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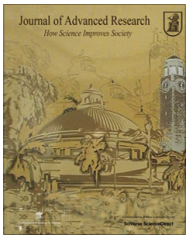

ORIGINAL ARTICLE

Tissue specific structural variations of mitochondria of fish ectoparasite *Argulus bengalensis* Ramakrishna, 1951 (Crustacea: Branchiura): Functional implications

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ABSTRACT

We studied the fine structure of some classical and six variant mitochondria from different tissues viz. proboscis gland, spinal gland, ovary, testis, and muscle of a fish ectoparasite, *Argulus bengalensis*. In the proboscis gland and spinal gland, mitochondria are protected within vesicle to preserve their structure and activity from exposure to glandular synthesis for its parasitic mode of feeding. In the oocytes, mitochondria are larger and cylindrical in appearance. Oocyte mitochondria are highly dynamic and exhibit frequent fission and fusion. Those are clustered in the cytoplasm of previtellogenic oocytes which prepare for different synthetic activities for successful reproductive investment. In contrast, mitochondrial abundance is less in the male gametic lineage. The spermatocytes and the nurse cells in the testis have an unusual type of mitochondria, nebenkern which is formed by the fusions of number of mitochondria. A completely different type of mitochondrion is discovered in the flagellum of the spermatozoa. It is provided with fifteen numbers of singlet microtubules at its outer periphery which is a salient feature of the flagellum of this Branchiuran genus. This unique mitochondrion uses the microtubule tract for its movement to distribute energy efficiently along the axoneme. Such mitochondrion and microtubular association provide evidence in favor of phylogenetic relationship between *Argulus* and pentastomid *Raillietiella*. In striated muscle of thoracic appendages, mitochondria maintain tight junctions with the endoplasmic reticulum and remain in close apposition of the myofibrils which helps in Ca^{2+} uptake for stimulating continuous muscular activity required for ventilation of respiratory structures of the parasites.

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Introduction

Recent studies have antiquated the classical structure of mitochondria as floating sausages of similar size with sheet-like baffles of cristae extending from the inner membrane as it was first proposed by Palade [1]. Rather, mitochondria in most tissues exist as a dynamic network, constantly undergoing fission

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and fusion [2,3]. Electron tomographical analyses of mitochondria show the cristae are originated from the inner membrane as collections of folds ranging from tubes to lamellae [4]. Mitochondria perform a number of cellular functions in ATP synthesis, ion homeostasis, lipid metabolism, cell fate determination, apoptosis, and aging [5–7]. *Argulus bengalensis* is an obligatory parasite which has a specialized feeding apparatus and a curious type of respiratory structure. Its parasitic fitness largely involves its efficient reproductive investment. To encompass their diverse functions, the mitochondria often establish specific numbers and locations, maintain specialized shapes as well as make unique associations with other structures in different cell types [2,8]. In the course of a comparative investigation of different cell types from those structures directly involved to its parasitic mode of life, some unusual mitochondrial forms along with the typical forms were observed. Those are reported and described here to elucidate the underlying strategies of ultrastructural variations in mitochondrial morphology which may focus our attention on some functional aspects of mitochondria not ordinarily considered.

Material and methods

Material

A. bengalensis were collected from "Barasagar Dighi" fish farm (24°58′08.86″N, 88°06′09.70″E) under Government of West Bengal located at Malda, West Bengal, India. A breeding colony of the parasite raised by cohabitation with the freshwater cyprinid host, *Cirrhinus mrigala* (Hamilton, 1822), was used for this study. The parasite was identified with the help of morphometric criteria following Ramakrishna [9].

Light microscopic study

For light microscopy, abdomen of the matured female parasites (age group of 29–32 days) was severed from the cephalothorax with the help of a sharp triangular surgical suture without affecting the ovary; thereafter, a small puncture was made to release the oocyte. Oocytes were then cleared by a solution containing ethanol, formalin, and acetic acid (6:3:1) and observed under microscope. For vital staining fresh oocytes were stained with 0.02% Janus green B (HiMedia Laboratories Pvt. Limited) in insect saline for 30 min and viewed under compound microscope (Prime, Dewinter Optical Inc., Italy).

Transmission electron microscopy

Several adult male and female parasites were anesthetized adding ethanol drop by drop in water and then transferred to 2.5% glutaraldehyde and 2% paraformaldehyde solution in cacodylate buffer (pH 7.4) to fix the specimens for overnight at 4 °C. The specimens were postfixed in 2% osmium tetroxide buffered solution and were embedded in epoxy resin. Subsequently, those were sectioned with a Leica Ultracut-UCT ultra microtome and stained with a saturated solution of uranyl acetate and lead citrate. Micrographs were produced using a JEM-2100 TEM (200 kV, Jeol).

Mitochondrial count

For counting mitochondria in the previtellogenic oocyte, image files of the electron micrograph of oocytes were opened with Adobe Photoshop CS4 software, and a grid was selected from the menu bar and superimposed on it. The grid was used as quadrate for sampling. Four chambers of the grid were selected randomly at each of five different sites, four at the corner and one at the center of the image. The number of mitochondria from four chambers was counted by putting individual marking to each with the eraser tool. Total number of the mitochondria was computed considering total number of chambers covering the entire area of the oocyte. An average number of mitochondria of four oocytes are presented here.

Schematic drawing

For schematic drawing, the micrographs were opened with Photoshop CS4 software, and drawing was done in different layer using the impressions from the image layer.

Results

Proboscis gland cell mitochondria

In the proboscis gland cell (Fig. 1a), the mitochondria are organized in two different forms (Fig. 1b and Table 1). Immediately surrounding the nucleus, there is a cluster of small mitochondria. Each of those mitochondria appears oval in cross section and provided by condensed cristae. Only very few mitochondria with orthodox cristae are distributed outside the cluster.

Spinal gland cell mitochondria

In the spinal gland cells (Fig. 1a), no free mitochondria are present in the cytoplasm rather, those are all vesicle enclosed (Fig. 1c). Those vesicle enclosed mitochondria are provided by orthodox cristae (Table 1).

Oocyte mitochondria

Janus green B staining of the previtellogenic oocyte reveals numerous spherical blue green bodies clustered in groups (Fig. 2a) in the vicinity of the nucleus. Transmission electron microscopy reveals the cluster contains mitochondria and electron-dense material (Fig. 2b). The mitochondria (Fig. 2c) appear round or oval in cross sections. The inner membrane is infolded perpendicular to the longitudinal axis to form a moderate number of cristae. The cristae extend at least three quarters of the distance across the mitochondrial diameter and have a tubular profile with bulged edges (Fig. 2c) (Table 1). Very often, the mitochondria are associated with rough endoplasmic reticulum through tethers (Fig. 2d). The inner matrix of the mitochondria is a homogeneous matter of finely granular material within which small numbers of variably sized, and dense granules of 180–220 Å diameters are visible. The mitochondrial clusters are intermingled with numerous small vesicles or granulo fibrillar material (GFM) approximately of 0.15–0.54 μm diameter. Several mitochondria are also observed in the state of both fission and fusion (Fig. 3).

Spermatocytes, nurse cell and spermatozoan mitochondria

The typical form of mitochondria is few in the spermatocyte; however, a large "nebenkern" is present near the nucleus of

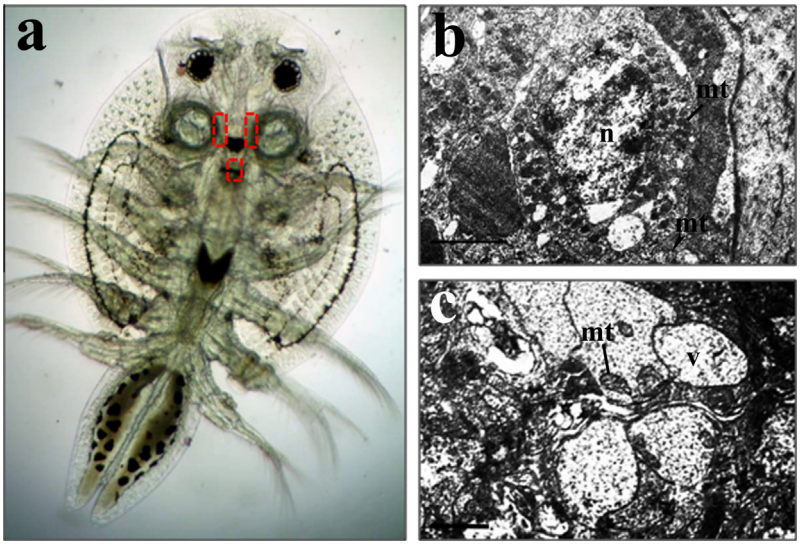

Fig. 1 Photomicrograph of *Argulus bengalensis* and transmission electron micrograph of mitochondrial forms in the glandular cells associated with feeding apparatus. (a) Ventral view of a male showing the anatomical position of proboscis gland (pg) indicated by paired side boxes and spinal gland (sg) indicated by lower median box. (b) Transmission electron micrograph of proboscis gland: mitochondria (mt) are arranged within a separate hub (h) surrounding the nucleus (n). The cristae of these mitochondria are of condensed type. The mitochondria distributed outside the hub are provided with orthodox cristae. Bar, 2 μm. (c) Transmission electron micrograph of spinal gland: mitochondria (mt) are enclosed within vesicles (v) in the cytoplasm. Bar, 1 μm.

Table 1 Comparative profile of mitochondrial forms in *Argulus bengalensis*.

Sources	Types	Mitochondria width (μm) [a]	Cristae types	Special features	Potential functions
Proboscis gland	A. Variant	0.29–0.33	Condensed	Clustered, confined within protected area	High ATP production for synthetic activity
	B. Classical	0.19–0.38	Orthodox	–	Low ATP production
Spinal gland	Variant	0.31–0.45	Orthodox	Vesicle enclosed	Protection of structure and function
Oocyte	Variant	0.32–2.03	Tubular	Inner compartment divided, Exhibit fission and fusion, ER associated	High energy production to carry out different synthetic activities
Sperm flagellum	A. Classical	0.15–0.17	Condensed diffused	–	High ATP production
	B. Variant	0.21–0.30	Stacked	Microtubule associated	Use of microtubuler tracts for efficient energy distribution
Spermatocytes	A. Variant nebenkern	9.05–13.78	Stacked	Highly packed cristae, obscured inner compartment	Later modified into flagellar mitochondria in spermatozoa
	B. Classical	0.52–1.25	Condensed	–	High ATP production
Striated muscle	Variant	0.67–1.25	Orthodox	ER associated	Mitochondrial Ca^{2+} uptake for continued muscular function

[a] Range of width (lowest–highest) of mitochondria from 10 ultrasections studied. For each presentation five measurements were made at different angles and averaged.

primary and secondary spermatocytes (Fig. 4b). A similar structure is also observed at the base of the cytoplasmic projection of the nurse cell (Fig. 4a). The nebenkern is provided with huge number of closely stacked zigzag cristae within a highly dense matrix (Fig. 4c and d). The zigzag cristae are extensive and profusely anastomotic or overlapped to each other. Apart from the nebenkern, very few classical forms of mitochondria are randomly distributed around the nebenkern, but a few are located at juxtaposition (Fig. 4b and Table 1).

In the flagellum of the spermatozoa (Fig. 5a), adjacent to the axoneme, there are three moderately sized mitochondria one with four numbers of orthodox cristae meeting at the center of the inner matrix and two others with condensed but diffused cristae (Fig. 5b). The medially located mitochondrion is pear shaped, but others two are oval in cross section. Serial sections of the flagellum reveal that these mitochondria are filiform and extend almost the entire length of it except the terminal part. One more unusual type of mitochondrion (Fig. 5c) is

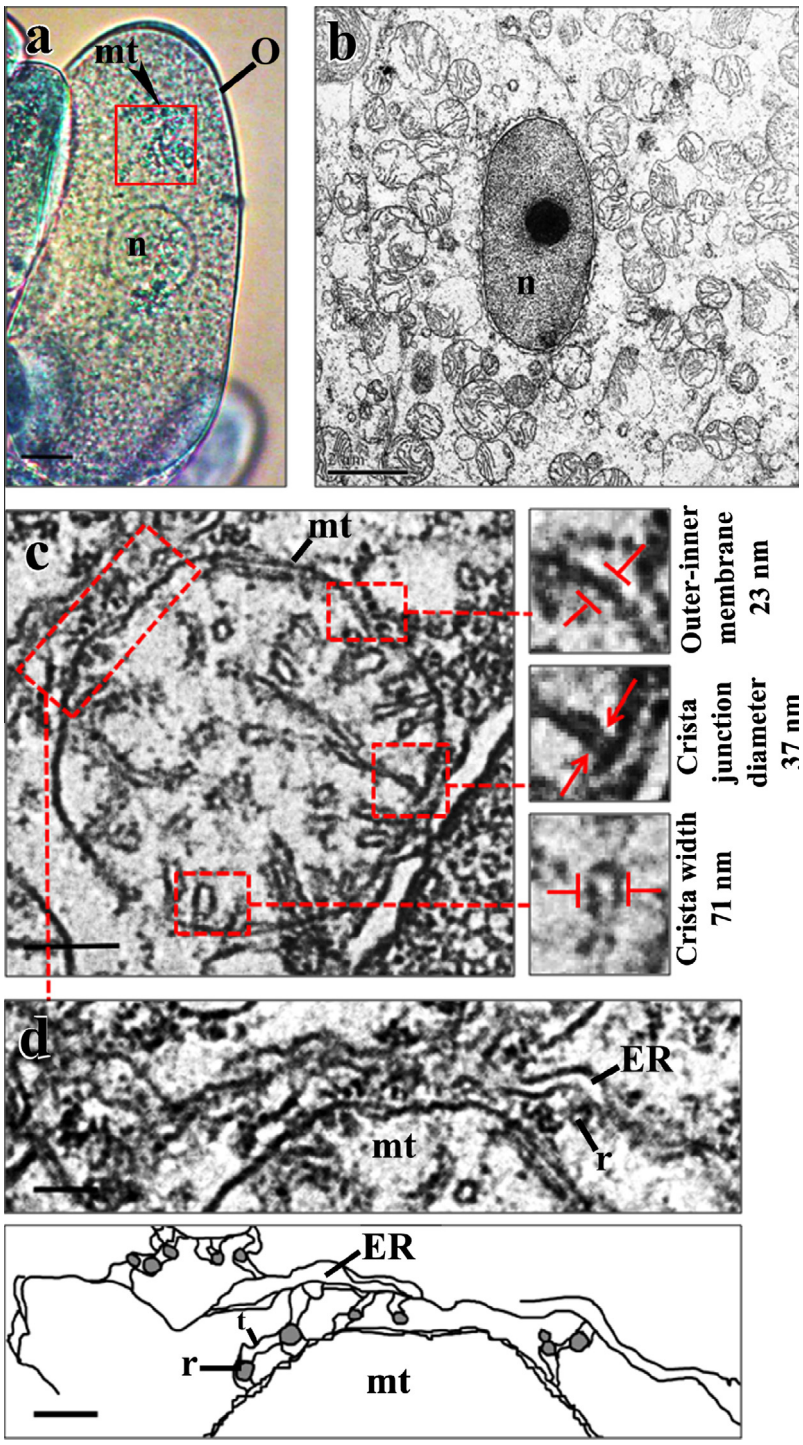

Fig. 2 Light and electron microscopy of mitochondria in the oocytes of *Argulus bengalensis*. (a) Light micrograph after mitochondria specific vital staining with Janus green B showing distribution pattern of mitochondria within the cytoplasm of an early previtellogenic oocyte (o); mitochondrial clouds (indicated by boxes) are differentiated beside the nucleus (n). Bar, 18 μm. (b) Transmission electron micrograph of an early previtellogenic oocyte showing similar mitochondria rich zone around the nucleus (n). Numerous small vesicles or granulofibrillar material (GFM) are distributed within this mitochondria rich zone. (c) Ultrastructure of mitochondrion of an early previtellogenic oocyte exhibiting its tubular cristae with dilated terminal. The mitochondrion exhibits a loose association with an ER. Numerous small but dense granules (g) are observed within the inner matrix. Bar, 0.34 μm. (d) A magnified view (2×) of the association of mitochondria with ER – showing tethers (t) and ribosomes (r) in the upper pannel, and the lower panel is the schematic diagram of the same. Bar, 0.2 μm.

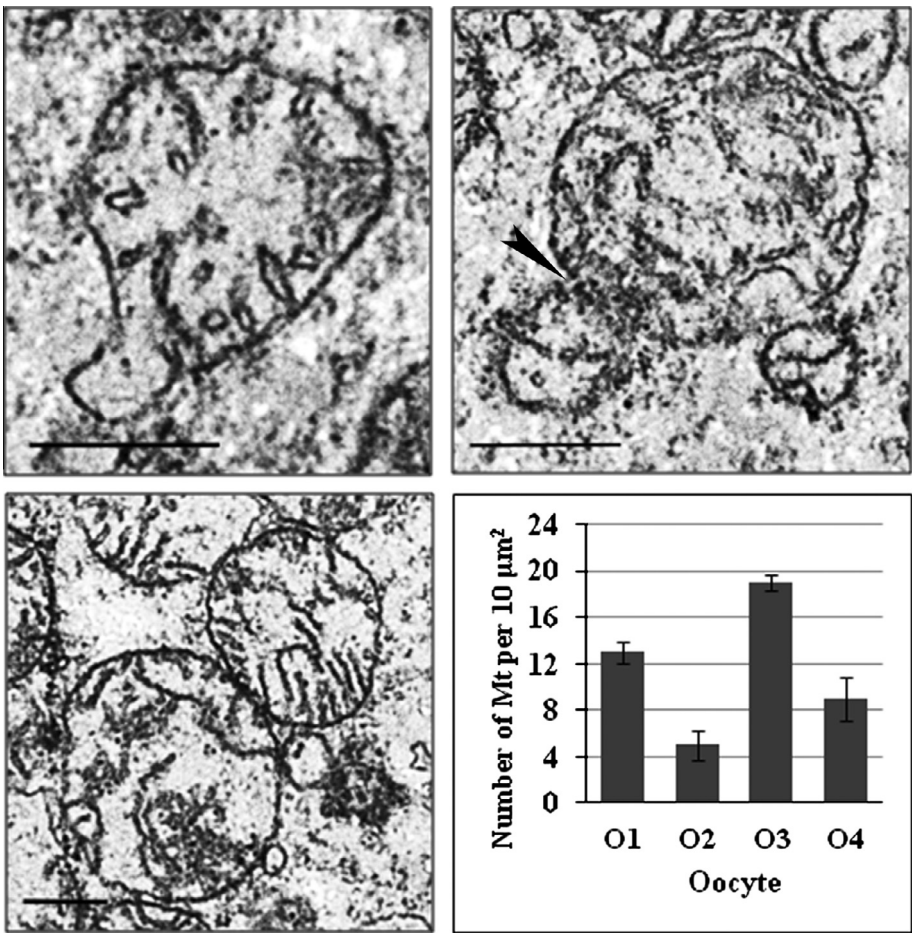

Fig. 3 Transmission electron micrograph of oocyte mitochondria at dynamic state in *Argulus bengalensis*. The upper left panel exhibits out pocketing, an indication of fission of mitochondria. The upper right panel exhibits mitochondrial fusion indicated by diffused membrane (arrow head) between mitochondria. The lower left panel shows two mitochondria immediately after completion of fission. Bar, 0.54 µm. The graphic in the lower right panel represents number of mitochondria (Mt) in four previtellogenic oocytes (O1, O2, O3, and O4); mitochondria undergoing fusion or fission are counted as a single unit. Mean of five readings with ± standard error is presented in the graphics.

found at a right angle to the medially located mitochondrion. It is pear shaped in cross section and spans about half of the flagellum. In its course through the flagellum, the alignment is changed with respect to the middle mitochondrion. The inner membrane of this mitochondrion is clearly distinguishable but looses its connection with the transverse cristae. The transverse cristae are closely stacked into a cluster, and 20 numbers of F_1 particles are aligned at regular intervals at the outer periphery of the cluster. Fifteen singlet microtubules, each comprises of 12 protofilaments, are attached to the circumference of this mitochondrion through motor proteins (Fig. 5d).

Striated muscle cell mitochondria

The mitochondria of striated muscles from the thoracic appendages are oval in shape and provided by orthodox cristae (Fig. 6a and Table 1). Inner matrix of those is compartmentalized further by the extension of some cristae. In the sarcomeres, the mitochondria are distributed adjacent to the myofibrils and are intimately associated with the ER through tight junction (Fig. 6c). Their associations with the vesicular tethering structures of endoplasmic reticulum (Fig. 6b) are also being observed. A gap of 78–86 nm is maintained between the mitochondria and the tethering vesicles where several ribosomes (Fig. 6b) are distributed.

Discussion

In *A. bengalensis* other than classical type, six mitochondrial variants are observed in different cell types to meet up the energy demands under varied physiological states of its parasitic mode of life.

Proboscis gland cell and spinal gland cell mitochondria

Feeding apparatus of *Argulus* spp. is a secondary acquisition and comprises of a proboscis and a preoral spine. A pair of proboscis gland consisting two giant cells is associated with the proboscis, and one spinal gland consisting four large cells is located at the base of the spine. The spine is used to pierce the host tissue, and the tissue fluid and blood ooze out are ingested through the proboscis. The spinal gland produces

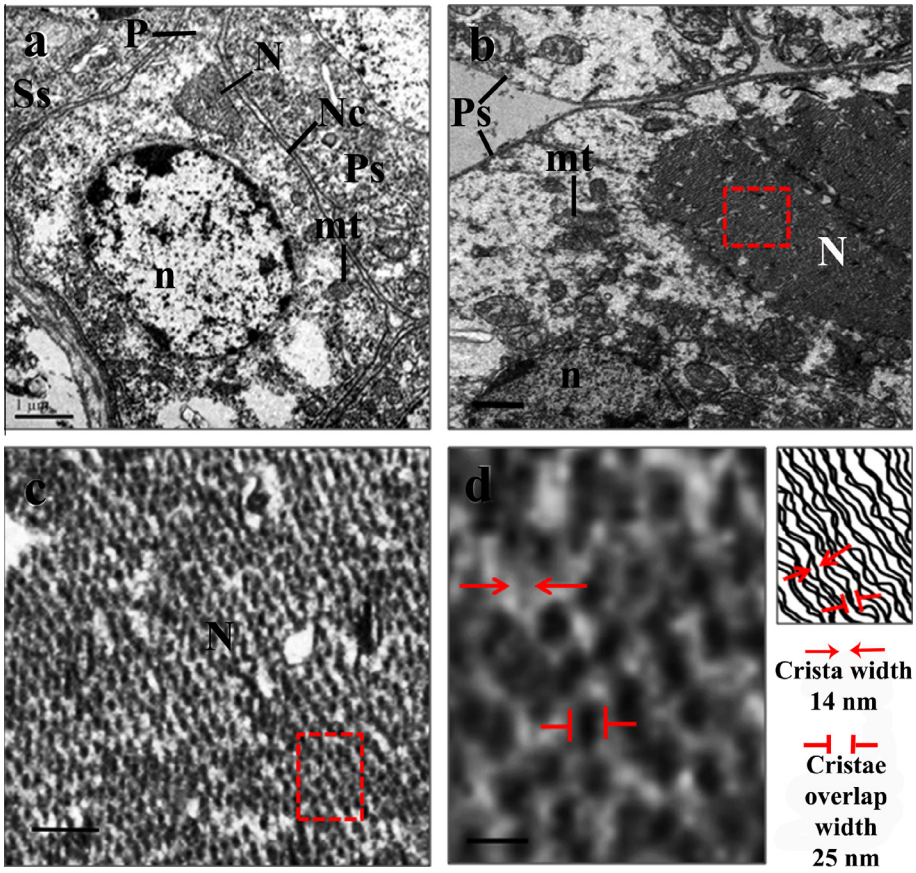

Fig. 4 Transmission electron microscopy of mitochondria in the testicular cells of *Argulus bengalensis*. (a) Nebenkern (N) in the nurse cell (Nc). Nurse cell is present in between the primary (Ps) and secondary spermatocytes (Ss). The nebenkern (N) is positioned at the base of the cytoplasmic projection (P). Small classical mitochondria (mt) are also randomly distributed beside the nucleus (n) of the nurse cell. (b) Primary spermatocyte also exhibits a large nebenkern (N) beside its nucleus (n) and small classical mitochondria (mt) are randomly distributed around the nebenkern. Bar, 1 μm. (c) Magnified view (4 ×) of the nebenkern showing its stacked zigzag cristae. Bar, 0.2 μm. (d) Highly magnified view (17×) of the nebenkern showing cristae with overlapping at regular interval. The right corner panel is a schematic diagram shows the arrangement of cristae and variation in cristae diameter. Bar, 54 nm.

an anesthetic substance which is injected into the fish's body for effortless feeding activity, and the proboscis glands produce an anticoagulant that prevents ingested blood from clotting within the gut [10]. Condensed state of cristae in the mitochondria found in these glandular cells correspond to their high workload of ATP production [11] required for their synthesis activities. An unusual type of closed membrane vesicles containing one or more mitochondria was observed in the spinal gland cells. Similar type of mitochondrial concealment was also observed in aging wheat coleoptiles and in neural tissue (Table 2) [12,13]. Concealment of the mitochondria within vesicles may preserve their structure and activity [12] and thereby protect them from exposure to the glandular synthesis (Table 1). In proboscis gland, cluster of small mitochondria is concealed in an area surrounding the nucleus. Similar type of mitochondrial cluster was also observed in human fetal and adult female germ cells (Table 2) [14]. Concealment of mitochondria within vesicles or in specialized area definitely protect the mitochondria from the detrimental effect of glandular synthesis and help to restore their structural and functional organization and thereby confers some adaptive advantages to the organisms in their parasitic mode of feeding or hematophagy.

Oocyte mitochondria

One of the most prominent features of the early previtellogenic oocytes of *A. bengalensis* is the mitochondrial aggregation into cluster known as Yolk nucleus and Balbiani body. The term mitochondrial cloud is often more appropriate to these clustered organelles and has frequently been used [15]. Light microscopy of the previtellogenic oocyte with Janus green B, which stains mitochondria supravitally [16], able to detect such clusters sometimes referred as the Balbiani body or Yolk nucleus in the oocyte of *Xenopus* sp. and human (Table 2) [14,15,17] which plays an important role in germinal granule localization in the vegetal pole [17]. The ultrastructure of the mitochondrial aggregates reveals that they consist of large numbers of discrete mitochondria, but the image is not quite that expected from a simple aggregate of separate mitochondria. However, during our study of the mitochondrial clusters of *Argulus* sp., we became impressed with the dynamicity

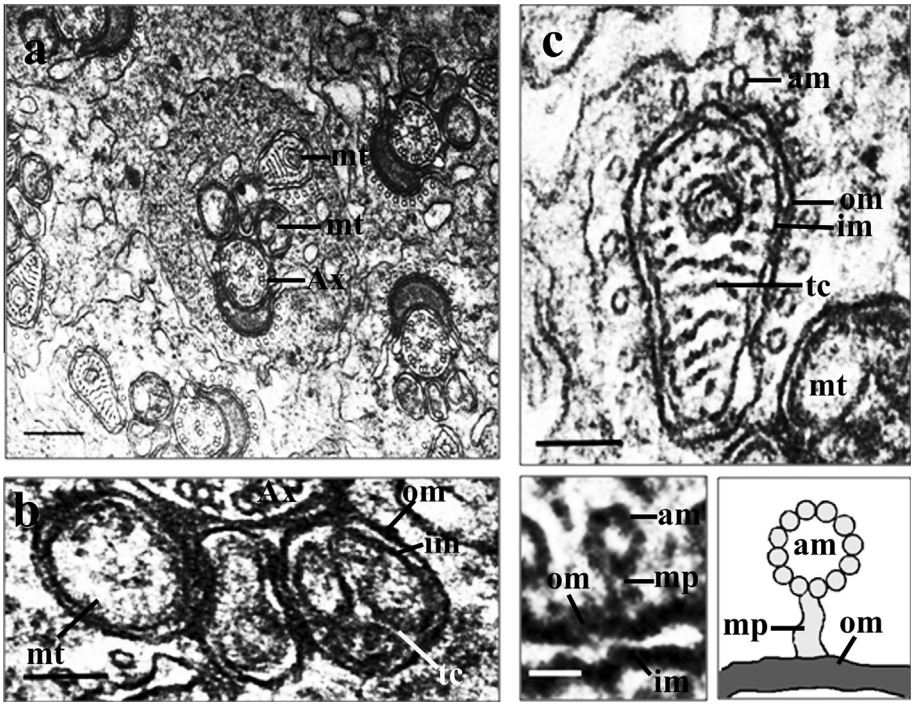

Fig. 5 Transmission electron micrograph of mitochondria in the sperm flagellum of *Argulus bengalensis*. (a) Mitochondrial (mt) alignment surrounding the axoneme (Ax). (b) Three vesicular filamentous mitochondria immediately adjacent to the axoneme with clearly distinguishable outer membrane (om), inner membrane (im) and transverse cristae (tc). Cristae are uniting at the central meeting point (mp) to compartmentalize the inner matrix. Bar, 74 nm. (c) An unusual association of mitochondrion with numerous microtubules (am). The transverse cristae (tc) are originated from the inner membrane and extending up to the inner membrane of the opposite side. Twenty F_1 particles are arranged near the base of each cristae. Bar, 74 nm. The bottom left panel is a magnified (6×) view showing the microtubular association with the outer membrane (om) by motor protein (mp). Bar, 13 nm. The bottom right panel is a diagrammatic representation of the microfilament. The microfilament is a complete circle of twelve protofilament (pf). It remains attached with the outer mitochondrial membrane (om) with a motor protein (mp).

accompanied with both fusion and fission leading profound morphogenetic changes, reflecting changing metabolic requirements. The complexity of the mitochondrial profiles is further validated by its association with other organelles. The endoplasmic reticulum composes a loose junction with the mitochondria which is occupied by granulo fibrillar material (GFM). Mitochondrial association with the endoplasmic reticulum will be important to supply energy for translation. One of the important features of an ectoparasite is its reproductive investment which involves much energy production and utilization to meet up the needs for maturation of gametes (Table 1). Previtellogenic stage is the most active stage in the maturation process when the oocytes become prepare for several synthetic activities including synthesis of Yolk to carry out the embryonic development of the parasite.

Spermatocytes, nurse cell and spermatozoan mitochondria

In the spermatocytes and nurse cell, a mitochondrial variant, nebenkern is observed like that of other insect spermatocytes. Nebenkern is formed through a multistep process by which the numerous mitochondria are clustered together and fused to produce a large spherical body [18–20]. The cristae of the ''nebenkern'' in the spermatocytes of *Argulus* are longer and more closely packed which indicates that the cells are in hyperactive metabolic state. Such type of zigzag orientation of cristae is also observed in hyper metabolically active tissue like cardiac muscle cells of the canary and other birds [21]. Dorogova et al. [22] explained that marlin protein in the nebenkern plays important role in spermatogenesis of *Drosophila*. The nebenkern also unfolds and extends along with growing axoneme in *Drosophila* sperm. Similar role of nebenkern in *Argulus* species can be apprehended.

One rare type of mitochondrion is found in the flagellum of the spermatozoa where it is associated with microtubules (Table 1). Microtubular association is also found *in vitro* with different cell types of the vertebrate like fibroblasts, macrophages, smooth muscle cells and in neuronal axons (Table 2) [23,24] but in those cases, mitochondria are associated with fewer microtubules, whereas *in vivo* argulid sperm is associated with numerous as more as 15 microtubules in a definite pattern. The physiological significance of such association is that mitochondrion uses these microtubular tracts for its movement [25] with the aid of motor proteins like dynein. During copulation of *Argulus,* sperm is donated as packets or spermatophores [26]. So, the individual sperm does not require active motility at this stage, but sudden active and regulated motility is required immediately after its release from the spermatophore just before fertilization. The movement of mitochondrion through the microtubular tracts must be related to energy distribution

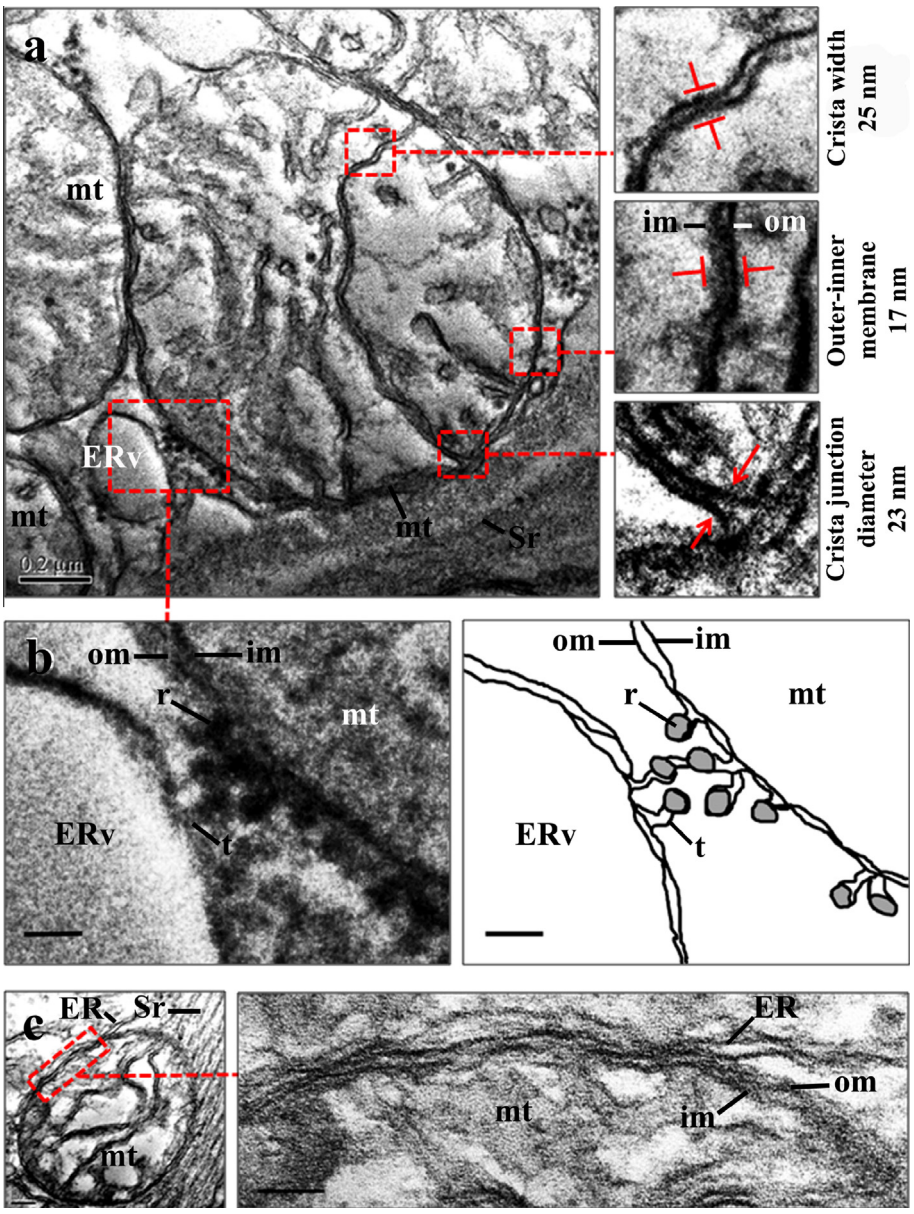

Fig. 6 Transmission electron microscopy of mitochondria in the striated muscle. (a) Mitochondrion (mt) in association with sarcoplasmic reticulum (Sr) showing attached putative ER vesicle (ERv). A narrow space is present in between the outer membrane (om) and the inner membrane (im). (b) Higher magnified (5×) view of the ER vesicle (ERv) showing tethers (t) and associated ribosome (r). Right panel is a schematic diagram showing molecular bridges that regulate the close contacts between ER and mitochondria. Bar, 43 nm. (c) Tight association of the ER with outer membrane (om) of a mitochondrion. Bar, 70 nm. Right panel is a magnified view of the same.

and utilization along the length of the flagellum resulting regulated sperm motility for successful fertilization in *Argulus*. This type of mitochondrial association with microtubules is also observed in the flagellum of tongue worm, Pentastomid *Raillietiella* in one of the publications of Wingstrand [27] that justify their phylogenetic relationship.

Striated muscle cell mitochondria

The mitochondria of the striated muscle cells of thoracic appendages are very large in comparison with the other types of mitochondria found in *Argulus* (Table 1). The physical association between the endoplasmic reticulum (ER) and mitochondria, which is known as the mitochondria-associated ER membrane (MAM), has important roles in various cellular "housekeeping" functions [28]. Close contacts between the ER membrane and the mitochondrial outer membrane have been visualized by various authors in rat liver tissue and in the pseudobranch gland of teleost (Table 2) [29,30]. ER and mitochondria are held together by different molecular chaperones as stated by Hayashi et al. [28] and Rizzuto et al. [31]. Other than the vertebrate system, mitochondrial association with ER is first time evident in an invertebrate like the parasitic *Argulus*. Mitochondria regulated efflux of endoplasmic Ca^{2+} and Ca^{2+} signaling thereof [28,31] helps in regulating muscle contraction, lipid transport [32], and cellular survival [33,34].

Table 2 Comparative account of mitochondrial variants of *Argulus bengalensis* with that of other referred organisms.

Mitochondrial variants	Source tissue of *A. bengalensis*	Other plant, invertebrate and vertebrate sources	References
Confined around nucleus	Proboscis gland	Human fetal and adult female germ cells	Motta et al. [14]
Vesicle enclosed	Spinal gland	Aging wheat coleoptiles, neural tissue	Bakeeva et al. [12], Mishchenko [13]
Mitochondria with fission and fusion and ER associated	Previtellogenic oocyte	Oocytes of vertebrates like *Xenopus laevis*	Billett and Adam [15], Motta et al. [14], Wilk et al. [17]
Nebenkern	Spermatocyte	Spermatocytes of various insects	Beams et al. [19], Tokuyasu [20].
Microtubule associated	Sperm flagellum (*in vivo* it is reported for the first time)	*In vitro* culture of. rat kidney cell, human fibroblasts, peritoneal macrophages and smooth muscle of mouse	Goldman and Follett [23], Heggeness et al. [24]
ER associated	Sarcomere	Liver cell, neuron and various other cell types of vertebrates	Copeland and Dalton [29], Morre et al. [30]

A continuous mitochondrial Ca^{2+} uptake occurs in the muscle tissue which in turn could facilitate mitochondrial Ca^{2+} overloading and membrane permeabilization [35]. Such type of ER-mitochondria tethering ensures the propagation of TP3R-linked Ca^{2+} signals to the mitochondria to coordinate ATP production with the stimulated state of the cell, and it protects the cell from energy depletion and maintain mitochondrial metabolism [34]. The respiratory structures of the parasite are located on the ventrolateral thoracic carapace, which is ventilated by the continuous movement of the three pairs of thoracic appendages when the parasite remains attached to the host body with a pair of suckers. Continuous movement of appendages needs uninterrupted muscular function. The physical association between the endoplasmic reticulum (ER) and the mitochondria must play important role in energy production and utilization to confer the stimulated state of the cells to bestow the parasitic adaptive advantages.

Conclusions

In *A. bengalensis,* mitochondria are highly dynamic structures and appear in varied forms and numbers in different cell types at varying physiological states. It readily undergoes fission and fusion in cells like oocytes and even can move on cytoskeletal track for efficient energy distribution and utilization in a specific cell type like argulid sperm. Muscle cells in continuous action can utilize the close association of mitochondria with the endoplasmic reticulum not only for efficient energy production and utilization but also for regulated contraction brought about by regulated Ca^{2+} release from the endoplasmic reticulum. The mitochondria of glandular cells associated with the feeding apparatus of *Argulus* are well protected within cytoplasmic vesicles. The tissue specific mitochondrial variability of this parasitic organism has its implication on the biology of the cell and hence on the biology of the organism which bestow several adaptive advantages to its parasitic mode of life. The phylogenetic relationship of argulids with pentastomids is a long pending issue; mitochondrial association with microtubules in the flagellum of the sperm adds further evidence in support of it.

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Conflict of interest

The authors have declared no conflict of interest.

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