Ultrastructure Based Morphofunctional Variation of Olfactory Crypt Neuron in a Monomorphic Protogynous Hermaphrodite Mudskipper (Gobiidae: Oxudercinae) (*Pseudapocryptes lanceolatus* [Bloch and Schneider])

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### Abstract

*Pseudapocryptes lanceolatus* (Bloch and Schneider) is a monomorphic protogynous hermaphrodite teleost that possesses ovotestis as gonadal unit of reproductive structure. At the onset of breeding season (i.e., June–July), the ovarian tissue is gradually differentiating into female-phased *P. lanceolatus*. At the same time, the pear-shaped crypt cells (a type of neuron) are frequently appeared at apical part of pseudostratified olfactory neuroepithelium of *P. lanceolatus*. The crypt neuron is characterized by the presence of sunken cilia and microvilli at the proximal region. The features of subcellular organelles are also explored in lieu of their probable functional significance. The nucleoplasm of mature crypt neuron shows chromatin granules having diameter: 15–25 nm. This cell undergoes neural apoptosis at the end of breeding phase (i.e., October–November). Fragmented chromatin fibers with numerous chromatin granules (diameter: 25–30 nm) in nucleoplasm and lysosomal diversity are the most notable characters of apoptotic crypt neuron. The large accumulation of heterochromatin chromatinis in nucleoplasm is also marked under fluorescence microscope. The frequent presence of acetylcholinesterase-positive vesicles in axoplasm of crypt neurons is also a prime subcellular indicator for inhibition of neural transmission of olfactory signals. Therefore, it is concluded that the sex differentiation in *P. lanceolatus* and occurrence of crypt neuron in olfactory neuroepithelium are interrelated events during the reproductive period. Consequently, we hypothesized that the crypt neuron plays an active role in the implementation of unique reproductive strategy through recognition of pheromonal cues within the social organization of *P. lanceolatus*.

### Keywords

Chromatin, crypt, hermaphrodite, *Pseudapocryptes lanceolatus*, protogynous

### Introduction

Olfactory chemoreception of pheromones includes cascades of reactions and plays a fundamental role for promoting reproductive synchrony in fish.\(^1,2\) Teleosts exhibit a diversity of sexual pattern.\(^3\) The hermaphroditism and bidirectional sex change in Family: Gobiidae is a unique phenomenon in zoological account. Several species among this family are protogynous hermaphrodites.\(^4-6\) It was previously reported that they are capable to change their sex in bidirection, i.e., male to female and vice versa throughout the lifespan. The ethological studies indicate that a dominant male controls a harem of several females.\(^7\) The repeated and reversible sex change in gobiid is regulated by hormones.\(^8\) The gonadal maturation occurs during sexual phases of life. During this phase, the crypt neuron may elicit the sexual behavior in fish (including gobiids) through the detection of sex pheromones at the time of nasal ventilation over olfactory neuroepithelium.\(^9\) Crypt neuron was exclusively found in the olfactory neuroepithelium of actinopterygian fishes and described by Hansen and Finger.\(^10\) This cell is a type of bipolar olfactory neuron and projects their axon to the ventral region of the olfactory bulb (OB) to make neural communication by forming lateral bundle of medial

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It is a basic key for understanding the ability of an animal to discriminate a wide range of odorants. The axons of crypt neurons expressing specific G-protein (Goα) and projecting to the ventral midline of the OB in catfish. These cells are found in the most superficial layer of the fish olfactory neuroepithelium. Crypt neuron is also showing seasonal variation in relation to their occurrence at variable depths of olfactory neuroepithelium in different prebreeding, breeding, and postbreeding seasons, respectively. No research-oriented data have been recorded in favor of seasonal variation of crypt neuron in gonadal maturation in protogynous hermaphrodites. Therefore, this research contribution is pertinent in raising the question that, how crypt neuron shows seasonal variation according to gonadal maturation protogynous hermaphrodites (male/female phase fish)?

**Material and Methods**

**Experimental specimen**

*P. lanceolatus* is a teleostean: Gobiid and considered as “Least Concerned” according to the Red List Category of International Union for Conservation of Nature (website: http://www.iucnredlist.org/details/169496/0). Live and adult specimens of *P. lanceolatus* (having total body length: 10–20 cm) were collected from the intertidal habitat of Hooghly River near Kanchrapara, North 24 Parganas, West Bengal, India (22.99°N 88.40°E) and brought to the laboratory. The specimens were acclimatized with the physical condition (temperature: 20°C to 25°C, humidity: >40%, etc.) for 48 h.

**Macro- and microanatomical study of the gonads**

The morphoanatomy of the gonadal structure in adult *P. lanceolatus* has been studied in consecutive reproductive seasons during 2010–2015. The studies were carried out in two phases, i.e., at before the onset of breeding season (i.e., June–July) and just after the breeding season (i.e., October–November) in a year. For anatomical study, the live specimens of *P. lanceolatus* of both the seasons were carefully sorted out and anaesthetized using MS-222 (dose: 100–200 mg/L). The gonadal structures were dissected through the ventral part *P. lanceolatus* and immediately fixed in 4% paraformaldehyde in 0.1 (M) phosphate buffer (pH 7.3) at 4°C for 2 h. The fixed tissues were then washed in the same buffer (3 changes at 30 min of interval) and cryoprotected in 15%–30% sucrose solution in 0.1 (M) phosphate buffer for 24 h at 4°C. The frozen sections (thickness: 15–20 µm) were cut using cryostat (Leica CM 1850; Leica Biosystems Nussloch GmbH, Germany) and carefully placed on gelatin-coated slides. The slides were stained with hematoxylin and Eosin, examined under trinocular light microscope (Primo Star; Carl Zeiss Microscopy, GmbH, Germany), and acquired images were analyzed by AxioVision LE (version 4.3.0.101) (Carl Zeiss Vision, GmbH, Germany).

**Light and fluorescence microscopical study on the seasonal variation of crypt neuron**

After procurement of the gonadal tissue, the dorsolateral part of the snout in the same anesthetized *P. lanceolatus* was again dissected out. The unilamellar olfactory apparatus was separately fixed in 4% paraformaldehyde in 0.1 (M) phosphate buffer (pH 7.3) at 4°C for 2 h. The fixed tissues were then washed in the same buffer (3 changes at 30 min of interval) and cryoprotected in 15%–30% sucrose solution in 0.1 (M) phosphate buffer for 24 h at 4°C. The frozen sections (thickness: 15–20 µm) were cut using cryostat (Leica CM 1850; Leica Biosystems Nussloch GmbH, Germany). The sections were separately incubated with Acidine Orange (AO) solution (6 µg/ml in 0.1 M phosphate buffer [pH 7.3] at 4°C for 15–30 min) and ethidium bromide (EB) solution (7 µg/ml in 0.1 M phosphate buffer [pH. 7.3] at 4°C for 30–45 min). The incubated sections were washed in the same buffer (3 changes), mounted on glass slides (equal volume of glycerol and buffer is used as mounting medium), and examined under light microscope (LM: Primo Star, Carl Zeiss, GmbH, Germany) and fluorescence microscope (Leica DM 3000; Leica Microsystems) through Blue filter. The acquired images were analyzed by AxioVision LE; Version 4.3.0.101; Carl Zeiss, Germany (for LM study) and Microscope Imaging Software; Leica Application Suite Advanced Fluorescence for fluorescence microscopical data.

**Transmission electron microscopical study**

For electron microscopical study, the fresh, adult (having total body length of 150–200 mm) specimens of *P. lanceolatus* were collected from local markets of South 24 Parganas, West Bengal, India and brought to the laboratory for acclimatization with the physical conditions (temperature: 20°C–25°C, humidity: >40%, time: 24 h, etc.). The specimens were anesthetized using MS-222 (dose: 100–200 mg/L) for microscopical studies. The olfactory apparatus of *P. lanceolatus* was dissected out from the anterodorsal side of the head and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2–7.4) at 4°C for 2 h. After primary fixation, the olfactory tissues were rinsed in the same buffer and then fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2–7.4) for 1 h at 27°C. The olfactory tissues were then rinsed in the same buffer and dehydrated in chilled acetone. The tissues were embedded in Araldite CY212 (TAAB, UK) and resin polymerized for 48 h.
at 60°C. The ultrathin sections were cut (thickness: 70–80 nm) using ultramicrotome (Leica Ultracut UCT), collected on copper grids, and stained with uranyl acetate and lead citrate, respectively. The sections were examined under Morgagni 268D transmission electron microscope (TEM, Fei Electron Optics, Eindhoven, The Netherlands) operated at 80 kV. Digital images were analyzed at using iTEM software (soft imaging system, Münster, Germany) attached to the microscope.

**Acetylcholine esterase activity under transmission electron microscope**

The olfactory apparatus of postbreeding female phases’ *P. lanceolatus* was separately dissected out and fixed in 2.5% glutaraldehyde and 4% paraformaldehyde (1:1) in 0.1 M phosphate buffer (pH 7.2–7.4) at 4°C for 2 h. The tissues were washed in same buffer and incubated in the medium as described by Karnovsky and Roots.[20] The incubated tissues were rinsed in DAB solution and processed for TEM study. For electron microscopical study, the tissues were secondarily fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2–7.4) for 1 h at 27°C. The tissues were then rinsed in the same phosphate buffer; dehydrated in graded chilled acetone, and embedded in araldite mixture for 48 h at 60°C. The sections (thickness: 70–90 nm) were cut using ultramicrotome (Leica Ultracut UCT), collected on copper grids, and viewed under TEM (Morgagni 268D) operated at 80 kV.

**Results**

*P. lanceolatus* is a monomorphic protogynous hermaphrodite species [Figure 1a]. This species possesses paired ovotestis as gonadal structure (examined at before the onset of breeding season) [Figure 1b]. The transparent tube-like paired ovary is present in association with small pair of testis. The sac-like testis is located at the posterior part of ovarian structures [Figure 1b]. The accessory gonadal structures are also present at the junction of testicular lobe and common genital sinus. Each gonad is separated into ovarian and testicular region [Figure 2a]. The immature ovary is consisting of small cluster of immature oogonia and vitellogenic oocytes [Figure 2a]. Each testis is small and comprises cluster of developing spermatogenic tissue. Spermatogonia-like appearances are also observed within the seminiferous tubules of testis [Figure 2b]. The sex-differentiated female-phased *P. lanceolatus* shows well-developed ovarian structure during breeding season [Figure 1c]. At the onset of breeding season, an ovoid-shaped special type olfactory neuron, namely, crypt neuron appeared in olfactory neuroepithelium of *P. lanceolatus* [Figure 3]. This type of cell is characterized by very short dendron, perikaryon, and axon [Figure 3]. Crypt neuron is generally observed in the apical part of olfactory neuroepithelium of *P. lanceolatus* [Figure 4a-g]. Generally, this cell is rare at pre- and postbreeding seasons but predominantly appeared during breeding season of *P. lanceolatus*. The proximal tip of extended perikaryon has a prominent apical invagination which is equipped with microvilli and sunken cilia [Figure 3]. The transverse sections of cilia show (9 + 0) arrangement of microtubules. Microvilli are present around the apical rim of crypt neuron [Figure 3]. The lateral margin of the perikaryon shows longitudinal arrangement of numerous neurofilaments along with vesicular structures having various morphometry and diameter (ranging from 10 nm to 50 nm) [Figure 5a-c]. Multivesicular bodies are also identified under TEM. Mitochondria with prominent cisterna and dense matrix are well marked within the cytoplasm of crypt neuron [Figure 5b]. Large, spherical nucleus is present at the lower-middle part of the cytoplasm [Figure 5c and d]. The heterochromatin materials are distributed at the peripheral part of nucleoplasm. The different regions of nucleoplasm in crypt neuron show various patterns of chromatin fiber condensation and accumulation of chromatin granules (diameter: 15–25 nm) [Figure 6]. The differential distributions of functional and repressive
chromatin structures are also marked under fluorescence microscope (stained with AO). Grater euchromatin materials within the nucleoplasm of crypt neuron are prominently indicating functional state during breeding season [Figure 4e]. The perinuclear cytoplasm of olfactory crypt neuron shows rough endoplasm reticulum (rER), free ribosomes, polyribosomes, secretory vesicles, etc., [Figure 5c and d]. Golgi complex with distinct secretory axis is clearly noted. Several phases of secretory vesicles (diameter ranges from 10 to 40 nm) are identified within the cytoplasm of crypt neuron [Figure 5c]. At the postbreeding season of _P. lanceolatus_, a large number of crypt neurons undergo neural apoptosis compared to breeding season (identified under trinocular light microscope and fluorescence microscope using EB staining) [Figure 4d, f, and g]. The apoptotic crypt neuron possesses functionally repressed heterochromatin materials within the nucleoplasm [Figure 4f and g]. This cell shows membrane blebs at perikaryon, several degenerating mitochondria with dilated cristae, loose and distorted neurofilaments, large number of vesicles at intertubular space, fragmented chromatin fibers with numerous chromatin granules (diameter: 25–30 nm) in nucleoplasm, etc., [Figure 7]. Perinuclear cytoplasmic organelles such as rough endoplasmic reticulums (rER), Golgi complex, and free ribosomes are also dilated in nature but lysosome shows structural variation in degenerating crypt neuron. The cytoskeletal integrity of neurofilaments and microtubules is also less compact in axonal region [Figure 7]. Large accumulation of acetylcholine esterase (AchE)-positive vesicles (substrate used: acetylthiocholine iodide) is observed at axoplasm of apoptotic crypt neuron during the postbreeding season [Figure 8].

**DISCUSSION**

The crypt neuron is one of the significant olfactory sensory receptor neuron that differs from other types (namely, ciliated and microvillous sensory receptor cells) in respect to their morphology, occurrence, location, number, etc._[10,21,22]_ This cell is originated from the globose basal cell (i.e., electron lucent basal cell) in cartilaginous fish, primitive and modern bony fishes._[23-25]_ This cell is still not recognized during the development of olfactory system in any actinopterygian fish._[26,27]_ Recent researches also indicate that crypt neuron is involved in imprinting of kin recognition of specific olfactory cues for mate selection._[22]_ Furthermore, the density of crypt neuron population is higher in female olfactory system than male during breeding season._[28]_ In hermaphrodite protogynous gobies, individuals are also attaining their sexual differentiation and maturity during specific breeding season._[29,30]_ The gonadal structure in _P. lanceolatus_ possesses both testicular and ovarian tissue. At the onset of breeding season, the female-phased _P. lanceolatus_ is differentiated and matured through the development of ovarian structures. The fate of testicular tissue in female-phased _P. lanceolatus_ is still not known to us. In other protogynous gobies, the testis degenerates when the individual differentiates into female phased and the reciprocal event is occurred in male phased._[19]_ This may be recognized as a unique reproductive strategy in gobies including _P. lanceolatus_ for
maintaining the definite sex ratio in their social organization.
The olfactory crypt neuron is functionally important for their involvement in definite reproductive functions through detection of sex pheromonal cues from external environment. This cell also shows G-protein-mediated signal transduction to the higher cortical areas of brain. Crypt neuron has expressed specific G-proteins (subunits: $G_{\alpha_o}$ and $G_{\alpha_q}$) mediated by single $V1R$-related $ora4$ gene. Form the ultrastructural aspect, we may assume that vesicular structures having variable morphometry in crypt neuron may play functional role in recognition and signal transduction of chemical cues during breeding period in $P. lanceolatus$. In ciliated olfactory sensory receptor cell, we have already described bidirectional transport of vesicular cargo which is subsequently crowded and docked at dendronic and axonal tip, respectively. The cytoskeletal structures may act as a roadway to transport the vesicular structures to their destiny. In nucleus, a comparative variation in chromatin structures has been seen in mature and apoptotic crypt neuron. The functional distribution of “permissive” euchromatin and “repressive” heterochromatin in nucleoplasm is a prime indicator of neural aging. The average diameter of first-order chromatin fiber is 10 nm. It is subsequently built-up from DNA wrapped around the nucleosomes and condensed by several factors (such as protein–DNA and protein–protein interactions).
interactions including individual nucleosomes, the linker histone H1 as well as other proteins) into a 30 nm chromatin fiber to form higher ordered chromatin structures. When the breeding season is over, the nucleus of crypt neuron shows features neural apoptosis that are characterized through TEM and confirmed under fluorescence microscope using EB as fluorochrome. During apoptosis, the AChE-positive vesicles in axons of crypt neuron may firmly demonstrating functional inhibition of neural signal propagation through a specialized cytological channel at the termination of breeding period of P. lanceolatus.

Conclusions
This study concluded that the sex differentiation and occurrence of crypt neuron in olfactory neuroepithelium are interrelated event of reproductive season examined in P. lanceolatus. Consequently, the crypt neuron may play a crucial role for implementation of unique reproductive strategy as well as reproductive success through recognition of specific olfactory cues within the social organization of P. lanceolatus.

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Conflicts of interest
There are no conflicts of interest.

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