

COMPARISON OF MICROTHRIX ULTRASTRUCTURE AND MORPHOLOGY ON THE PLEROCERCROID AND ADULT SCOLEX OF *CALLIOBOTHRIUM* CF. *VERTICILLATUM* (TETRAPHYLLIDEA: ONCHOBOTHRIIDAE)

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ABSTRACT: The distal bothridial surfaces of adult trilobulate onchobothriids are covered with short structures that have been tentatively classified as very short filitriches, but this hypothesis has never been tested. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to investigate microthrix morphology in the plerocercoid and adult forms of *Calliobothrium* cf. *verticillatum*, a trilobulate onchobothriid tapeworm from Long Island Sound (Connecticut). Plerocercoids of *C. cf. verticillatum* were collected from the anterior midgut caeca of *Pagurus pollicaris* Say, 1817 (flat-clawed hermit crab), and adults were collected from the spiral intestine of the dusky smooth hound *Mustelis canis* (Mitchell, 1815). Two plerocercoids and 2 adults were examined using SEM; 2 plerocercoids and 2 adults were examined using TEM. Microthrix distribution and morphology (including measurements of total length, base length, shaft length, and base width) were investigated on all surfaces of the plerocercoid and adult scolex. Slender filitriches and large bladelikey spinitriches were observed extending from the tegument of plerocercoid and adult forms. The filitriches were found to have significantly narrower bases than the spinitriches (65–167 nm vs. 466–1,936 nm, respectively). The scolex proper of the plerocercoid and adult forms were found to have filitriches of medium-length and bladelikey spinitriches. The distal bothridial surfaces differed dramatically in microthrix morphology between plerocercoid and adult forms; on the distal surfaces of the plerocercoids were long filitriches and bladelikey spinitriches. However, the distal surfaces of the adults had short structures (previously hypothesized to be short filitriches) and a few bladelikey spinitriches. Serial transverse sections revealed that the short structures on the distal bothridial surfaces of the adults were homologous with filitriches. They included all of the structural components of a filitrix as well as a base width that conformed to the filitriches found on other surfaces. The bothridial margins of the plerocercoid and adult forms had a microthrix pattern similar to that seen on the proximal bothridial surfaces except that the filitriches on the margins were significantly longer than those found anywhere else on the bothridia. The most dramatic difference between the plerocercoid and adult forms occurred on the distal bothridial surfaces, where the filitriches of the adult cestodes were significantly shorter and narrower, and the spinitriches were almost entirely lacking.

Cestodes are unique among platyhelminthes in their possession of specialized tegumental extensions called microtriches (Rothman, 1959, 1963). Numerous studies examining tapeworm ultrastructure have revealed that conspicuous microtrichial polymorphism exists among taxa and among regions in a single individual (e.g., Berger and Mettrick, 1971; Featherston, 1972; Hess and Guggenheim, 1977; Voge et al., 1979; Thompson et al., 1980, 1982; MacKinnon and Burt, 1983; Novak and Dowsett, 1983). Despite their wide morphological diversity, all microtriches have been shown to conform to one basic structure (Jha and Smyth, 1969) consisting of a proximal base with a cytoplasmic core, an electron-dense distal shaft, and a base plate that separates the base from the shaft. The entire structure is enveloped in a plasma membrane. A number of authorities (e.g., Hess and Guggenheim, 1977; Voge et al., 1979; Thompson et al., 1982; Novak and Dowsett, 1983) have suggested that microtriches can be divided into 2 shape categories: long slender and short robust. Of particular interest in this study are the very short structures found on the distal bothridial surfaces of trilobulate onchobothriid genera, e.g., *Acanthobothrium* Van Beneden, 1850 and *Calliobothrium* Van Beneden, 1850, which have been examined extensively using scanning electron microscopy (SEM) and which appear to be, but have never been confirmed as, short and slender microtriches (Caira and Keeling, 1996; Nasin et al., 1997; Caira et al., 1999; Caira and Burge, 2001; Caira and Zahner, 2001; Ghoshroy and Caira, 2001; Fyler and Caira, 2006). These structures have been coded as such in morphological phylogenetic analyses (Caira et al., 1999, 2001); however, given that the internal features of these

short structures have never been characterized, their homology with microtriches remains unconfirmed.

Caira and Ruhnke (1991) made a comparison between the plerocercoid and adult forms of *Calliobothrium* cf. *verticillatum*, a representative trilobulate onchobothriid cestode, using SEM. Interesting differences in the microthrix patterns between plerocercoid and adult forms were observed. The occurrence of short structures on the distal bothridial surfaces of the adult was described, but the nature of these structures as microtriches was not confirmed.

In the present study, the ultrastructure of these surface features was investigated for the first time in the plerocercoid and adult forms of *C. cf. verticillatum* using transmission electron microscopy (TEM). TEM allowed the following issues to be addressed: the homology of the short structures on the distal bothridial surfaces of the adult with microtriches, differences in the microthrix patterns between plerocercoid and adult forms, and quantification of the differences between the 2 microthrix forms.

MATERIALS AND METHODS

Four flat-clawed hermit crabs, *Pagurus pollicaris* Say, 1817, were collected from the Long Island Sound region off the coast of Connecticut. One of 4 hermit crabs examined was found to host 8 plerocercoids of *C. cf. verticillatum*. The plerocercoids were obtained live from the lumen of the anterior midgut caeca of the infected individual. Two dusky smooth hounds *Mustelus canis* (Mitchell, 1815), were collected from the Long Island Sound region and upon necropsy were found to host over 30 live adults each of *C. cf. verticillatum* in their spiral intestines.

The terminology of cestode surface structures used in the present study follows Faliex et al. (2000), who first proposed the contraction filitriches for the long and slender microtriches (previously filiform microtriches), and spinitriches for the shorter and more robust microtriches

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(previously spiniform microtriches). Additionally, this paper adopts the singular form microtrix from Smyth (1969) and likewise uses filitrix (singular for filitriches) and spinitrix (singular for spinitriches).

The species of cestode examined in the present study had been previously identified as *C. verticillatum* (see Caira and Ruhnke, 1991), but recent research in this genus suggests that *C. verticillatum* is a species complex in need of taxonomic revision (Nasin et al., 1997; J. Caira, pers. comm.). Thus, for the purposes of this paper, this cestode species is referred to as *C. cf. verticillatum*. Strobilae of adult cestodes were prepared as whole mounts using conventional methods and deposited as voucher specimens at the United States National Parasite Collections in Beltsville, Maryland (USNPC-99241-99242). Two voucher specimens each of the plerocercoid and adult stages were prepared for SEM and have been retained at the University of Connecticut.

Two plerocercoids and the scolices of 2 adults were prepared for SEM. Live specimens were fixed in 10% buffered formalin, transferred to 70% ethanol, hydrated in a graded ethanol series, transferred to 1.5% osmium tetroxide overnight, dehydrated in a graded ethanol series, and placed in hexamethyldisilazane (HMDS, Ted Pella Inc., Redding, California) for 15 min. They were allowed to air dry and were subsequently mounted on carbon tape and grounded with carbon paint on aluminum stubs. Specimens were sputter-coated with ~20–30 nm of gold/palladium, and bothridial surfaces were examined with a LEO/Zeiss DSM 982 Gemini Field Emission Scanning Electron Microscope.

Two plerocercoids and the scolices of 2 adults were prepared for TEM. Live specimens were fixed for several days in 1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1M HEPES buffer at pH 7.4, followed by several rinses in 0.1M HEPES buffer. Specimens were post-fixed in 1% osmium tetroxide and 0.8% potassium ferrocyanide in 0.1M HEPES buffer for 1 hr at 4 C. They were subsequently rinsed in distilled water 3 times for 10 min. The material was dehydrated in a graded ethanol series and cleared in 100% propylene oxide (PO). Specimens were infiltrated with an epoxy resin mixture of Araldite 506, SPI-PON 812, and DSSA in a 1:2 ratio of resin + DMP30:PO for 4 hr, followed by a 1:1 ratio of resin + DMP30:PO for 14 hr, and finally in 100% resin + DMP30 for 5 hr. Specimens were suspended in 100% resin and polymerized at 60 C for 48 hr.

To address the identity of the structures on the bothridial surfaces, serial transverse sections 100 nm in thickness were cut at multiple points through the scolex using an LKB Ultratome III. Sections were placed on Cu/Pd grids, stained in 4% uranyl acetate in 50% ethanol for 7–10 min followed by 1% lead citrate for 3–5 min, and examined using either a Philips EM300 or an FEI Tecnai Biotwin electron microscope at 80 kV.

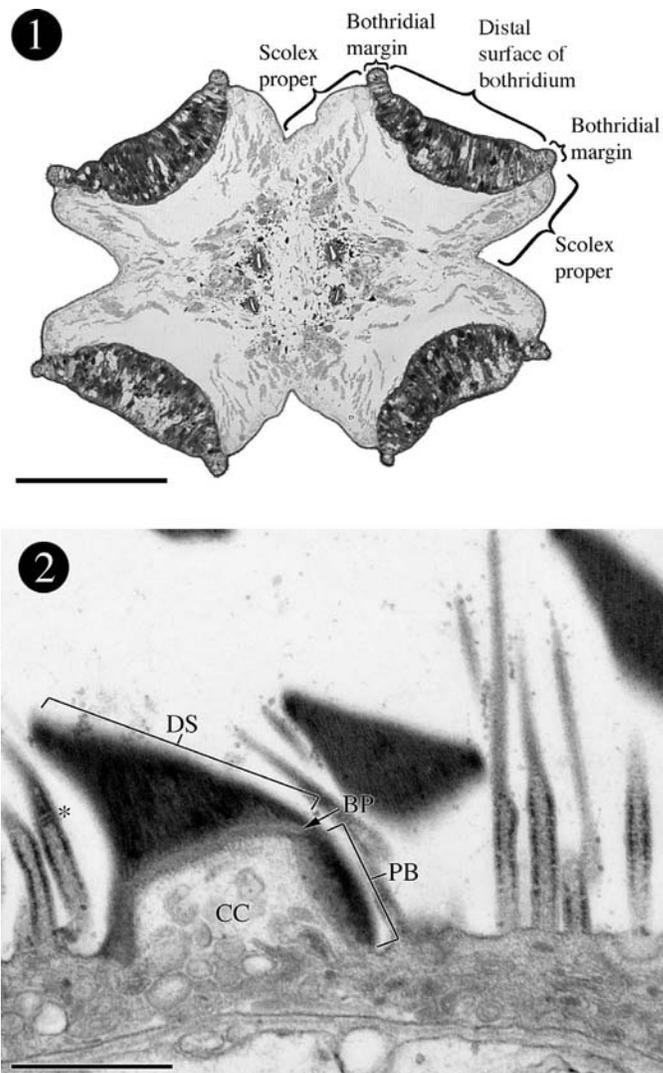
For each region, i.e., scolex proper, distal surface of bothridium, and bothridial margin, of the plerocercoid and adult forms, between 15 and 30 filitriches and spinitriches were measured for total length (TL), base length (BL), shaft length (SL), and base width (BW). Two-sample *t*-test comparisons were performed to quantify variation between filitriches and spinitriches, and to explore differences in microtrix morphology between the plerocercoid and adult forms. All measurements are reported in nm unless otherwise stated.

RESULTS

Larval and adult cestodes were confirmed as *C. cf. verticillatum* using SEM, based on their possession of a relatively unique muscular pad bearing 3, rather than a single apical sucker.

Three distinct regions of the scolex were recognized based on microtrix composition. These were scolex proper, distal surface of bothridium, and bothridial margin (Fig. 1). In total, 134 filitriches and 106 bladelike spinitriches were measured for this study (Fig. 2). The microtrix patterns on each of these 3 regions are characterized separately below for the plerocercoid and adult forms.

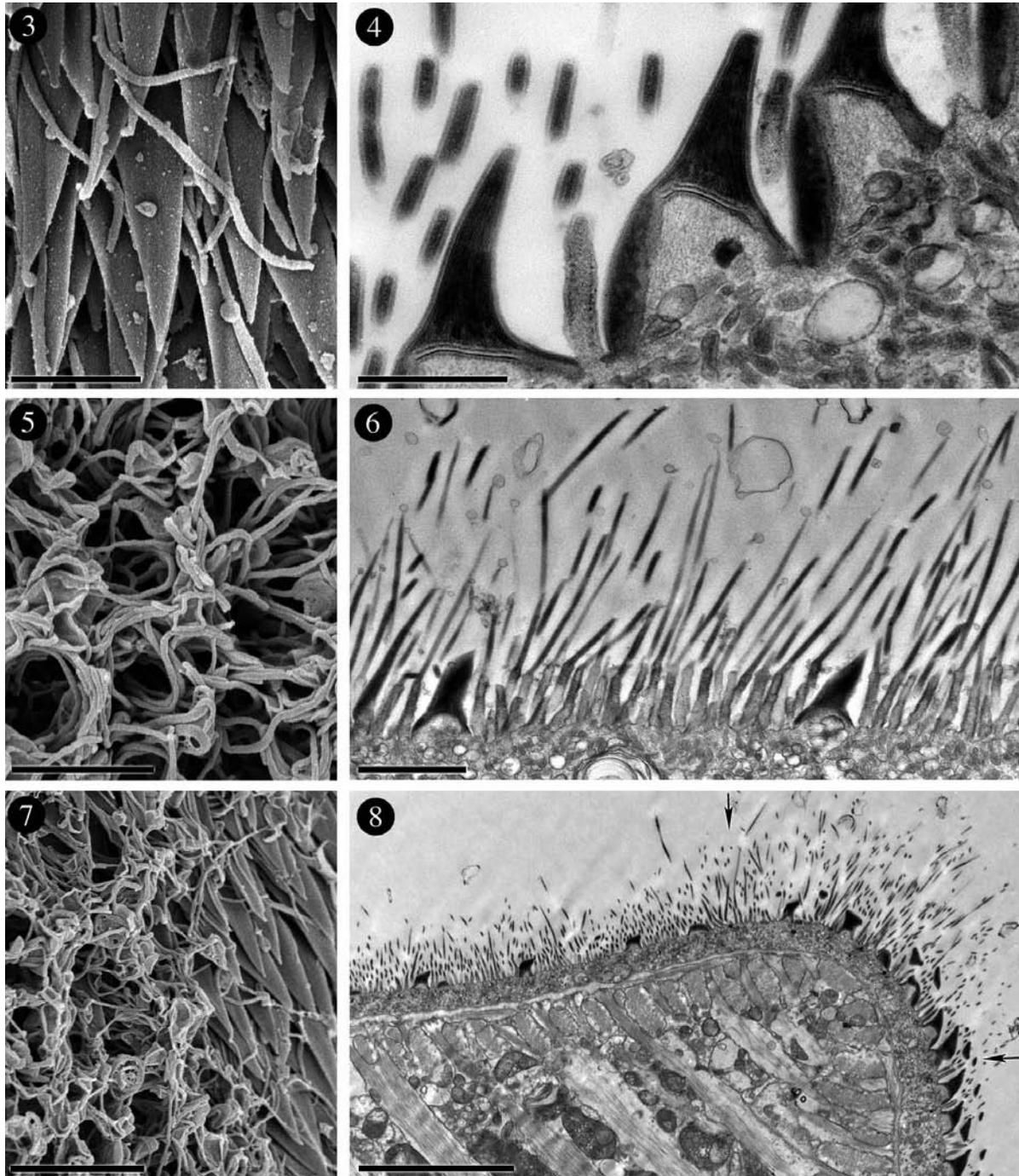
The scolex proper of plerocercoids (Figs. 3, 4) possessed medium-length filitriches (TL 738–1,356; BL 544–869; SL 111–585; BW 92–148), and bladelike spinitriches (TL 1,087–1,862; BL 351–707; SL 499–1,316; BW 519–883). The distal



FIGURES 1–2. Adult of *Calliobothrium cf. verticillatum*. (1) Light micrograph of transverse section through the scolex showing 3 regions based on distinct microtrix composition: scolex proper, distal surface of bothridium, and bothridial margin. Bar = 100 μ m. (2) Transmission electron micrograph of the distal margin of the bothridium showing the main components of a microtrix on a bladelike spinitrix. Several slender filitriches are also shown (note the base plate of a single filitrix marked with an asterisk). Bar = 0.5 μ m. BP = base plate; CC = cytoplasmic core; DS = distal shaft; PB = proximal base.

surfaces of the bothridia of plerocercoids (Figs. 5, 6) had long filitriches (TL 1,792–3,766; BL 426–692; SL 1,321–3,295; BW 100–167) and bladelike spinitriches (TL 763–1,789; BL 307–536; SL 364–1,270; BW 466–744). The bothridial margin (Figs. 7, 8) had long filitriches (TL 2,411–6,369; BL 365–629; SL 2,027–5,326; BW 81–107) that were significantly longer than those found anywhere else on the scolex and bladelike spinitriches (TL 914–1,646; BL 391–656; SL 431–1,123; BW 500–805).

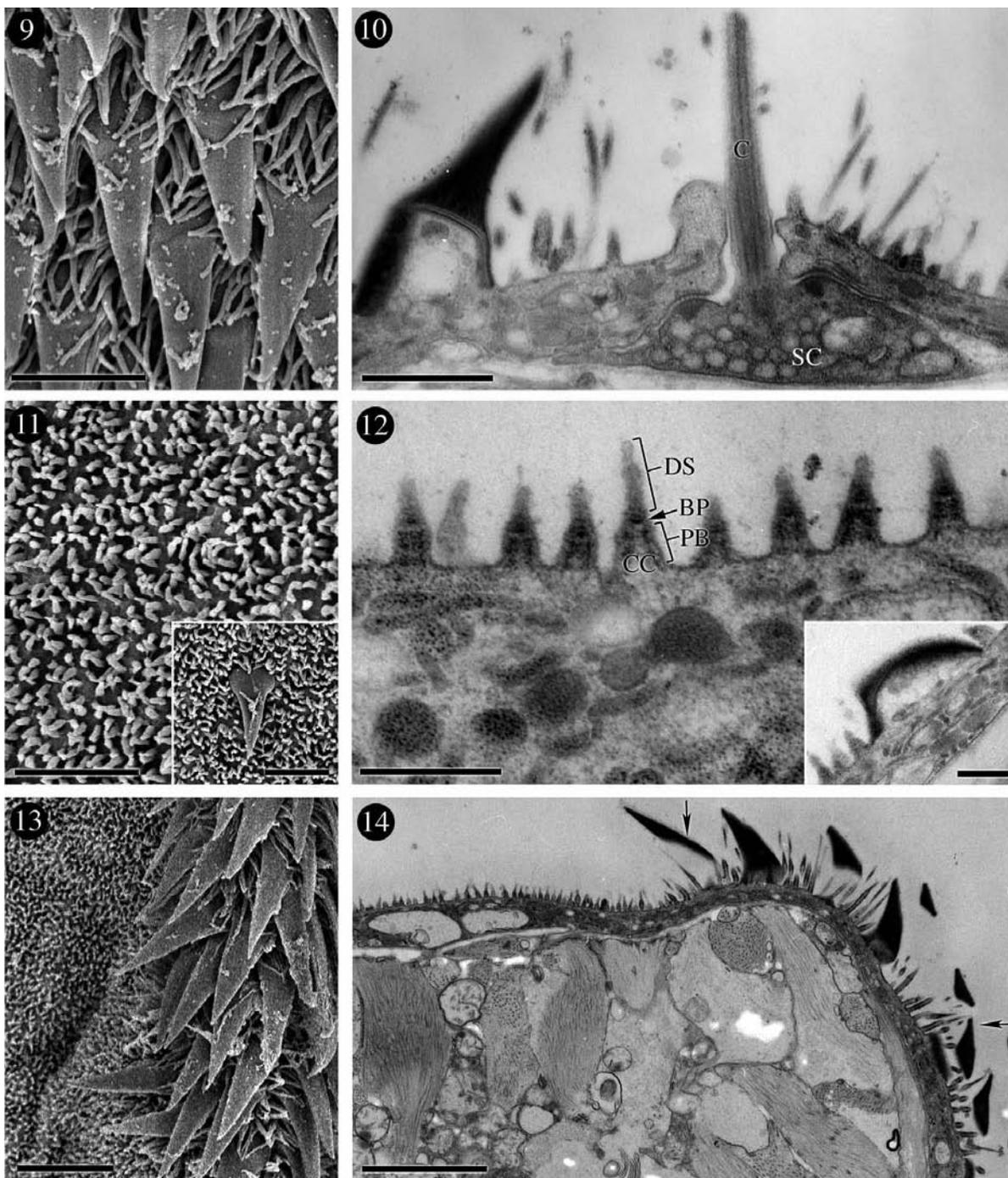
The scolex proper of adults (Figs. 9, 10) possessed medium-length filitriches (TL 465–940; BL 276–384; SL 190–604; BW 84–116) and bladelike spinitriches (TL 934–1,479; BL 416–600; SL 342–1,003; BW 625–894). The distal surfaces of the bothridia of adults (Figs. 11, 12) exhibited small structures (TL



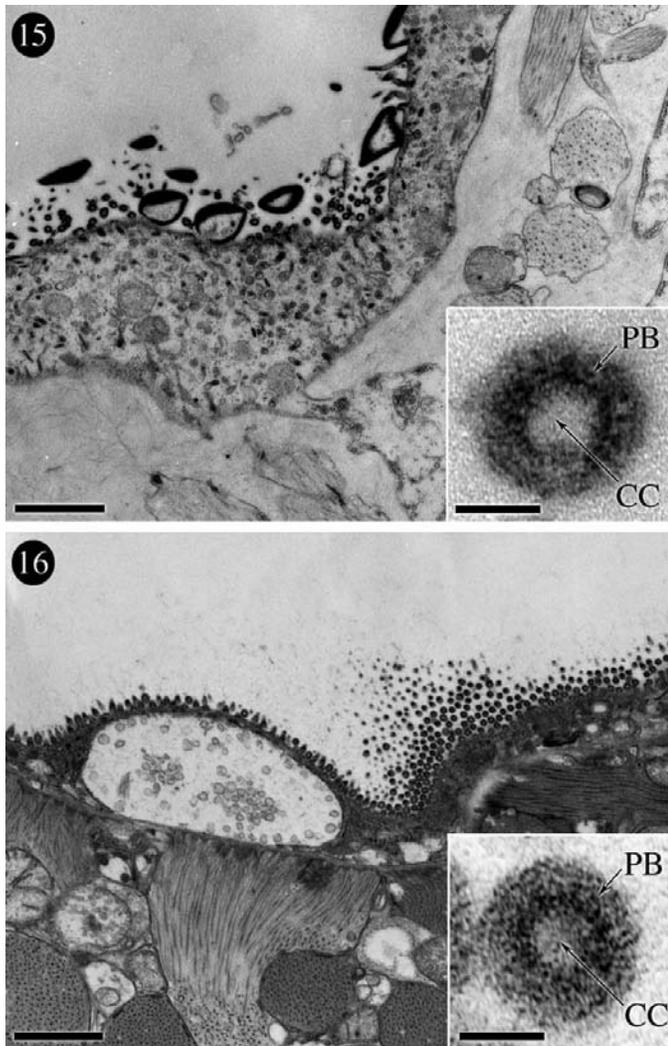
FIGURES 3–8. Plerocercoid of *Calliobothrium* cf. *verticillatum*. (3) Scanning electron micrograph of the scolex proper of the bothridium showing medium-length filitriches and bladelike spinitriches. Bar = 1.0 μm . (4) Transmission electron micrograph of the scolex proper of the bothridium showing medium-length filitriches and bladelike spinitriches. Bar = 0.5 μm . (5) Scanning electron micrograph of the distal surface of the bothridium showing long filitriches and bladelike spinitriches. Bar = 1.0 μm . (6) Transmission electron micrograph of the distal surface of the bothridium showing long filitriches and bladelike spinitriches. Bar = 1.0 μm . (7) Scanning electron micrograph of the distal margin showing the interface between distal surface and distal margin. Bar = 2.0 μm . (8) Transmission electron micrograph of the distal margin showing the interface between distal surface and distal margin (arrows mark boundaries of distal margin). Bar = 5.0 μm .

150–258; BL 60–109; SL 82–154; BW 65–109) that TEM revealed consisted of a proximal base with a cytoplasmic core, an electron-dense distal shaft, and a base plate, consistent with microthrix morphology. Three bladelike spinitriches were also found on the distal surfaces, and their presence in transmission

electron micrographs, albeit sparse, was confirmed using SEM (insets Figs. 11, 12). Because of their limited number ($n = 3$), measurements were not taken for spinitriches in this region. The bothridial margins (Figs. 13, 14) exhibited long filitriches (TL 820–1,414; BL 347–532; SL 429–1,066; BW 70–100) that



FIGURES 9–14. Adult of *Calliobothrium* cf. *verticillatum*. (9) Scanning electron micrograph of the scolex proper of the bothridium showing medium-length filitriches and bladelike spinitriches. Bar = 1 μ m. (10) Transmission electron micrograph of the scolex proper of the bothridium showing a bladelike spinitrix, multiple filitriches, and a sensory structure with cilium and sensory cells. Bar = 0.5 μ m. C = cilium; SC = sensory cells. (11) Scanning electron micrographs of the distal surface of the bothridium showing short filitriches. Bar = 1.0 μ m. Inset shows a single bladelike spinitrix from the distal surface. Inset Bar = 1.0 μ m. (12) Transmission electron micrographs of the distal surface of the bothridium showing short filitriches. Bar = 0.25 μ m. BP = base plate; CC = cytoplasmic core; DS = distal shaft; PB = proximal base. Inset shows a single bladelike spinitrix from the distal surface. Inset Bar = 0.25 μ m. (13) Scanning electron micrograph of the distal margin showing the interface between distal surface and distal margin. Bar = 2.0 μ m. (14) Transmission electron micrograph of the distal margin showing the interface between distal surface and distal margin (arrows mark boundaries of the distal margin). Bar = 2.0 μ m.



FIGURES 15–16. Adult of *Calliobothrium cf. verticillatum*. (15) Scolex proper of bothridium showing medium-length filitriches in transverse section. Bar = 1.0 μm . Inset shows a single medium-length filitrix in transverse section. Inset bar = 0.05 μm . CC = cytoplasmic core; PB = proximal base. (16) Distal surface of bothridium showing short filitriches in transverse section. Bar = 1.0 μm . Inset shows a single short filitrix in transverse section. Inset bar = 0.05 μm . CC = cytoplasmic core; PB = proximal base.

were significantly longer than those found anywhere else on the scolex and bladelike spinitriches (TL 1,026–2,619; BL 327–587; SL 570–2,259; BW 533–1,036). Transverse sections through the medium length filitriches on the scolex proper (Fig. 15) and through the small structures on the distal surface (Fig. 16) were indistinguishable.

Filitriches and spinitriches were found to differ significantly in base width, with the filitriches being significantly narrower than the spinitriches (see Table I). The comparisons between filitriches and spinitriches of subsequent life stages confirmed that a number of microthrix components differed significantly from the plerocercoid to the adult stage (Table II). However, the differences observed on the distal surfaces were most dramatic; the filitriches decreased significantly in total length, shaft

TABLE I. Microthrix comparison between filitriches and spinitriches of *C. cf. verticillatum*.*

Character	Filitrix	Spinithrix	<i>p</i> Value
Total length	1,448 \pm 1,304; 125	1,226 \pm 298; 96	0.067
Base length	419 \pm 205; 128	480 \pm 90; 104	0.003
Shaft length	972 \pm 1,134; 122	745 \pm 316; 97	0.037
Base width	98 \pm 20; 134	695 \pm 119; 106	0.000†

* Measurements are given as mean, followed by standard deviation and number of microtriches examined; all measurements are in nanometers.

† $p < 0.001$ is considered statistically significant.

length, base length, and base width, and the spinitriches disappeared almost entirely.

DISCUSSION

The short structures found on the distal bothridial surfaces of adult trilobulate onchobothriid cestodes generally have been interpreted to represent short filitriches (Caira and Keeling, 1996; Nasin et al., 1997; Caira et al., 1999; Caira and Burge, 2001; Caira and Zahner, 2001; Ghoshroy and Caira, 2001; Fyler and Caira, 2006), but this hypothesis has not been tested. In the present study, the ultrastructure of the short elements found on the distal bothridial surfaces of adult *C. cf. verticillatum* was found to be consistent with previously published data on microthrix morphology. These structures possessed all of the components of a microthrix, including a proximal base, a distal shaft, a base plate, and a cytoplasmic core (Fig. 12). In fact, transverse sections through the base of these structures were indistinguishable from transverse sections through the base of medium-length filitriches on the scolex proper (Figs. 15, 16).

Previous studies comparing scolex ultrastructure between the plerocercoid and adult forms of cestodes are limited. Andersen (1974) showed remarkable similarity in the microtriches of the plerocercoid and adult scolices of *Diphyllbothrium dendritum* (Nitzsch, 1824), *D. latum* (L. 1758), and *D. ditremum* (Creplin, 1825). Likewise, MacKinnon et al. (1985) reported similarity in the plerocercoid and adult scolices of *Haplobothrium globuliforme* Cooper, 1914. Coggins (1980) found that larval microtriches of *Proteocephalus ambloplitis* Leidy, 1887 were different from those found in the adult, but it is unclear in his study which sites were being compared between plerocercoid and adult forms. In the present study, the microthrix pattern of the life cycle stage immediately preceding the adult, i.e., the plerocercoid, differed in a number of respects from that seen in the adult; the most dramatic differences were seen on the distal bothridial surfaces. Whereas the plerocercoid exhibited long filitriches and bladelike spinitriches on the distal surfaces, the adult had very short filitriches and a small number of bladelike spinitriches. The infrequent occurrence of spinitriches on the distal surfaces of trilobulate onchobothriid cestodes as adults has been documented before (Nasin et al., 1997; Caira and Zahner, 2001) and is interpreted here as remnants of the previous life cycle stage.

The present study supports the previous hypothesis (Hess and Guggenheim, 1977; Voge et al., 1979; Thompson et al., 1982; Novak and Dowsett, 1983) that microtriches in general can be divided into 2 shape categories, i.e., filitriches and spinitriches, based on the terminology of Faliex et al. (2000), and suggests

TABLE II. Microthrix comparison between plerocercoid and adult forms of *C. cf. verticillatum*.*

	Character	Plerocercoid	Adult	<i>p</i> Value
Proximal surface				
Filitriches				
	Total length	982 ± 171; 16	641 ± 109; 22	0.000†
	Base length	723 ± 105; 18	330 ± 31; 22	0.000†
	Shaft length	235 ± 112; 18	313 ± 104; 20	0.033
	Base width	117 ± 15; 19	96 ± 9; 24	0.000†
Spiniriches				
	Total length	1,373 ± 192; 30	1,083 ± 164; 15	0.000†
	Base length	510 ± 95; 30	526 ± 56; 15	0.465
	Shaft length	869 ± 228; 30	561 ± 199; 15	0.000†
	Base width	716 ± 106; 29	753 ± 83; 17	0.189
Distal surface				
Filitriches				
	Total length	2,510 ± 612; 19	190 ± 33; 22	0.000†
	Base length	546 ± 69; 22	76 ± 14; 22	0.000†
	Shaft length	2,006 ± 594; 18	114 ± 23; 22	0.000†
	Base width	126 ± 18; 21	84 ± 14; 22	0.000†
Spiniriches				
	Total length	1,023 ± 294; 18	NA‡	NA
	Base length	414 ± 68; 21	NA	NA
	Shaft length	597 ± 271; 18	NA	NA
	Base width	600 ± 88; 22	NA	NA
Distal margin				
Filitriches				
	Total length	3,790 ± 1,254; 18	1,112 ± 165; 28	0.000†
	Base length	484 ± 85; 17	425 ± 54; 27	0.017
	Shaft length	3,135 ± 1,078; 16	690 ± 177; 28	0.000†
	Base width	93 ± 9; 19	81 ± 7; 29	0.000†
Spiniriches				
	Total length	1,130 ± 188; 15	1,383 ± 401; 18	0.025
	Base length	517 ± 78; 17	439 ± 81; 21	0.005
	Shaft length	611 ± 193; 15	942 ± 433; 19	0.006
	Base width	667 ± 110; 18	743 ± 138; 20	0.065

* Measurements are given as mean, followed by standard deviation and number of microtriches examined; all measurements are in nanometers.

† *p* < 0.001 is considered statistically significant.

‡ NA = measurements are not indicated because of insufficient sample size.

that the smaller form is readily distinguished from the larger form in its significantly narrower base width, i.e., 65–167 nm versus 466–1,936 nm, respectively. Several authors have postulated that the 2 distinct microthrix forms have 2 distinct functions, i.e., the robust and rigid spiniriches being used for abrasion and attachment and the elongate, slender, and flexible filitriches to increase surface area and facilitate absorption (Hess and Guggenheim, 1977; Novak and Dowsett, 1983). Consistent with the previous hypotheses, the bladelike spiniriches seen here seem appropriately configured for attachment, and their posteriorly pointing tips add additional support for their use in maintaining the worm's position in the intestine. Many filitriches, however, are equipped with long electron-dense shafts (Figs. 2, 6, 8, 14), which seem poorly configured for the previously proposed function of increasing surface area to facilitate absorption. The present study supports the multifunctional hypotheses of Rothman (1963) and Hayunga (1991), which suggest that the electron-dense distal shaft of filitriches is used in attachment, while the electron-lucent base is used in nutrient absorption. Assuming an attachment function for the filitrix

shaft, it is apparent from these results that the plerocercoid scolex of *C. cf. verticillatum* has a greater attachment potential than that of the adult scolex of *C. cf. verticillatum* since all significant changes in filitrix shaft length resulted in a decrease from plerocercoid to adult stages (Table II). It is likely that the adult, which, with the development of its strobila, is conspicuously longer than the plerocercoid, is no longer reliant solely on the scolex for attachment purposes. The surface of the strobila of adult cestodes (including *C. cf. verticillatum*) is typically covered with densely arranged elongate posteriorly directed microtriches (e.g., Andersen, 1974; Thompson et al., 1982; Cairns et al., 1999), adding additional support for their use in maintaining position.

The phylogenetic relationships between onchobothriid cestodes are poorly known. Robust molecular phylogenies for the onchobothriids and their relatives do not exist. Morphological phylogenies suggest the monophyly of the Onchobothriidae, but the evolutionary relationships among the onchobothriid genera are not well understood. In the most complete phylogenetic analysis to date (based on 157 morphology characters in 32

onchobothriid taxa), a trilobulate clade was found nested within the unilobulate and bilobulate groups (Caira et al., 2001), yet the presence of trilobulate bothridia remains the only robust synapomorphy uniting these taxa. The present study, along with the previous morphological analyses (Caira et al., 1999, 2001), reveals an interesting trend in the microtrich patterns of the distal bothridial surfaces of adult onchobothriid cestodes. Unilobulate genera such as *Balanobothrium* Hornell, 1911, *Pachybothrium* Baer and Euzet, 1962, *Pedibothrium* Linton, 1908, and *Potamotrygonocetus* Brooks and Thorson, 1976, have been found with bladelike spinitriches and long filitriches on their distal bothridial surfaces. In contrast, species of trilobulate genera, i.e., *Acanthobothrium*, *Acanthobothroides* Brooks, 1977, *Calliobothrium*, *Dicranobothrium* Euzet, 1953, and *Platybothrium* Linton, 1890, always exhibit short filitriches and occasionally a few bladelike spinitriches on their distal surfaces as adults. The bilobulate genera have a less consistent pattern, with different genera displaying various microtrich patterns, i.e., *Phoreiobothrium* Linton, 1889, with short filitriches and sparse bladelike spinitriches and *Biloculuncus* Nasin, Caira and Euzet, 1997, with spinitriches and long filitriches. It is worth noting that the plerocercoid of *C. cf. verticillatum* was found to have a scolex condition similar to that seen in the adults of unilobulate genera, i.e., spinitriches and long filitriches on the distal bothridial surfaces. It is possible that the change in scolex microtrich composition between the plerocercoid and adult forms represents a developmental synapomorphy uniting the trilobulate cestodes. Future work examining the plerocercoid stage of other onchobothriid cestodes and a robust phylogeny of the Onchobothriidae is necessary to address this hypothesis more fully. Microtriches of cestodes have been previously hypothesized to be of systematic utility (MacKinnon and Burt, 1983; Richmond and Caira, 1991; Beveridge and Jones, 2000), and the present study supports this hypothesis; microtriches have the potential to be important taxonomic and phylogenetic markers and should continue to be explored in this context.

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