A new species of *Lepidodasys* (Gastrotricha, Macrodasyida) from Panama with a description of its peptidergic nervous system using CLSM, anti-FMRFamide and anti-SCP$_B$

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Abstract

A new species of *Lepidodasys* (Gastrotricha: Macrodasyida: Lepidodasyidae) is described from sublittoral sediments in the Bocas del Toro archipelago in Panama and represents the first species of Lepidodasyidae described from the Caribbean. The new species possesses keeled scales that form a crossed-helical pattern across its dorsal and lateral surfaces and ventral scales that form a herringbone pattern between and lateral to the ciliary columns. A bilateral pair of three ventral adhesive tubes at the posterior end further differentiates this new species from its seven congeners. A confocal laser scanning microscope examination of the nervous system using antibodies to small cardioactive peptide B (SCP$_B$) and FMRFamides reveals a dumbbell-shape cerebral ganglion, paired pharyngeal neurites and paired posterior nerve cords. Expression patterns of immunoreactivity to both classes of neuropeptides show a high degree of similarity. Only within the lateral somata of the cerebral ganglion and a single median pharyngeal neurite is there a difference in immunoreactivity to FMRFamide (positive) compared to SCP$_B$ (negative). Results from this investigation reveal that neuropeptides, among other neuronal markers, might provide phylogenetically informative characters in macrodasyidan gastrotrichs, especially regarding the topology of the cerebral ganglion.
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1. Introduction

Marine gastrotrichs are cryptic, microscopic invertebrates that live within the interstitial spaces between sand grains on coastal beaches down to the deep sea. Over 400 species have been described from around the globe (Hummon, 2009), with most descriptions coming from the European coastline; comparatively few species are known from the tropics, specifically the Caribbean (Renaud-Debyser, 1963; Thane-Fenchel, 1970; Hummon, 1974, 2010; Schoepfer-Sterrer, 1974; Kisielewski, 1984; Decho et al., 1985; Evans and Hummon, 1991; Evans, 1992, 1994; Todaro, 1994; Todaro et al., 1995; Hochberg, 2008; Hochberg and Atherton, 2010). However, recent studies of various Caribbean islands have revealed a wealth of new species (e.g., see Hummon, 2010), hinting at the vast unexplored diversity that likely awaits future explorations.

To date, no species of *Lepidodasys* are reported from the Caribbean. All records of the genus come from either the European coastline (Remane, 1926, 1927; Fregni et al., 1999; Clausen, 2000; Hummon, 2009), its inland seas (Roszczak, 1939; Balsamo et al., 1995; Clausen, 2000, 2004; Hummon, 2009) or from Japan (Lee and Chang, in press). Additional species are known but not yet described (see Clausen,

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2000; Hummon, 2009). Apart from their restricted distribution, which is undoubtedly a consequence of sampling bias, species of *Lepidodasys* are peculiar in their possession of a suite of unusual characteristics that exclude them from categorization with other macrodasyidan gastrotrichs including the structure of their cuticle (Rieger and Rieger, 1977), the absence of striated myofibrils in their pharyngeal epithelium (Ruppert, 1991), the absence of pharyngeal pores, and their process of spermatogenesis (Guidi et al., 2004). These characteristics, among others, led Hummon and Todaro (2010) to emend the Lepidodasyidae, removing six genera to a new family (Cephalodasyidae) and retaining only the type genus *Lepidodasys*. Despite these peculiarities, species of *Lepidodasys* do share a few characteristics, considered synapomorphies by Travis (1983), with the most speciose family of Macrodasyida, the Thaumastodermatidae: electron-dense (myofilament-containing) Y-cells and the absence of somatic circular muscles in the lateral body regions. A third potential synapomorphy considered by Travis (1983), the sculptured cuticle, is rejected on the basis of ultrastructural data by Rieger and Rieger (1977; see also Remane, 1927).

Even with all of the accumulated data on species of *Lepidodasys* since their first description by Remane (1926), including taxonomic characters (reviewed in Hummon and Todaro, 2010), ultrastructural characters (Ruppert, 1978; Travis, 1983), reproductive features (Guidi et al., 2004), and more recently, molecular data (Todaro et al., 2003, 2006), there is still no consensus on where the taxon fits into the phylogenetic framework of the Gastrotricha. The most modern analysis by Kieneke et al. (2008) lent only marginal support to the hypothesis of Travis (1983) – that *Lepidodasys* might be the sister group of Thaumastodermatidae – a hypothesis also supported by an earlier study by Hochberg and Litvaitis (2001). In the current study, we provide a new set of morphological characters for species of *Lepidodasys* that might be phylogenetically informative and assist future efforts at reconstructing macrodasyidan phylogeny. Herein, we describe a new species of *Lepidodasys* from the Bocas del Toro archipelago in Panama, and present the first description of its nervous system using antibodies to small cardioactive peptide B (SCP$_B$) and FMRFamide combined with confocal laser scanning microscopy.

2. Materials and methods

Sediments were collected via SCUBA from 15 m depth at Wild Cane Reef (9°21.039′N, 82°10.345′W) in Bocas del Toro, Panama. Extraction of gastrotrichs from their sediments used the following protocol: (1) 100 cm^3 of sediment was combined with ca. 900 cm^3 of 7% MgCl$_2$ in a 1 L Erlenmeyer flask and allowed to rest for 15 min; (2) the flask was gently shaken and the supernatant was decanted over a 63 μm mesh; and (3) the mesh was gently washed with seawater into a Petri dish. Specimens were sorted with a stereomicroscope, transferred to a glass slide, and viewed with a Zeiss A1 compound microscope equipped with DIC (differential interference contrast) and a Sony Handycam digital camera. Measurements of all specimens were performed with an ocular micrometer. Lengths and positions of various organ systems are described in terms of percentage body units, with total body length from anterior (U00) to posterior (U100) as 100 units.

For cyto- and immunohistochemistry, a total of seven specimens were anaesthetized (7% MgCL$_2$) and fixed in 4% paraformaldehye in 0.1 M PBS for >72 h. Specimens were rinsed (2×) over the course of 24 h in 0.1 M PBS and then processed for cyto- or immunohistochemistry. Four specimens were first incubated in 1% bovine serum albumen in 0.1 M PBS for 24 h to minimize non-specific immunostaining. Specimens were then transferred to separate microcentrifuge tubes containing either rabbit anti-FMRFamide (Immunostar) or mouse anti-SCP$_B$ (courtesy of Dr. Scott Santagata), both diluted 1:500 in PBT (0.1 M PBS plus 0.5% Triton X-100). All stainings were performed at 4 °C on an orbital shaker for 48 h. Specimens were next rinsed (3×) in 0.1 M PBS over 24 h and transferred to either goat anti-rabbit or goat anti-mouse Alexa Fluor 546 (Sigma–Aldrich; dilution 1:200; Absorption/emission spectra at 556 nm/573 nm). Specimens were stained in the dark at 4 °C on an orbital shaker for 24 h and then rinsed in 0.1 M PBT for 48 h. All specimens were mounted in Fluormount G (Electron Microscopy Sciences). Two specimens were processed as controls for non-specific staining; they were omitted from the primary antibody, incubated in the secondary antibody, and mounted in Fluormount G. One specimen was stained with Alexa 488 Phalloidin (Invitrogen) and mounted in ProLong Gold antifade reagent with DAPI (Invitrogen).

Gastrotrichs were examined on a Zeiss LSM 510 confocal microscope system at the Smithsonian Marine Station in Fort Pierce, Florida. For confocal microscopy, Zeiss Zen, 2009 software (Carl Zeiss Microimaging, Thornwood, NY) was used to collect a series of 0.2 μm optical sections with maximum intensity projection along the *z*-axis. Confocal images were saved as TIF files. No manipulations of the original images were made other than changes of color (false color, grey-scale, background coloring) or cropping. The program Carnoy V 2.0 (© 2001 Peter Schols) was used to make measurements of neurons in some digital images. The DAPI stained specimen was viewed on a Zeiss M1 epifluorescence microscope equipped with a MRm camera. A single mounted specimen was selected as holotype and video recorded (Sony Handycam) while illuminated with DIC microscopy (Zeiss A1 Microscope). Videos files (.mov) were placed on CD and deposited in the National Museum of Natural History, Smithsonian Institution, Washington, DC.

The holotype specimen was removed from the mounting medium and prepared for museum archival using the following protocol: rinse with PBS for 1 h; expose to 1% OsO$_4$ in 0.1 M PBS for 1 min (to increase contrast); rinse in PBS for 15 min; dehydrate through an ethanol series; transfer to propylene oxide for 30 min; embed in epon on a glass micro-
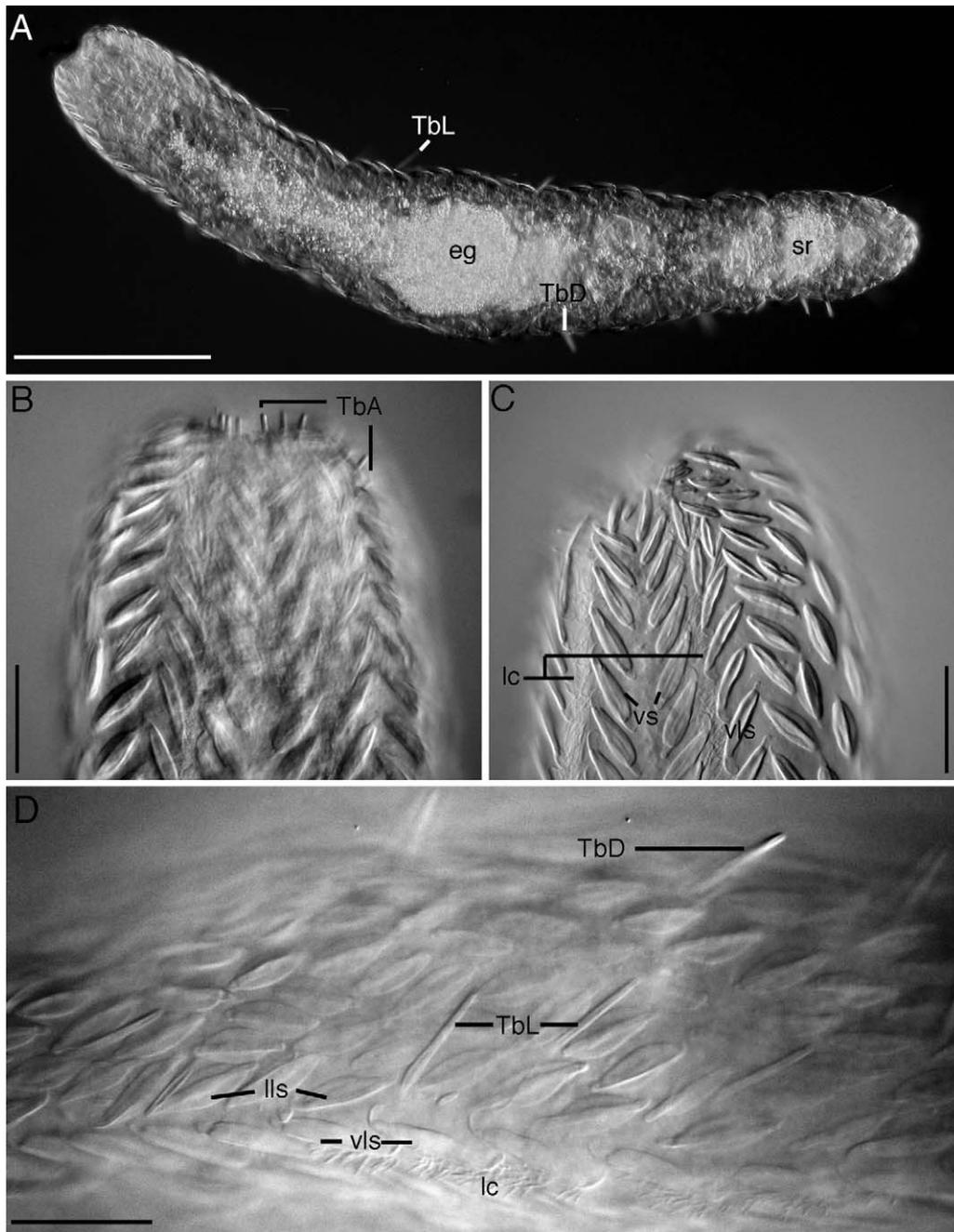
Fig. 1. *Lepidodasys worsaae* n. sp. (holotype, USNM 1149369). (A) Dorsal view of live specimen. Scale bar = 140 μm. (B) Close-up of anterior end, ventral view, with focus on TbA. Scale bar = 20 μm. (C) Close-up of anterior end, ventral view, with focus on ventral locomotory cilia. Scale bar = 20 μm. (D) View of the scales on the dorsolateral side of the body. Scale bar = 20 μm. *Abbreviations*: eg, egg; lc, locomotory cilia; lls, lower lateral scales; sr, seminal receptacle; TbA, anterior adhesive tubes; TbD, dorsal adhesive tubes; TbL, lateral adhesive tubes; TbP, posterior adhesive tubes; vls, ventrolateral scales; vs, ventral scales.

scope slide and place in an oven at 60 °C for 24 h. The holotype is deposited in the National Museum of Natural History, Smithsonian Institution, Washington, DC.

Abbreviations used in the text: PIJ, pharyngeointestinal junction; TbA, anterior adhesive tubes; TbD, dorsal adhesive tubes; TbL, lateral adhesive tubes; TbP, posterior adhesive tubes; TbV, ventral adhesive tubes.
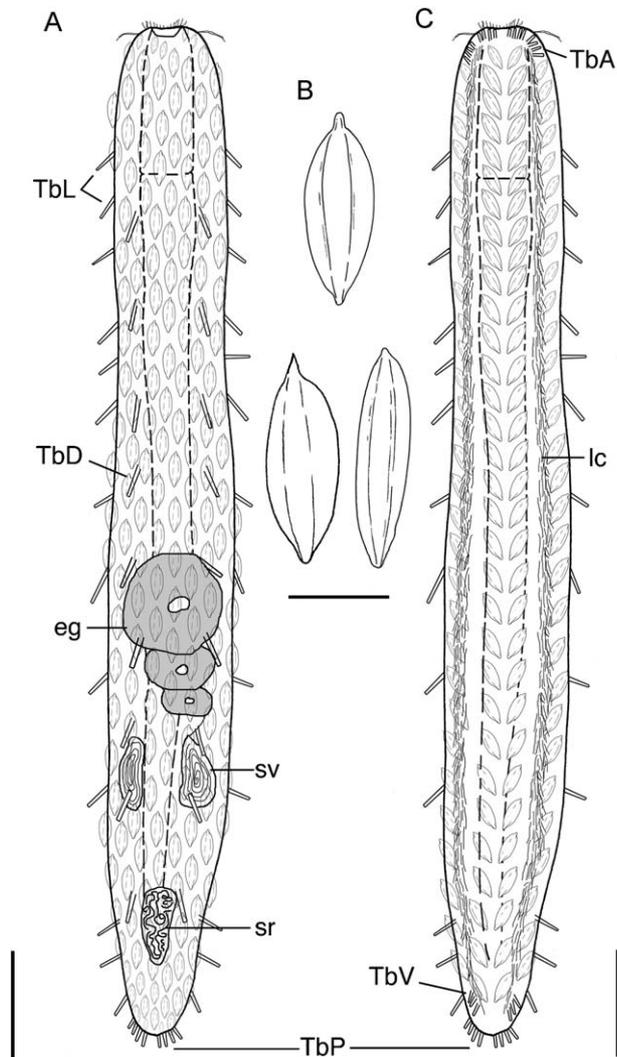
3. Systematics

Family Lepidodasyidae Remane, 1927 [Hummon and Todaro, 2010]
Lepidodasys Remane, 1926
***Lepidodasys worsaae* n. sp.**
Figs. 1–6.

Fig. 2. *Lepidodasys worsaae* n. sp., habitus. (A) Dorsal view. (B) Variations in scales shape. (C) Ventral view. Scale bars for A and C = 80 μm. Scale bar for B = 7 μm. *Abbreviations*: lc, locomotory cilia; eg, egg; sr, seminal receptacle; sv, seminal vesicle; TbA, anterior adhesive tubes; TbD, dorsal adhesive tubes; TbL, lateral adhesive tubes; TbV, ventral adhesive tubes; TbP, posterior adhesive tubes.

3.1. Diagnosis

Lepidodasys with an adult body length from 425 μm to 530 μm long. Maximum body width at mouth/PIJ/midpoint of body is 15/38/100 μm. Pharynx up to 88 μm long. Body covered in elliptical scales with a crossed-helical pattern across the dorsal and lateral surface, and herringbone patterns on the ventral surface. Up to eight TbA per side insert directly on body surface at mouth rim and arch posteriorly. TbL present, up to 13 per side. TbD present, 10 per side, beginning in pharyngeal region. TbV present at posterior end, 3 per side. Eight TbP insert terminally on rounded caudal end. Hermaphroditic, with paired anterior testes with posterior

seminal vesicles. A single non-muscular seminal receptacle and single ovary are present.

3.2. Description

The description is based on the holotype (adult, 530 μm long), with ranges provided from specimens measured *in vivo*. Body strap-shaped and 425–530 μm long (Fig. 1A). Widths of mouth at U01/pharynx region at U08/trunk at U50 are 15/38/100 μm. Pharynx 63–88 μm long with no pharyngeal pores. Few stiff sensory hairs to 10 μm long line the mouth at U01. Lateral sensory hairs to 25 μm long sparse, inserting in a somewhat dorsolateral position. Ventral locomotory cilia present as two columns from ca. U05 and extending to the caudum (Figs. 1B, D, C and 2C).

3.3. Cuticular armature

Both crossed-helical and herringbone patterns of scales are present (Figs. 1B and C, 2 and 3). Dorsally, several columns of elliptical scales, which are more-or-less parallel to the body axis, form a crossed-helical pattern across the surface and extend to the lateral body wall (Fig. 2A). The lateral body wall bears a single column of scales that are parallel to the body axis. The lower lateral body surface bears three columns of scales (lls) in a crossed-helical pattern with their anterior ends tilted medially (Figs. 1C and D and 3); the most ventro-medial column forms a herringbone-pattern with the ventrolateral column of scales that is lateral to the ventral ciliary field (Figs. 1C and D and 3). Two columns of ventral scales between the ciliary fields are oriented in a herringbone pattern (Figs. 1B, 2B and 3). All scales bear a median keel that extends anteriorly and posteriorly as a notch (Fig. 2B). The length and shape of the notch is variable, as is the general shape of many scales (Fig. 2B). Scale length varies from the anterior to the posterior end of an animal: anterior scales around the mouth are 6–8 μm long, while scales on the trunk are 12–16 μm long, and scales toward the posterior are 8–12 μm long. Notch length of individual scales is generally longer (2–3 μm) at the anterior end compared to the posterior end (1–2 μm).

3.4. Adhesive tubes

Approximately 7–8 anterior adhesive tubes (TbA) per side up to 10 μm long: 3–4 TbA are distributed along the mouth margin in a single row and 4 TbA are arranged in a posterior arc, but pointing anterolaterally (Figs. 1B and 2C). Approximately 13 TbL per side, up to 25 μm long, beginning at ca. U30 and pointing in a dorsolateral position (Figs. 1A and D and 2A). TbV present as 3 tubes per side, oriented posteriorly, and inserting at ca. U90 (Fig. 2C); TbV were only observed in fixed specimens viewed with DIC. Approximately nine TbD per side, up to 20 μm long, begin posterior of the PIJ at approximately U30 (Figs. 1A and 2A).
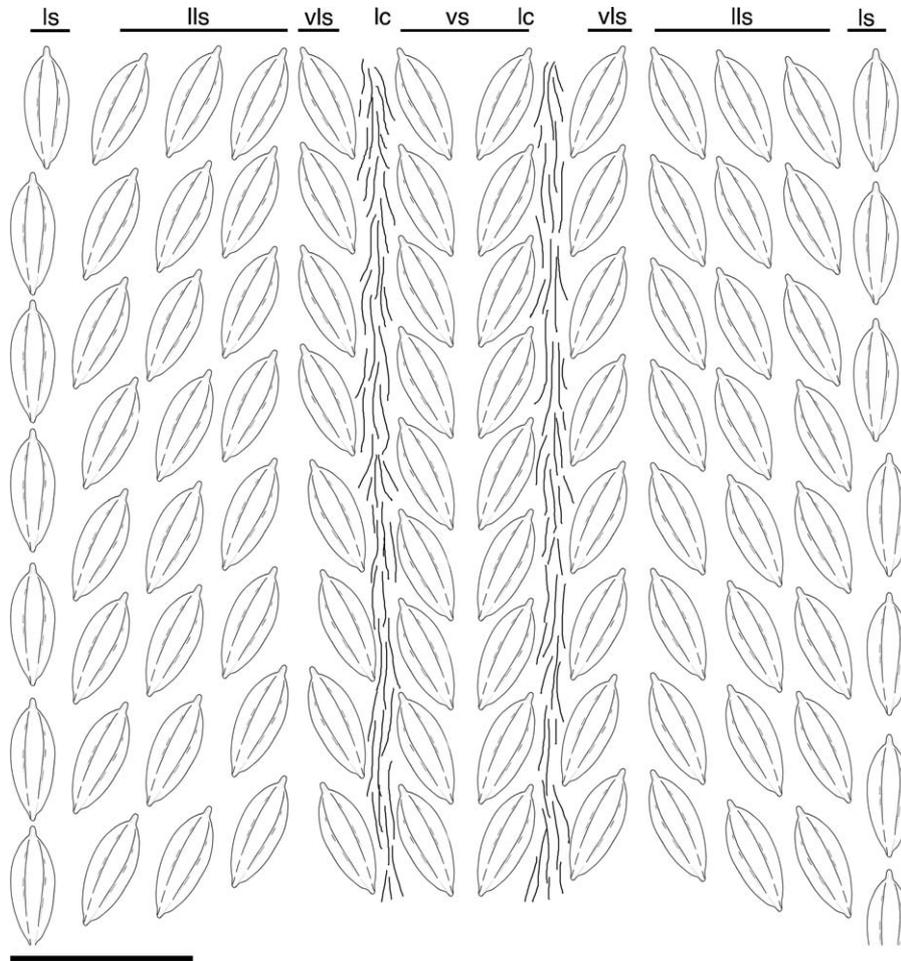
Fig. 3. *Lepidodasys worsaae* n. sp., pattern of scales on lateral and ventral surfaces. Scale bar = 20 μm. Abbreviations: lc, locomotory cilia; ls, lateral scales; lls, lower lateral scales; vls, ventrolateral scales; vs, ventral scales.

Eight TbP, to 15 μm long, distributed on rounded caudum (Fig. 2C).

3.5. Digestive tract

Mouth terminal to 15 μm wide. Pharynx to 20 μm wide and 88 μm long. Intestine narrow to 25 μm wide and tapering toward posterior end. Diatoms present in a single specimen. Anus at U88.

3.6. Reproductive system

Hermaphroditic. Female system consists of one (?) ovary with oocytes maturing in a dorsal and anterior direction (Figs. 2A and 4A). A sac-like organ containing sperm representing a potential seminal receptacle (sr) present at body midline at ca. U90 (Figs. 2A and 4A); sac-like organ is non-muscular (Fig. 4B). Male system consists of two testes; the anterior region of each testis was not observed. Vasa deferentia extend from at least ca. U50 posteriorly toward

a pair of dilated, sperm-containing seminal vesicles (sv) (Figs. 2A and 4A and C).

3.7. Holotype

Wholemount of adult specimen in lateral orientation; resin preparation. Digital videos (five .mov files) of fixed specimen prepared prior to resin preparation. Wholemount and CD received the same catalog number: USNM # 1149369.

3.8. Type locality

Station BRS2010–110, Wild Cane Rock, Bocas del Toro, Panama, 15 m depth, medium coarse sand plain, 9°21.039′N, 82°10.345′W. Sediment collected by Daniel Gouge and Dr. Ashleigh Smythe on June 8, 2010.

3.9. Etymology

Dedicated to Dr. Katrine Worsaae, who found the first specimens of the new species.

3.10. The nervous system

Immunoreactivity to anti-FMRFamide (FMRFamide-like IR) and anti-small cardioactive peptide B (SCP$_B$-IR) is present throughout the central nervous system of all specimens examined (Fig. 5). The negative control specimens showed very little background staining. DAPI staining revealed nuclei across the entire body of a single specimen, with a large cluster at the anterior end (Fig. 5F). The center of this cluster is present around U10, which overlaps with the position of both FMRFamide-like IR and SCP$_B$ IR in the cerebral ganglion.

The relative abundance and distribution of immunoreactivities to both antibodies was extremely similar among all specimens. The only significant difference between the anti-FMRFamide and anti-SCP$_B$ staining was observed in the number of lateral somata in the cerebral ganglion and the presence of a single median pharyngeal neurite, both characterized by FMRFamide-like IR only. For these reasons, we provide a single description of the nervous system based on observations of four specimens, noting where appropriate when differences between neurotransmitter phenotypes was evident.

The CNS shows strong immunoreactivity (IR) within the cerebral ganglion and nerve cords. The cerebral ganglion occurs at ca. U11 and is defined by a total of 10 SCP$_B$-IR somata, approximately 16 FMRFamide-like IR somata, two dorsal commissures and one ventral commissure; both the somata and the commissures are positioned close to the posterior border of the cluster of nuclei defined by the DAPI staining (compare Fig. 5A and F). The thickest dorsal commissure is the suprapharyngeal commissure (spc), defined by strong IR and consisting of several neurites that arch across the dorsal portion of the pharynx at ca. U11. A single pair of IR somata, the suprapharyngeal commissure somata (spcp), are located posterior of the suprapharyngeal commissure and arranged bilaterally on either side of the midline (Fig. 5A and D). Each perikaryon has a pair of thin frontal connections to the commissure (Fig. 5C). Laterally, the suprapharyngeal commissure becomes noticeably thinner on both sides of the pharynx and extends posteriorly as a single neurite within the nerve cords (pc-mn); this neurite parallels a second more lateral neurite within the nerve cords (pc-ln) that extends from the anterior pharyngeal commissure (Fig. 5).

The anterior pharyngeal commissure (apc), which arches dorsally over the pharynx immediately anterior to the suprapharyngeal commissure, consists of one or two thin IR fibers that extend from a single large perikaryon (apc-lp) on either side of the pharynx (Figs. 5B–D and 6). This perikaryon also gives rise to one of the two posterior neurites that define the nerve cords (pc-ln; Figs. 5B and C and 6). At the center of the commissure and positioned anteromedially to it is a single pair of extremely weak IR somata (apcp), each bearing a pair of thin connections to the commissure (Figs. 5D and 6). The connections were not evident with anti-FMRFamide staining. Laterally, a single pair of IR fibers extends off the
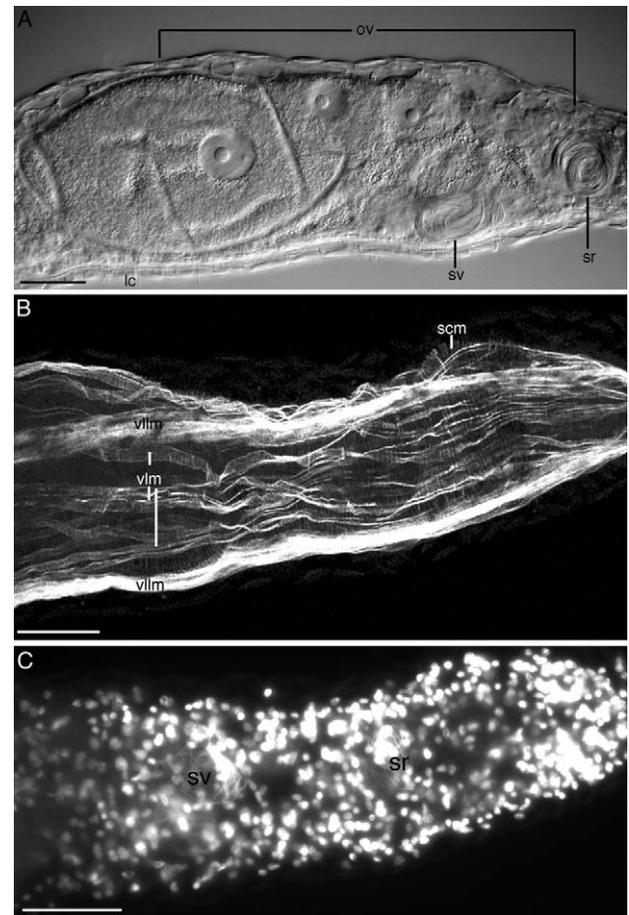

Fig. 4. *Lepidodasys worsaae* n. sp., reproductive system. (A) Close-up of the posterior one-third of the body, lateral view with ventral side down. Seminal vesicles are paired but only one is present in this view; the singular seminal receptacle is shown by the presence of sperm. Scale bar = 30 μm. (B) Close-up of the same specimen in (A), showing the same body region after staining with phalloidin to reveal the musculature. Note the lack of any muscular organ that might represent a caudal organ. Scale bar = 30 μm. (C) Close-up of a DAPI-stained specimen with a focus on the same general body region as in (A) and (B). Note the presence of sperm in the seminal receptacle and seminal vesicle. Scale bar = 25 μm. *Abbreviations*: lc, locomotory cilia; ov, ovary; scm, splanchnic circular muscles; sr, seminal receptacle; sv, seminal vesicle; vlm, ventral longitudinal muscle; vllm, ventrolateral longitudinal muscle.

anterior pharyngeal commissure and innervates a perikaryon along the lateral pharynx wall (not shown, but see Fig. 6). A single thin IR neurite (pn, Figs. 5A and B and 6) extends from each of these perikarya toward the mouth, but their point of synapse at the anterior end was undetermined.

Approximately three somata, defined only by the presence of FMRFamide-like IR, are present on either side of the dorsal commissure (lp$_{fm}$, Fig. 6). Individual connections among the somata were not evident.

A third IR commissure is present around U10 and extends off of a pair of small somata present on either ventrolateral
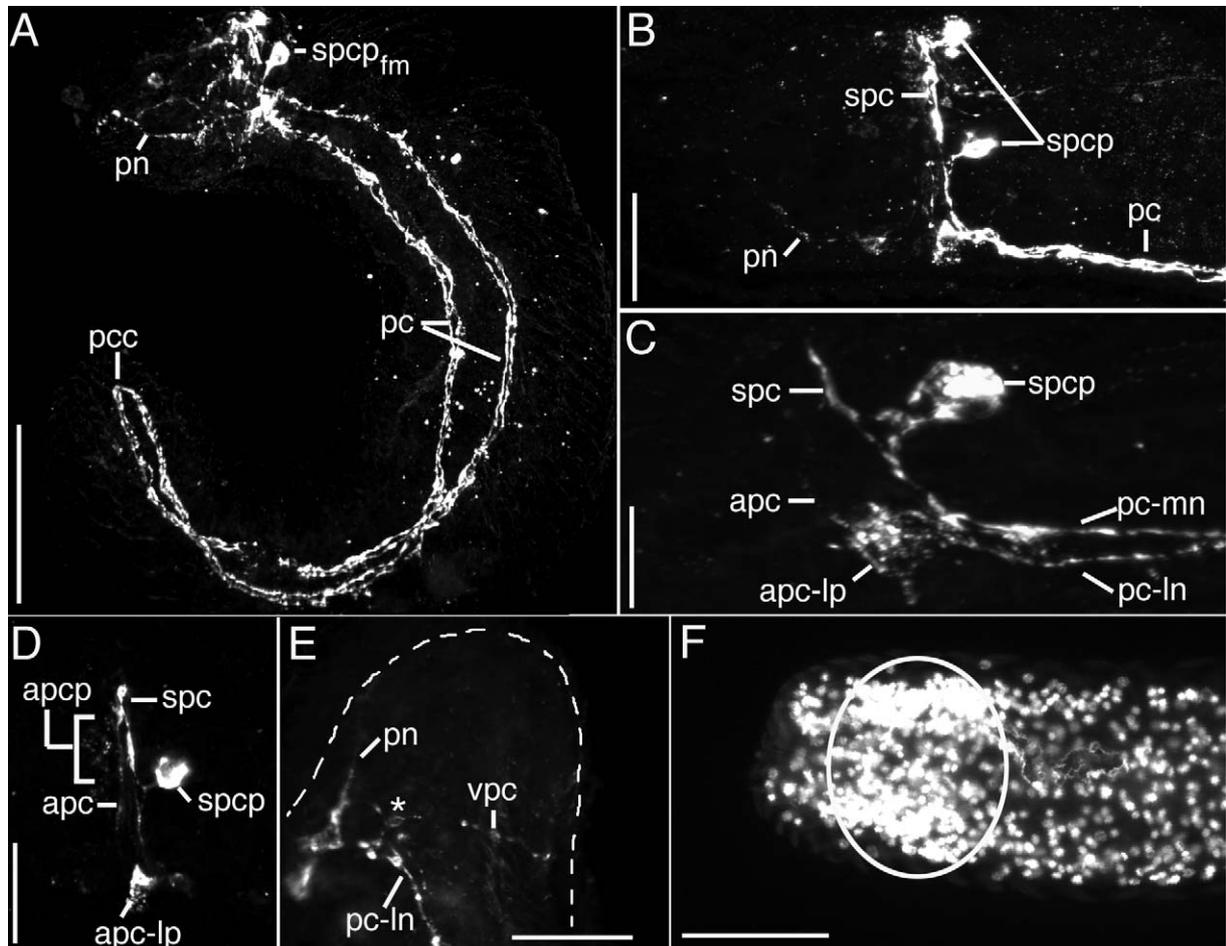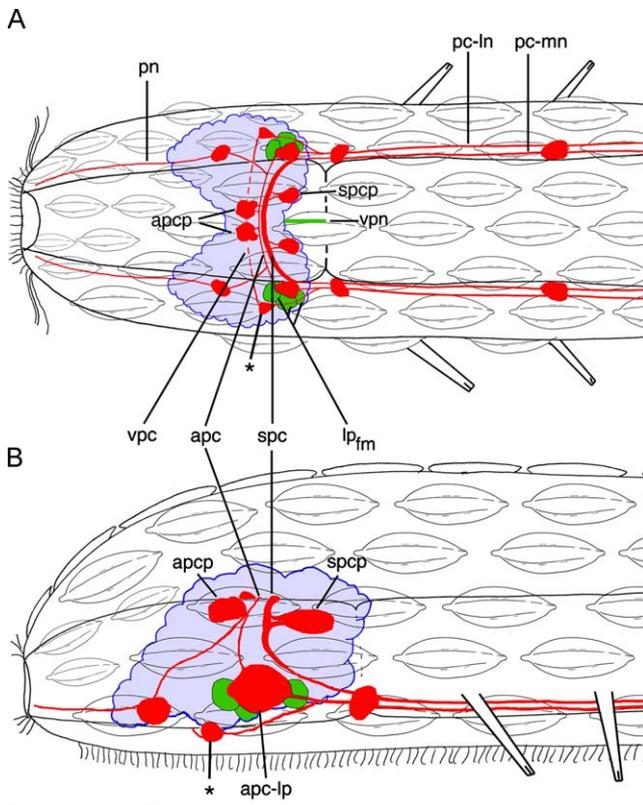
Fig. 5. The peptidergic nervous system of *Lepidodasys worsaae* n. sp. (A) Specimen oriented on its side, anterior on the top left, stained with anti-FMRFamide. Scale bar = 100 μm. (B) Close-up of the head of a specimen stained with anti-SCP$_B$, dorsolateral view, anterior to the left. Focus on the suprapharyngeal commissure. Scale bar = 20 μm. (C) Close-up of a different specimen stained with anti-SCP$_B$, with a focus on the left lateral side of the dorsal commissure. Anterior to the left. Scale bar = 6 μm. (D) Same specimen as in (A), with a focus on the anterior pharyngeal commissure. Scale bar = 5 μm. (E) Close-up of specimen in (A) with a focus on the ventral pharyngeal commissure. Anterior is up, dorsal to the left. Head shape is outlined in dashes. Scale bar = 20 μm. (F) DAPI-stained specimen, with a focus on the anterior end revealing the cluster of nuclei in the region of the cerebral ganglion. Scale bar = 25 μm. *Abbreviations*: *, ventrolateral perikaryon on either side of the ventral pharyngeal commissure; apc, anterior pharyngeal commissure; apcp, dorsal perikarya of the anterior pharyngeal commissure; apc-lp, lateral perikarya of the anterior pharyngeal commissure; pc, posterior nerve cord; pcc, posterior commissure of the posterior nerve cords; pc-ln, lateral neurite of the posterior nerve cord; pc-mn, medial neurite of the posterior nerve cord; pn, pharyngeal neurite; spc, suprapharyngeal commissure; spcp, posterior perikaryon of the suprapharyngeal commissure; spcp$_{fm}$, posterior perikaryon of the suprapharyngeal commissure, anti-FMRFamide-IR (assumed to be the same as the spcp in specimens stained with anti-SCP$_B$); vpc, ventral pharyngeal commissure.

side of the pharynx (asterisk, Figs. 5E and 6). This commissure consists of a single thin neurite that arches around the ventral wall of the pharynx (vpc, Fig. 6). The ventrolateral somata have a second connection to the lateral neurites of the posterior nerve cords (pc-ln; see Fig. 5E). In addition, a single medial neurite, defined only by FMRFamide-like IR, was present along the ventral side of the pharynx (Fig. 6). The connection of the neurite to the cerebral ganglion could not be determined.

As mentioned, the posterior nerve cords consist of at least two IR neurites. The neurites have numerous varicosities along their length that might represent small somata (see Fig. 5A), but without DAPI staining to show nuclei, some of their identities remain in question. The varicosities defined by anti-FMRFamide-like IR were more prevalent than those observed with anti-SCP$_B$ IR. At least one pair of weak IR somata is present around the PIJ, and a second pair of somata are present at about U30 (Fig. 6). It is undetermined if any of these somata innervate one or both of the stained neurites that are part of the nerve cords. The nerve cords unite at the posterior end of the body, forming a u-shape neural coalescence (pcc, Fig. 5A).

Fig. 6. Schematic of the peptidergic nervous system in the head region of *Lepidodasys worsaae* n. sp. in dorsal (A) and lateral (B) views. Colors correspond to the different stains employed: green, anti-FMRFamide only; red, both anti-FMRFamide and anti-SCP$_B$; blue, DAPI. Scale bar = 15 μm. *Abbreviations*: *, ventrolateral perikaryon on either side of the ventral pharyngeal commissure; apc, anterior pharyngeal commissure; apcp, dorsal perikarya of the anterior pharyngeal commissure; apc-lp, lateral perikarya of the anterior pharyngeal commissure; lp$_{fm}$, lateral perikarya with anti-FMRFamide-like IR only; pc, posterior nerve cord; pcc, posterior commissure of the posterior nerve cords; pc-ln, lateral neurite of the posterior nerve cord; pc-mn, medial neurite of the posterior nerve cord; pn, pharyngeal neurite; spc, suprapharyngeal commissure; spcp, posterior perikaryon of the suprapharyngeal commissure; vpc, ventral pharyngeal commissure; vpn, medial pharyngeal neurite on ventral side of pharynx.

4. Discussion

4.1. Taxonomic remarks

This report constitutes the first description of a species of *Lepidodasys* from the Caribbean, all other described species being reported from the Faroe Islands in the North Atlantic (Clausen, 2004), various parts of the European coast (Remane, 1926, 1927; Fregni et al., 1999; Clausen, 2000; Hummon, 2009) including its inland seas (Roszczak, 1939; Balsamo et al., 1994; Clausen, 2000, 2004; Hummon, 2009), and two species from Japan (Lee and Chang, in press). A single species is also reported from Hawaii (Hummon, 2009) but has yet to be formally described.

Species of *Lepidodasys* are the only members of the Macrodasyida to possess cuticular scales that are either rounded or elliptical in shape, which according to Rieger and Rieger (1977), probably evolved independently of the complex scales present in other macrodasyidan gastrotrichs including species of Thaumastodermatidae. To date, there are seven described species of *Lepidodasys*, most of which can be distinguished based on the structure and orientation of their scales: *L. arcolepis* Clausen, 2004, *L. castoroides* Clausen, 2004, *L. laeviacus* Lee and Chang, 2011, *L. martini* Remane, 1926, *L. platyurus* Remane, 1927, *L. tsushimaenensis* Lee and Chang, 2011 and *L. unicarenatus* Balsamo, Fregni and Tongiorgi, 1994. Among these species, only *L. arcolepis*, *L. martini*, *L. platyurus* and *L. unicarenatus* bear scales with distinct keels similar to that of *L. worsaae* n. sp. The keel is a median ridge that extends down the length of the scale and often projects as an anterior and/or posterior notch at the end of the scale. In *L. worsaae* n. sp., the single keel extends off both ends of the scale, though the anterior notch is often more pronounced than the posterior notch. Only *L. martini* and *L. unicarenatus* also bears scales with a distinct keel that extends as a notch at both ends. However, *L. worsaae* n. sp. differs from *L. martini* in the arrangement of its scales, which are oriented in a crossed-helical pattern across the dorsal and lateral surfaces as opposed to a herringbone pattern that is characteristic of *L. martini*. Alternatively, the scale patterns of *L. worsaae* n. sp. and *L. unicarenatus* are similar on their dorsal surfaces but differ on their ventral surfaces (more below).

Among the more interesting cuticular features of *L. worsaae* n. sp. is its possession of two types of scale patterns: the crossed-helical array on the dorsal surface, and a herringbone pattern on the ventral surface (see Fig. 3). In most species described to date, the ventral scale patterns either match the dorsal patterns (e.g., the dorsal and ventral herringbone pattern of *L. laeviacus*) or are not illustrated (Balsamo et al., 1994), and therefore the exceptionality of the dual pattern remains to be determined. It should be mentioned that Hummon's (2009) database contains unpublished illustrations of specimens of *L. martini* from Helgoland, Germany and *L. unicarenatus* from the Mediterranean Sea, each possessing a dorsal crossed-helical array and some resemblance of a ventral herringbone pattern; however, in *L. martini*, the dorsal pattern also contains two medial columns that form a herringbone pattern, not present in *L. worsaae* n. sp., and in *L. unicarenatus*, the ventral scales that form the herringbone pattern are separated by smaller scales that are oriented parallel to the body axis, thereby distinguishing that specimen from *L. worsaae* n. sp.

Apart from the cuticle, which bears the most distinguishable taxonomic characteristics, *L. worsaae* n. sp. also possesses some features that may be exceptional. First, specimens of *L. worsaae* n. sp. are some of the smallest members of the genus, reaching only 530 μm in length, with most specimens under 500 μm. Other species with similarly small bodies include *L. castoroides* and *L. unicarenatus*. The small body size of all species is reflected in the lengths of their pha-

rynges. The pharynx of *L. worsaae* n. sp. reaches a maximum length of 88 μm, while in comparably sized relatives such as *L. castoroides* (470 μm) and *L. unicarenatus* (550 μm), the pharynges are 122 μm (Clausen, 2004) and 114 μm long (Balsamo et al., 1994), respectively. Another remarkable feature is the possession of a distinguishable patch of smaller TbV at the posterior end, which is not a continuum of another series of adhesive tubes such as the TbL (see Fig. 6).

The hermaphroditic reproductive system of *L. worsaae* n. sp. consists of two exceptional attributes that are reminiscent of the condition present in *L. arcolepis*. First, there are two large swellings of the vasa deferentia that may represent seminal vesicles. Second, no muscular caudal organ was observed (with light microscopy or phalloidin staining), but a large sac-like organ containing sperm was present and probably represents a seminal receptacle. Lastly, only a single ovary was observed in all specimens. This observation needs further confirmation, but so far oocytes have only been observed to be produced on one side of the body; however, paired ovaries that alternative oocyte production cannot be ruled out.

4.2. Topology of the nervous system

The gastrotrich nervous system has been the subject of an increasing number of investigations in recent years due to its potential for revealing phylogenetically informative characters (Joffe and Kotikova, 1987; Joffe and Wikgren, 1995; Hochberg and Litvaitis, 2003; Hochberg, 2007a; Rothe and Schmidt-Rhaesa, 2007, 2008, 2010). To date, all investigations have relied on the distribution of structural proteins such as alpha tubulin and/or neurotransmitters such as serotonin (5-HT) and FARPs (FMRFamide-like peptides) as a means of mapping neuronal architecture. Antibodies to these compounds highlight the structure of the cerebral ganglion and the distribution and abundance of major and minor nerves, and provide a foundation for understanding organ system function and physiology. In this study, we also utilize anti-FMRFamide to study the gastrotrich nervous system, but in addition, use a new neuropeptide, small cardioactive peptide B (SCP$_B$), which has never been studied in gastrotrichs. SCP$_B$ was originally isolated from *Aplysia brasiliana* Rang, 1828 (Morris et al., 1982) and has been implicated in modulating muscle activity among other functions in mollusks (e.g., Lloyd et al., 1984, 1985). The presence and distribution of SCP$_B$ in most meiofaunal organisms is relatively unknown compared to the aforementioned neurotransmitters (for meiofaunal platyhelminths see e.g., Reuter and Palmberg, 1989; Hochberg, 2009; for a benthic freshwater rotifer see Hochberg, 2007b). However, several studies have shown that SCP$_B$ is often colocalized with FARPs such as FMRFamide (Callaway et al., 1987; Hochberg, 2009), perhaps because they both function to modulate Cl$^-$ currents but in opposite directions (Buttner and Siegelbaum, 2003). In this respect, the presence of one neurotransmitter phenotype may function as a proxy for the other, with some limitations.

In *L. worsaae* n. sp., both FMRFamide-like IR and SCP$_B$-IR are present throughout the nervous system including the cerebral ganglion, major nerve cords, and a few peripheral neurites that presumably innervate the pharynx or sensory structures on the head. While colocalization of both neuropeptides was not performed, our observations indicate a high degree of overlap in their phenotypic expression based on the position of neuronal somata and their associated neurites. Results from *L. worsaae* n. sp. combined with data from previous investigations (Hochberg, 2007a; Rothe and Schmidt-Rhaesa, 2008, 2010) reveal that all gastrotrichs possess a characteristic "dumbbell-shaped" cerebral ganglion defined by the presence of lateral somata and a ring-like commissure across the pharynx. Immunoreactivity to SCP$_B$, like that of serotonin (Rothe and Schmidt-Rhaesa, 2008), appears more restricted within the cerebral ganglion compared to FMRFamide-like IR. Based strictly on neuropeptide expression, the cerebral commissure forms a thick dorsal neuropil generally consisting of several neurites. In most species, the neurites are divided into two distinct bundles: a thick, posterior commissure and a thinner anterior commissure (Hochberg, 2007a; Rothe and Schmidt-Rhaesa, 2008, 2010). Only in *Oregodasys cirratus* Rothe and Schmidt-Rhaesa, 2010 is there no distinction between the dorsal bundles; instead, the neurites form a thick solitary commissure (Rothe and Schmidt-Rhaesa, 2010). Ventrally, a subpharyngeal commissure is present in most macrodasyidans except *O. cirratus*. In the case of *L. worsaae* n. sp., the ventral commissure is anterior to the dorsal commissure, instead of directly beneath it, which may argue against its homology with the ventral commissures observed in such species as *Turbanella* cf *hyalina*, *Xenodasys riedli* (Schöpfer-Sterrer, 1969) (Hochberg, 2007a) and *Dactylopodola baltica* (Remane, 1926) (Rothe and Schmidt-Rhaesa, 2008).

The remainder of the nervous system defined by FMRFamide-like IR and SCP$_B$-IR in *L. worsaae* n. sp. follows a similar topology to that described in other species. At least one pair of bilateral neurites extends from the anterior pharyngeal commissure and parallels the pharynx toward the mouth. A second neurite, defined only by FMRFamide-like IR, is present at the ventral midline of the pharynx and probably innervates the pharyngeal myoepithelium. Additionally, two pairs of bilateral neurites exit the cerebral ganglion and parallel the intestine toward the anus, where they eventually coalesce into a single u-shaped commissure. Both neurites on either side of the digestive tract are in proximity to one another, and are likely to represent individual axons within a single pair of nerve cords. However, as mentioned by Rothe and Schmidt-Rhaesa (2010), statements about the number of nerve cords based on limited data (e.g., neurotransmitter expression) should be interpreted with caution, since additional markers might reveal neurites in other cords that have not been visualized. Furthermore, as mentioned by Rothe and Schmidt-Rhaesa (2010), there is a distinction among researchers about what constitutes a nerve cord, since examinations with transmission electron microscopy and confocal

microscopy rarely agree (e.g., compare observations of *D. baltica* with electron microscopy (4 nerve cords, Teuchert and Lappe, 1980; Travis, 1983) and confocal microscopy (2 nerve cords, Rothe and Schmidt-Rhaesa, 2008). In any case, the proximity of the neurites in *L. worsaae* n. sp. suggests that at least one pair of nerve cords is present, which is in line with Travis' (1983) observation of a single pair of nerve cords in species of *Lepidodasys*.

4.3. Phylogenetic conclusions

To date, the macrodasyidan brain has been shown to have a relatively conservative organization (Hochberg, 2008), and while differences among species are evident (Hochberg, 2008; Rothe and Schmidt-Rhaesa, 2007, 2008, 2010), it has not been used as a source of phylogenetic characters. Teuchert (1977) provided the first evidence for regionally specialized somata along the anterior-posterior axis of the brain in gastrotrichs, and Wiedermann (1995) provided further evidence that patterns of cerebral neurites innervating the head and pharynx might also be phylogenetically informative. Unfortunately, comparing species based on similar ultrastructural data would be a daunting and time-consuming task given that cell positions may be variable among species and their identities might be hard to homologize without further cytochemical evidence. Alternatively, the use of neurotransmitter expression patterns in wholemount specimens as performed in this study and others (Hochberg, 2008; Rothe and Schmidt-Rhaesa, 2007, 2008, 2010) have the ability to generate phylogenetically informative characters because of their relative ease of use and high specificity. In fact, Rothe and Schmidt-Rhaesa (2007) have used expression patterns of 5HT-IR to distinguish morphologically similar species of *Turbanella*. The interspecific variation present among species of *Turbanella* suggests that characters such as the number and shape of cerebral somata and the presence of a ventral commissure might have phylogenetic utility once intraspecific variation is assessed. Using this idea as the basis for comparing *L. worsaae* n. sp. to other macrodasyidans, we find that the pattern of IR staining in the dorsal commissure in *L. worsaae* n. sp. is more similar to that observed in species of *Dactylopodola* (Rothe and Schmidt-Rhaesa, 2008), *Turbanella* and *Xenodasys* (Hochberg, 2007a) than to species of *Oregodasys* (Rothe and Schmidt-Rhaesa, 2010). Past cladistic studies (Hochberg and Litvaitis, 2000, 2001) have argued for a close relationship between *Lepidodasys* and the Thaumastodermatidae, a family that includes *Oregodasys*. However, molecular studies such as Todaro et al. (2003) have proposed that species of *Lepidodasys* might be in a more basal position within the Macrodasyida than previously thought, and therefore further removed from the Thaumastodermatidae than cladistic studies have hypothesized. Whether or not the brain of *L. worsaae* n. sp., with its particular IR pattern in the dorsal commissure, represents a homologous condition to that of species of other clades of Macrodasyida remains to be determined, but provides new characters for future investigations of neurophylogeny within the Gastrotricha.

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