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Systematic distribution of birefringent bodies in Rotifera and first evidence of their ultrastructure in *Acyclus inquietus* (Gnesiotrocha: Collothecaceae)

Hui Yang · Rick Hochberg · Elizabeth J. Walsh · Robert L. Wallace

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Abstract Small birefringent concretions have been reported in rotifers for over a century and often hypothesized as energy sources. Here, we provide an update on their distribution in superorder Gnesiotrocha and the first data on their ultrastructure. Within Gnesiotrocha, these birefringent bodies (BRB) are known from at least ten species of Collothecaceae and 14 species of Flosculariaceae, both of which include planktonic and sessile species. Among sessile species, the predator *Acyclus inquietus* contains a single BRB that has been described as starch-like. We examined larvae of *A. inquietus* with transmission electron microscopy and revealed the BRB to have an irregular, electron-dense margin that surrounds a speckled core. The core appears mostly amorphous, but contains numerous, very small electron-dense spots and thin electron-dense fibers; there is no evidence of any crystalline lattice. The intestinal lumen contains smaller concretions that are probably the result of BRB metabolism. The thin epithelium contains abundant electron-dense granules but relatively few organelles. We hypothesize that the BRB is a unique form of extracellular glycogen that functions as an energy source in larvae for their dispersal and metamorphosis. In adult *A. inquietus*, the BRB may provide energy permitting reproduction when prey are no longer available.

Keywords Anisotropic · Glycogen · Indirect life cycle · Lecithotrophic · Planktonic · Sessile

Introduction

Williamson (1853) was the first to report small brown concretions in the digestive tract of the sessile rotifer *Floscularia ringens* (Linnaeus, 1758), a member of the superorder Gnesiotrocha. Since then, similar structures have been noted in several species of gnesiotrochan rotifers, across all life stages including both females and males (reviewed in Wallace, 1993). While their distribution is not systematically ubiquitous, their unusual nature as solid, mostly extracellular concretions is noteworthy and has garnered some interesting hypotheses on their structure and potential functions. Several researchers have noted the pigmented

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H. Yang · R. Hochberg (✉)
Department of Biological Sciences, University of Massachusetts Lowell, Lowell, MA, USA
e-mail: rick_hochberg@uml.edu

E. J. Walsh
Department of Biological Sciences, University of Texas, 500 W. University Ave, El Paso, TX, USA

R. L. Wallace
Department of Biology, Ripon College, Ripon, WI, USA

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appearance of some concretions: e.g., dark brown (Williamson, 1853), yellow (Leidy, 1882), and orange (Donner, 1964). Other researchers report a transparent halo (Weber, 1888) or considered the concretions to be crystalline in structure (de Beauchamp, 1909). Wallace (1993) was the first to describe their anisotropic (birefringent) properties when viewed with polarized light microscopy, and so coined the term anisotropic crystalline structure (ACS) for them. He described their presence from 18 species, noting that the ACS is variable in both size and position (in different organ systems) in different species. To date, neither the chemistry nor ultrastructure of these concretions have been examined, but functionally, they have been hypothesized to be waste products (Joliet, 1883; Weber, 1888; de Beauchamp, 1909, 1912), statocyst organs (Hünerhoff, 1931), and energy sources (Weber, 1888; Wallace, 1993).

In this study, we provide an update on the systematic distribution of these birefringent concretions—herein called birefringent bodies (BRB) until their crystalline nature has been verified—in superorder Gnesiotrocha based on live observations and a literature review. We also provide the first data on the ultrastructure of these concretions based on observations of larvae of the predatory rotifer *Acyclus inquietus* (Leidy, 1882), which is described to have a BRB that is starch-like in appearance (Wallace, 1993). Importantly, larvae of this rotifer are short-lived and metamorphose into adults after only a few hours in the plankton (Hochberg et al., 2010). The larvae always settle in the colony of their sessile prey, the rotifer *Sinantherina socialis* (Linnaeus, 1758), where they undergo a rapid and dramatic transformation to the adult stage (Hochberg et al., 2010). The adult stage feeds on embryos and juveniles of *S. socialis*, but to date, there is no evidence that the larvae of *A. inquietus* are planktotrophic. *Acyclus inquietus* possess a BRB well into their adulthood (Wallace, 1993).

Methods

Specimens of *A. inquietus* were collected from colonies of *Sinantherina socialis* in Flint Pond, Tyngsboro, MA (42°40′28.14″N, 71°25′35.09″W). Live observations were also made of the following species: *Collotheca ferox* (Penard, 1914) from White River Marsh State Wildlife Area, Green Lake Co., WI, USA (43°54′31.1″N, 89°5′43″W); *Hexarthra* sp. and *Lacinularia flosculosa* (Müller, 1773) from Hueco Tanks State Park and Historic Site, El Paso Co., TX, USA (*Hexarthra*: 31°55′28.5″N, 106°2′33.19″N; *L. flosculosa*: W31°55′30″N, 106°2′48″W); *Limnias melicerta* Weisse, 1848, *Octotrocha speciosa* Thorpe, 1893, *Stephanoceros fimbriatus* (Goldfuβ, 1820), and *S. millsii* (Kellicott, 1885) from Mascuppic Pond, Tyngsboro, MA, USA (42°40′42.15″N, 71°24′07.64″W); *S. ariprepes* Edmondson, 1939 from Moon Lake, Marquette Co., WI, USA (43°48′10″N, 89°22′6″W); and *Testudinella patina* (Hermann, 1783) from La Mancha Wetland, Doña Ana Co., NM, USA (31°18′12″N, 106°33′14″W).

Larvae of *A. inquietus* were removed from culture dishes of rotifer colonies and placed in small glass bowls with 0.5% bupivacaine. Anesthetized larvae were photographed with a Zeiss A1 compound microscope equipped with differential interference contrast (DIC) and a Sony Handycam digital camera. Two specimens were processed for transmission electron microscopy (TEM). Larvae were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 2 h at room temperature on a rotator. They were then rinsed (3 × 30 min) in 0.1 M sodium cacodylate buffer, postfixed in 1% OsO_4 in 0.1 M cacodylate buffer (2 h), and rinsed again in sodium cacodylate buffer (3 × 30 min). Larvae were dehydrated by sequentially moving them through four ethanol baths (70, 90, 100, 100%) for 10 min each, transferred through two changes of propylene oxide (15 min each), and then placed in a propylene oxide:epoxy resin mixture (Araldite, EMbed 812; Electron Microscopy Sciences) of 3:1 (1 h), 2:1 (1 h), 1:1 (1 h), and then 1:2 (1 h), followed by pure epoxy resin overnight on a rotator at room temperature. Afterward, the larvae were placed in pure resin in a BEEM capsule and cured at 60°C for 48 h. Blocks were sectioned on a Reichert ultramicrotome and the sections were collected on copper grids stained with uranyl acetate (2 min) and lead citrate (2 min). Sections were examined on a Philips CM10 (80 kV) equipped with Gatan Orius 813 digital camera at the Core Electron Microscope Facility at the University of Massachusetts Medical School in Worcester, MA. A total of 23 ultrastructural Sects. (70 nm) of the BRB were analyzed. Measurements of ultrastructural features in the digital images were made with Image J using the scale bar in the TEM images as the bases for

Table 1 List of gnesiotrochan rotifer species surveyed for the presence of birefringent bodies (BRB) in their embryos, larvae, or adults

Taxa	Taxonomic authority	BRB	Selected references
Order Collothecacea			
Family Collothecidae			
Collotheca campanulata (gracilipes)	(Edmondson, 1939)	Amictic embryos	Wallace (1975, 1993)
Collotheca coronetta	(Cubitt, 1869)	Amictic embryos	Wallace RLW, pers. obs.
Collotheca. ferox	(Penard, 1914)	Larvae, adult	Penard (1914), Wallace (1993)
Collotheca hoodiia	(Hudson, 1883)	In adults clusters "of yellow globules"	Hudson (1883), Hudson and & Gosse (1886, p. 56; Pl II.5)
Collotheca moselii	(Milne, 1905)	Larvae "a few brown concretions"	Milne (1905)
Collotheca ornata	(Ehrenberg, 1830)	Amictic embryos	Wallace (1993)
Stephanoceros fimbriatus	(Goldfußs, 1820)	Amictic embryos	Hochberg & Hochberg (2017)
Stephanoceros millsii	(Kellicott, 1885)	Amictic embryos	Hochberg, pers. obs.
Family Atrochidae			
Acyclus inquietus	(Leidy, 1882)	Amictic embryos, adults	Leidy (1882), Wallace (1993)
Cupelopagis vorax	(Leidy, 1857)	Amictic embryos, adults	Wallace (1993)
Order Flosculariacea			
Family Conochilidae			
Conochilus unicornis	(Rousselet, 1892)	NP	Wallace (1993)
C. dossuarius	(Hudson, 1885)	NP	Wallace (1993)
Family Flosculariidae			
Beauchampia crucigera	(Dutrochet, 1812)	NP	Wallace (1993)
Floscularia conifera	(Ehrenberg, 1832)	Amictic embryos	Wallace (1993)
Floscularia melicerta	(Ehrenberg, 1832)	Amictic embryos	Wallace (1993)
Floscularia janus	(Hudson, 1881)	Amictic embryos	Walsh, pers. obs.
Floscularia ringens	(Linneaus, 1758)	Amictic embryos	Wallace (1993)
Lacinularia flosculosa	(Müller, 1773)	Amictic embryos, larvae	Wallace (1993); Walsh, pers. obs.
Limnias melicerta	Weisse, 1848	NP	Wallace (1993); Walsh, pers. obs.
Limnias ceratophylli	Schrank, 1803	Amictic embryos	Wallace (1993)
Octotrocha speciosa	(Thorpe, 1893)	Amictic embryos	Wallace (1993)
Ptygura beauchampi	(Edmondson, 1940)	Amictic embryos	Wallace (1993)
Ptygura brevis	(Rousselet, 1893)	Amictic embryos	Wallace (1993)
Ptygura libera	(Myers, 1934)	Amictic embryos	Wallace (1993)
Ptygura pectinifera	(Murray 1913)	Larvae	Koste (1974, 1978)
Ptygura pilula pilula	(Cubitt, 1872)	Amictic embryos	Wallace (1993)
Sinantherina socialis	(Linneaus, 1758)	Amictic embryos; not present in adults	Wallace (1993)
Sinantherina ariprepes	(Edmondson, 1939)	Amictic embryos, larvae; not present in adults	Walsh, pers. obs.

Table 1 continued

Taxa	Taxonomic authority	BRB	Selected references
Family Hexarthriidae		NP	Walsh, pers. obs.
Hexarthra n. sp.			
Family Testudinellidae	(Hermann, 1783)	NP	Walsh, pers. obs.
Testudinella patina			

The data in this table have been expanded modified from Wallace (1993)

NP = BRB not present based on personal observations or referenced literature

measurement. Multiple measurements were made and averages were calculated to include ± 1 standard deviation (SD). No digital images were manipulated beyond basic changes to brightness and contrast.

We also searched the rotifer literature for any illustrations, photographs, or descriptions of structures in the rotifer digestive tract and/or body cavity that matched the descriptions of birefringent bodies based on the study of Wallace (1993). While such structures cannot be identified with certainty based on most historical descriptions, we include them in Table 1 for future reference in the event that further studies become warranted.

Results

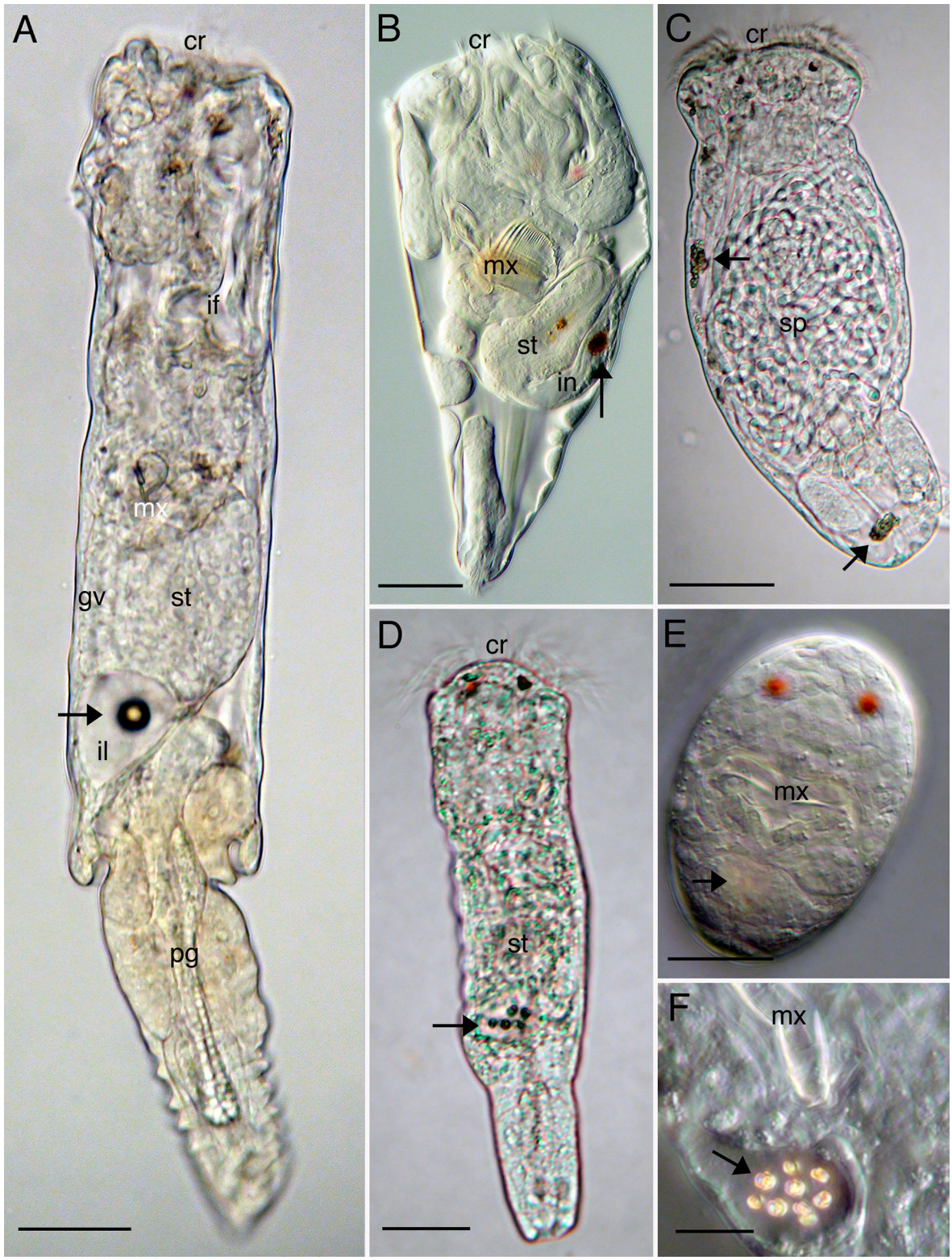
Systematic distribution

Live observations and surveys of the literature revealed 24 species of Gnesiotrocha to possess BRB-like structures (Table 1). In most cases, not all life stages were examined, so the distributional data on the presence of BRBs among life stages (amictic and mictic embryos, larvae, adults) and sexes may be incomplete. When present, the BRBs are generally solitary extracellular concretions in the intestinal lumen. We note the presence of a single brown BRB in the intestinal lumen of larvae of *Stephanoceros fimbriatus* (not shown), while amictic embryos and larvae of *S. millsii* have multiple refractive BRBs (Fig. 1D–F). Extracellular BRB-like concretions have also been observed in the blastocoel of male specimens of *Octotrocha speciosa*, where they are present around the testis and copulatory organ (Fig. 1C); these concretions appear more granular than those described for most other species. Wallace (1993) notes the absence of BRBs from *Beauchampia crucigera* (Dutrochet, 1812), *Conochilus unicornis* Rousselet, 1882, *C. dossuarius* (Hudson, 1885), and *Limnias melicerta*, while Walsh (pers. obs.) notes that BRBs are absent from *Hexarthra* sp., *Limnias melicerta*, and *Testudinella patina*.

Fig. 1 Light microscopy of gnesiotrochan rotifers with birefringent bodies (arrows). **A** Larva of *Acyclus inquietus*, brightfield microscopy. **B** Larva of *Floscularia conifera*, differential interference contrast microscopy (DIC). **C** Male of *Octotrocha speciosa*, brightfield microscopy. **D** Larva of *Stephanoceros millsii*, brightfield microscopy. **E** Late-stage embryo of *S. millsii*, DIC. **F** Close-up of intestinal lumen and BRBs from specimen in D, DIC microscopy. *cr* corona, *gv* germovitellarium; in developing infundibulum, *il* intestinal lumen; in intestine, *mx* mastax, *pg* pedal gland, *sp* sperm, *st* stomach. *Scale bars* **A** 20 um; **B** 35 μm; **C** 35 μm; **D** 25 μm; **E** 30 μm; **F** 15 μm

Acyclus inquietus

A single BRB was present in the intestinal lumen (Fig. 1A) of both specimens. With differential interference contrast, the center of the BRB appeared different from the periphery (Fig. 2A). The maximum size of the BRB in section was 5.7 μm long by 3.5 μm wide. The BRB was irregular in shape due to the presence of numerous small granules that lined its margin (Fig. 2B, C). Individual granules ranged in size from 61 to 151 nm in diameter ($\bar{x}$ = 83.3 nm ± 23.3 nm, n = 22 sections examined), and though larger granules were also present, they appeared to consist of several smaller granules fused together (Fig. 2C). All granules were electron dense throughout their structure. The BRB had an electron-dense border that was highly irregular in outline making precise measurements of its thickness difficult, so we chose to measure areas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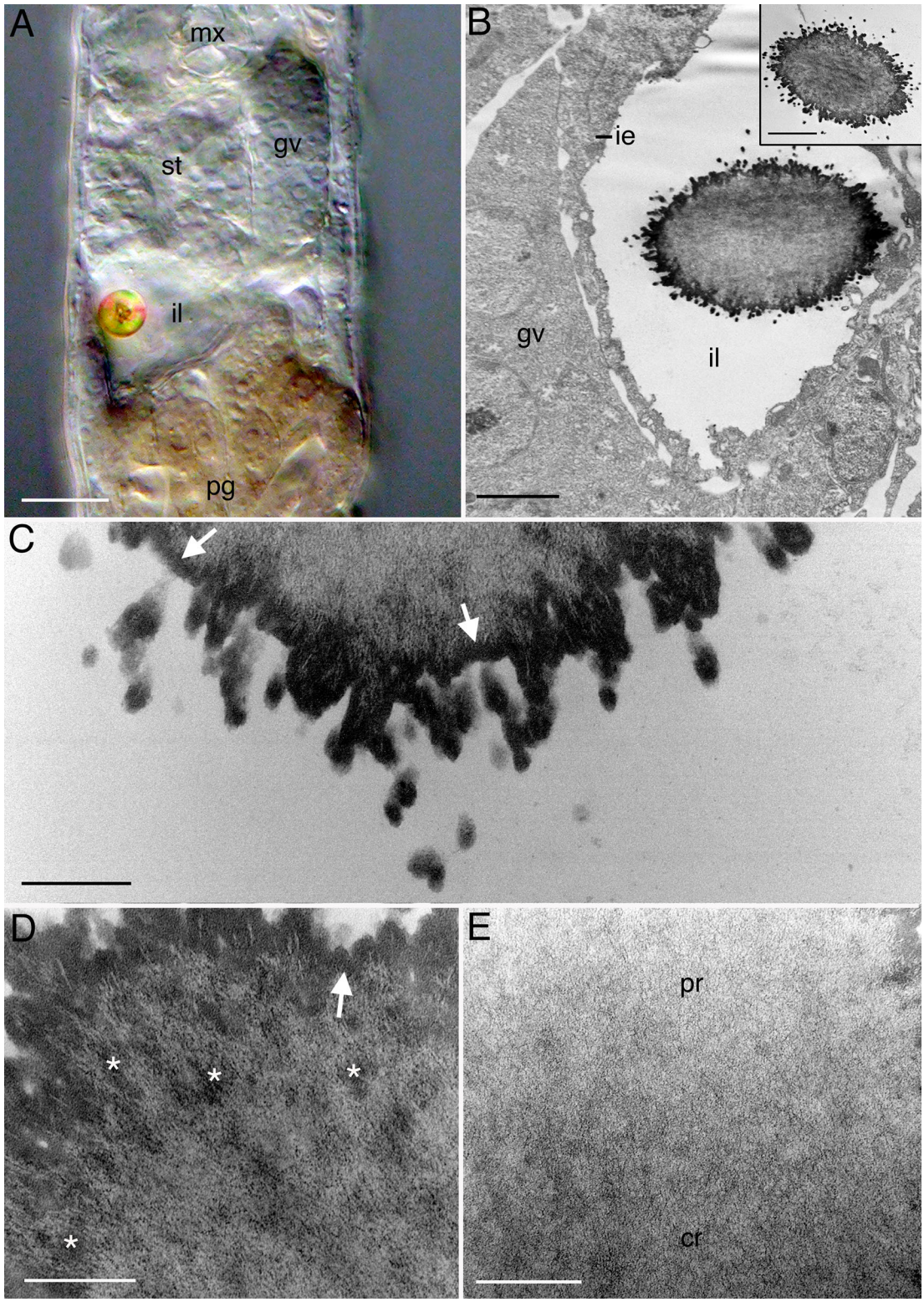
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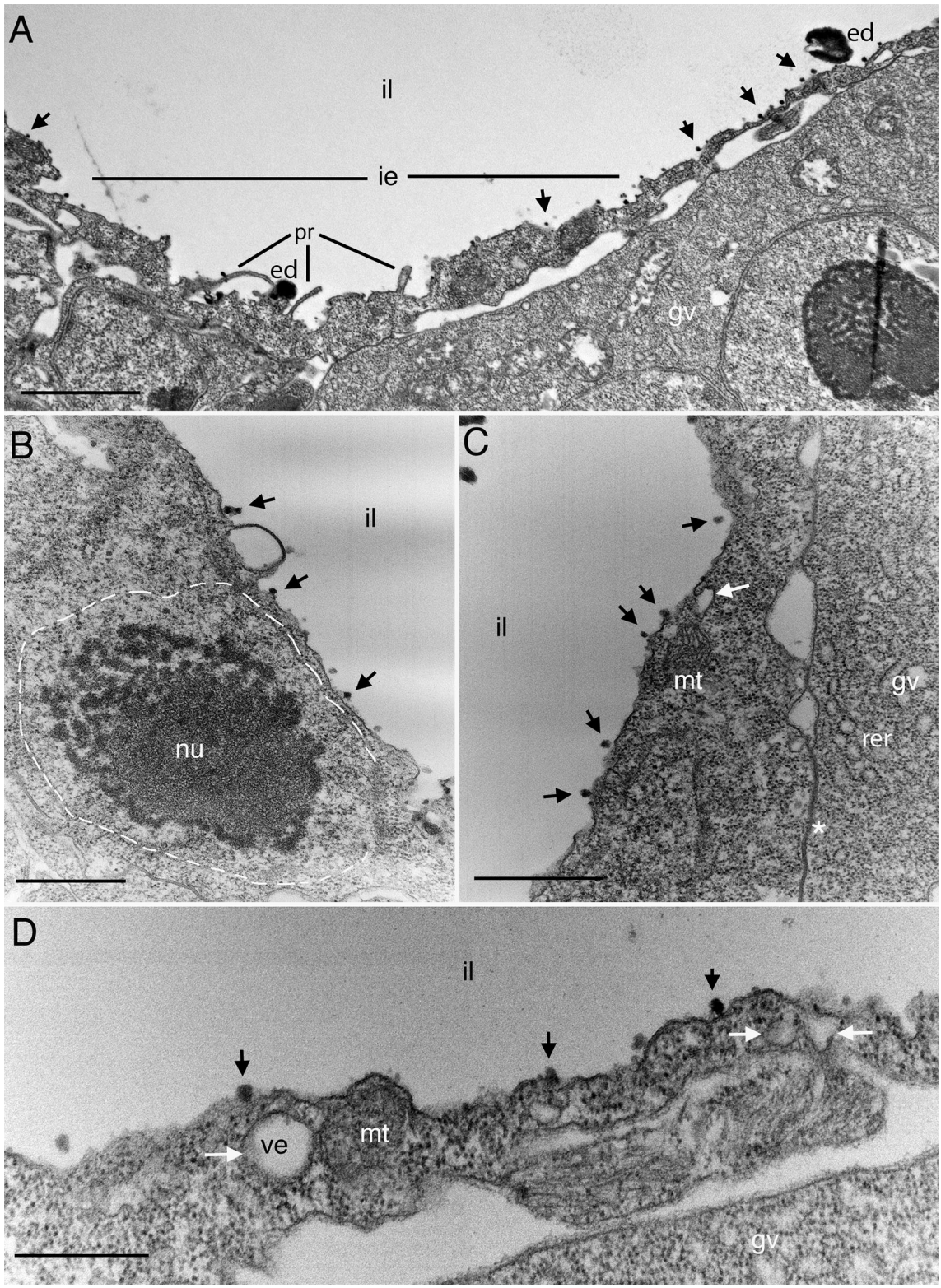
◀**Fig. 2** The BRB of larval *Acyclus inquietus*. **A** Focus on the BRB in the intestinal lumen of a live specimen, DIC microscopy. **B** Ultrastructure of the BRB from the same specimen in A, longitudinal section through the larva. Inset: Ultrastructure of the BRB from second larva showing an electron-dense line extending through the center. **C** Ultrastructure the margin of the BRB from same specimen in A. Note the presence of several smaller granules around the periphery. **D** Ultrastructure of the region just internal to the margin revealing a series of electron-dense patches of similar size to the granules that line the margin. **E** The amorphous core of the BRB. The peripheral regions (pr) are slightly more transparent to electrons than the central core (cr). Very fine fibers and electron-dense dots are present throughout the periphery and core. Symbols and abbreviations: arrows, peripheral regions of the BRB that were measured for thickness; *gv* germovitellarium, *i.e.,* intestinal epithelium, *il* intestinal lumen, *mx* mastax, *pg* pedal gland, *st* stomach. *Scale bars* **A** 10 μm; **B** 2 μm; **C**–**E** 200 nm

that had no granules touching the surface (short arrows, Fig. 2C, D; 35–83 nm thick, $\bar{x}$ = 50.1 ± 12.6 nm (1SD); n = 16 sections). Just inside the border, the matrix of the BRB consisted of many electron-dense shapes, most of which had an indistinct outline, but were similar in size and appearance to the granules on the outside of the BRB (asterisks, Fig. 2D). Slightly more internal to these shapes were numerous electron-dense dots (granules) that provided a speckled appearance at high magnification (Fig. 2D). These dots were between 3.8 and 6.4 nm in diameter ($\bar{x}$ = 5.2 ± 0.7 nm, n = 20). More internally toward the core of the matrix, these electron-dense dots became more transparent and the BRB became somewhat amorphous, but with very fine electron-dense lines or fibers throughout (Fig. 2E). At high magnification, these lines were numerous, but it was difficult to discern whether they formed any patterns; they did not appear regular nor give the appearance of a crystalline lattice. Some sections had an extremely electron-dense line extending down the center of the core (Fig. 2B inset). Apart from the BRB and the granules that bordered it, the only other items in the intestinal lumen were electron-dense granules proximal to the intestinal epithelium (Fig. 3). Most of these granules were 36–64 nm diameter ($\bar{x}$ = 50.3 ± 10.5 nm; short arrows, Fig. 3A), but other larger granules (> 100 nm diameter) were also present (ed, Fig. 3A). Our sections did not reveal a stomach lumen, and so no granules were observed anywhere else within the alimentary canal.

The intestinal epithelium was an extremely thin syncytium that ranged from 206 nm thick (containing cytoplasm with mitochondria and electron-dense granules only) to 1894 nm thick (containing a solitary nucleus). The size of the intestinal lumen varied depending on the region of the section. The border of the epithelium was irregular in outline with occasional finger-like projections (pr, Fig. 3A), but there was no evidence of any cilia or microvilli on the apical border (Fig. 3). Just beneath the plasma membrane and throughout the cytoplasm were abundant granules of 16–33 nm diameter ($\bar{x}$ = 21.9 nm ± 5.7 nm; n = 54; measured at 64,000X only) that gave the cytoplasm a speckled appearance (Fig. 3B–D). These granules varied in electron density; most were electron dense and with an indefinite outline, but some had a stippled appearance. Mitochondria were abundant, but nuclei and rough endoplasmic reticulum were rare (Fig. 3B–D). Membrane-bound vesicles were present, but never abundant and always had electron-lucent cores (Fig. 3C, D). The germovitellarium was in proximity to the intestine in most sections and had abundant rough endoplasmic reticulum that formed tubular membranes with many bound ribosomes; bound ribosomes measured 15–21 nm in range ($\bar{x}$ = 17.7 ± 2.3 nm; n = 25; measured at 64,000X only). Many large nuclei were also present (Fig. 3A, C, D). The plasma membrane of the germovitellarium was proximal to the intestinal epithelium in many areas but gaps between the two organs were present (Fig. 3C, D). Specific epithelial junctions (e.g., gap junctions) were not observed.

Discussion

Wallace (1993) was the first to describe the birefringent qualities of BRBs in rotifers, and called them anisotropic crystalline structures, though their refractive nature is not always obvious and may require specialized microscopy such as polarized light microscopy or DIC. In our studies of gnesiotrochan BRBs, we found that they may be highly refractive (Fig. 1A, F) or not (Fig. 1B) with DIC. We also found male *O. speciosa* to contain small brown BRBs in its blastocoel, (Fig. 1C) and the larvae of *S. millsii* to contain multiple refractive BRBs in its intestine (Fig. 1D, F). This latter condition is noted for late-stage amictic embryos, newly hatched larvae, and larvae > 4 h old,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◀**Fig. 3** Ultrastructure of the intestinal epithelium of a larval *Acyclus inquietus* showing electron-dense granules (black arrows) that line the apical plasma membrane. **A** The thin syncytial epithelium of the intestine. A large nucleus of the germovitellarium lies proximal to the intestine. **B** Close-up of the intestinal lining showing a nucleus (nuclear membrane is artificially outlined) and the granular cytoplasm. **C** Membrane-bound secretion vesicles (white arrows) are rare, but glycogen granules are abundant. Note the closely apposed germovitellarium (Asterisk) and large gaps between intestine and germovitellarium. **D** Close-up of epithelium showing a large membrane-bound secretion vesicle next to a mitochondrion. *ed* electron dense, *er* endoplasmic reticulum, *gv* germovitellarium, *i.e.*, intestinal epithelium, *il* intestinal lumen, *mt* mitochondrion, *nu* nucleus, *pr* finger-like projections of the intestinal epithelium, *rer* rough endoplasmic reticulum, *ve* secretion vesicle. *Scale bars* **A** 500 nm; **B** 500 nm; **C** 700 nm; **D** 450 nm

and therefore is unlikely to be the result of digestive processes. Of significance for our interpretations is that Wallace (1993) observed that not all BRBs are crystalline in appearance, and he specifically described the BRBs of *A. inquietus* and other species of Atrochidae to appear as "starch grains or translucent glass beads." We also note that the BRB-like concretions in male *O. speciosa* are granular in appearance and therefore unlike those described for most other gnesiotrochans. Whether this difference is significant (e.g., chemically, functionally) or not remains to be determined. Based on these observations and those from the literature (see references in Wallace, 1993), the BRB can be highly variable in size, shape, appearance, and position in the body. With this in mind, it is important to state that our data on the ultrastructure of the BRB in larvae of *A. inquietus* may not pertain to the ultrastructure of the BRB in other species. We are also uncertain whether the BRB has evolved only once or multiple times, the answer to which will require additional observations of live specimens and a well-supported phylogeny.

The BRB of larval *A. inquietus* is a large, singular, extracellular concretion that is only present in the lumen of the intestine (Figs. 1A, 2A). It has an irregular electron-dense border and more electron-lucent core; the core appears to consist of very fine fibers and electron-dense dots (Fig. 2B–E). Though we did not test for glycogen, we can only speculate that the electron-dense dots are γ-like glycogen granules due to their size range (3.8–6.4 nm diameter; compare to 3 nm γ-glycogen from vertebrates, Prats et al., 2018), and that they condense toward the margin of the BRB and form larger glycogen granules, and all held together by a fibrous protein matrix. The electron-dense margin of the BRB probably consists of highly condensed granules that are digested away from the BRB during the life of the larva. The granules are probably slowly removed from the BRB and broken down into smaller subunits that eventually come to lie proximal to the intestinal epithelium (Fig. 3); at that point they are further digested and translocated across the plasma membrane and stored in the cytoplasm. The cytoplasmic electron-dense granules are 16–33 nm in diameter (21.9 ± 5.7 nm) and we assume most are glycogen. While glycogen granules stain similarly to free ribosomes and overlap in size with them (20–25 nm; Revel et al., 1960; Revel, 1964), we think that a majority of the cytoplasmic granules are probably ß-granules for three reasons. (1) They are widely distributed throughout the cytosol and not obviously localized near lamellae or microtubules, as free ribosomes may be (Suprenant, 1993; Noma et al., 2017). (2) They are more variable in size than is known for most free ribosomes (Vournakis & Rich, 1971) including the bound ribosomes we measured on the RER of the germovitellarium. (3) They have an indefinite outline, which characterizes glycogen granules in ultrastructural sections of many animals (Revel, 1964; Revel et al., 1960). If our interpretation is correct, then the BRB is probably the source of these granules because rotifer larvae are assumed to be non-feeding (Young et al., this volume) and no other food was observed in the intestinal lumen.

Prats et al. (2018) described glycogen granules as glycosomes because they contain glucose and a variety of proteins, the core of which is glycogenin that is covalently bonded to the glucose polymer. They defined *y*-glycogen (3 nm) as the smallest subunits of ß-glycogen, which is the primary source for rapid energy. ß-glycogen can also form larger aggregations to make α-glycogen rosettes as demonstrated in limpets and rats (Revel, 1964; Sullivan et al., 2010); the largest diameter of α-glycogen is 300 nm and known only from mammalian liver cells (Sullivan et al., 2010). Our ultrastructural data on the BRB of *A. inquietus*, which can reach 30 μm in diameter in adult females (Wallace, 1993), are probably not pure glycogen and cannot be directly related to the largest glycogen granules in other animals. The fact that none of the smaller electron-dense granules in the rotifer's

intestine ever appeared as rosettes or took on any form other than as an amorphous circular shape makes their classification as α-glycogen unwarranted. We hypothesize that the BRB could be a collection of glycosomes that are held together by a protein matrix, but this will require further biochemical studies in addition to electron microscopy.

While the results of this ultrastructural examination pertain to only a single species and a single life-cycle stage, we speculate that the BRB functions as an energy source during the larval life span of various gnesiotrochan rotifers (Weber, 1888; Wallace, 1993). Many species appear to be non-feeding, and while their lifespan is short (hours), they must require an energy source for dispersal, during which time they seek out various substrata that will be paramount for a successful adult life (Wallace, 1993). However, it is clear that not all larvae have a BRB (see "Results"), so we are uncertain whether some species are planktotrophic or are provided with another form of energy source by their mother. In the case of *A. inquietus*, larvae are non-feeding, obligate symbionts of the colonial rotifer *S. socialis*, and so must swim until they find a viable colony (Hochberg et al., 2010). Once settled, the eggs and neonates of the colony become their food source. Interestingly, adults of *A. inquietus* have larger BRBs than the larvae (Wallace, 1993), leading us to hypothesize that these predatory rotifers augment their BRB during adulthood in the event of an early death of the colony or an end to its reproduction, i.e., when their prey source are no longer available. And while the function of the BRB in other species of Gnesiotrocha remains unknown, we speculate that it functions as an endogenous energy source for larval dispersal. In species of Collothecaceae, this temporary form of energy may also be important for their further development. All collothecid rotifers produce a larva that undergoes a rapid and dramatic metamorphosis to the adult stage after settlement (Kutikova, 1995; Hochberg & Hochberg, 2017; Hochberg et al. this volume). This metamorphosis involves replacement of the larval head with a newly generated adult head called the infundibulum, and we assume this process of replacement requires a great deal of energy.

Lastly, Wallace (1993) notes that BRBs are generally absent from early-stage embryos of most rotifers that possess them as larvae, but they do appear just prior to hatching (see Fig. 1E). This would seem to indicate that the process of BRB production takes several hours and may involve the slow accumulation of BRB precursors during the development of the larval intestine. We think transmission electron microscopy of embryos in different stages of development may be useful to witness the assembly of these BRB precursors and may therefore answer the question about the size distribution of granules that form the developing BRB. We also think that histological staining (e.g., Buchner et al., 1970) and biochemical analyses (e.g., enzyme digestion) of in situ and extracted BRBs may be necessary to ultimately determine their chemical composition, and therefore, their ultimate function(s).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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