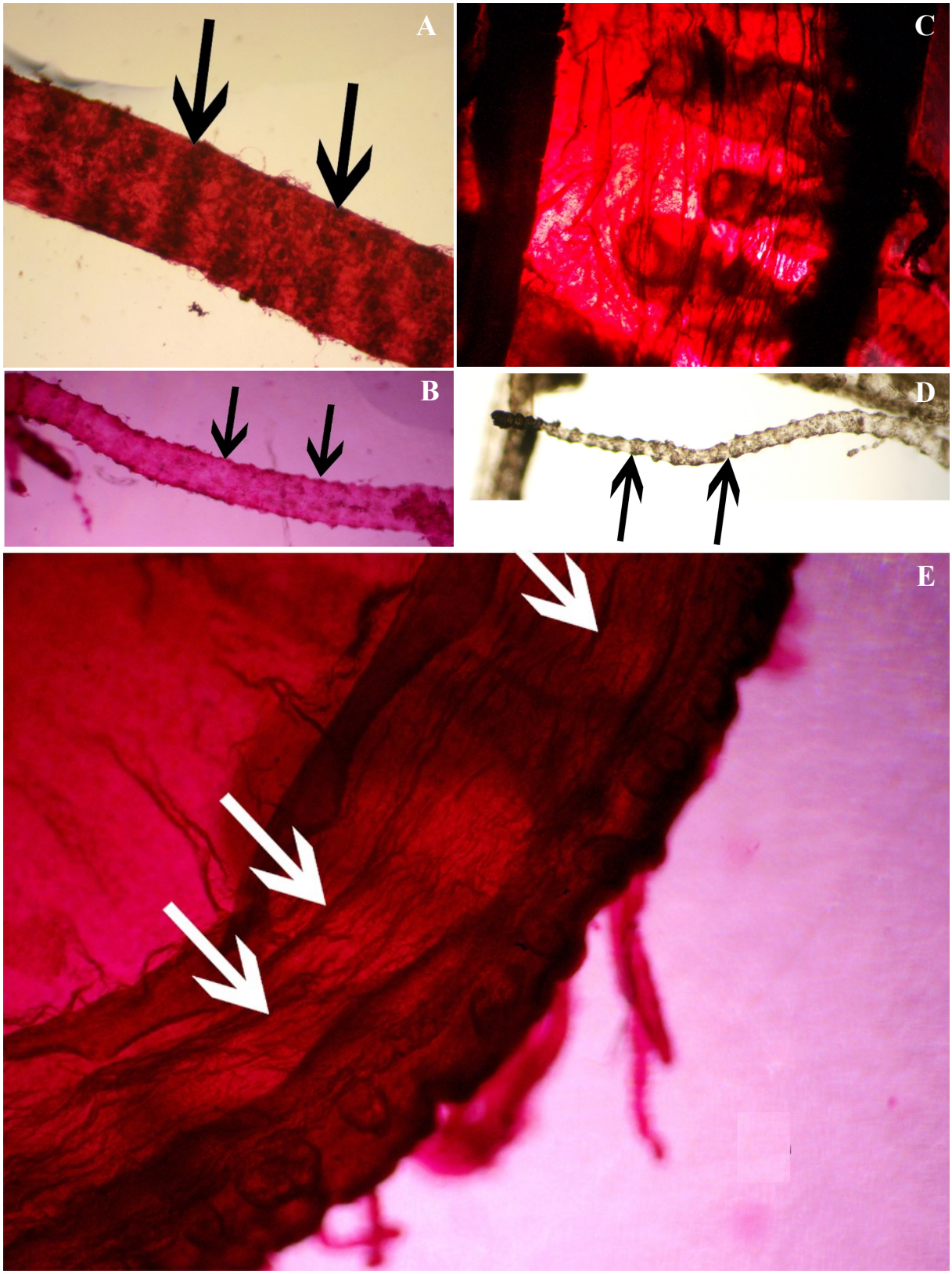

Figure 3: Light microscopy images of stained *L. indica* tissues. (A) HE and S-stain with tentacles having rings of collagen (black arrows). (B) H and E stain with collagen blocks unseen (black arrows). (C) HE and S stain with edge of the bell with thick muscular ring stained dark red, mesoglea stained reddish-yellow. (D) Silver stain with discontinuous dotted brown patches on tentacle (black arrows). (E) HE and S stain with part of velum showing red-stained connective tissue fibers (white arrows).

The diploblastic body wall is formed of an outer ectodermis and inner endodermis separated by a large noncellular mesoglea. Mesogleal space did not show affinity to MBS (Fig. 1C) or $AgNo_3$ but stained pale pink with H and E (Fig. 3B) and orange-red with HE and S (Fig. 4A). The surface of the ectodermal epithelium appeared as a smooth sheet of epithelial cells, each having a nucleus. The cell borders and nerve endings were distinctly stained by $AgNo_3$ (Fig. 6).

The SEM images (Fig. 5A, B) confirmed that the body of the medusa is of a bilayered cellular organization, outer ectoderm and the inner endoderm, separated by a noncellular mesoglea. All the epithelial cells were lined with a basal membrane and myofibrils on the basal side of both layers. Both the tissue layers were found to have basal muscle fibers running parallel to each. Neuronal cells are found scattered in both the tissue layers. The ectodermis and endodermis are predominantly formed of elongated epithelial cells. The ectodermal outer surface is formed of a sheet of epithelial tissue. The neuronal cells and nerve cell ends intermixed with the cell layer were found to be scattered irregularly, as revealed by $AgNo_3$ staining (Fig. 6). The lower edge of the bell of the medusa showed the details of how the muscular ring is formed. In this region, the mesogleal layer is reduced. The basal borders of both epithelial layers, supported by neuronal bundles and muscle fibers, run towards the edge and are entwined with each other to form a large muscular ring. The cut section of the muscle ring is seen as a thick ridge-like structure covered by a cell layer. The tentacles though, have a clear epithelial covering that showed a rough, crinkled appearance due to extracellular deposition of materials (Fig. 5C).

Regarding the movement of the jellyfish tentacles, it was observed that while descending in the water column, the jellyfish held its primary tentacles upright. This behavior was identical in the natural habitat and laboratory. Movements of the primary and secondary tentacles were also observed while the jellyfish was swimming in the laboratory container. While swimming, the primary tentacles of the jellyfish exhibited lateral, upward, and downward movements, whereas the secondary tentacles showed only forward and backward movements. While sinking down into water the primary tentacles were held upward, parallel to each other in a straightened posture, while at the tip of each a curved hook-like faced outward (Fig. 1D).

Discussion

In this study it was observed that the radially symmetrical discoid structure of the *L. indica* medusa has a soft but stable structure within its body. Flexibility and stability of its structures must be maintained while flexing itself during swimming movements; for this, added rigidity along with provisioned flexibility is a required criterion. Three supporting components—chitin, collagen, and extracellular fibers—have been identified via staining analysis which support the structural organization of the medusa. Prior to this study, the arrangement of structures within the medusa were analyzed (see Jankowski et al., 2008) without consideration of the organization of chitin and collagens. These comparatively hard components are arranged with a gap in between so that flexible movements of structures such as tentacles are possible and flexing of the body of the medusa is not interrupted.

The diploblastic body wall, interlaid by a noncellular mesoglea, gives hydrostatic support for structural form and stability of tissues in cnidarians. That it is the storehouse macromolecules (Davis and Haynes, 1968) that constitute the connective tissue fibers has been confirmed in this present work by tracing the presence of collagen and chitin in the mesoglea. The presence of chitin in the mesoglea is uniquely found as scattered granular structures nearer to the basal rim of the medusa; this may be because of its role in maintaining the wear and tear of the basal region of tentacles. The other supporting component, extracellular collagen fibers, and other unstained fibrous material placed parallel to each fiber around the length of the velum to maintain its structure, act as a supporting fibrous network. The muscle rings are innervated by neuronal dendrons and have additional support of parallel-running extra tissue fibers. The muscle ring is formed by the entwined parallel muscle fibers resting just above the rim which are covered by the epithelial tissue layer. The ectodermis and gastrodermis have muscle cell layers below the basement membrane and both the cell layers have elongated epithelial cells and intermixed nerve cells. The epithelial cells of the gastrovascular cavity did not possess flagellar structures. These results have shown that the arrangement of the medusa's organs is consistent with the body plan.

The primary and secondary tentacles extending from the muscular ring had additional deposition of collagen and chitin, as indicated by the HE and S and LCB stains conducted in this study. As stated above, the movement of the tentacles warranted more analysis in order to confirm the components of the tentacles. This is because muscle fiber alone cannot hold the tentacles in an uplifted position or enable their extension laterally, parallel to each other. MBS indicated the presence of a muscle and neural tissue combination through the entire length of tentacles of both the types. LCB and HE and S staining revealed the presence of segmented blocks of chitin and collagen deposited at the base of nematocysts, providing stiffness to the tentacles. $AgNo_3$ also showed a dotted, broken segment-like distribution pattern of the terminals of neuronal cells ending and concentrated around the base of the nematocysts for normal sensory and response functioning. The deposition of chitin and collagen might provide additional support while nematocyte thread is shot out at a very high speed.

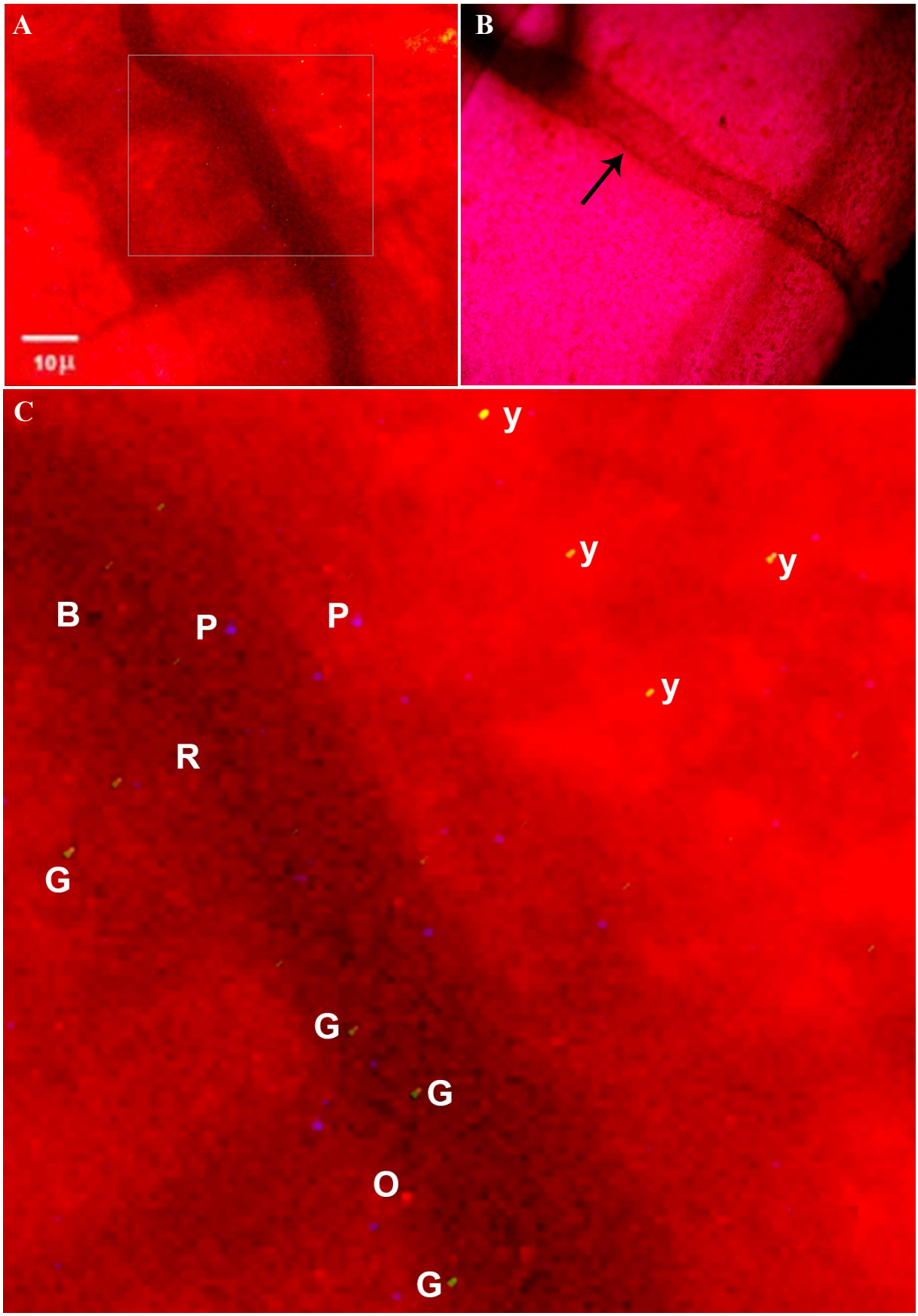

Figure 4: Stained *L. indica* tissues under light microscopy images. (A) Radial canal stained with HE and S showing noncellular collagenous covering around it. (B) Radial canal stained with H and E did not show the noncellular covering. (C) An enlarged view of the portion of the collagen covering shows different colored granular material. Bl—blue, R—red, Pl—purple, O—orange, G—green, Y—yellow.

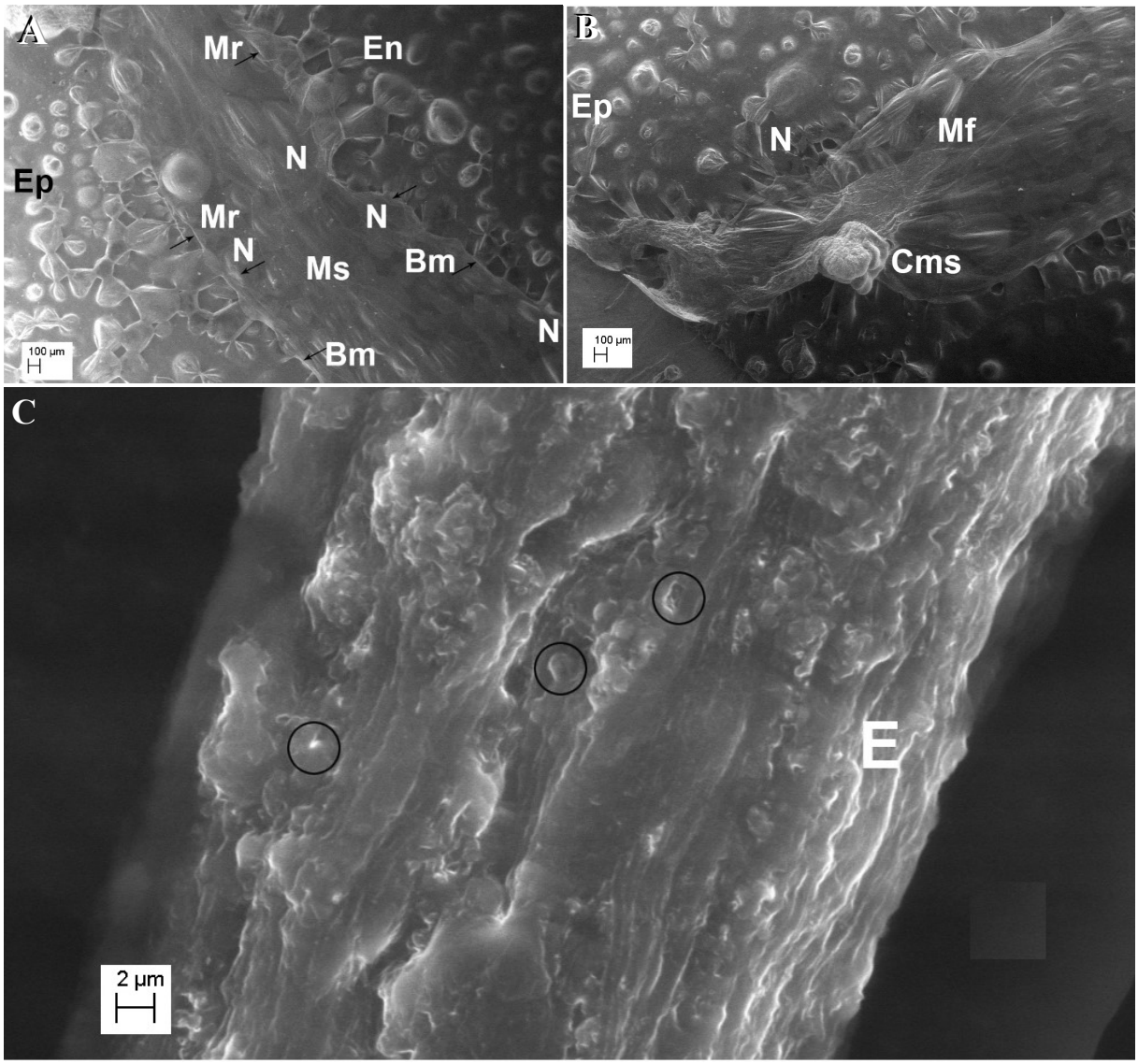

Figure 5: SEM images of *L. indica* tissues. (A) Diploblastic organization of body layers. Ec—ectodermal epithelium, En—endodermal epithelium, Ms—mesoglea, Mr—muscle fibers, N—nerve cells, Bm—basement membrane. (B) Section showing the organization of muscle fibers to form muscle ring. N—nerve cells, Mf—muscle fibers, Ep—epithelial cells. (C) Crinkled surface of the tentacle showing nematocytes. E—epithelium.

Among metazoans, chitin is the major supportive component in hard and flexible structures. The distribution pattern of chitin is dissimilar among taxa. For example, it is found in the internal skeletal tissues of corals (Bo et al., 2012), and in grasshoppers chitin is placed in the exoskeleton (Kaya et al., 2015). Vendepas et al. (2023) have confirmed through their studies that there is a wide distribution of chitin across cnidarians and other invertebrate taxa, with different patterns present among the species examined. Further, it was affirmed that for Cnidaria, as a sister group in evolutionary divergence from bilaterians, understanding of the pattern of chitin distribution and the genes responsible carries phylogenetic significance. Understanding the distribution of chitin and its utility in simple cnidarians such as the freshwater jellyfish *L. indica* may provide a new insight into the evolutionary approach with regard to the origin of these molecules, and may also provide new opportunities for technological applications because of the industrial, biotechnological, and medical applications (Kumar, 2000) because of their properties (Yan et al., 2021) and high thermal stability (Koll et al., 1991).

HE and S staining, introduced by Edston and Grafti (1997) to substitute other combinations for staining specific tissues, provided impressive results. Natural saffron solution is used to identify collagen fibers, as it gives reproducible and reliable results (Ceccopieri et al., 2021). Collagen is one of the tissue components that adds strength to the organs and it protects structures, gives strength and elasticity, and helps the tissues to bear physical stress and tissue damages via its strong stiffened network.

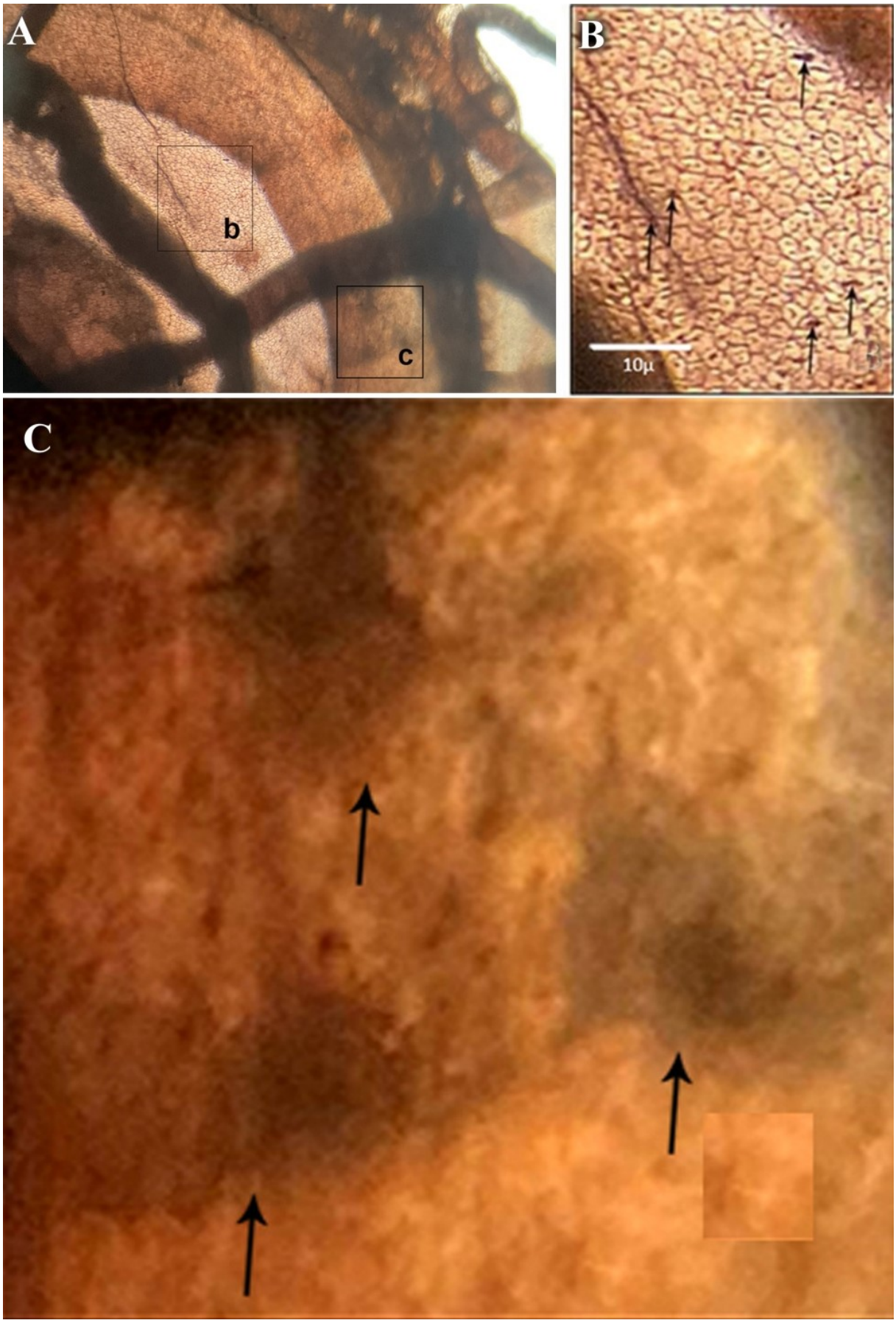

Figure 6: Light microscopy images silver-stained *L. indica* tissue. (A) Aboral surface of the medusa. (B) Sheet of epithelium of ectodermis with nerve ends (long arrows), (C) Sub-epithelial nerve cells (short arrows).

It is a load-bearing biopolymer that exists in different types across taxa but the three major types—I, II, and III—occur in a large quantum. Type IV is found as a two-dimensional reticulum, while the others are associated with fibril types, as found in vertebrates (Smita et al., 2017). Collagen stained with Sirius red gives different colors to collagen fibers under polarized light, ranging from yellow, pink (purple), to orange, and thin fibers appear green due to bi-refringence, representing different collagen types (Junqueira et al., 1979; Lattouf et al., 2014; Smita et al., 2017). In this study, all the mentioned colors manifested under normal light. Saffron imparted red, blue, green and yellow colors to the extracellular deposition of noncellular fibrous structure around the radial canal; nowhere else in the mesoglea did such deposition occur, except for a few spots of mesoglea that had yellow patches. Such a character of a stain producing more than one color to the tissue components is referred to metachromasia. Several stains are known to generate the metachromasia effect in tissues, which is mainly due to polymerization of the affinity molecules (D'Mello et al., 2016). In general, one or two metachromatic colors are generated in a tissue stained by a particular dye under different conditions. In this case it was different, as neither the tissue nor the cells were metachromatic but the extracellular components of collagen showed five different colors at different spots in the same region. This is unique.

The combined analyses of this study determined that this coelenterate has a body plan typical to limnomedusae; it possesses added features such as tentacles with chitin and collagen depositions, velum with supportive collagen fibers, radial canals extended within the mesoglea supported by collagen covering, and collagen molecules imparting different colors with saffron stain. Thus, the structures involved in organizing the body plan and strengthening this arrangement have a unique support of macromolecules. These molecules and their association with anatomical structures may provide a clue to the understanding of the evolutionary mechanisms, as cnidarians occupy a basal position in the radiation of radially symmetrical tissue grade animals, as well among bilaterian triploblastic animals. The unique collagen organization is particularly interesting and requires a detailed molecular analysis.

Conclusion

L. indica has a unique status, being opportunist in its appearance, and has attracted greater interest in its structural studies. This study determined that the species has a body plan typical to hydrozoan medusa and has a unique architecture of macromolecular support in its structures. Moreover, the organization of the macromolecules chitin and collagen is interesting because both of these molecules have biomedical applications and further analysis on the properties and molecular organization of these molecules in this animal may open new opportunities of research.

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Author contributions

All three authors made equal contributions to this work. T. S. J. and P. P. G. had a key role in the collection of specimens and microphotographic recordings. The work was designed by H. C. All the three authors were involved in the preparation of this article.

Conflict of interest

The authors declare that there is no conflicting issue related to this research article.

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