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THE HISTOLOGICAL STRUCTURE OF THE ANDROGENIC GLAND AND CELLULAR CORD OF THE MALE REPRODUCTIVE SYSTEM OF ADULT *LITOPENAEUS* AND *RIMAPENAEUS BYRDI*

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ABSTRACT

The current knowledge of the precise extension and location of the androgenic gland (AG) in the genera *Litopenaeus* and *Rimapenaeus* is partial. Therefore, we analyzed the complete structure of the AG and cellular cord associated with the reproductive system of *Litopenaeus setiferus*, *L. occidentalis*, *L. stylirostris*, and *Rimapenaeus byrdi* at the stereoscopic microscopy and histological level. Our observations clearly show that the adult male reproductive system has a cellular cord that runs along the entire vas deferens. This cord is a blood vessel that runs along the ampoule, the descending and the ascending medial vas deferens. Terminal ampoules of the four species investigated have a gland-like tissue associated with the blood vessel that is attached to the external surface near to the chamber containing sperm; this structure was identified as the AG and is formed by a homogeneous mass of cells characterized by a small size (7 μm), a cytoplasm with discrete cell boundaries, and an oval nucleus (4 μm). The blood vessel is surrounded by massive layers of adipose tissue at the distal region of the ascending medial vas deferens, near to the large lumen that contains sperm. This tissue has large cells (25-40 μm), with eosinophilic cytoplasms, and small nuclei (4-5 μm) in *L. setiferus*; *L. stylirostris* and *L. occidentalis* also show a cellular structure at the same location, identified as white adipose tissue. This anatomical description further improves the current knowledge of the general organization of the male reproductive system of *Litopenaeus* and *Rimapenaeus*.

KEY WORDS: androgenic gland, *Litopenaeus*, Penaeidae, reproductive system, *Rimapenaeus*

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INTRODUCTION

The androgenic gland (AG) of crustaceans is the organ that controls masculinization by releasing an androgenic gland hormone (AGH) that regulates primary and secondary male sexual characteristics (Charniaux-Cotton, 1954; Charniaux-Cotton and Payen, 1985). This hormone is a peptide, purified from three isopods (Hasegawa et al., 1987; Martin et al., 1999; Ohira et al., 2003; Sagi and Aflalo, 2005), but it has not yet been isolated in decapods. Recently, an insulin-like gene expressed exclusively in the AG of *Cherax quadricarinatus* (von Martens, 1868) was discovered (Manor et al., 2007). The AGH seems to function as a sex-differentiating factor in crustaceans but not a sex-determining factor (LeBlanc, 2007).

The AG has been identified in many malacostracans, including the orders Amphipoda, Isopoda, and Decapoda. In general, these findings have suggested the existence of only one kind of gland, organized as thin strands or compacted lobes of cells (Kleinholz and Keller, 1979), associated to the subterminal region of the sperm duct in amphipods or at the end of each testicular utricle in isopods (Okumura and Hara, 2004).

In *Procambarus clarkii* (Girard, 1852) and *Macrobrachium rosenbergii* (De Man, 1879) different cell-type populations have been identified from the AG (Taketomi, 1986; Sagi, 1988; Sagi and Aflalo, 2005). Murakami et al. (2004) discovered two kinds of glands associated with the subterminal ejaculatory region of *P. clarkii*; the typical AG of malacostracans is located inside the body cavity, and the other gland is inside the coxa, while *M. rosenbergii* has its AG attached to the terminal ejaculatory bulb (terminal ampoule; Nagamine et al., 1980; Okumura and Hara, 2004; Ventura et al., 2009).

The morphological, physiological, and behavioral effects of the gland observed to date have raised the question of whether one molecule is responsible for all the effects in higher crustaceans (Sagi and Aflalo, 2005). Although the AGH is a peptide, other molecules have been isolated from the AG: farnesylacetone and steroids, which may play complementary roles (Sagi, 1988).

Gland-like tissues associated with the male reproductive system of Penaeidae have been identified in *Melicertus kerathurus* (Forskål, 1775) (cf. Charniaux-Cotton and Payen, 1985), *Marsupenaeus japonicus* (Bate, 1888) (cf. Nakamura et al., 1992), *Fenneropenaeus chinensis* (Osbeck, 1765) (cf. Li and Li, 1993), *Litopenaeus vannamei* (Boone, 1931) (cf. Alfaro, 1994; Campos-Ramos et al., 2006; Garza-Torres et al., 2009), and *L. stylirostris* (Stimpson, 1871) (cf. Alfaro, 1994). These studies indicate that the AG is a cord-like cellular mass attached to the distal region of the medial vas deferens, agreeing with the description of the genital organ by King (1948). Complementarily, in *L. stylirostris* (Alfaro, 1994), *L. vannamei* (Campos-Ramos et al., 2006),

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and *F. chinensis* (Li and Xiang, 1997), the cellular cord extends in both directions of the distal medial vas deferens. It has been observed that the AG of *M. japonicus* (Charniaux-Cotton and Payen, 1985; Nakamura et al., 1992) and *L. vannamei* (Garza-Torres et al., 2009) may not be involved in sex determination because the gender differentiates earlier, soon after transformation of larvae.

The descriptions of the cellular cord and the location of the AG published to date are partial concerning Penaeidae. Therefore, a research program was implemented to analyze the complete structure of the cellular cord associated with the reproductive system of three open-thelycum species: *L. setiferus* (Linnaeus, 1767), *L. occidentalis* (Streets, 1871), and *L. stylirostris* at the stereoscopic microscopy and histological level. Additional observations were made on the close-thelycum species: *Rimapenaeus byrdi* (Burkenroad, 1934). For the nomenclature of penaeoid shrimps, we have adopted the taxonomy of Pérez-Farfante and Kensley (1997).

Materials and Methods

Vasa deferentia of male reproductive systems of *L. occidentalis*, *L. stylirostris*, and *R. byrdi* were studied at stereoscopic level. The vas deferens is organized in four morphologically distinct regions, similar to the descriptions of *L. setiferus* (Ro et al., 1990; Bauer and Cash, 1991) and *L. vannamei* (Garza-Torres et al., 2009). Here, the four sections are named based on King (1948) and Ro et al. (1990) as proximal vas deferens (Fig. 1B), ascending vas deferens (Fig. 2A), descending vas deferens (Fig. 2B), distal vas deferens (Fig. 3), and terminal ampoule (Fig. 4).

L. setiferus Samples

Adult males of *L. setiferus* (body weight: b.w. = 40 g) were collected in the Gulf of Mexico, off Port Aransas (27°5′N; 97°3′W), Texas, in 1989, and transported alive to the Texas A&M University Nutrition Laboratory at Port Aransas; then the entire vas deferens of the reproductive system was dissected by cutting the pleon and gently pulling out with dissecting tweezers the terminal ampoule. Tissues were fixed in Davidson' solution for 24 h and stored in 50% ethyl alcohol, according to Bell and Lightner (1988). Terminal ampoules, distal vas deferens, descending and ascending vas deferens of four reproductive systems were paraffin-embedded, sectioned in cross orientation and stained in hematoxylin plus eosin at the Department of Veterinary Anatomy, Texas A&M University, College Station, TX.

L. occidentalis, *L. stylirostris*, and *R. byrdi* Samples

Adult males of *L. occidentalis* (b.w. = 37 g), *L. stylirostris* (b.w. = 41 g), and *R. byrdi* (b.w. = 6.5 g) were captured in the Gulf of Nicoya on the central Pacific coast of Costa Rica (10°N; 85°W), in 2009-2010. Animals were transported alive to Estación de Biología Marina (EBM) at Puntarenas, Gulf of Nicoya. Dissections of reproductive systems from six males of each species were performed under stereoscopic microscopy, complemented with histological preparations processed by the Histology Laboratory, EBM, and a private Laboratory: Andromeda. Cross sections were prepared from terminal ampoules, distal regions of descending vas deferens, proximal regions of descending vas deferens, and distal regions of ascending vas deferens. Tissue structure analysis was based on the descriptions provided by Bell and Lightner (1988).

Results

Our observations from *L. setiferus*, *L. occidentalis*, and *L. stylirostris* clearly show that the adult male reproductive system has a cellular cord that runs along the entire vas deferens (Figs. 1 and 2). This cord is a blood vessel, which is difficult to observe under the stereoscope and runs along the ampoule, the descending and the ascending medial vas deferens (Fig. 2A and B), where blood vessel ramifications are projected from the main vessel. The histology of these regions reveals the structure of the blood vessel, which clearly takes an oval shape, transversally, showing the three common layers: acellular intima, endothelium, and elastin fibers (Fig. 2A-D).

Terminal ampoules (Fig. 4) of the three *Litopenaeus* analyzed, as well as *R. byrdi*, have a gland-like tissue attached to the external surface near to the chamber containing sperm; in *Litopenaeus*, the gland is located at the opposite side of the adhesive glands (Bauer and Cash, 1991). This structure has been defined as the AG based on its histological characteristics (Fig. 3A-D), and it is associated with various blood vessels that merge and extend towards the distal vas deferens; blood vessels show their normal structure, composed by the acellular intima and the endothelium. The AG is formed by a homogeneous mass of cells, characterized by a small size (7 μm), a cytoplasm with discrete cell boundaries, and an oval nucleus (4 μm). Based on the staining of nuclei, two cell types were observed: cells with strong basophilic nuclei, and cells with weak basophilia of nuclei showing distinctive nucleolus. Connective tissue attaches this gland-blood vessel complex to the ampoule (Fig. 3E). In *R. byrdi*, the AG is a more compacted mass of epithelial cells, stained blue with hematoxylin, and without the vascularization and connective tissue observed in *Litopenaeus* (Fig. 3F). Along the distal vas deferens (Fig. 3), the cord is formed by a major blood vessel without gland-like tissue.

The cellular cord at the distal region of the descending vas deferens (Fig. 1D) only reveals the presence of the blood vessel; no glandular tissue was observed in any cross section taken from this region in *L. occidentalis*, *L. stylirostris*, and *R. byrdi* (Fig. 2A, B). At the level of the middle region of the descending vas deferens, the cord is also formed by the blood vessel.

The blood vessel is surrounded by massive layers of connective-adipose tissue (Fig. 4) at the distal region of the ascending medial vas deferens (Fig. 2A), near the junction with the descending vas deferens and close to the large lumen, which contains sperm. This tissue surrounds the blood vessel and has large cells (25-40 μm), with eosinophilic cytoplasms, and small nuclei (4-5 μm) in *L. setiferus* (Fig. 4A); *L. stylirostris* (Fig. 4B), and *L. occidentalis* (Fig. 4C and D) also show a similar structure at the same location; the processing of samples, however, seems to have induced histological alterations, leaving empty spaces (20-30 μm) with nuclei (4-5 μm) at the periphery, similar to the white adipose tissue, described by

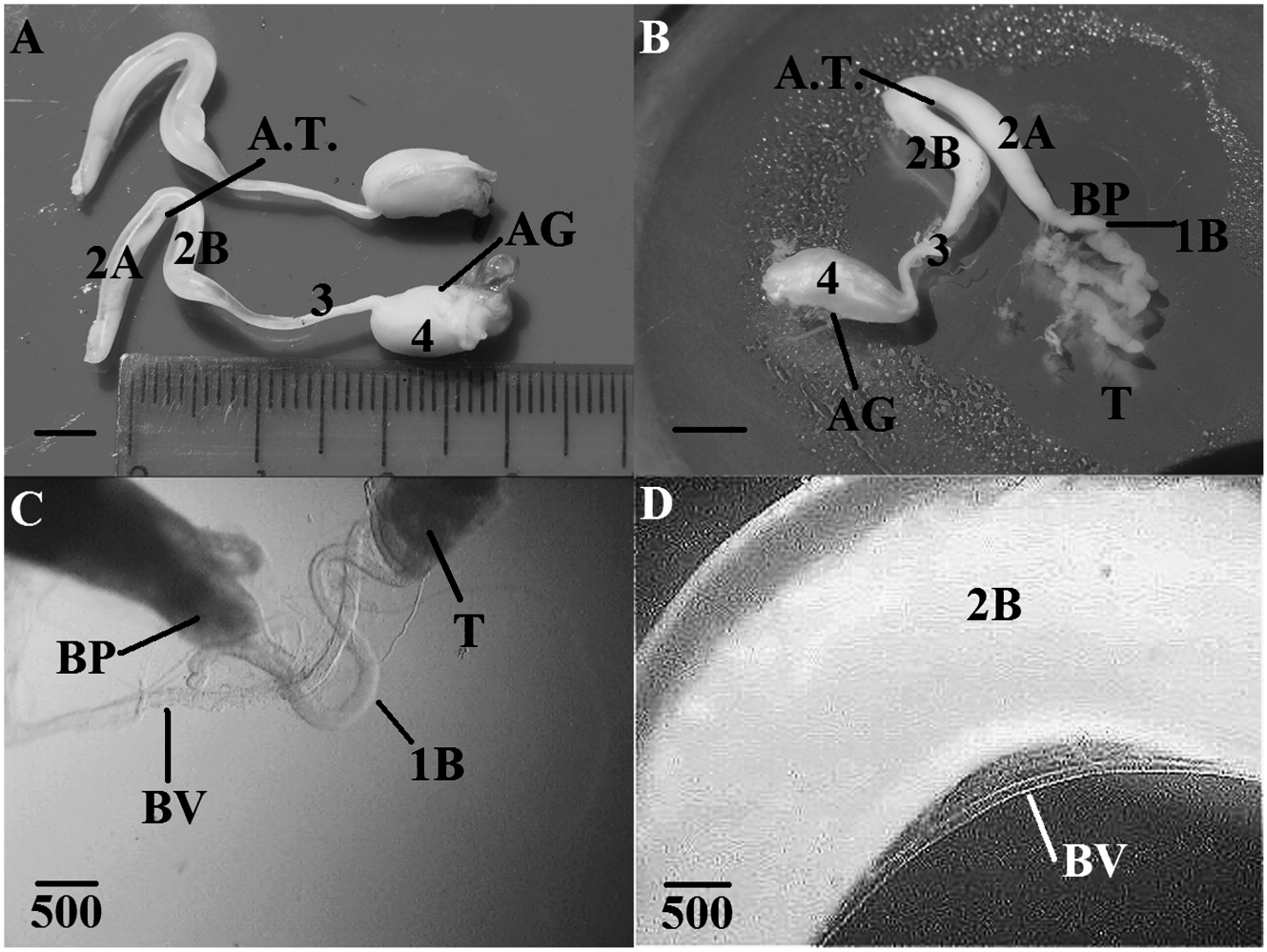

Fig. 1. Male reproductive system. A, *Litopenaeus occidentalis*, scale bar units = 5 mm; B, *Litopenaeus stylirostris*, scale bar units = 5 mm; C, *L. stylirostris*, blood vessel at the junction with testes, scale bar units = μm; D, *L. occidentalis*, blood vessel at distal region of descending vas deferens, scale bar units = μm. T = testes; 1B = proximal vas deferens; BP = blind pouch; 2A = ascending vas deferens; 2B = descending vas deferens; 3 = distal vas deferens; 4 = terminal ampoule; AG = androgenic gland; A.T. = adipose tissue; BV = blood vessel.

Garza-Torres et al. (2009). However, based on Bell and Lightner (1988), this tissue associated with blood vessels is classified as spongy connective tissue.

The cellular cord at the level of the middle region of the ascending vas deferens shows no connective-adipose tissue associated with the blood vessel (Fig. 2C, D). The blood vessel continues along the blind pouch at the proximal end of Fig. 2A and connects to testes lobes, similar to the connection of the proximal vas deferens (Fig. 1B) to testes (Fig. 1C). The entire cellular cord and the AG from terminal ampoules were easily removed from vasa deferentia under stereoscopic microscopy, corroborating its loose association with the male reproductive system.

Discussion

The discovery of a gland-like structure located at the terminal ampoule and the blood vessel associated with the vas deferens of the genera *Litopenaeus* and *Rimapenaeus* improves our knowledge on the general anatomy of the male reproductive system of penaeoid shrimp. The blood vessel in *L. setiferus* was previously observed associated with the proximal vas deferens (Fig. 1A, B) by Ro et al. (1990), but it was not as completely described as this contribution has done.

Most descriptions of the decapod AG correspond to small epithelial cells, around 10-20 μm (Taketomi et al., 1990; Fowler and Leonard, 1999; Okumura and Hara, 2004; Simeó et al., 2009). In penaeid shrimps, Li and Xiang (1997) reported 6-10 μm for *F. chinensis*, and Campos-Ramos et al. (2006) observed AG cells of 5-7 μm for pre-adult *L. vannamei*. However, Okumura et al. (2005) reported AG cells of larger dimensions (46 $\times$ 10 μm^2) in the protandric shrimp, *Pandalus hypsinotus* Brandt, 1851. It is suggested that the epithelial cell masses localized at the surface of terminal ampoules of *Litopenaeus* and *R. byrdi* correspond to the AG of most Malacostraca.

Previous studies on the AG of *Litopenaeus* were partial descriptions (Alfaro, 1994; Campos-Ramos et al., 2006; Garza-Torres et al., 2009). The distal region of the descending vas deferens in pre-adult (Campos-Ramos et al., 2006) and adult (Garza-Torres et al., 2009) *L. vannamei* was considered as the location of the AG. However, in adult males

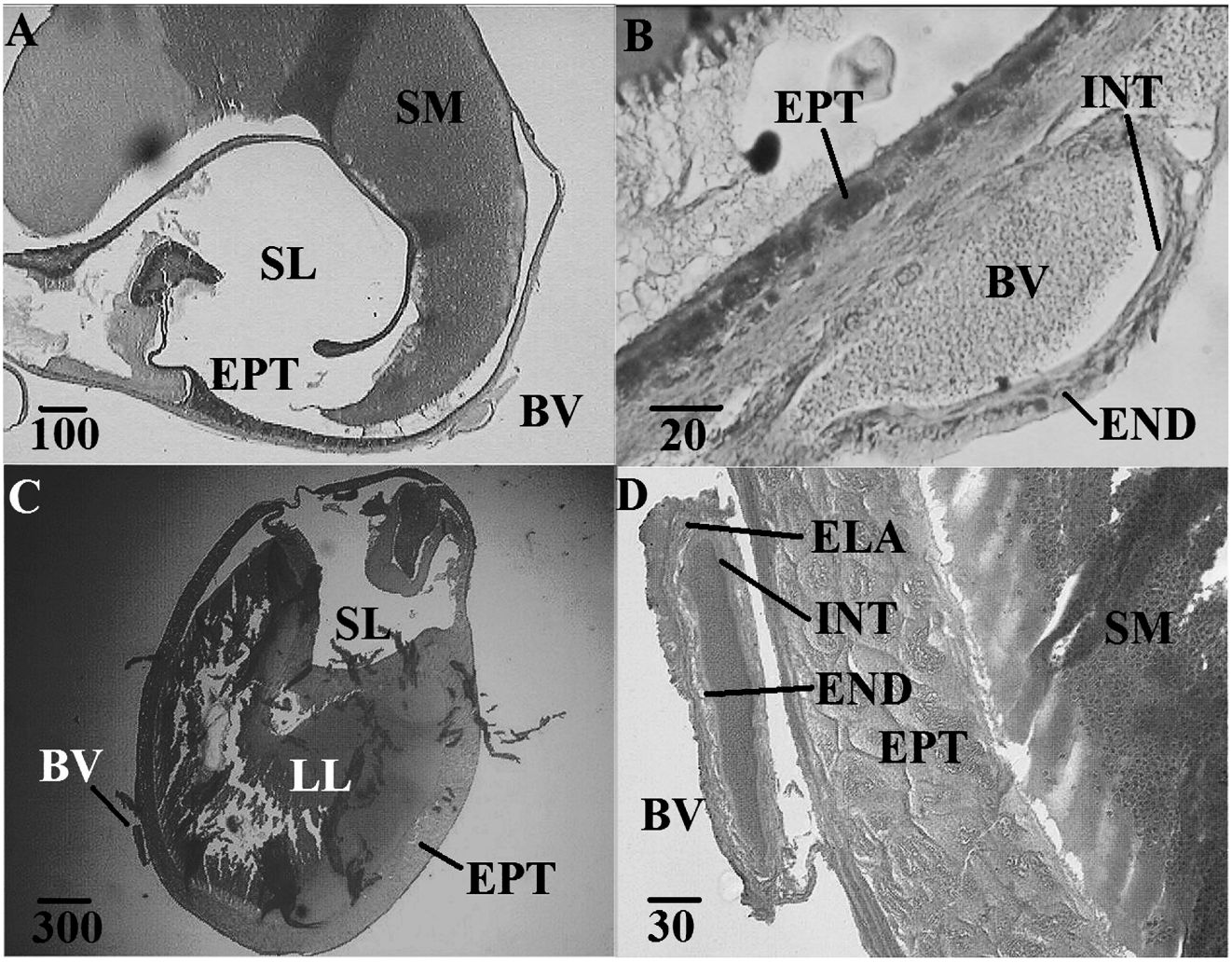

Fig. 2. A and B, *Litopenaeus occidentalis*, histology of blood vessel (cross sections) associated with distal region of descending vas deferens; C and D, *Litopenaeus setiferus*, middle region of ascending vas deferens. MSX = muscle layer; EPT = epithelial lining; BV = blood vessel; INT = intima; END = endothelium; ELA = elastin fibers; LL = large lumen; SL = small lumen; SM = sperm mass. Bar units = μm.

of the three species analyzed in this study, that region only reveals the presence of the blood vessel, which was not observed in cross sections of *L. vannamei* postlarvae (Garza-Torres et al., 2009). These differences may be related with species-specific anatomical organization, or a progressive maturation of the male reproductive system according to age as documented by Alfaro-Montoya (2010) and Ceballos-Vásquez et al. (2010), or that additional glandular tissue located at the distal region of the descending vas deferens was separated and lost during dissection. The form of the AG varies according to the developmental stage; Li and Xiang (1997) reported that the AG was arranged in cords in juveniles and in masses for adults of *F. chinensis.* In this species, the AG covers the surface between terminal ampoule and vas deferens. In *M. rosenbergii*, the AG is attached to the terminal ampoule (Nagamine et al., 1980; Okumura and Hara, 2004; Ventura et al., 2009).

The tissue located at the distal region of the ascending vas deferens is characterized by large cells (25-40 μm) with eosinophilic cytoplasms in *L. setiferus*, and spongy connective tissue in *L. stylirostris* and *L. occidentalis*, suggesting an adipose nature. This adipose tissue, in addition to the known connective and fat-storage functions, could play other roles in reproduction similar to subepidermal adipose tissue (SAT), which is involved in vitellogenin and protein synthesis by lipocalin (Fainzilber, 1989; Wang et al., 2007; Wade et al., 2009); further analysis is required for defining possible complementary functions of this adipose tissue.

There is not much information from decapod crustaceans on the organization of the AG in association with a blood vessel-cellular cord of the male reproductive system. In the crayfish, *Orconectes limosus* (Rafinesque, 1817), Charniaux-Cotton et al. (1966) described blood vessels associated with the vas deferens and the irrigation of the AG. Similarly, the AG of the three species of *Litopenaeus* shows a high degree of vascularization, which is not a generalized feature among decapod crustaceans.

The findings of the location of the AG at the terminal ampoule, and the associated blood vessel of the reproductive

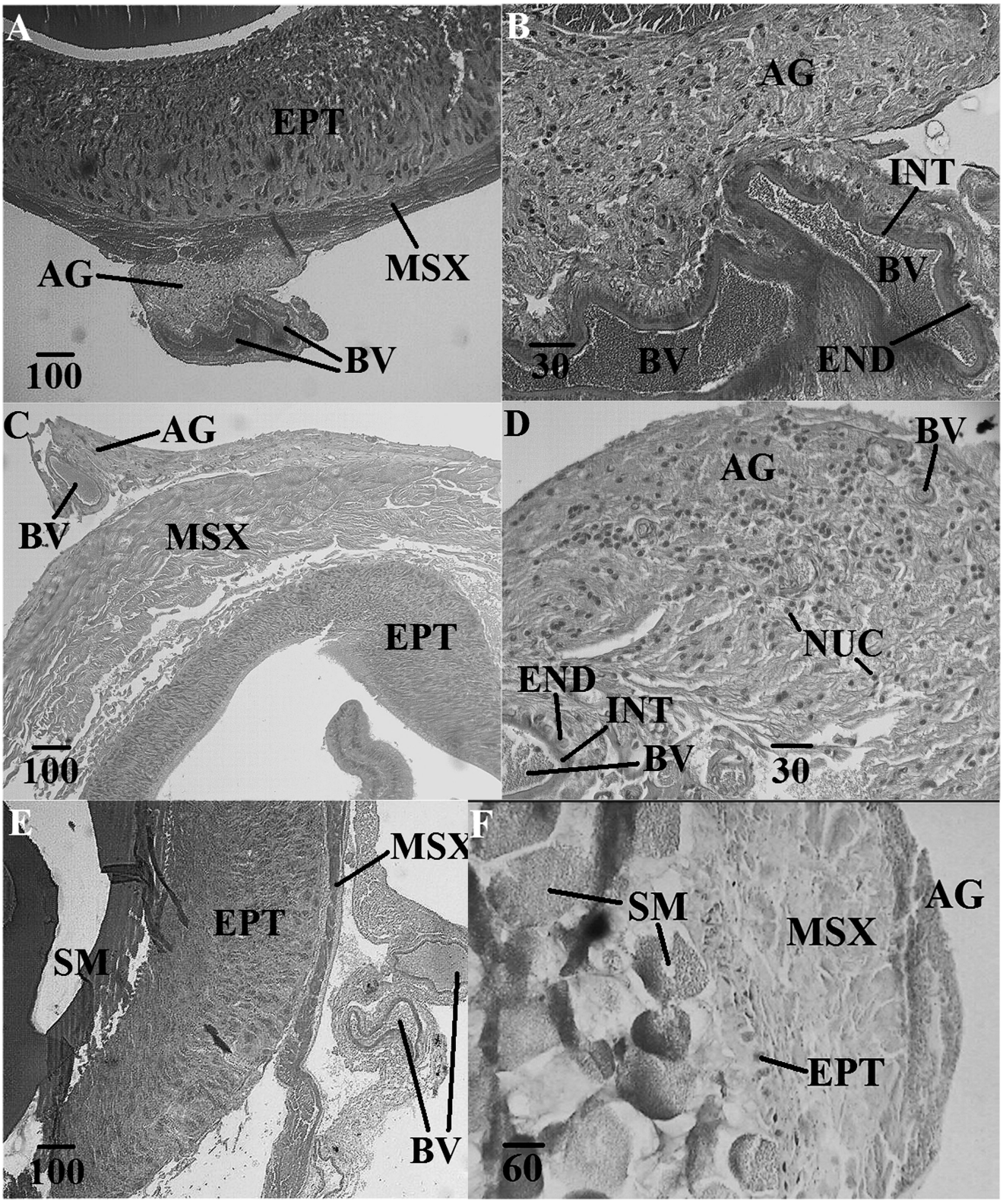

Fig. 3. Histological preparations of complex blood vessel (BV) – androgenic gland (AG) from cross section through terminal ampoule. A and B, *Litopenaeus setiferus*; C, *Litopenaeus stylirostris*; D, *Litopenaeus occidentalis*; F, *Rimapenaeus byrdi*; E, connective tissue attached to complex of terminal ampoule in *L. setiferus*. MSX = muscle layer; EPT = epithelial lining; SM = sperm mass; INT = intima; END = endothelium; NUC = nucleus. Scale bar units = μm.

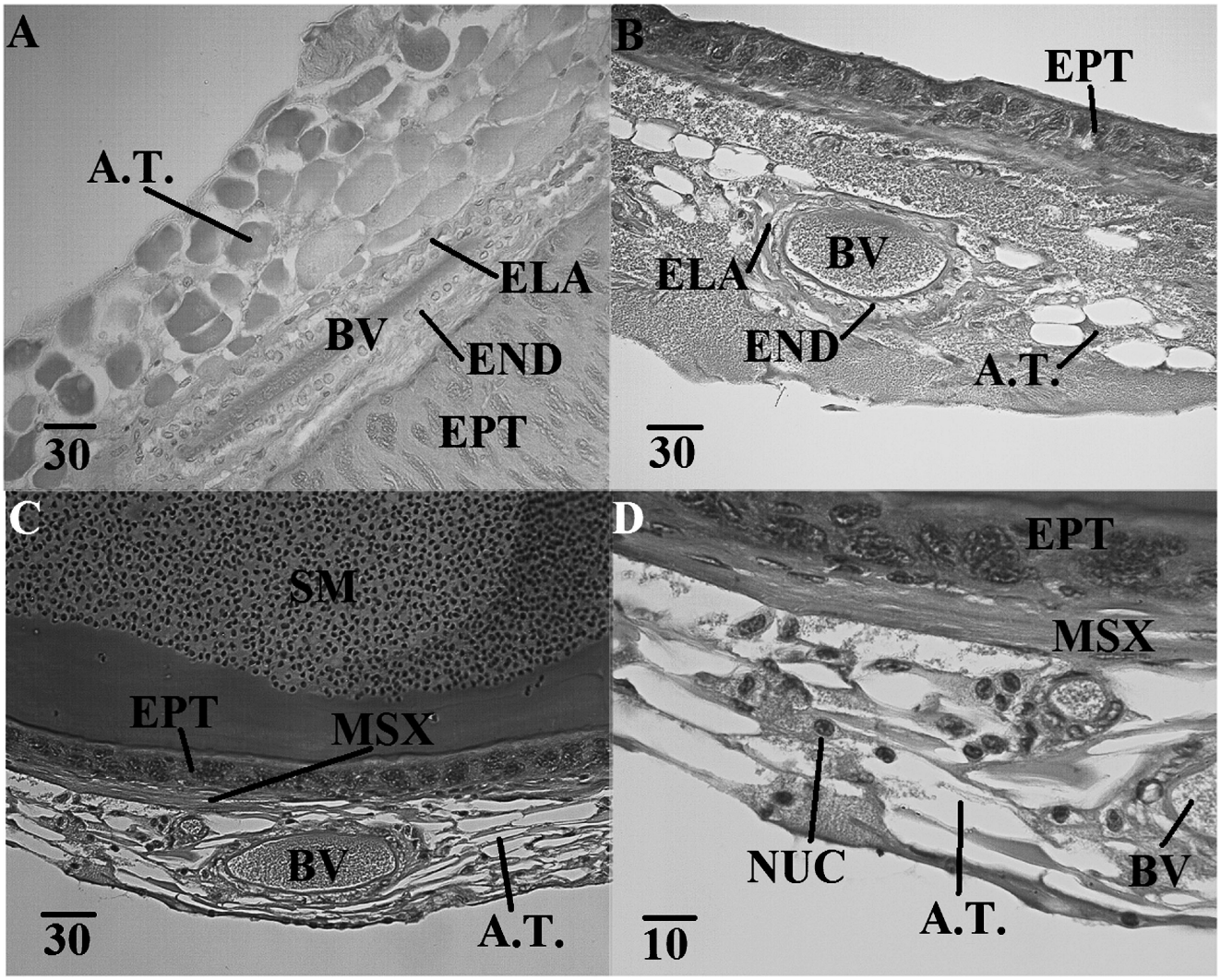

Fig. 4. Histological preparations of blood vessel (BV) and adipose tissue (A.T.) at distal region of ascending vas deferens from a longitudinal section. A, *Litopenaeus setiferus*; B, A cross section of *Litopenaeus stylirostris*; C and D, *Litopenaeus occidentalis*. MSX = muscle layer; EPT = epithelial lining; SM = sperm mass; INT = intima; END = endothelium; ELA = elastin fibers; NUC = nucleus. Scale bar units = μm.

system have practical implications for fundamental science and biotechnological management of the androgenic gland in marine shrimps. Considering previous reports from *L. vannamei*, it seems that this species has a different distribution of AG epithelial cells; however, a more detailed histological evaluation of the cellular cord from *L. vannamei* is needed.

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