Revisiting *Acanthocephalus dirus* (Van Cleave, 1931) (Acanthocephala: Echinorhynchidae) Using SEM and Energy Dispersive X-Ray Analysis (EDXA) for the First Time

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Abstract

Acanthocephalus dirus (Van Cleave, 1931) Van Cleave and Townsend, 1936 is the most widely distributed and studied species of acanthocephalans in North America. *Acanthocephalus jacksoni* Bullock, 1962 from New England and *Acanthocephalus parksidei* Amin, 1975 from Wisconsin are junior synonyms. Line drawings were used in these and all subsequent descriptive accounts since. We are reporting SEM images of *A. dirus* for the first time from 2 Wisconsin populations from *Catostomus commersoni* Lacépède showing new details that could not have been seen only with optical microscopy. The new SEM observations include new features that were not previously reported such as apical sensory pores, micropores variably distributed on the trunk and on the proboscis and hooks, hooks of variable size, and bursa with 2 circles of sensory papillae and 2 pedunculated suckers. We are also providing a detailed elemental analysis of middle hooks using Energy Dispersive x-ray analysis (EDXA) showing high levels of sulfur in all samples especially hook tips.

Keywords: Acanthocephala; *Acanthocephalus dirus* Revisited; Wisconsin population; First SEM & EDXA Analysis

Introduction

Van Cleave [1] first described *Acanthocephalus dirus* as *Echinorhynchus dirus* from the freshwater drum *Aplodinotus grunniens* Rafinesque in the Yazoo River, Mississippi before Van Cleave and Townsend [2] reassigned it to the genus *Acanthocephalus* Koelreuter, 1771 after validating the posterior position of the cerebral ganglion and re-evaluating the length of the lemnisci. Van Cleave and Townsend [2] reported the wider distribution of *A. dirus* in the Ohio and Illinois rivers and tributaries from other species of fish, and noted that this is the first occurrence of a species of the genus *Acanthocephalus* in North America. Amin [3] accounted for the distribution of *A. dirus* in the Mississippi River drainage system, or waters previously connected to it in the Mobile Bay drainage, Great Lakes, and in New

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England. *Acanthocephalus dirus* has the "widest host (65 fish species in 16 families) and geographical distribution (in 10 states) and exhibits the greatest morphological variability compared to the 2 other species of *Acanthocephalus* in North America, *A. tahlequahensis* Oetinger and Buckner, 1976, and *A. alabamensis* Amin and Williams, 1983 (see Amin [3], p. 210). About the same time, Amin [4] provided an expanded diagnosis of *A. dirus* within which he assigned *Acanthocephalus jacksoni* Bullock, 1962 from New England and *Acanthocephalus parksidei* Amin, 1975 from Wisconsin and Lake Michigan as junior synonyms based on the use of (1) comparative morphometrics of populations from Mississippi (2 collections), Kentucky (2 collections), Illinois (3 collections), West Virginia, Wisconsin, and Ohio, (2) stratified histograms, (3) canonical discriminant function analysis, and (4) cluster analysis. Within the Mississippi River populations, Amin [4] identified clinal variations showing that the more northern populations had the largest hooks and fewer hooks per row compared to the more southern Mississippi populations.

Our studies of *A. dirus* began with investigating its Wisconsin population from various fishes in waters of southeastern Wisconsin tributaries of the Mississippi River and southwestern Lake Michigan. The Wisconsin population is most intensively studied morphologically, ecologically, biologically, developmentally, and from an evolutionary and cladistic standpoint, compared to other populations and species of the genus *Acanthocephalus* in North America. The following is a chronological review of research on the Wisconsin population of *A. dirus* since its initial description as *A. parksidei* from 11 species of fish in the Pike River where Amin [5] (Table 1, p. 304) distinguished between it and *A. jacksoni* Bullock [6] from New England and Van Cleave's [1] Mississippi population using distinct variations in trunk dimensions and proboscis armature; line drawings of 17 figures were included. In the same year, Amin [7] described the variability in size and shape of trunk, proboscis, receptacle, lemnisci, eggs, number, arrangement and size of hooks, and reproductive structures in males and females, as well as abnormalities in hooks and body wall, and monorchidism. "Most of these variations were related to worm sex and age as well as to host species" (Amin [7], p. 307). Ecological associations in 11 species of fish were found to be related to host species, age and sex, concurrent infections, collection site, and season (Amin [8] p. 318) who also found that parasite distribution in host intestine to be related to intensity of infection, posterior migration and concurrent infection. An additional collection of helminths from 26 species of fish in 10 families from 2 streams in SE Wisconsin included moderate to heavy infections of *A. dirus* in 11 fish species (Amin [9], p. 226). A study of 8 species of helminth parasites of 18 species of SW Lake Michigan fishes revealed the presence of a viable population of *A. dirus* there, mostly in the white sucker *Catostomus commersoni* (Lacépède) (Catostomidae) (the type host of *A. parksidei*) and slimy sculpin *Cottus cognatus* Richardson (Cottidae) (Amin [10]); mixed infections with *Echinorhynchus salmonis* Müller, 1784 were common. Cystacanth infections and developmental cycle appeared to be associated with those of the isopod intermediate host *Caecidotea militaris* (Hay) (Crustacea) in a SE Wisconsin stream with 1 breeding season observed suggesting that *A. dirus* has a well-defined life cycle of 1 year in both intermediate and definitive hosts (Amin et al. [9]). Amin et al. [11] noted that cystacanths were over-dispersed fitting a negative binomial or truncated negative binomial distribution. Amin [4] provided an expanded description of *A. dirus* including the full spectrum of variabilities in all populations in North America encompassing 775 worms in 10 collections using various computer-based analyses justifying the designation of *A. parksidei* and *A. jacksoni* as junior synonyms, with the Mississippi River population being the most ancient and diversified. Amin, et al. [12] demonstrated interspecific variability in the genus *Acanthocephalus* using stratified histograms contrasting means of 20 variables and included a key to the species. In his discussion of the hosts and geographical distribution of *Acanthocephalus* in North America, Amin [3] Table 2 further differentiated between the 3 known species of *Acanthocephalus*, elaborated on the origin, dispersal, and diversification of *A. dirus*, and declared that "the discovery of species of *Acanthocephalus* in new hosts from the Mississippi River drainage system which might have evolved through host isolation is not unlikely" (p. 218). "Cladistic analysis provided greater understanding and further support of the evolutionary relationships among the species and populations of the genus *Acanthocephalus* from North American freshwater fishes" Amin [13]. A survey with line drawings of abnormalities in some helminth parasites of fish including body form, body wall, and proboscis anomalies in *A. dirus* from Wisconsin was reported by Amin [14]. The seasonal distribution of *A. dirus* from 9 species of fish in 5 families in Tichigan Lake exhibited considerably higher parameters in 1984 than in 1977-1978 leaving the question open as to whether *A. dirus* "undergoes annual cycles in fecundity and abundance" [15].

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Element*	Whole hook	Site of sample			Longitudinal sections		
		Hook tip	Hook middle	Hook base	Hook tip	Hook middle	Hook base
Magnesium (Mg)	0.48	0.00-0.23	0.14-0.19	0.13-0.15	0.08	0.09	0.06
Sodium (Na)	0.76	0.00-0.01	0.00-0.13	0.09-0.13	0.03	0.00	0.00
Phosphorous (P)	0.46	0.45-1.15	0.86-3.94	0.60-1.31	0.38	0.43	0.42
Sulfur (S)	8.1	9.72-23.15	9.09-11.35	6.28-12.25	19.18	11.59	9.00
Calcium (Ca)	0.7	0.25-0.26	0.14-0.19	0.15-0.38	0.27	0.28	0.25

*Palladium (Pd) and Gold (Au) were used to count the specimens and the Gallium for the cross cut of the hooks. These and other elements (C, O, N) common in organic matter are omitted. Data is reported in weight (WT%).

Table 1: Chemical localization of elements in various parts of middle hooks of females of *Acanthocephalus dirus* from *Catostomus commersoni* collected in the Pike River, SE Wisconsin.

The above review addresses only the Wisconsin population of *A. dirus* which is being investigated herein for SEM-based description and Energy Dispersive x-ray analysis (EDXA). We have studied many species of acanthocephalans using X-ray scans (EDXA) of FIB-sectioned hooks and spines for metal composition. The biological significance of EDXA as a diagnostic tool is exemplified by the observation that populations of an acanthocephalan species will consistently have similar EDXA spectra irrespective of host species or geography [16]. Results of the X-ray analysis of the FIB-sectioned hooks (dual beam SEM) of *A. dirus* show differential composition and distribution of metals in different hook parts characteristic of the species being examined.

Ten surveys and reports from 7 states along the Mississippi River drainage system providing host and locality data and some descriptive information are included in Amin [3,4]. Kennedy [17] reviewed the evolutionary ecology of *A. dirus* based on some relevant publications by Amin and Amin et al., quoted above. Our most recent work on *A. dirus* from the Wisconsin material by Amin, et al. [18] is a cross sectional study of males and females showing many internal anatomical details not previously reported.

Materials and Methods

Collections of the Wisconsin Population of *A. dirus* and Locations

A total of 4159 ethanol-fixed and preserved specimens of *A. dirus* were originally collected from 737 examined fishes of 11 species and 6 families seined from 7 sites on the Pike River in Racine and Kenosha counties, Wisconsin (42°38′40″N,87°51′09″W) in 1972, 1973 [5]. A total of 1583 additional specimens were collected from 117 infected of 845 examined fish of 16 species and 7 families in Tichigan Lake, a relatively large eutrophic impoundment on the Fox River which is a tributary of the Mississippi River in Racine County (42°48′20″N,88°13′00″W) in 1977, 1978, and 1984 [15]). A total of 224 acanthocephalans were collected from 12 species of fish gill-netted or trawled in the coastal waters of Lake Michigan off Kenosha County at the Wisconsin-Illinois state line (42°34′33.59″N, 87°50′26.39″W) in 1980-1981 (Amin [10]). In a separate Mississippi River collection, White, et al. [19] provided specimens of *A. dirus* from their 1005 specimens reported as *A. jacksoni* collected in 1973 from *Catostomus commersoni* from tributaries of the Kentucky and Ohio rivers (Madison and Fayette counties) (37°43′12.00″N,84°16′48.00″W) in Kentucky Amin [4]. Our specimens from the Pike River and Tichigan Lake have direct continuity with those from the Mississippi River which connects with Lake Michigan via the Illinois River.

A total of 60 specimens of *A. dirus* from 2 collections from white suckers, *C. commersoni*: 30 specimens (15 males, 15 females) in each collection from the Tichigan Lake (coll. M-98a in 1984), and from the Pike River (coll. M-11, 1985) were used for SEM and EDXA studies. All males had everted bursae.

Scanning Electron Microscopy (SEM)

Specimens that had been fixed and stored in 70% ethanol were processed for SEM following standard methods Lee [20]. These included critical point drying (CPD) (Tousimis Automandri 931.GL; Rockville, Maryland) and mounting on aluminum SEM sample mounts (stubs) using conductive double-sided carbon tape. Samples were

sputter coated with an 80%-20% gold-palladium target for 3 minutes using a sputter coater (Quorum (Q150T ES) www.quorumtech.com) equipped with a planetary stage, depositing an approximate thickness of 20 nm. Samples were placed and observed in an FEI Helios Dual Beam Nanolab 600 (FEI, Hillsboro, Oregon) Scanning Electron Microscope (FEI, Hillsboro, Oregon). Samples were imaged using an accelerating voltage of 5 kV, and a probe current of 86 pA, at high vacuum using a SE detector.

Focused Ion Beam (FIB) Sectioning of Hooks

A dual-beam SEM with gallium (Ga) ion source (GIS) is used for the LIMS (Liquid Ion Metal Source) part of the process. The gallium beam (LIMS) is a gas injection magnetron sputtering technique whereby the rate of cutting can be regulated. Hooks were sectioned at two positions (Tip and Middle) using the FEI Helios Dual Beam Nanolab mentioned above. The dual-beam FIB/SEM is equipped with a gallium (Ga) Liquid Ion Metal Source (LIMS). The hooks of the acanthocephalans were centered on the SEM stage and cross-sectioned using an ion accelerating voltage of 30 kV and a probe current of 2.7 nA. Following the initial cut. The time of cutting is based on the nature and sensitivity of the tissue. The sample also goes through a cleaning cross-section milling process to obtain a smoother surface. Cuts were analyzed with an X-ray normally at the tip, middle, and base of hooks for chemical ions with an electron beam (Tungsten) to obtain an X-ray spectrum. The intensity of the GIS was variable according to the nature of the material being cut. Results were stored with the attached imaging software then transferred to a USB for future use.

Energy Dispersive X-Ray Analysis (EDXA)

The Helios Nanolab 600 is equipped with an EDXA (Mahwah, NJ) TEAM Pegasus system with an Octane Plus detector. The sectioned cuts were analyzed by EDXA. Spectra of selected areas were collected from whole specimens and from the center and the edge of each cross-section. EDXA spectra were collected using an accelerating voltage of 15 kV, and a probe current of 1.4 nA. Data collected included images of the displayed spectra as well as the raw collected data. Relative elemental percentages were generated by the TEAM software.

Results and Discussion

Amin [5] described and measured 100 males (from *C. commersoni* and *Semotilus atromaculatus* Mitchill) and 130 females (from 5 host species) of the Wisconsin population of *A. dirus*, originally named *A. parksidei*, during November and May from the Pike River, Kenosha County. A detailed description was provided with 7-line drawings of 2 males, male and female reproductive systems, an egg, and 2 proboscides. Six other line drawings comparing the body form of *A. parksidei* with *A. jacksoni* were also included. The following is an SEM description of specimens of *A. dirus* from this collection in Wisconsin revisiting external features first seen with SEM that were not possible to observe with light microscopy that was used for the original line drawings. Detailed anatomical description of internal structures and measurements reported by Amin [5] are not repeated herein.

New Morphological SEM Description of *A. dirus* from Wisconsin

Specimens cylindrical, small to medium in length, wider near middle, and tapering towards both ends. Males (Fig. 1) smaller than females. Proboscis nearly cylindrical, usually directed ventrad, with 10-16 (10-14 in males) rows of 8-11 hooks each (Figs. 2, 3). Proboscis with well-defined apical sensory pore (Fig. 4) and micropores (Fig. 10). Middle hooks most robust (Fig. 6) gradually becoming more slender and smaller anteriorly (Fig. 5) and posteriorly (Fig. 7). Apical and basal hooks smallest. All hooks with thin cortical layer and thick core (Fig. 8) and micropores (Fig. 9) comparable to those of the proboscis. Anterior trunk integument with micropores (Fig. 17) that vary from those in the middle and posterior trunk (Fig. 18) as well as from those on the proboscis and hooks in diameter and distribution. Bursa normally tilting ventrad (Fig. 11), with large spheroid base and the multi-layered corrugated distal end housing the partially retracted elevated inner wall with sensory papillae (Figs. 12-13, arrows). Extended inner bursal wall shows 2 clusters of sensory papillae (Fig. 14, arrows) and source attachment of suckers to ventral aspect of bursa. Two adjacent inflatable suckers (Fig. 15) pedunculated at ventral aspect of bursa characterize *A. dirus* males. High magnification of 1 sucker (Fig. 16) demonstrating 1 sensory papilla (Fig. 16, arrow). Female gonopore terminal with plump vulva (Fig. 19) with prominent orifice. Eggs elongate with dense network of fibrils (Fig. 20).
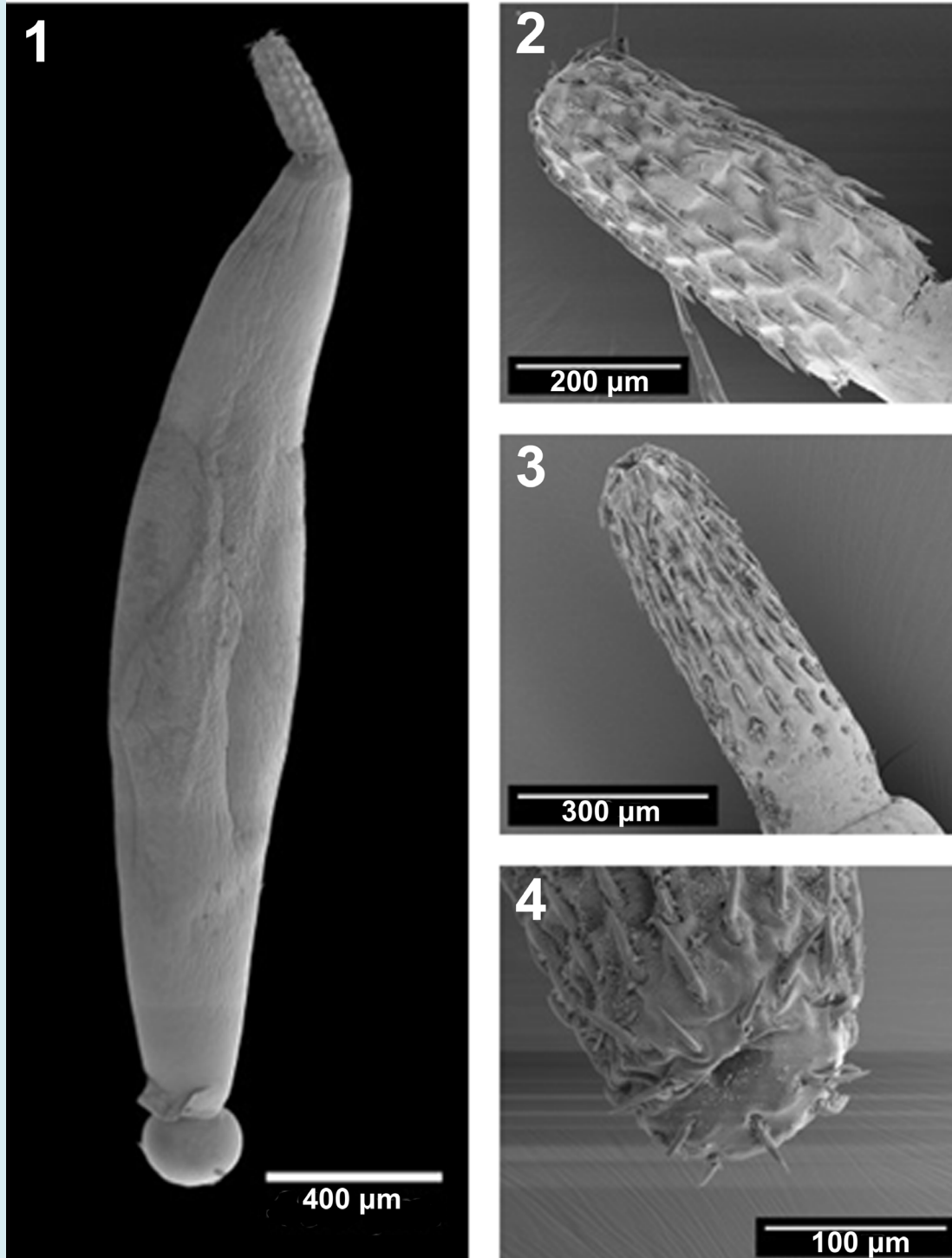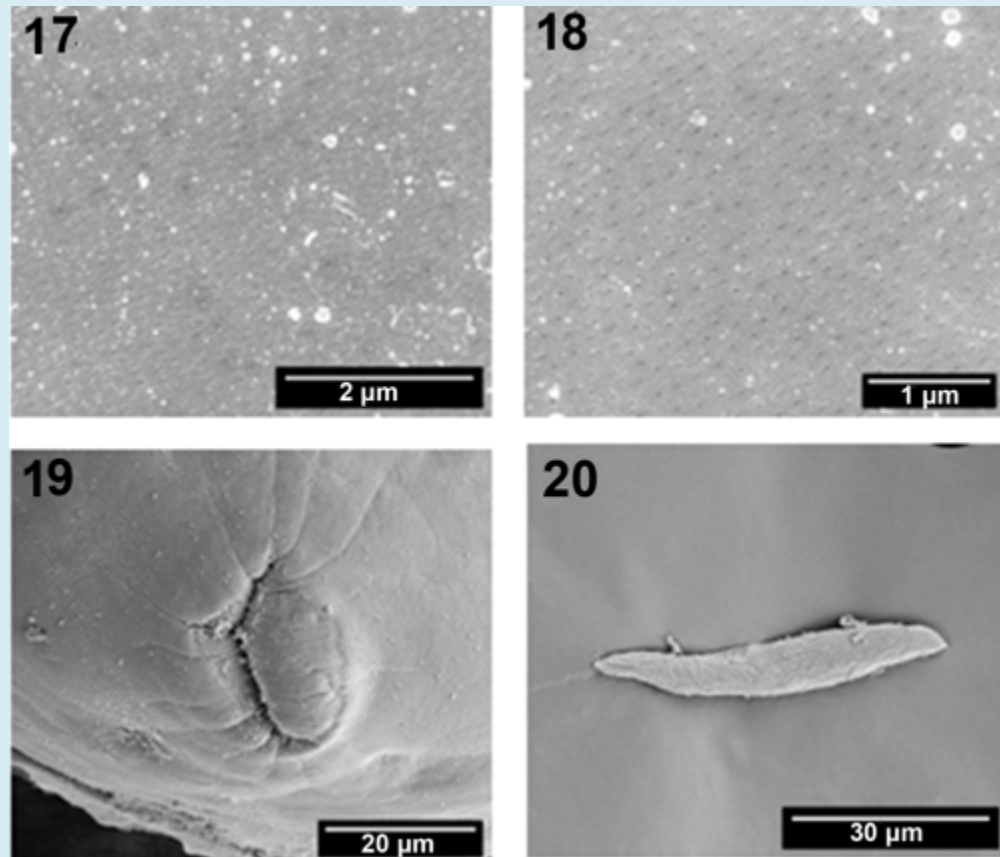
Figures 1-4: SEM of specimens of *Acanthocephalus dirus* from the Wisconsin population originally obtained from *Catostomus commersoni* in the Pike River. 1. A whole male. Note the overall shape and the size of proboscis and bursa compared to the trunk. 2. A proboscis with 8 hooks per row. 3. Another proboscis with 10 hooks per row. The number of hooks per row in this population varied between 8 and 11 (usually 9 or 10). 4. The anterior end of a proboscis showing the recessed apical sensory pore.
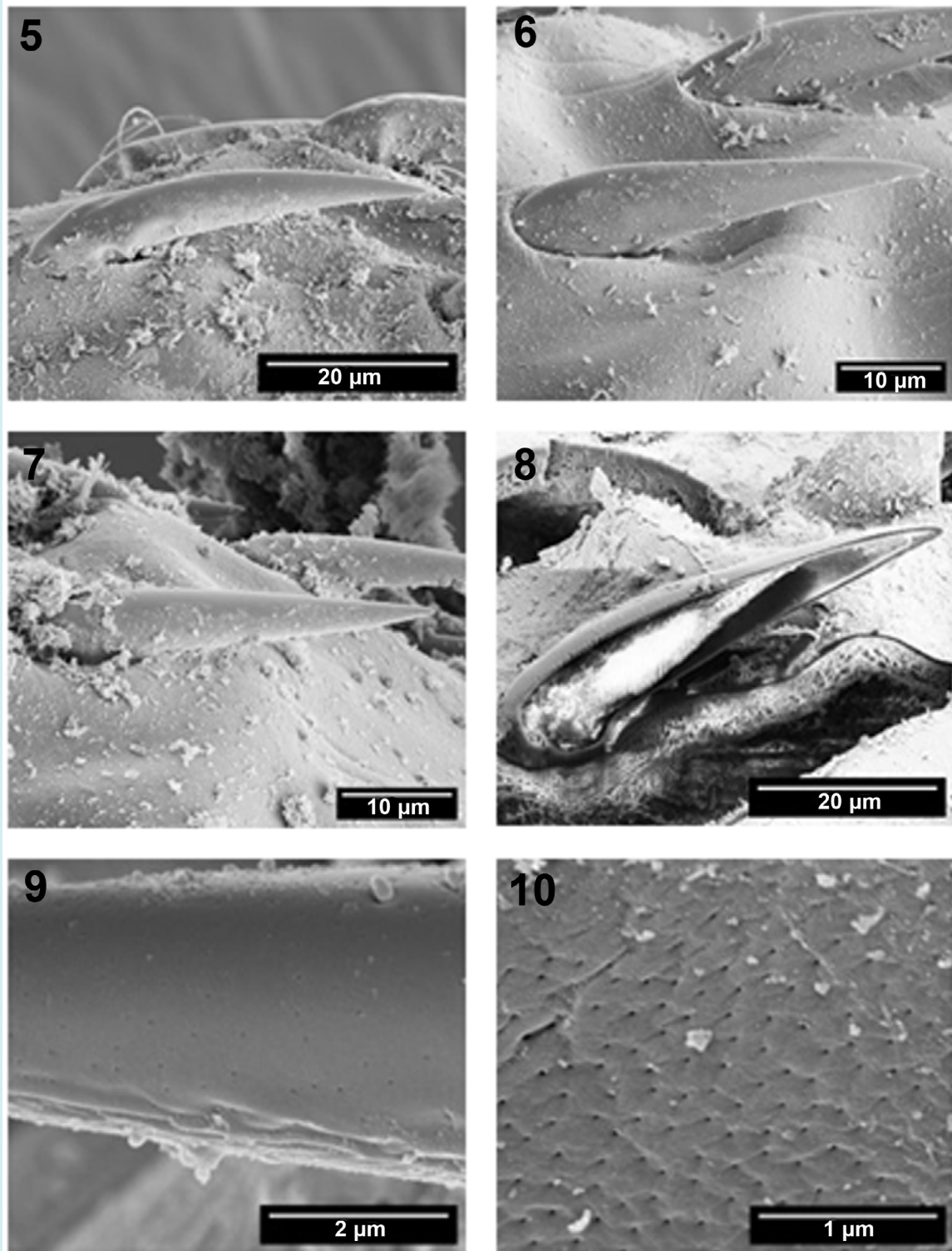
Figures 5-10: SEM of specimens of *Acanthocephalus dirus* from the Wisconsin population originally obtained from *Catostomus commersoni* in the Pike River. The proboscis. 5-7. Detail of sub-apical, middle, and pre-basal hooks, respectively, showing differences in size and thickness. Middle hooks appeared more robust. 8. A Gallium-cut section of a middle hook with a thin cortical layer and thick separated core. The simple elongate root is masked by the thick tegumental layer. 9. A highly magnified hook showing micropores continuous with micropores on the proboscis proper. 10. Micropores on the surface of the proboscis.
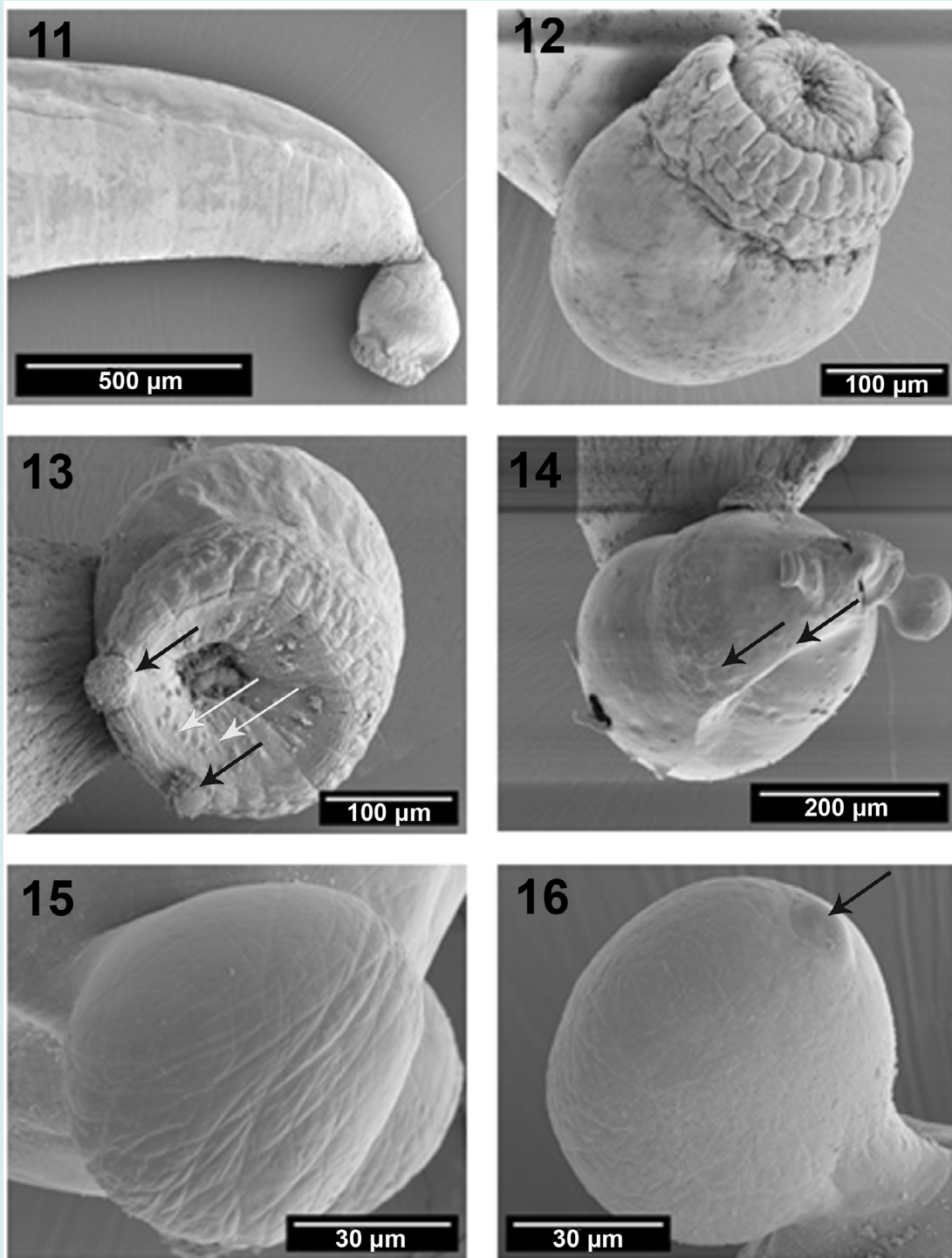
Figures 11-16: SEM of specimens of *Acanthocephalus dirus* from the Wisconsin population originally obtained from *Catostomus commersoni* in the Pike River. The bursa. 11. The terminal bursa in its usual ventrally oriented position. 12. A closer perspective of a bursa with the large spherical base and the multi-layered corrugated distal end housing the partially retracted elevated inner wall with sensory papillae. 13. A terminal perspective of another bursa showing the inner sensory papillae (white arrows) and the two suckers (black arrows). 14. A lateral perspective of a bursa with totally evaginated inner wall showing the distribution of the two zones of sensory papillae (arrows) and the two pedunculated suckers; one is broken. 15. Lateral view of the two suckers. 16. An inflated pedunculated sucker and its sensory papilla from the bursa proper (arrow).

Figures 17-20: SEM of specimens of *Acanthocephalus dirus* from the Wisconsin population originally obtained from *Catostomus commersoni* in the Pike River. 17, 18. The differential distribution of micropores in the Anterior vs. the posterior integument, respectively. 19. The shape of the female genital opening with the vulva extended. 20. A ripe egg. Note the subcutaneous filaments not fully emerged.

Micropores

Micropores normally found in the various trunk regions in variable distributions and diameter, were also unusually detectable on the hooks and proboscis (Figs. 9, 10).

Energy Dispersive X-Ray Analysis (EDXA)

The spectra of EDXA summarized in Table 1 indicate that sulfur was the only dominant element in all sample sites of all hook parts and in longitudinal sections from the same parts reaching 23.15 WT% in hook tips. Calcium was negligible from all sites and the WT% of phosphorous was very low reaching a maximum of 3.94 from the middle of 1 hook of 1 specimen (Table 1).

Morphology

This is the first morphological account of *A. dirus* from Wisconsin, or from anywhere, using SEM. The first description by Amin [5] was quite detailed but the light microscopy account and the resulting line drawings were limited and missed many features observable only by SEM. We have demonstrated the presence of an apical sensory pore on the proboscis of *A. dirus* for the first time. Our Wisconsin specimens had proboscides with 8-11 hooks per row (Figs. 2, 3) which appears to be at the middle of a clinal variation between the northern New England populations of *A. jacksoni* with 7-10 hooks per row and the southern Mississippi population of *A dirus* with 11-13 hooks per row. Bullock (1962, line drawings Figs. 12-18) reported intraspecific variability in proboscis armature of *A. jacksoni* as did Amin (1975b, line drawings Figs. 1-8) [7] in *A. parksidei*. Intraspecific variability by age, sex, host, and geographical location has been documented in Amin [7]. The layers of hooks and the presence of micropores not only on the hooks and the proboscis but also on the different regions of the trunk at variable densities and diameters indicate that practically all the integument surface participate in the process of nutrient absorption; see below. The morphology of the bursa is delineated for the first time showing the presence of 2 clusters of sensory papillae and

the true appearance of the characteristic 2 suckers with their own sensory papillae. Only Amin, et al. [21] and Doyle and Gleason [22] previously showed microscopic images of *A. dirus* suckers, compressed under cover slips, appearing as concentric double walls first reported by Doyle and Gleason [22] from the northern hogsucker *Hypentelium nigricans* Lesueur in Gasper River, Kentucky. The morphology of the female genital orifice is seen for the first time when extended. The fibrillar matrix of the ellipsoid eggs is evident through the thin outer membrane (Fig. 20). Bullock [6] and Oetinger and Nickol [23] recognized the fibrillar coat on the inner surface of the outer egg membrane of *A. jacksoni* in egg suspensions. The latter authors [23] (p. 1056) related the function of the filaments to "entangling and clumping… of eggs (for) enhancing infection of the intermediate host or they might retard passage through the isopod alimentary canal giving acanthors additional time in which to hatch." Whitfield [24] described the ultrastructure of filaments from other acanthocephalan species.

Micropores

We have reported micropores in a large number of acanthocephalan species [25] and in a few more since, and demonstrated the tunneling from the cuticular surface into the internal crypts by TEM. The micropores of *A. dirus*, like those reported from other species of the Acanthocephala, are associated with internal crypts and vary in diameter and distribution in different trunk regions, proboscis, and hooks, corresponding with differential absorption of nutrients. Amin, et al. [26] gave a summary of the structural-functional relationship of the micropores in various acanthocephalan species. Wright and Lumsden [27] and Byram and Fisher [28] reported that the peripheral canals of the micropores are continuous with canalicular crypts. These crypts appear to "constitute a huge increase in external surface area . . . implicated in nutrient up take." Whitfield [29] estimated a 44-fold increase at a surface density of 15 invaginations per 1 μm^2 of *M. moniliformis* tegumental surface. The micropores and the peripheral canal connections to the canaliculi of the inner layer of the tegument were demonstrated by transmission electron micrographs in *Corynosoma strumosum* (Rudolphi, 1802) Lühe, 1904 from the Caspian seal *Pusa caspica* (Gmelin) in the Caspian Sea (Figs. 19, 20 of Amin, et al. [30]) and in *Neoechinorhynchus personatus* Tkach, Sarabeev, Shvetsova, 2014 from *Mugil cephalus* Linn. in Tunisia (Figs. 26, 29, 30 in Amin, et al. [21]).

Energy Dispersive X-Ray Analysis (EDXA)

Our studies of acanthocephalan worms have usually involved X-ray scans (EDXA) of FIB-sectioned hooks and spines [31-34]. Sulfur is usually seen at the outer edge of large hooks and Calcium and Phosphorus are major ions in the base and middle of hooks where tension and strength are paramount for hook function. The following results of the X-ray analysis of the FIB-sectioned hooks (dual beam SEM) are limited to those from our Wisconsin specimens of *A. dirus*. The tip of middle hooks of *A. dirus* showed the highest level of Sulfur reaching 23.25 WT%. Comparable or higher levels of sulfur were observed in other species of acanthocephalans. For instance, *Cavisoma magnum* (Southwell, 1927) Van Cleave, 1931 from *Mugil cephalus* in the Arabian Sea, has a similar pattern but considerably higher levels of sulfur in hook tips (43.51 wt. %) and edges (27.46 wt. %) (Amin, et al. [35]). Our results are comparable to those of mammalian teeth enamel. The chemical elements present in the hooks are typical for other acanthocephalans [32-33]. X-ray scan analysis provides insight into the hardened components, e.g., calcium, sulfur, and phosphorus, of acanthocephalan hooks. The EDXA appears to be species-specific, as in fingerprints. For example, EDXA is shown to have significant diagnostic value in acanthocephalan systematics. For example, *Moniliformis cryptosaudi* Amin, Heckmann, Sharifdini, Albayati, 2019 from Iraq is morphologically identical to *Moniliformis saudi* Amin, Heckmann, Mohammed, Evans, 2016 from Saudi Arabia, and it was erected based primarily on its distinctly different EDXA pattern (Amin, et al. [36]) as a cryptic species. Our methodology for the detection of the chemical profile of hooks in the Acanthocephala has also been used in other parasitic groups including the Monogenea [37-38] and Cestoda [39].

Biological Significance of EDXA

The taxonomic identity of species is deep-seated at the genetic level which is expressed by the organism's morphology and biochemistry as revealed, in part, by its elemental spectra. Amin, et al. [16,40-42] discussed in detail the biological significance of EDXA as a diagnostic tool exemplified by the observation that populations of an acanthocephalan species will consistently have similar EDXA spectra irrespective of host species or geography. Metal analysis of hooks has become a diagnostic standard since hooks have the highest level of elements compared to the mid- and posterior trunk regions of the acanthocephalan body (Heckmann et al., [33]). Specifically, the Sulfur content in the proboscis is paramount in the composition of disulfide bonds in the thiol groups for cysteine and cystine of the polymerized protein molecules [43]. The formed disulfide bonds are direct by-products of the DNA-based process of protein synthesis which makes up the identity of a biological species. Accordingly, the level of sulfur in our EDXA profiles will indicate the number of sulfur bonds that along with the levels of calcium phosphates, will characterize the elemental identity of a species based on its nuclear DNA personality. Variations in chemical compositions probably indicate differences in allele expression. The DNA-generated sulphide

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bonds evident in our EDXA profiles have an important role in the stability and rigid nature of the protein accounting for the high sulfur content of the proboscis. The above processes explain the observed species-specific nature of EDXA profiles noted in our many findings.

Compliance with Ethical Standards

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