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Seasonal changes in the structure and secretory activity of the androgenic gland of *Travancoriana schirnerae*

Research Article

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Abstract: This study investigated the seasonal variation in the structure and secretory activity of the androgenic gland (AG) in the freshwater crab: *Travancoriana schirnerae*. The androgenic gland is an elongate structure, attached to one side on the wall of the ejaculatory duct. Histological studies showed the presence of three cell types, which differ in size, shape of nuclei, and presence or absence of secretory vesicles. Type I cells are small with large nuclei whereas type II cells are large with small nuclei. Type III cells are intermediate in size and exhibited streak-like nuclei and transparent cytoplasm. Seasonal changes were discerned in the morphology, histology and secretory activity of the gland. March-June appeared to be the active season with type II cells containing secretory vesicles. The mode of release of secretion found to be holocrine. The secretory activity almost completed by July-August (the mating season) with vacuolization of type II cells. The gland remained inactive from September-December with abundance of vacuoles, scattered pycnotic nuclei, indistinct cell membranes and total cellular degeneration. January-February was the revival period with type I cell proliferation. The present study revealed that the secretory activity of the gland is in tune with the male reproductive cycle.

Keywords: *Androgenic gland • Freshwater crab • Posterior vas deferens • Secretory vesicles • Seasonal variation • Travancoriana schirnerae • Vacuoles*

1. Introduction

Unlike other arthropods, sex differentiation in male crustaceans is not controlled by the gonad, but by a unique gland-the androgenic gland (AG) [1-7]. It is the only endocrine gland in crustaceans, which is exclusively linked to sexual functions [8-10]. The androgenic gland was first described in the blue crab *Callinectes sapidus* by Cronin [11]. Charniaux-Cotton [1] carried out the first experimental study that elucidated the role of androgenic gland in amphipod, *Orchestia gammarella*. Since then, this gland has been described in several other malacostracans, including the isopods and decapods [12-18]. However, the androgenic gland has been identified in a variety of crustaceans, its location and morphology vary in different species. The androgenic gland of the freshwater prawn *Macrobrachium lamerri* is located close to the seminal vesicle along the wall of the vas deferens [19]. Li and Xiang [20] reported the location and light microscopic structure of the androgenic gland of the shrimp *Penaeus chinensis*. The androgenic gland structure of the brown mud crab *Scylla serrata* was investigated by Rangneker *et al.* [21]. The location, morphology, microstructure and ultrastructure of the androgenic gland were studied in the swimming crab *Portunus trituberculatus* [22]. Seasonal morphology and histology of androgenic gland in the crayfish, *Orconectes nais* have been described by Carpenter and De Roos [23].

The androgenic hormone (AH) secreted by the androgenic gland plays a key role in the regulation of male sexual differentiation in crustaceans [9,12,24-29]. King [30] and Sun *et al.* [31] reported a proteinaceous or polypeptidic nature for androgenic hormone in decapods. Berreur-Bonnenfant *et al.* [32] extracted a lipoidal substance with a molecular weight of 200-250 daltons from the androgenic gland of the crab *Carcinus maenas*. The ultrastructural studies on the androgenic gland of the crayfish *Procambarus clarkii* revealed a peptidergic-proteinaceous nature for the androgenic hormone [16,33]. Sagi and Khalaila [27] isolated, purified and characterized the androgenic hormone

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from the isopod *Armadillidium vulgare* and found that it is a glycosylated protein composed of two peptide chains connected together by disulphide bonds.

Most studies on the decapod androgenic glands have focused on prawns, shrimps and crayfishes [34-41]. Though the structure and function of the androgenic glands have been well described in many marine, estuarine and intertidal crabs [13,15,24,30,42,43], reports on freshwater crabs are limited [44]. Therefore, the present study was undertaken to investigate the seasonal changes in structure and secretory activity of the androgenic gland in the freshwater crab, *Travancoriana schirnerae*, a common species found in Wayanad, Kerala, India.

2. Experimental Procedures

Travancoriana schirnerae is widely distributed in the paddy fields, areca and banana plantations of Wayanad, Kerala, India. Male crabs (carapace width 3.2-5.8 cm) were collected monthly (n=5-10) during the period January 2010-December 2011. Crabs were dissected out under a stereozoom microscope and androgenic gland along with posterior vas deferens (PVD) were removed and fixed in Bouin's fluid. The tissue was dehydrated in ethanol series, cleared in xylene and embedded in paraffin. Tissue sections of 5 µm thickness were cut, stained with hematoxylin-eosin for histological observations. For histochemical studies, the sections were stained with mercuric bromophenol blue (MBB), periodic acid-Schiff (PAS), Sudan black B, alcian blue and aldehyde fuchsin. Stained sections were observed under a Leica research microscope and photographed using DG 330/120 camera and Biowizard software. The number of cells belonging to each cell type was counted in five visual fields to determine the major cell types of the androgenic gland of crabs collected in different months. From each field, 60 cells were counted, totaling 300 cells per specimen. The diameter of the androgenic gland cells, their nuclei and secretory vesicles were measured using Biowizard software.

3. Results

3.1 Structure of the androgenic gland

The androgenic gland in *T. schirnerae* appeared as an elongate thickening on the wall of the ejaculatory duct, towards the terminal end of the posterior vas deferens (PVD), in the coxal muscles of the 5th pereiopod (Figure 1). The gland was attached to one side of the PVD and was shown to be surrounded by a connective tissue layer (Figure 2). The size, appearance, proportion of cell types, cell membranes and secretory activity of the gland varied greatly in accordance with the seasons. Histological examinations revealed three cell types in the androgenic gland-type I, type II and type III whose proportions varied in different parts of the androgenic gland in different seasons. The three cell types could be distinguished based on the size, relative proportion of nuclei and presence or absence of secretory vesicles.

Type I: Type I cells were small (6.4-12.7 µm) with large nuclei and small amounts of cytoplasm (Figure 3A). These cells were arranged compactly with distinct cell membranes. Each cell had a round or oval,

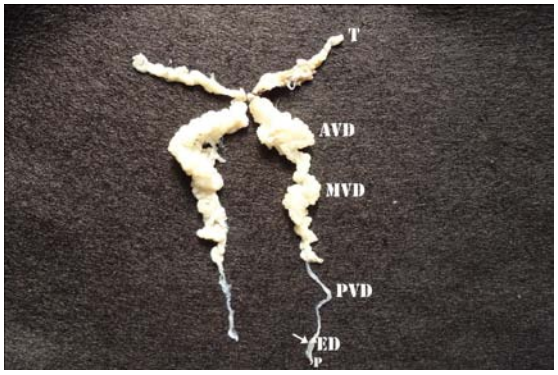

Figure 1. Male reproductive system of adult *Travancoriana schirnerae*. Androgenic gland (arrow), anterior vas deferens (AVD), ejaculatory duct (ED), median vas deferens (MVD), posterior vas deferens (PVD), penis (P), testis (T).

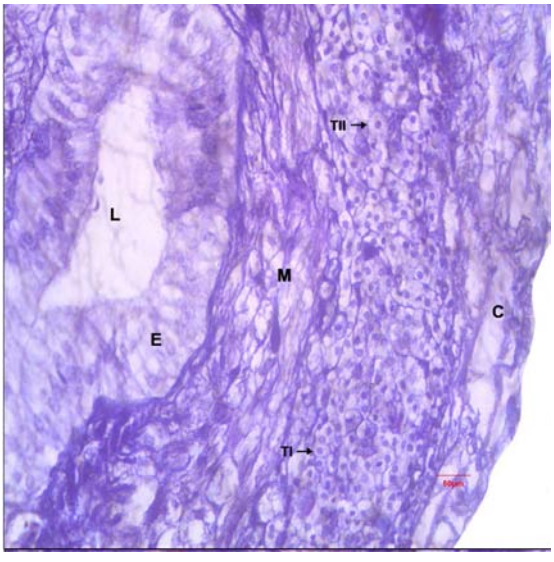

Figure 2. Histology of the androgenic gland of adult male *Travancoriana schirnerae* during the active season (March). Connective tissue layer (C), type I (TI) and type II cells (TII) of the androgenic gland; muscle layer (M), epithelium (E) and lumen (L) of the posterior vas deferens.

uni- or multi-nucleolated (1-3) nucleus, and measured 3.8-7.8 µm in diameter. The nuclei were positioned mostly within the centre of the cells. Their nucleoli occupied central or peripheral positions. In some type I cells, the basophilia of the nuclei was very strong and the nucleoli could not be seen, however, few cells showed moderate basophilia and prominent nucleoli. Type I cells were characterized by a nucleocytoplasmic ratio (NPR) of 0.50-0.72 (Table 1). Their nuclei and cytoplasm exhibited moderate reactions towards MBB and negative reactions to PAS, alcian blue, aldehyde fuchsin and Sudan black B (Figure 3C).

Type II: Type II cells formed the major cell types of the androgenic gland. These cells were noticeably large, polygonal and measured 13.0-24.0 µm in diameter (Figure 3A). The nuclei (2.5-6.4 µm) were round or oval, acentric with distinct nuclear membranes. The highly basophilic nucleoli (1-3) occupied central or peripheral positions. There was much more cytoplasm in type II cells than type I. Type II cells were seen laden with small to large, oval secretory vesicles (7.5-21.2 µm in diameter) during the active season (March-June) and vacuoles during the inactive season (September-December) (Figure 4A,C). Each type II cell carried a single, large secretory vesicle, which occupied most of the intracellular space. An acinar pattern of arrangement of type II cells with a bi- or multi-nucleate condition was noticeable during the months of April-June. Type II cells had low NPR (0.2-0.5) compared to type I (Table 1). Their nuclei and cytoplasm were moderately positive to MBB but showed negative reactions with PAS, alcian blue, aldehyde fuchsin and Sudan black B (Figure 3C).

Type III: Type III cells were few in number, detected only during the active season. They were medium sized (11.10-19.20 µm) cells, seen scattered among type I and II (Figure 3B). Their nuclei were elongated and streak-like (2.0-3.6 µm), and were found moderately stained with hematoxylin and MBB (Figure 3C). The cytoplasm showed a weak reaction to MBB. The NPR was found to be very low (0.13-0.30) (Table 1).

Cell types	Cell diameter (µm)	Nucleus diameter (µm)	NPR
Type I	6.4-12.7	3.8-7.8	0.50-0.72
Type II	13.0-24.0	2.5-6.4	0.20-0.50
Type III	11.1-19.2	2.0-3.6	0.13-0.30

Table 1. Comparison of the three cell types in androgenic gland of *Travancoriana schirnerae*.

NPR, nucleocytoplasmic ratio.

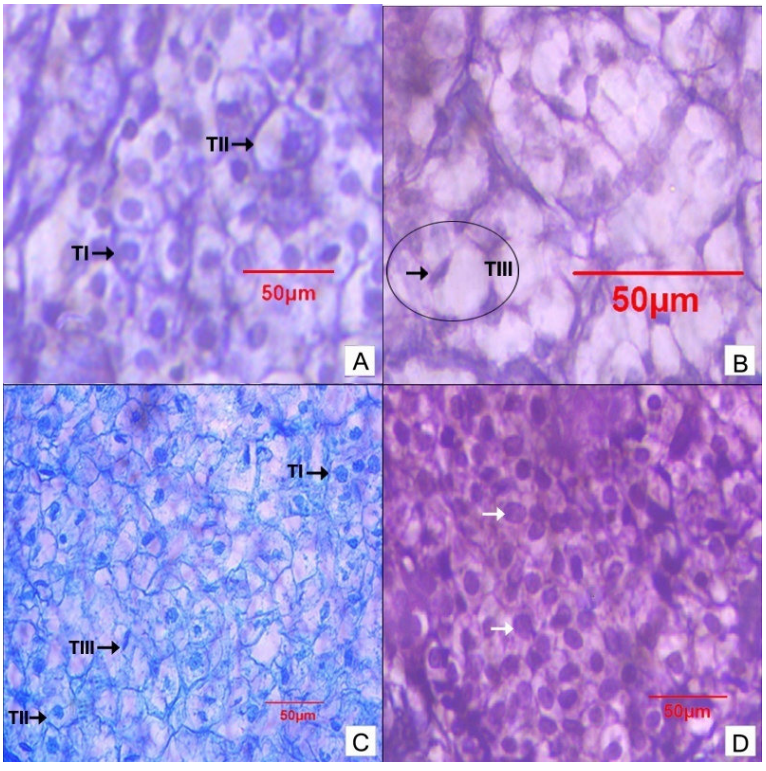

Figure 3. Different cell types in the androgenic gland of *Travancoriana schirnerae*. A, Type I (TI) and type II cells (TII) in the active season (March); B, Type III cells (TIII) in June; C, Mercuric bromophenol blue staining of type I (TI), type II (TII) and type III (TIII) cells in March; D, Androgenic gland cells of immature crab, nucleus of immature cell (arrow).

The androgenic gland in immature crabs (carapace width 3.2-3.6 cm) appeared small (length - 571.7±5.6, width - 348.9±80.1), comprised of a single type of cell and packed compactly (Figures 3D, 5A). These immature gland cells (5.2-9.6 µm) were similar to type I of the androgenic gland of mature crabs (carapace width 4.0-5.8 cm), possessed large nuclei (3.6-7.01 µm) and small amounts of cytoplasm. Their nuclei exhibited strong basophilia but the cytoplasm showed moderate basophilia. The NPR was found in the range 0.54-0.78.

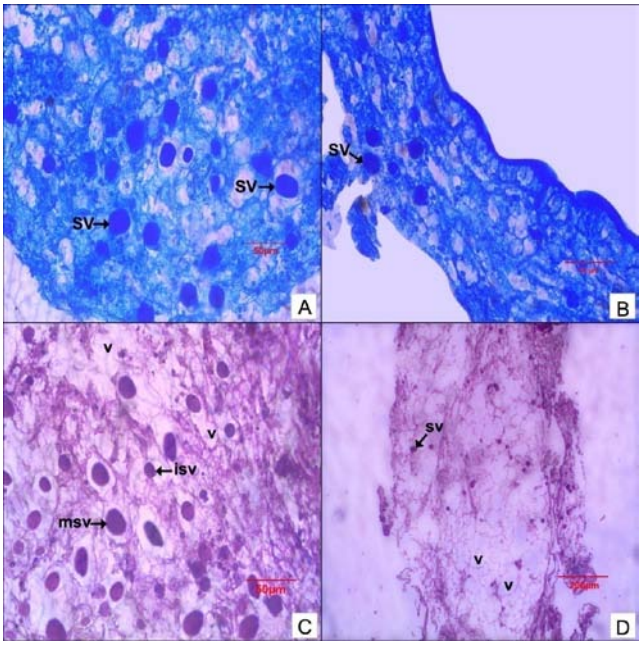

Figure 4. Seasonal variation in the secretory activity of the androgenic gland. A, The androgenic gland with secretory vesicles (sv) in the active season (March) (Mercuric bromophenol blue staining); B, Holocrine pattern of release of secretion of type II cells in June, secretory vesicles (sv); C, Secretory vesicles and vacuoles (v) in the active season (June), immature (isv) and mature (msv) secretory vesicles; D, Vacuolated appearance of the androgenic gland in the mating season (July), secretory vesicle (sv), vacuole (v).

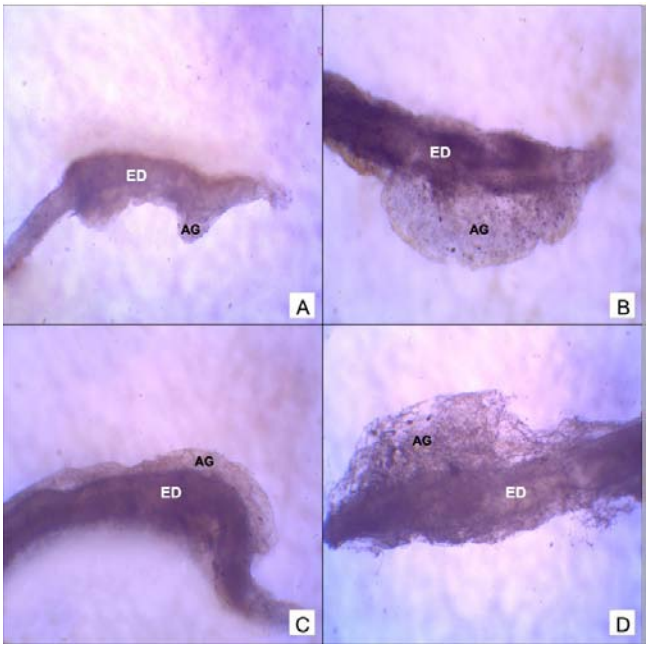

Figure 5. Seasonal variation in the morphology of the androgenic gland in *Travancoriana schirnerae*. A, Androgenic gland (AG) of immature crab attached to the ejaculatory duct (ED); B, Morphology of androgenic gland of mature crab in the active season (March); C, Morphology of androgenic gland in the inactive season (December); D, Androgenic gland in the revival period (February).

3.2 Seasonal changes in the androgenic gland

Seasonal changes were observed in size, appearance of the gland, and cell membranes, proportion of cell types and in the secretory activity of androgenic gland. During the active season, the gland reached its maximum size (Figure 5B). Its length was noted to be 2128.00±185.59 µm (1958-2326 µm). The anterior (230.20±59.23 µm), middle (363.00±51.79 µm) and posterior (167.10±32.61 µm) width of the gland reached its maximum in this period. The gland showed a reduction in size with length (903.33±306.04 µm) (781.3-1328.1 µm) and width (anterior width 48.16±57.02 µm; middle 208.73±106.42 µm and posterior 145.76±75.57 µm) during the inactive season (Figure 5C). The gland had a length of 1184.1±203.61 (1040.2-1559.0 µm) and a width of 213.15±55.21 µm during the revival period (January-February) (Figure 5D).

The appearance of the gland was found to vary according to seasons. All through the active season, the gland appeared as an elongated structure, fully packed with cells. The gland was firmly attached to the posterior vas deferens with a thick, intact connective tissue layer (20.26±9.29 µm). The gland had an elongated, shrunken appearance during the mating season. A major portion of the gland was filled with vacuolated type II cells with indistinct membranes. The gland was seen attached loosely to the posterior vas deferens and the connective tissue layer appeared thin (10.47±1.76 µm). The cells were arranged loosely with prominent intercellular spaces. From September-October (the first half of the inactive season), the gland was characterized by large vacuolated areas indicating cellular degeneration (Figure 6A). Intact cells with membranes were scarcely detected. Pycnotic nuclei were seen scattered inside the gland. In November-December (the second half of the inactive season), the gland was found detached from the wall of the posterior vas deferens, composed of parallel protoplasmic strands and pycnotic nuclei (Figure 6B). The connective tissue layer remained indistinct.

Seasonal variation was also noticed in the proportion of cell types of the androgenic gland. In the active season, all the three cell types were observed. The proportion of type II cells with and without secretory vesicles were found to be high (71%) all through the season, followed by type I (20%) and type III (9%) (Figures 4A, 7A). The androgenic gland in the mating season was characterized by a low percentage of type II cells (27%). Type I cells were the major cell types (51%)

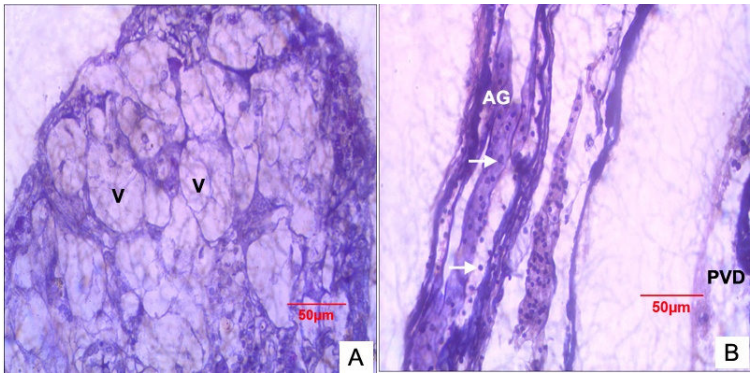

Figure 6. Histology of the androgenic gland in the inactive season. A, Vacuolated appearance of the gland in October, vacuole (v); B, Stranded appearance of the gland in November, androgenic gland (AG), posterior vas deferens (PVD), pycnotic nucleus (arrow).

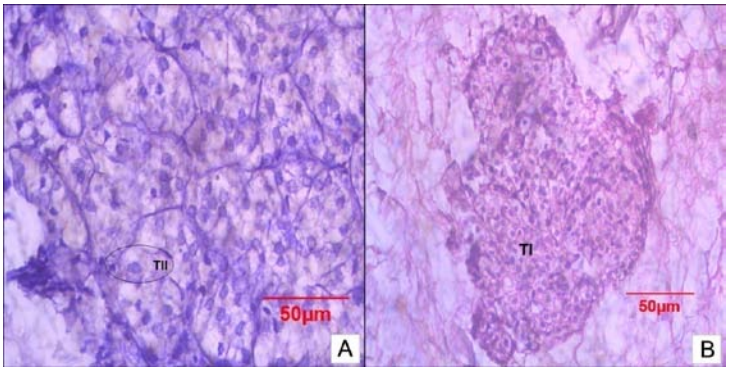

Figure 7. Seasonal variation in the proportion of cell types of the androgenic gland in *Travancoriana schirnerae*. A, Acinar pattern of arrangement of Type II (TII) cells in the active season (June); B, Type I (TI) cell proliferation in the revival period (January).

in the revival period (as proliferation of these cells was the most striking feature of this period; Figure 7B). Only very few type II cells could be perceived among this newly formed type I cells; type III seldom was noticed.

The membrane of androgenic gland cells displayed variations in different seasons. The gland was composed of all the three cell types that were packed closely with distinct cell membranes in the active season (Figure 3 A,C). During the mating season, type II cells were shown to be vacuolated and loosely packed, most of them with indistinct cell membranes. In a large number of type II cells, the cell membranes were found to be broken. No intact cells were found within the gland all through the inactive season (Figure 6A). The androgenic gland in the revival period possessed many newly formed type I cells with intact membranes (Figure 7B). A small percentage of type II cells with distinct membranes were also perceptible in this period.

Differences were also perceived in the secretory activity of the gland according to seasons. The secretory activity was noticeable from March-June. During this period, a majority of type II cells were laden with small to large, oval secretory vesicles. The secretory vesicles stained strongly with hematoxylin and MBB (Figure 4A,C). In March-April, 51.25% (46.5-56.0%) of the type II cells showed secretory vesicles (Figure 4A). The percentage of type II with secretory vesicles (65.8%) was found to have increased in May. The secretory activity reached its peak in June with 80% type II cells carrying secretory vesicles (Figure 4C). The cell membranes were found broken in the outer periphery of the gland where mature secretory vesicles were noted. After the release of secretion, type II cells were seen vacuolated; their membranes became indistinct and finally degenerated, thus suggesting a holocrine mode of release of secretion (Figure 4B). The secretory activity was almost completed by July-August (10 and 6% respectively) (Figure 8). The gland was characterized by an abundance of vacuoles and very few secretory vesicles in these months (Figure 4D). Thereafter, no secretory vesicles were apparent in the gland until December.

4. Discussion

As reported for other brachyurans [13,43], the androgenic gland in *T. schirnerae* is an elongate, thread-like structure, located in a subterminal position on the wall of the ejaculatory duct near the penis. In the warty crab *Eriphia verrucosa* [15] and in the Chinese mitten crab *Eriocheir sinensis* [14], the gland was formed of cords of cells located on the wall of the posterior vas deferens. In the crayfish *Cherax destructor*, multiple cords of epithelial cells of the androgenic gland were attached to the posterior vas deferens [17]. The androgenic gland in *M. rosenbergii* was made up of strands of cells in pyramidal cluster, loosely associated with the terminal vas deferens [31]. On the other hand, the androgenic gland in *M. lamerri* was made up of cords of cells arranged loosely along the wall of the posterior vas deferens [19]. The androgenic gland cells originate from the epithelium of the ejaculatory bulb, in the protandric shrimp *Pandalus platyceros* [45] and in *M. rosenbergii* [46]. In *T. schirnerae*, the androgenic gland seemed to be originated from the epithelium of the terminal vas deferens.

In the present investigation, the size of the gland appeared to vary according to seasons. Similar reports were made in *C. maenas* [47], *C. sapidus* [48], in the ghost crab *Ocypode platytarsis* [13], *M. rosenbergii* [18], *C. destructor* [17] and in the green mud crab *S. paramamosain* [43]. There observed differences in the cell types that form the androgenic gland in decapods. As reported for *O. platytarsis* [13] and *S. paramamosain* [43], three cell types could be distinguished in the androgenic gland of *T. schirnerae.* On the other hand, the androgenic gland was composed of two cell types in *P. clarkii* [16], *P. chinensis* [20,38], *P. trituberculatus* [22], *E. verrucosa* [15] and five cell types in *O. nais* [23]. The size of the androgenic gland cells of *T. schirnerae* was comparable to that of other decapods such as *Pachygrapsus crassipes* [30], *Rithropanopeus harrisii, C. sapidus* [42], *P. clarkii* [16,49], *C. destructor* [17], *S. paramamosain* [43] and marine shrimps [37].

The three distinct cell types in the androgenic gland of *T. schirnerae* were based on the differences in their size, size and shape of nuclei and presence or absence of secretory vesicles. The larger size of nuclei

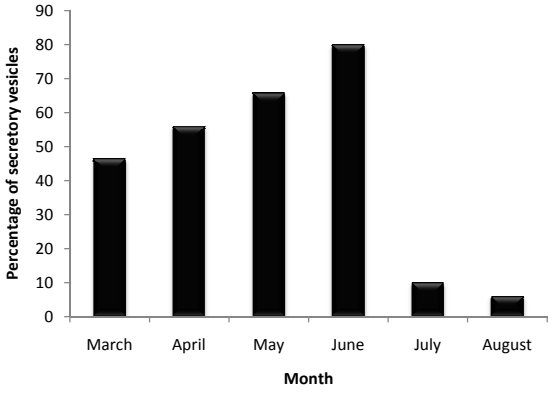

Figure 8. Seasonal variation in the secretory activity of the androgenic gland of *Travancoriana schirnerae*.

of type I possibly suggest the transcription of large number of mRNAs for the production of secretion. The type I and II of androgenic gland cells of *T. schirnerae* were comparable to those of *S. paramamosain* [43]. In *S. paramamosain,* type III formed the largest and seemed to represent the degenerative phase of the gland [43]. In the present study also, type III cells with streak-like nuclei possibly represent the degenerative phase of type II. In *O. platytarsis,* the three cell types were distinguished based on the nature of cytoplasm and presence or absence of vacuoles [13]. But in *E. verrucosa*, the differentiation of two cell types was based on size, shape of nucleus and presence or absence of secretory vesicles and vacuoles [15]. In *P. chinensis*, the two cell types were identified by their size [20]. Veith and Malecha [46] observed three cell types in the androgenic gland of *M. rosenbergii*. According to them, type I cells are small, bi-nucleate with dense cytoplasm; type II cells are slightly larger and vacuolated and type III are large with vacuoles filling most of the intracellular space. It is suggested that the structural differences observed between cell types in the present study seemed to be correlated with the different stages in the secretory cycle of the androgenic gland. Further research is required at the ultrastructural level to determine whether these structurally different cell types are functionally distinct or not. The acinar pattern with bi- or multi-nucleate condition described for type II cells in the androgenic gland of *T. schirnerae* was comparable to that explained for type I cells of *S. paramamosain* [43]. Bi-nucleate condition of small cells was perceptible in the androgenic gland of *O. platytarsis* [13].

The present study could elucidate seasonal variations in the cell membranes of the androgenic gland cells. The cell membranes were distinct during the active season or secretory phase and indistinct during the inactive season. Similar pattern was described in *M. kistensis* [35], *P. chinensis* [38] and *P. trituberculatus* [22]. Liu *et al.* [43] reported that the cell membrane of the androgenic gland cells of *S. paramamosain* varied in different months.

In *T. schirnerae*, the gland cell proportions varied according to seasons. The active season was characterized by an abundance of type II cells and the inactive season by a decrease in type II proportion. Similar observations were made in *M. kistensis* [35], *S. paramamosain* [43] and in *P. chinensis* [20,38,50]. In *P. clarkii*, the highly active cells increased during summer and their number found decreased during winter [4]. Thampy and John [13] reported that the proportion of cell types of the androgenic gland in *O. platytarsis* was varied according to seasons. In *M. rosenbergii*, the proportion of three cell types found varied among male morphotypes [31].

There is considerable difference in the activity and release of secretory product in the androgenic gland of various crustaceans according to seasons. In *T. schirnerae*, the secretory activity of the gland was perceptible from March-June, *i.e.* during the reproductively active phase of the male. The mating season coincided with the destruction of type II cells after releasing their secretory product ie. the activity of the gland ended just before the mating season. In *E. verrucosa,* the major mating season is between late July and the end of August, which corresponded to the destruction of type II cells after releasing their contents [15]. In *M. lamerri*, the androgenic gland showed signs of increased secretory activity during the sexually active phase of the animal [19]. In contrast, in *S. paramamosain* [43] and *P. chinensis* [50], the gland was found to be most active during the mating season. The results of our study indicated a holocrine mode of release of secretion for the androgenic gland. Holocrine pattern of secretion was noted in many decapod crustaceans such as *M. rosenbergii* [31,46], *E. sinensis* [14], *E. verrucosa* [15] and *P. trituberculatus* [22]. Conversely, there was no evidence for holocrine pattern of secretion in *P. crassipes* [30].

The seasonal variations in size, structure and secretory activity of the gland in *T. schirnerae*, seemed to correlate with the sexually active and inactive phases of the male reproductive cycle. The gland attained maximum size and activity during the reproductively active season, ended its activity before the mating season and showed signs of degeneration during the reproductively inactive season. Variation in size, structure and activity of the androgenic gland in relation to the male reproductive cycle has been documented in *O. nais* [23], *Potamon koolooense* [14], [44] and in *S. paramamosain* [43]. In *M. kistensis*, the androgenic gland attained a maximum size during the breeding season and became reduced during non-breeding months [35]. Okumura and Hara [51] found that in *M. rosenbergii*, the androgenic gland appeared larger in blue-clawed males which was the final morphotypic stage having high mating activity. Seasonal variations in structure and activity of the androgenic gland have been reported in other decapod crustaceans [4,12,16,22,38,50].

The histochemical findings of the present study revealed a proteinaceous or polypeptidic nature for the secretory product as reported for *E. verrucosa* [15]. In *P. crassipes*, the presence of a considerable amount of protein in the secretory vesicles suggests that the androgenic hormone may be a protein or polypeptide [30]. Several authors established the proteinaceous nature of the androgenic hormone in *M. rosenbergii* [18,31,52]. On the contrary, Veith and Malecha [46]

suggested a steroidogenic nature for androgenic hormone in *M. rosenbergii*. Ferezou *et al*. [53] reported the presence of terpenoids in the androgenic gland of *C. maenas*. The lipoidal substance extracted and purified from the androgenic gland in *O. gammarella* inhibited vitellogenesis when injected into females [32]. The ultrastructural studies on *P. clarkii* revealed the peptidergic-proteinaceous nature of the androgenic hormone [16,33]. In *E. sinensis*, the androgenic hormone was composed of protein and lipid [14].

The androgenic gland of *T. schirnerae* presents seasonal variations in morphology, histology and secretory activity. The three cell types identified in the androgenic gland of *T. schirnerae* may possibly represent the different stages of the secretory cycle and not functional cell types. Similar conclusions are made in *S. paramamosain* [43], *O. platytarsis* [13] and *E. verrucosa* [15]. Further research is required (at the ultrastructural level) to confirm whether the three cell types are functionally different cell types or not. The histochemical findings indicate a proteinaceous nature for the androgenic gland secretion. To conclude, variations in the structure and activity of the androgenic gland are directly correlated to the testicular activity of the male.

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