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EPIMERITE–HOST EPITHELIUM RELATIONSHIPS AMONG EUGREGARINES PARASITIZING THE DAMSELFLIES *ENALLAGMA CIVILE* AND *ISCHNURA VERTICALIS*

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ABSTRACT: The host–parasite interface between 2 species of damselflies and 4 species of eugregarines was examined at the ultrastructural level. *Nubenocephalus nebraskensis* organisms attached to the host midgut epithelium by means of a sucker-like protomerite; the space between the epicytic folds and host epithelium was filled with electron-dense material interpreted to be adhesive in nature. *Actinocephalus carrilynnae* organisms attached by means of the epimerite, which had no epicytic folds, and by the fluted stalk with characteristic epicytic folds; host cell and parasite membranes appeared fused at some places on the epimerite. *Hoplorhynchus acanthatholius* organisms attached by means of an ovoid epimerite with backward-pointing digitations; the entire epimerite was embedded in a host cell, and host cell microvilli surrounded the stalk. *Steganorhynchus dunwoodyi* organisms attached by means of an ovoid stalk papilla enclosed in a retractable globular sheath; the papilla was covered with epicytic folds, but the sheath was not, and the sheath had a single membrane, whereas the epicytic folds had 2 or 3 membranes. The entire apparatus was inserted between epithelial cells, and the sheath was highly folded at its surface. The ultrastructural observations suggest that actinocephalid gregarines have evolved 2 general strategies for attaching to the host epithelium, that is, suckerlike protomerites, as in the case of *N. nebraskensis,* and deeply embedded epimerites inserted within or between host cells, as in the other species studied.

Actinocephalid gregarines (Apicomplexa: Eugregarinorida: Actinocephalidae) are relatively large protistan parasites, typically possessing elaborate epimerites. The actinocephalid fauna of Odonata (dragonflies and damselflies) is especially diverse, with parasite species differing in the structure of their epimerites, as well as in a number of other characters such as oocyst morphology (Ellis, 1914; Devdhar and Deshpande, 1971; Narasimhamhurti and Ahamed, 1980; Sarkar and Haldar, 1980; Sarkar and Mazumber, 1983; Kori and Amoji, 1986; Richardson and Janovy, 1990; Clopton et al., 1993; Clopton, 1995; Percival et al., 1995). The diversity of epimerite structures makes odonate—gregarine systems useful for studies concerning host–parasite relationships, especially those involving tissue-level interfaces. This structural diversity, manifested in holdfasts, also presents interesting clues as the nature of gregarine adaptations, convergence and homology, and subcellular differentiation.

In the present study, 2 species of damselflies (Odonata: Zygoptera: Coenagrionidae)—*Enallagma civile* Hagen, 1861, and *Ischnura verticalis* Charpentier, 1840—each possessing a 4-species gregarine assemblage, were used to describe and interpret the ultrastructural details of the host–parasite interface. Both host species occur across most of the United States, with *E. civile* occurring throughout much of North America and *I. verticalis* common east of the Rocky Mountains (Johnson, 1972; Dunkle, 1990). In western Nebraska, these damselflies also are infected with 5 species of large, structurally complex, septate gregarines that often exhibit persistent epimerites with hooks, papillae, and membranous folds (Richardson and Janovy, 1990; Clopton et al., 1993; Clopton, 1995; Percival et al., 1995). Four of these parasite species are included in the present study: *Nubenocephalus nebraskensis* Clopton, Percival, and Janovy 1993; *Hoplorhynchus acanthatholius* Percival, Clopton, and Janovy 1995; *Steganorhynchus dunwoodyi* Percival, Clopton, and Janovy 1995; and *Actinocephalus carrilynnae* Richardson and Janovy 1990.

Odonate midgut epithelium, to which these gregarines attach during the trophozoite portion of their life cycles, is a complex structure containing tall columnar enterocytes with an extensive brush border, regenerative cells, mucocytes, and endocrine cells (Needham, 1898; Chapman, 1969; Andries, 1976; Komnick and Kukulies, 1987). Needham (1898) considered the epithelium a holocrine organ and also described striking structural changes resulting from feeding and starvation cycles, whereas Komnick and Kukulies (1987) found evidence for apocrine secretion. The question of whether the odonate midgut is structurally and functionally dynamic enough to account for damselfly gregarine diversity remains to be answered, but that diversity, especially in the holdfast structure, is much greater than, for example, that of the Gregarinidae of orthopterans. In order to begin understanding the evolution and adaptive significance of damselfly gregarine holdfasts, the following questions were asked. Do these parasites differ in their attachment mechanisms? Do the epimerites of these parasites differ ultrastructurally in ways that suggest different functions?

MATERIALS AND METHODS

Adult damselflies were collected at Oak Lake, Lancaster County, Nebraska (section 14, township 10 north, range 6 east, 40°49.950′N, 96°42.467′W), and the following locations in Keith County, Nebraska: Beckius Pond (sec. 32, T14N, R38W, 41°12.501′N, 101°37.066′W), McGinley Pond (sec. 28, T15N, R38W, 41°15.000′N, 101°40.694′W), Dunwoody Pond (sec. 32, T15N, R37W, 41°13.967′N, 101°34.717′W), Sillasen Ranch pond (sec. 11, T14N, R36W, 41°12.426′N, 101°15.110′W), and Lake Ogallala (sec. 2, T14N, R38W, 41°12.762′N, 101°38.138′W), and taken to the laboratory at the University of Nebraska (Lancaster County specimens) or the Cedar Point Biological Station (Keith County specimens). All damselflies were dissected, some parasites were detached and identified, and pieces of midgut with parasites attached were fixed in alcoholic Bouin's fixative for paraffin sectioning or in 3% phosphate-buffered glutaraldehyde for electron mi-

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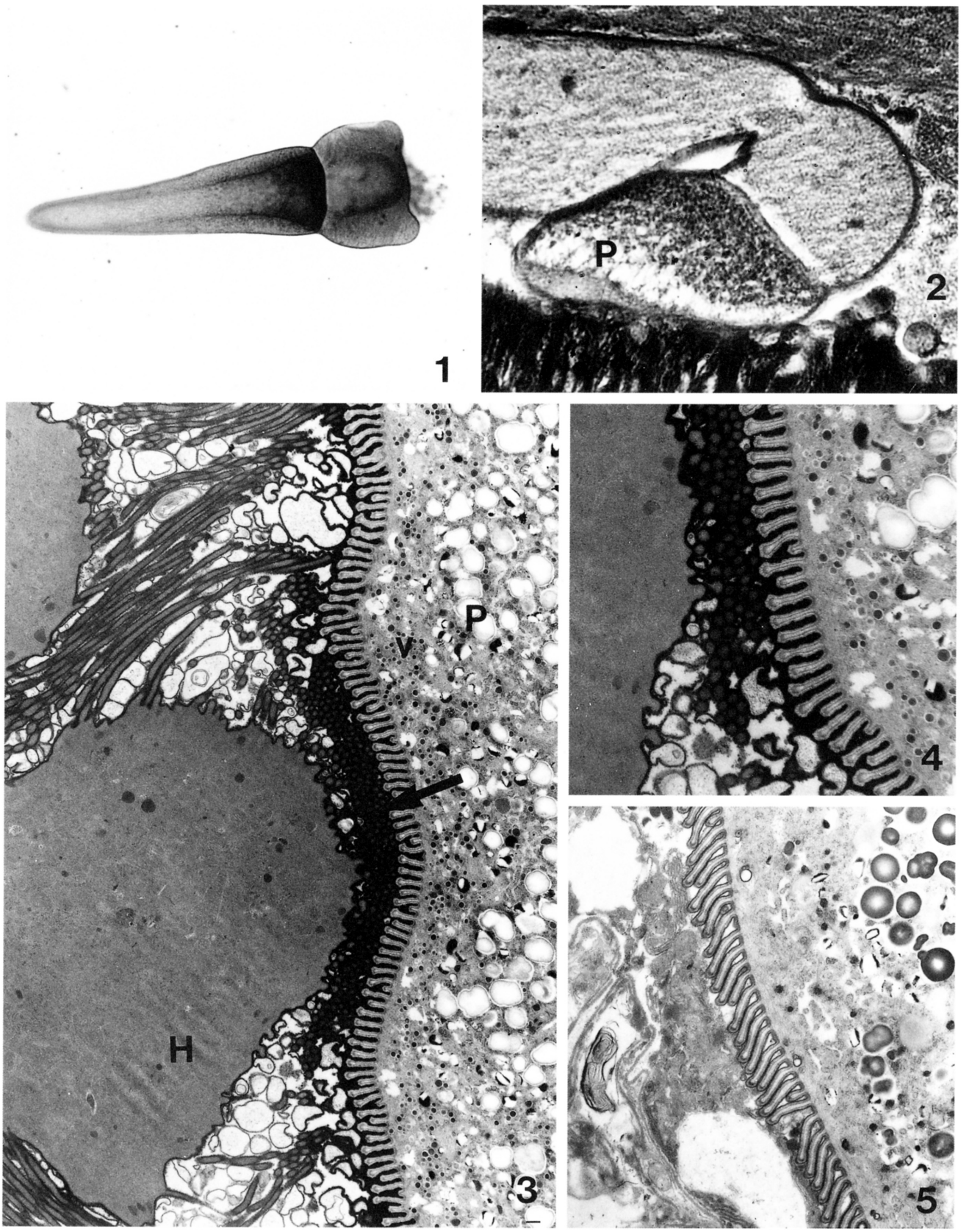
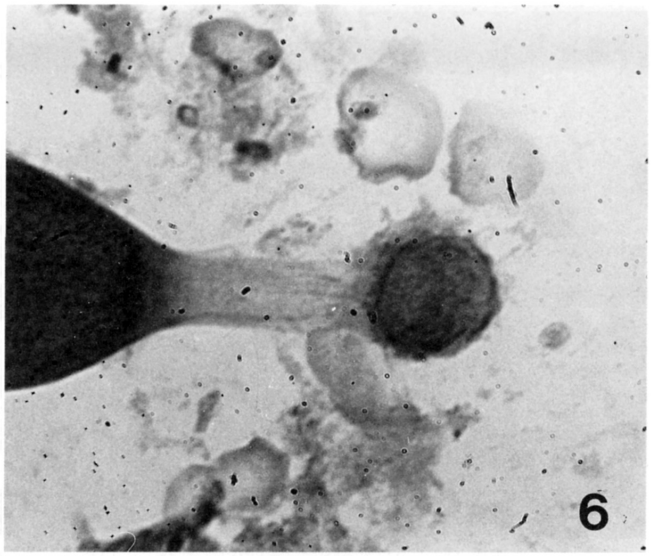
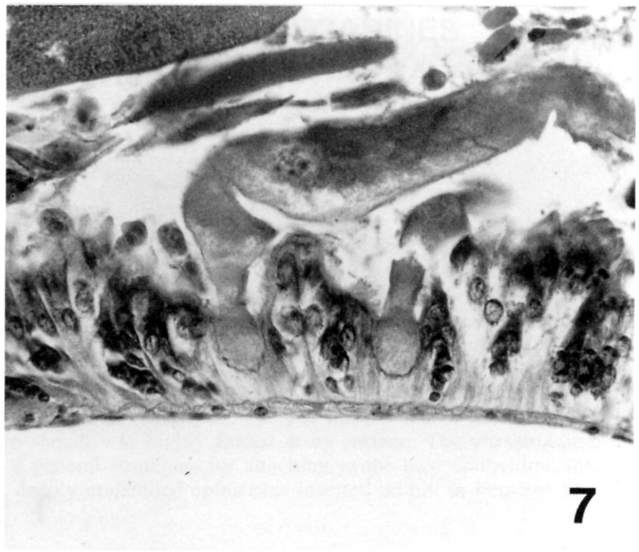
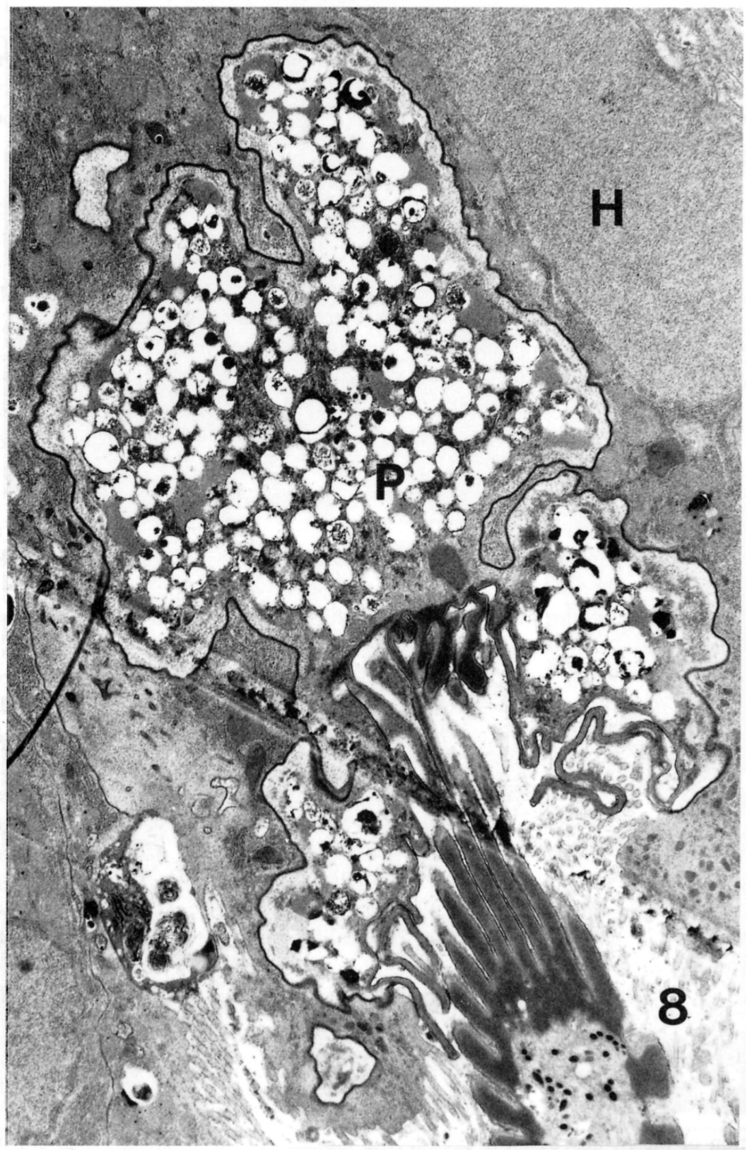
FIGURES 1–5. **1.** *Nubenocephalus nebraskensis,* showing host tissue still attached to the protomerite with suckerlike concavity visible through the cell. **2.** Histological section of *Enallagma civile* gut showing the *N. nebraskensis* mode of attachment, with protomerite adhering to the host

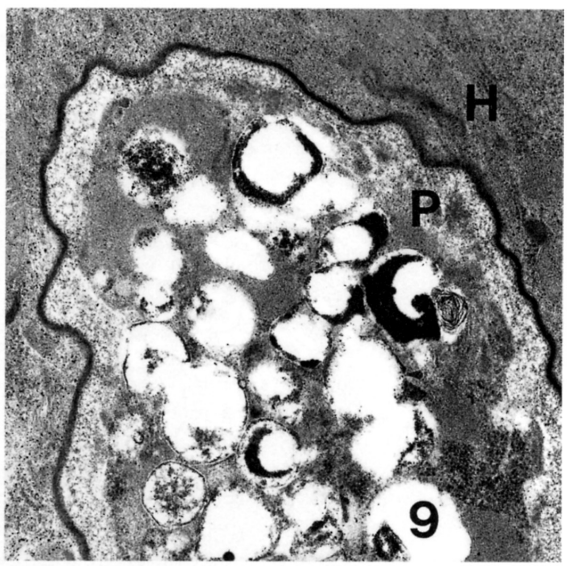


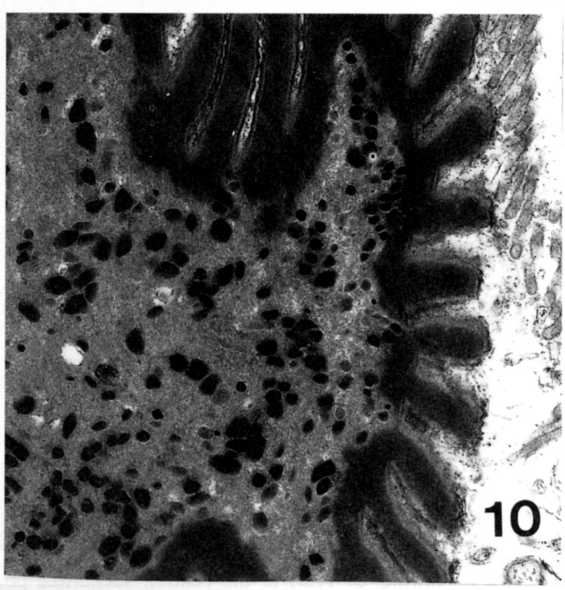
epithelium; P, parasite. **3.** TEM of the central, suckerlike portion of the *N. nebraskensis* protomerite attached to the *E. civile* midgut, showing electron-dense material (arrow) between the parasite (P) and host epithelium (H) and host microvilli pressed against the epicytic folds. **4.** Higher magnification of the host–parasite interface, showing microvilli embedded in the electron-dense material. **5.** TEM of the edge of the *N. nebraskensis* protomerite, lacking the electron-dense material.











FIGURES 6–10. **6.** *Actinocephalus carrilynnae,* epimerite. **7.** Histological section of 2 *A. carrilynnae* attached to *Enallagma civile* gut, showing epimerites embedded between host cells. **8.** TEM of interface between *A. carrilynnae* epimerite (P) and host gut epithelium (H), showing stalk

croscopy. Whole, heavily infected guts were also fixed in Bouin's for paraffin sectioning. Serial paraffin sectioning was done according to standard methods, and sections were stained with hematoxylin and eosin (Galigher and Kozloff, 1971; Pritchard and Kruse, 1982).

Specimens for transmission electron microscopy (TEM) were washed for 1 hr in 5 changes of phosphate buffer, pH 7.0, postfixed in osmium tetroxide for 2 hr, rinsed in distilled water for 30 min, and dehydrated in an ethanol series. TEM specimens were then cleared with propylene oxide and embedded in Epon 812. Silver sections were cut with an LKB Ultratome III and a diamond knife. Sections were mounted on formvar-coated grids, stained with 2% uranyl acetate and lead citrate, and carbon coated. Observations were made on a Philips 201 TEM operated at 60 kV.

RESULTS

Serial paraffin sections of guts sectioned in a longitudinal plane showed no obvious and repeated characteristic regional distribution of any of the four parasite species, although most parasites were attached in the anterior ⅔ of the gut. Damselflies were sometimes infected with more than 1 species of parasite, although never with all 4, and there was no obvious evidence for competition or displacement of 1 species by another. However, the methods were not designed specifically to detect such potential interactions. *Actinocephalus carrilynnae* was distinguished by its very long deutomerite and *N. nebraskensis* by its mode of attachment; final distinction between attached *S. dunwoodyi* and *H. acanthatholius* required sectioning, but epimerite differences were easily seen in both paraffin and epon sections at both light and EM levels. Host gut epithelium height and folding varied more between individual hosts than along the length of any one gut.

Nubenocephalus nebraskensis trophonts lose their small epimerites early in gamont development and subsequently attach to the host midgut epithelium by means of a suckerlike protomerite (Clopton et al., 1993; Figs. 1, 2). Epicytic folds persisted across the attachment surface, and in the central part of the protomerite, there was electron-dense material that appeared to engulf the host epithelium microvilli (Fig. 3, arrow). There was also a zone of relatively small, approximately uniform-sized vesicles proximal to the central protomerite pellicle (Fig. 3). The microvilli-entangling electron-dense material was absent from the region between host epithelium and the edge of the protomerite, as was the zone of small vesicles proximal to the pellicle (cf. Figs. 4, 5).

Actinocephalus carrilynnae trophonts attached to the host midgut by means of a globular, hemispherical epimerite located at the end of a fluted stalk and covered with stublike projections (Richardson and Janovy, 1990; Figs. 6, 7). The epimerite lacked epicytic folds, and its cytoplasm was filled with a variety of vesicles (Figs. 8, 9). The multiple membranes of the *A. carrilynnae* somatic pellicle extended into the epimerite. The region of contact between host and parasite cell membranes was ill-defined, and it was impossible to distinguish between the 2 membranes in electron micrographs (Figs. 8, 9). Epimerite cytoplasmic contents exhibited zonation, namely (from host to parasite) a homogeneous host cell cytoplasm, host and parasite membranes tightly appressed or fused, an unmodified inner parasite pellicle membrane, and a subpellicular vacuolated zone (Figs. 8, 9).

The *A. carrilynnae* stalk exhibited typical epicytic folds (Figs. 8, 10), with large-scale ridges interpreted to be the flutes easily observed in light microscopy. The epicytic folds, however, were structurally distinct from those covering the protomerite and deutomerite, in that the stalk epicytic folds had thicker outer membranes and denser cytoplasmic contents and lacked the microtubules of the somatic folds. Spaces between the stalk epicytic folds were filled with material similar in density to the cytoplasm (Fig. 10). Stalk cytoplasm lacked the large vacuoles of the epimerite but contained a profusion of small, extremely dense, irregularly shaped and sized particles or vacuoles (Fig. 10). The stalk did not have direct contact with the host epithelium, and host cell microvilli could be seen in the spaces surrounding the stalk (Fig. 8). *Nubenocephalus nebraskensis* and *A. carrilynnae* sporozoites were found in both host species; no differences in parasite ultrastructure or in host–parasite relationships were observed in material from the 2 hosts.

Hoplorhynchus acanthatholius trophonts attached to the host by means of an epimerite with digitations embedded deeply in the gut epithelium (Figs. 11, 12). The entire epimerite was covered with loose, ultrastructurally amorphous material and was surrounded at varying distances by host cell cytoplasm, including both intact membranes and membrane pieces (Fig. 13, arrow; Fig. 14). TEM of longitudinal sections through *H. acanthatholius* showed no evidence of the membrane fusion or close appression typical of *A. carrilynnae* (Figs. 13, 14). The epimerite appeared inserted within host epithelial cells, as suggested by the well-formed rough endoplasmic reticulum adjacent to the digitation (Fig. 14) and by the epimerite base where it joined the stalk, being surrounded by tightly packed host cell microvilli (Fig. 13).

The anterior digitations of *H. acanthatholius* are permanent, regular, cytoplasmic extensions covered by the 2 or 3 membranes typical of the pellicle, with a regular, dense layer of cytoplasm immediately proximal to the pellicle (Figs. 13–15). This proximal dense cytoplasmic layer was thickest in the extreme anterior edge of the epimerite but absent in the posterior part of the epimerite (Fig. 13). The interior of the epimerite was filled with many small, round, electron-dense, membrane-bound vesicles (Fig. 13), which were quite different in structure from those found in *A. carrilynnae.*

The epimerite of *Steganorhynchus dunwoodyi* was structurally more complex than those of the other species studied. The stalk, covered with epicytic folds, ended in an ovoid papilla (Figs. 16, 17). Approximately ¾ the way up the stalk, on either side, was an outpocketing of epicytic folds that curved anteriorly and away from the stalk, creating a sheath (Fig. 18). At its base, the sheath had 2 membranes, but only 1 in the more distal regions; this membrane was folded into many small, irregular

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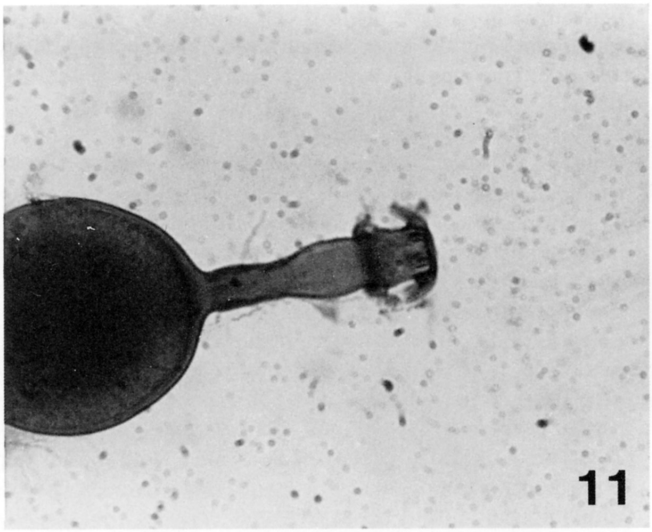
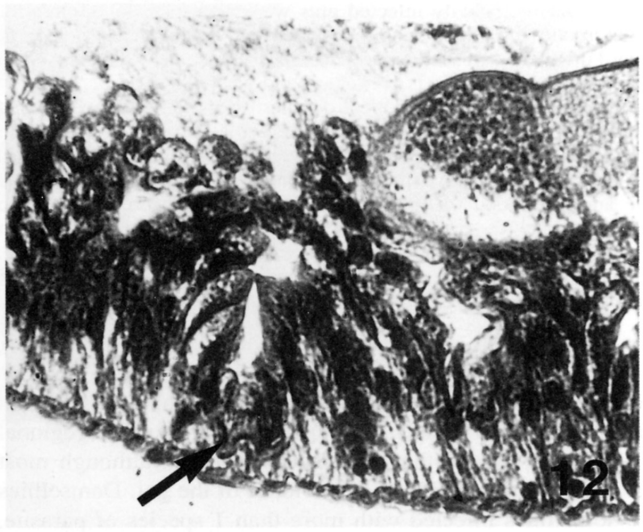
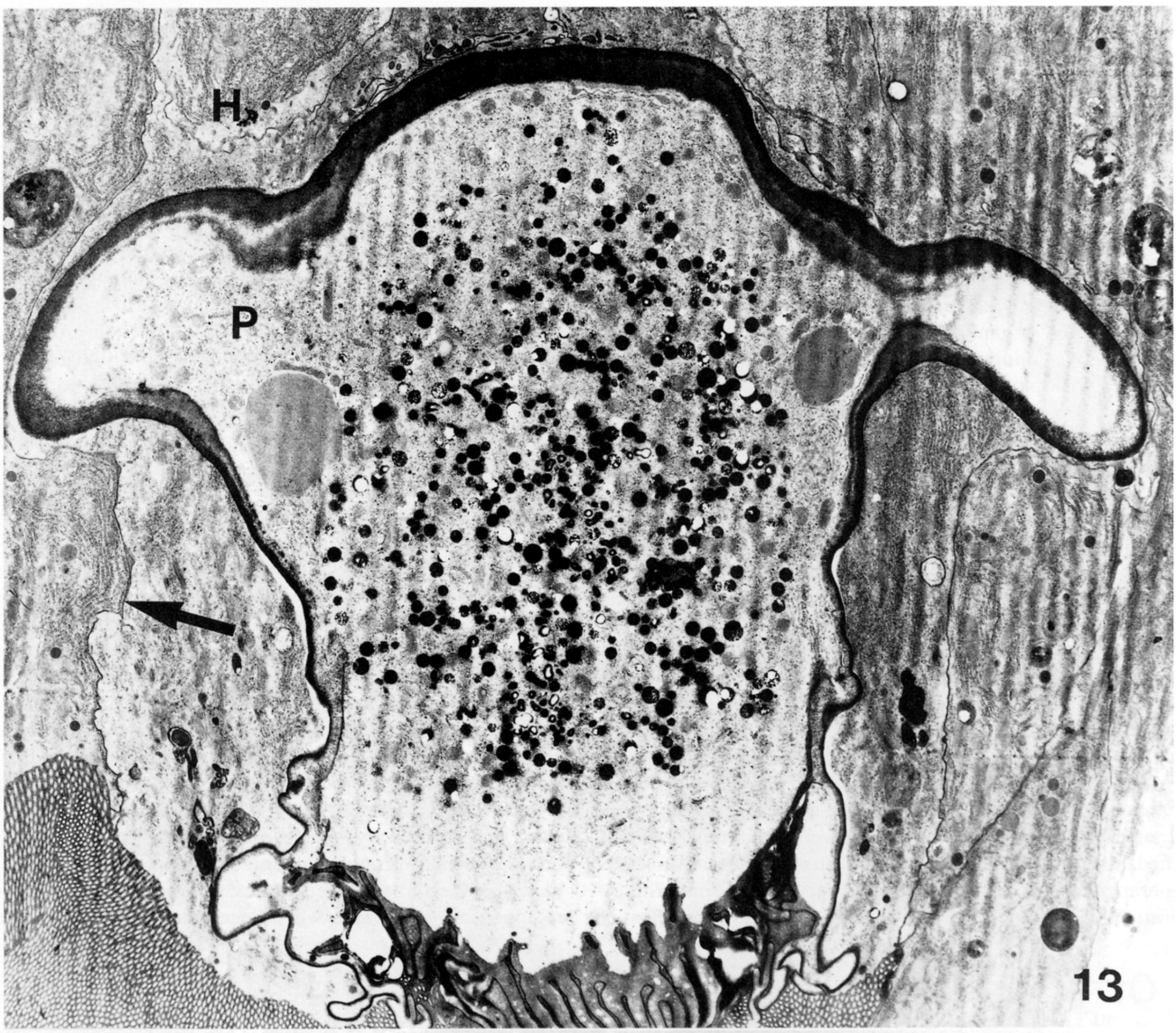
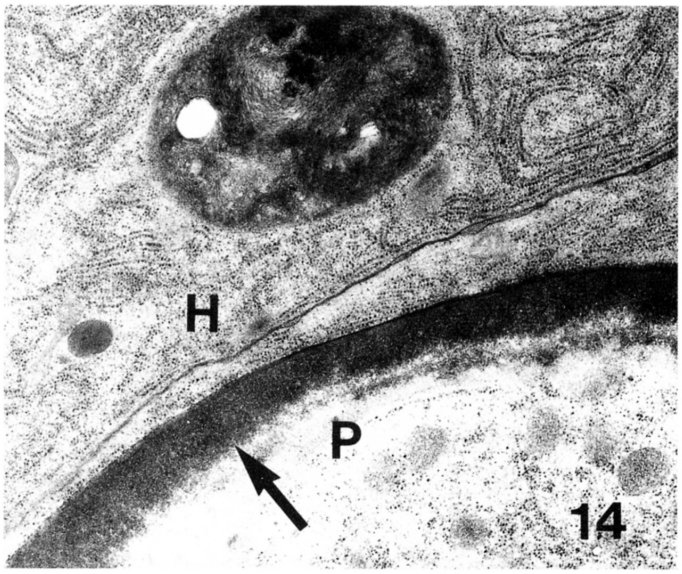
epicytic folds and differences between stalk and epimerite cytoplasm. **9.** Higher magnification TEM of interface between *A. carrilynnae* epimerite (P) and host epithelial cells, showing vacuolated parasite cytoplasm, finely granular layer proximal to the parasite-limiting membrane, and thickened union between host and parasite membranes. **10.** Stalk region of *A. carrilynnae* epimerite, showing epicytic folds and dense, nonvacuolated cytoplasm.

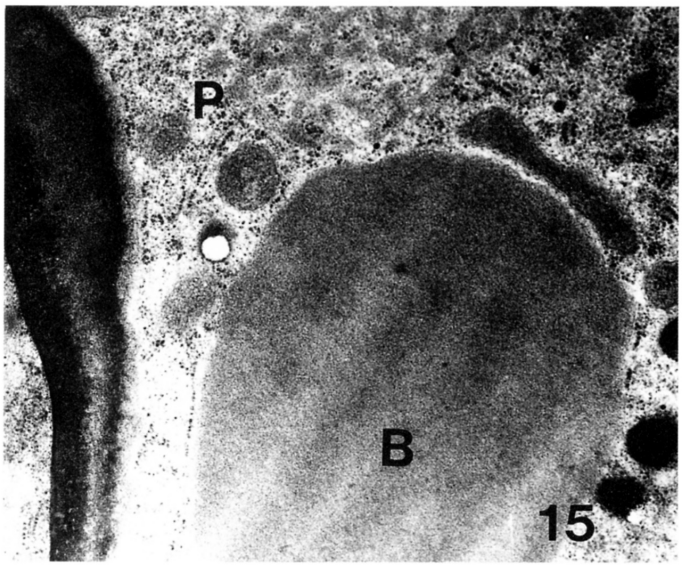






FIGURES 11–13. **11.** *Hoplorhynchus acanthatholius,* hooked epimerite. **12.** Histological section of *H. acanthatholius* attachment to *Enallagma civile* gut epithelium, showing hooks near the basement membrane (arrow). **13.** TEM of *H. acanthatholius* attachment to *E. civile* gut, showing epimerite (P) embedded within a host cell (H); host cell membrane (large arrow) surrounds epimerite. Host cell microvilli are closely applied to the stalk at the lower right, and at the lower left, microvillus lumens appear continuous with the cytoplasm surrounding the epimerite (small arrow).





FIGURES 14–15. **14.** Higher magnification of the *Hoplorhynchus acanthatholius–Enallagma civile* interface showing host cell cytoplasm (H), with rough endoplasmic reticulum (ER), directly in contact with the parasite (P), and the thickened pellicle, lacking epicytic folds, of the epimerite (arrow). **15.** Epimerite edge directly posterior to the hook, showing parasite cytoplasm (P) and the dense body (B) near the hook base.

digitations distally (Figs. 18, 19). In living *S. dunwoodyi* trophonts, the papilla was often seen extruded from the anterior end of the sheath, but it is not known whether the stalk is retractable or if the sheath simply collapsed once the parasite was free from host tissue. The sheath itself was highly flexible and, in some fresh preparations of live organisms, could be seen completely everted, forming a cup or cone with the apex at the tip of the papilla. Above the tip of the stalk, where the edges of the epimerite met, was a seam leading down into the stalk (Fig. 18). The margins of the seam were heavily lined with cytoplasmic digitations, and the tip of the papilla was covered with epicytic folds.

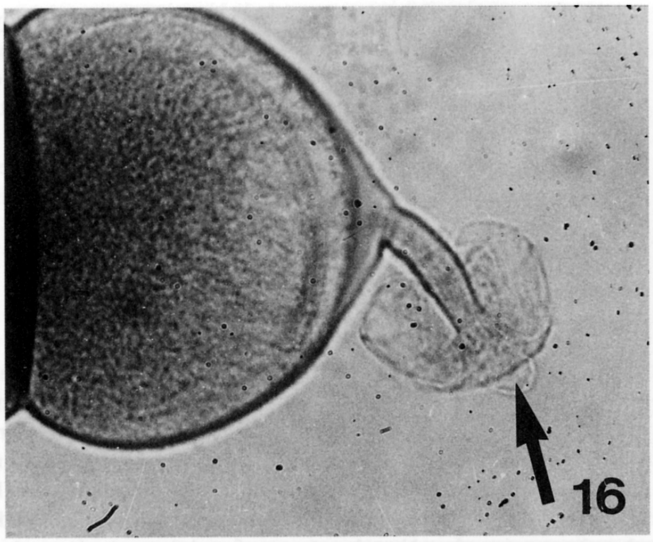
The granulation patterns of the epimerite (sheath), stalk, and papilla were very different from one another. Epimerite cytoplasm was filled with many small, membrane-bound vesicles of varying electron densities (Fig. 18), whereas papilla vesicles were all small and very electron dense, and the cytoplasm itself was relatively electron lucent with fibers oriented along the stalk (Fig. 20). Papilla cytoplasm was also more heterogeneous than that of the epimerite, and the papilla cytoplasm was filled with ribosome-sized granules.

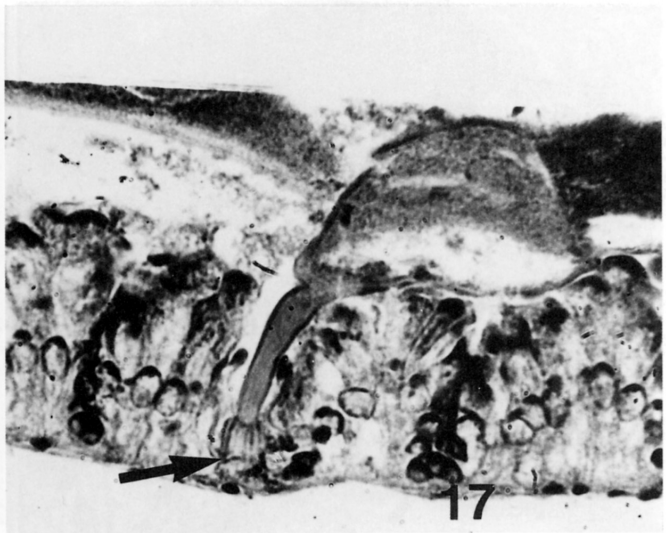
DISCUSSION

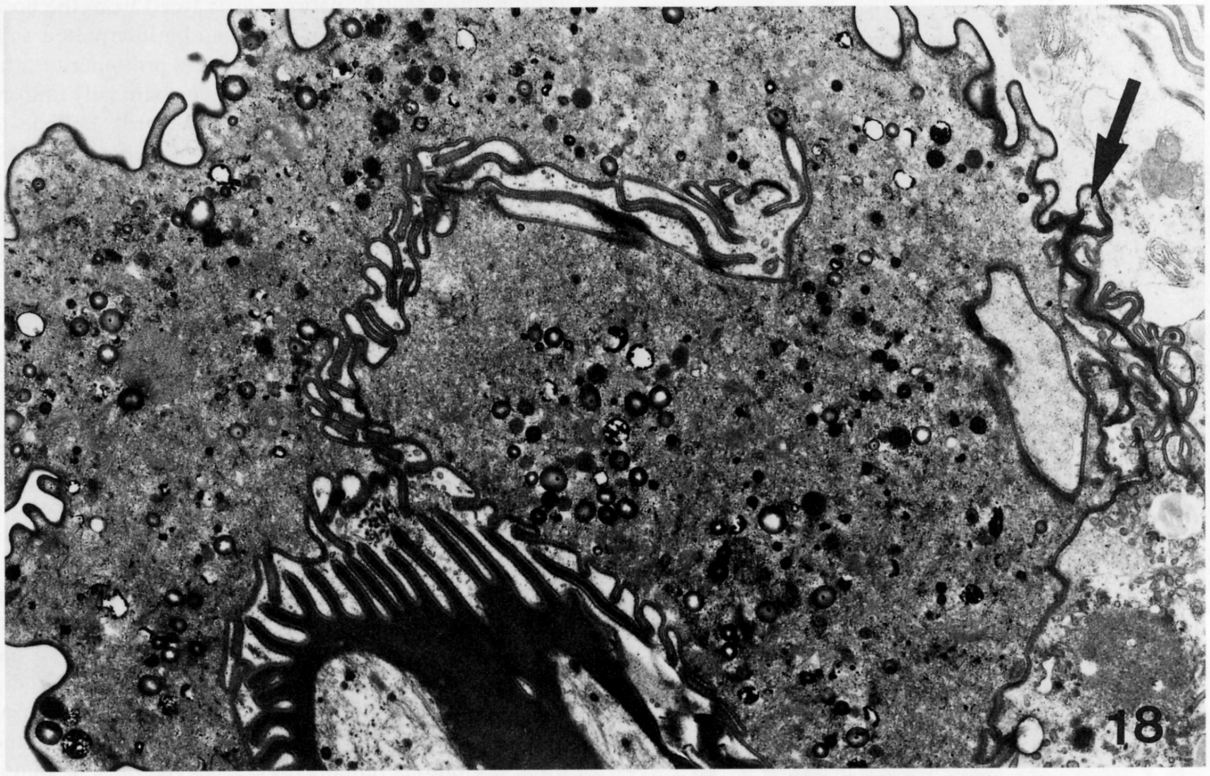
The major contribution of this paper is the demonstration that confamilial parasites, attaching to the same host tissue, have evolved rather strikingly different mechanisms for maintaining that attachment. In the case of *N. nebraskensis,* the electron-dense material, located in the central region of the protomerite, may function in binding the parasite to the host microvilli (Fig. 3). The adhesive function of this material is also suggested by the observation that *N. nebraskensis* freed from the host almost invariably retains something that can be interpreted as host epithelium on the central portion of the protomerite attachment surface (Fig. 1). Finally, the small, approximately uniform-sized vesicles proximal to the central protomerite pellicle (Fig. 3), could be exocytic vesicles, and their varying electron density suggests a developmental sequence for the adhesive that evidently functions in parasite attachment.

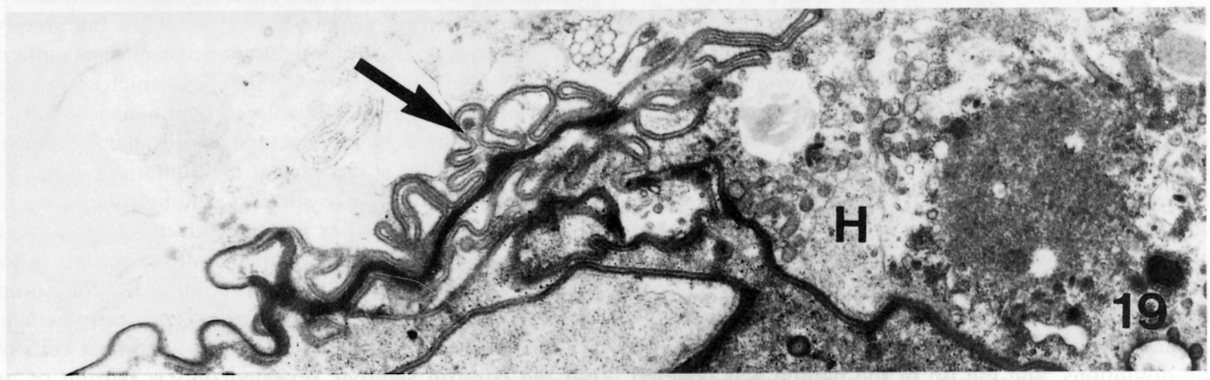
In contrast to *N. nebraskensis, A. carrilynnae* attaches by means of a globular, hemispherical epimerite at the end of a fluted stalk. Previous gregarine studies have suggested membrane fusion and junctional complexes between hosts and parasites (MacMillan, 1973; Marques, 1979). In the present study, the difficulty of distinguishing between host and parasite membranes also suggests membrane fusion, although no junctional complexes were apparent (Figs. 8, 9). The diversity of structural features in the *A. carrilynnae* epimerite, however, indicates functional complexity, perhaps involving exocytosis, endocytosis, and compartmentalization of cytoplasmic contents.

The genus *Hoplorhynchus* is distinguished from similar forms almost entirely by structure of the epimerite, which is a flattened bulb bordered by digitations (Carus, 1863; Levine, 1988). In species descriptions, the anterior digitations have been variously described as hooks, spines, and stumpy digitiform processes (Hoshide, 1953, 1954; Obata, 1953; Sarkar and Haldar, 1980; Percival et al., 1995). Until now, however, the nature of these digitations remained unclear. The present study reveals that these structures are not hooks or spines, but simply extensions of the cell, albeit relatively permanent ones quite different from the papillae seen under the light microscope on the *A. carrilynnae* epimerite. The role or significance of the 2 structures, represented by approximately circular, somewhat electron-dense profiles at the base of the digitations (Figs. 13, 15B), is not obvious from these studies, although they may represent a supporting ring that lies just beneath the digitations. Because the odonate midgut is lined with a stratified columnar holocrine-type epithelium (Needham, 1898; Andries, 1976; Komnick and Kukulies, 1987), it is difficult to determine whether the *H. acanthatholius* epimerite caused damage to host cells or tissue, but the paraffin sections revealed only a parting of epithelial cells in the region of attachment (Fig. 12). Drawings of epithelium sections showing embedded epimerites have been pub-









FIGURES 16–19. **16.** *Steganorhynchus dunwoodyi* protomerite and epimerite showing stalk, papilla (arrow), and attachment sheath. **17.** *Steganorhynchus dunwoodyi* epimerite embedded in *Ischnura verticalis* gut epithelium; sheath enclosing papilla indicated by arrow. **18.** TEM of sheathlike functional portion of the *S. dunwoodyi* epimerite, showing highly folded parasite membranes (arrow) and groove or canal through sheath. **19.** Higher magnification of interface between *S. dunwoodyi* sheath membrane (arrow) and host gut (H).

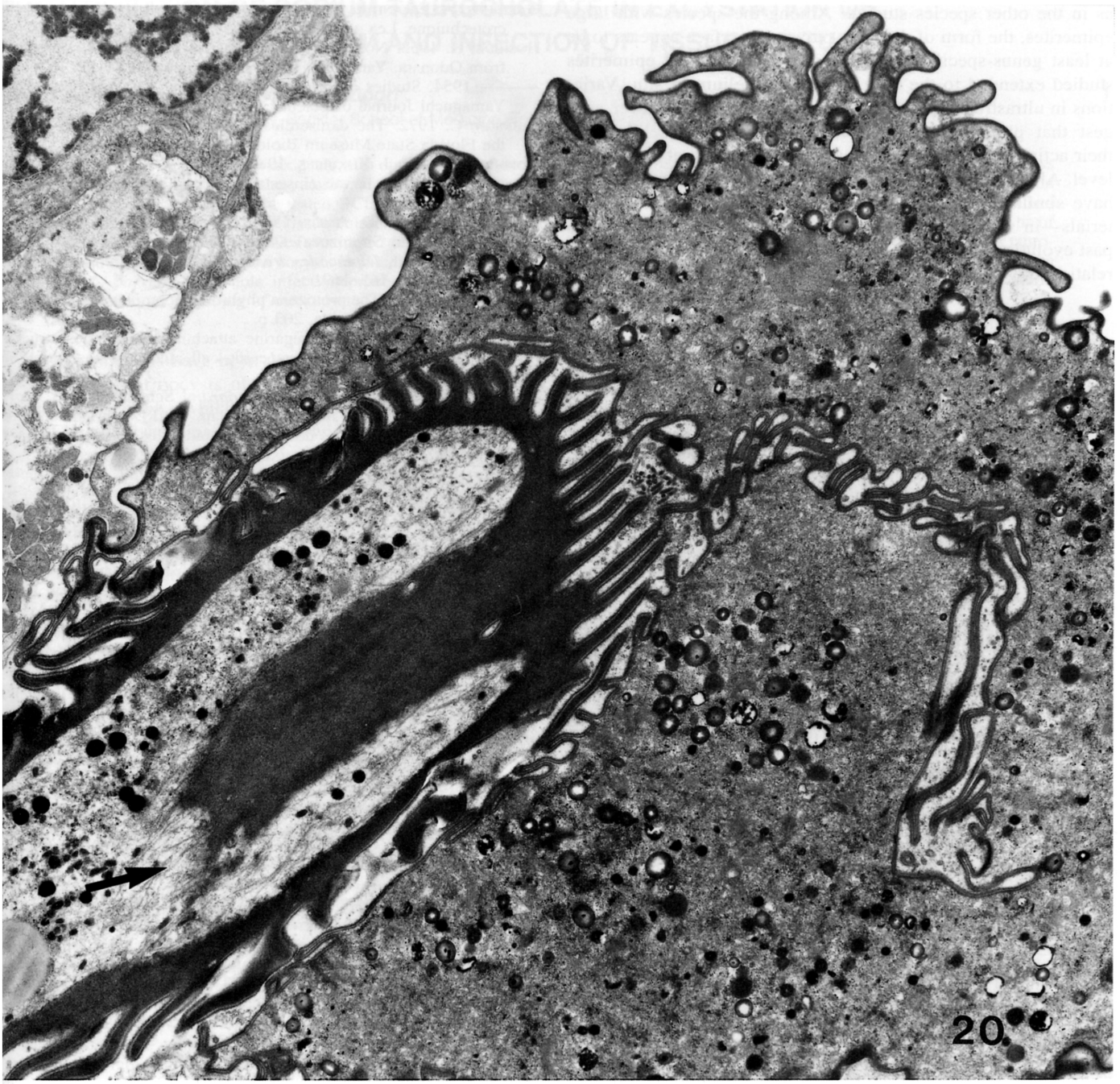


FIGURE 20. *Steganorhynchus dunwoodyi* stalk, papilla, and sheath, showing the cytoplasmic differences between epimerite and stalk cytoplasm and the fibrous cytoplasm of the stalk (arrow).

lished previously (Ahamed and Narasimharmurti, 1979; Sarkar and Haldar, 1981), but no tissue damage is obvious from these drawings, and the authors did not mention pathology in the accompanying text.

In the case of *S. dunwoodyi,* the observation that epimerite (sheath) cytoplasm was somatic, whereas that of the stalk was clearly not, suggests functional differences between these two regions of the cell. It is possible that once the epimerite is inserted between epithelial cells and attachment is firmly established, the papilla is everted and functions in nutritional uptake. The attachment organelle of *S. dunwoodyi* thus appears to be a very dynamic structure worthy of additional study.

The observed structural differences between species and between regions of single epimerites suggest that the interactions taking place between parasite and host are quite complex and may vary between genera. The ultrastructural observations reveal that actinocephalid gregarines have evolved at least 2 general strategies for attaching to host epithelium: namely suckerlike protomerites, as in the case of *N. nebraskensis,* and deeply embedded epimerites inserted within or between host cells,

as in the other species studied. Among the species with large epimerites, the form of the host–parasite interface appears to be at least genus-specific, but all of the large, stalked epimerites studied extended to the base of host epithelium in situ. Variations in ultrastructural details of these epimerites, however, suggest that physiological interactions between damselflies and their actinocephalid gregarines also differ at least at the generic level. Alternatively, actinocephalid gregarine epimerites may all have similar functions—that is, uptake of particular host materials—in which case their diversity may be more related to past evolutionary events than to current, biochemically intimate relationships with their hosts.

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