Ultrastructure of digenean trematode eggs (Platyhelminthes: Neoophora): A review emphasizing new comparative data on four European Microphalloidea

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Abstract

Despite their tremendous diversity and their medical and veterinary importance, details of egg ultrastructure among the digenean trematodes has been studied rather little. The available literature is spread over several decades and several species, but has not been adequately reviewed to reveal patterns of similarity and divergence. We present this review to synthesize and analyse what is known from the available literature reporting studies using both transmission electron microscopy (TEM) and scanning electron microscopy (SEM). To support our general review of existing literature, we also have synthesized our own previously published descriptions, and present herein our new previously unpublished data. From these new electron micrographs, we provide a comparative analysis of the intrauterine eggs of four digenean species, representing four genera and three families of the superfamily Microphalloidea, collected from four different host wildlife species in four European countries: 1) *Mediogonimus jourdanei* (Prosthogonimidae) from *Myodes glareolus* (Mammalia: Rodentia), collected in France; 2) *Maritrema feliui* (Microphallidae) from *Crocidura russula* (Mammalia: Soricimorpha), collected in Spain; 3) *Brandesia turgida* (Pleurogenidae) from *Pelophylax ridibundus* (Amphibia: Anura: Ranidae), collected in Russia; and 4) *Prosotocus confusus* (Pleurogenidae) from *Rana lessonae* (Amphibia: Anura: Ranidae), collected in Belarus. All were studied by preparing whole worms by various techniques for TEM, so that eggs could be studied in situ within the uterus of the parent worm. Based on the literature review and the new data presented here, we describe basic similarities in patterns of embryogenesis and egg formation among all trematode species, but substantial variations in timing of larvigenesis, sculpturing of egg shell surfaces, and some other features, especially including accessory cocoon coverings outside the egg shells of *B. turgida* and *P. confusus*. In the future, many more studies are needed to explore egg ultrastructure in other digenean taxa, to explore potential phylogenetic patterns in egg development and structure, and to correlate structure with function in the life cycle.

Keywords
development, egg, embryo, larva, miracidia, reproduction, Trematoda, ultrastructure, vitellocyte

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Introduction

Life cycle schematics have long played a prominent role in forming the conceptual framework of how parasites circulate in the environment between hosts. Most published life cycle schematics, as well as written descriptions, primarily emphasize those developmental stages that occur within the hosts, or that directly invade hosts. For digenean trematodes, this includes stages such as adults within vertebrate hosts, intramolluscan stages within gastropod molluscs, and metacercariae (Martin and Conn 1990) and other transitional stages (Conn 2007a, 2010; Goater et al. 2005; Conn et al. 2008) within various second-intermediate hosts. Secondary but significant emphasis has been placed on free-living stages that are directly infective to hosts, including miracidia that invade molluscs (Karatayev et al. 2012) and cercariae that invade either intermediate hosts (Conn and Conn 1995), or that directly invade definitive hosts (e.g., the extensively studied schistosomatids). In contrast, relatively little emphasis has been placed on the egg stage, although there has been an increase in the recognition that cestode eggs are highly diverse in both structure and role within the life cycle (see review by Conn and Świderski 2008).

Because trematode eggs are very small – usually microscopic – little can be see using light microscopy methods. Thus, electron microscopy is necessary to reveal the essential features of the structure of the eggs and the embryos or larvae they contain. To further complicate their study, because the highly resistant trematode egg shells are designed to protect the embryo and larva from harsh environmental conditions outside the host and outside the parent worm, preparing trematode eggs for ultrastructural studies is technologically challenging. Thus, egg ultrastructure in this important group of parasites has received relatively little study, even when compared to their closely related class of parasitic Platyhelminthes, the cestodes (Burt 1986; Świderski 1996; Świderski and Conn 2000, 2001; Conn 2007b, 2016).

The purpose of this paper is to provide a brief review of the known studies on digenean trematode egg ultrastructure, while providing up-to-date context by reporting new comparative data and a new synthesis of information on four microphalloid trematodes from Europe, which until now have been the subject of only a cursory comparison (Świderski and Conn 2014).

Materials and methods

Adult specimens of *Brandesia turgida* (Brandes, 1888) were obtained from crypts in the intestinal wall of naturally infected mars frogs, *Pelophylax ridibundus* (Pallas, 1771) (Amphibia: Ranidae), collected near the Rybinsk Reservoir on the Volga River, Russia during Adults of *Maritrema feliui* were collected live from the intestine of the greater white-toothed shrew,

Crocidura russula (Hermann, 1780) (Eulipotyphla: Soricidae), captured in La Ricarda, a marshy nature reserve close to the estuary of the River Llobregat (Barcelona, Spain), during October, 2010.

Naturally infected bank voles, *Myodes glareolus* (Schreber, 1780) (Rodentia: Cricetidae), were captured in the Nature Reserve of Py (Pyrenean Mountains, France) during June, 2009. Live mature specimens of *Mediogonimus jourdanei* Mas-Coma et Rocamora, 1978 were collected from the liver upon necropsy and dissection of voles at the laboratory of "Centres Científics i Tecnològics" of the University of Barcelona (CCiTUB) in order to apply high pressure freezing fixation and freeze substitution (see below).

Adult, live specimens of *Prosotocus confusus* (Looss, 1894) were collected from the intestine of naturally infected pool frogs, *Pelophylax lessonae* (Camerano, 1882) (Amphibia: Ranidae), during April 2008 in the Bugskiy landscape reserve (Southwest Belarus).

Conventional TEM methodology

For *Maritrema feliui* and *Prosotocus confusus*, live worms were first placed in a 0.9 % NaCl solution. Later, they were fixed in cold (4 °C) 2 % paraformaldehyde and 2.5 % glutaraldehyde in a 0.1M sodium cacodylate buffer at pH 7.4 for a minimum of 2 h, rinsed in a 0.1M sodium cacodylate buffer at pH 7.4, postfixed in cold (4 °C) 1 % osmium tetroxide in the same buffer for 1 h, rinsed in MilliQ water, dehydrated in an ethanol series and propylene oxide, and finally embedded in Spurr's resin. Ultrathin sections were obtained using a Reichert-Jung Ultracut E ultramicrotome, placed on copper grids and double-stained with uranyl acetate and lead citrate. Ultrathin sections were examined using a JEOL 1010 TEM operated at an accelerating voltage of 80 kV in the CCiTUB (Barcelona, Spain). For *Brandesia turgida*, materials were embedded in a mixture of Araldite and Epon. Ultrathin sections were cut on a Leica Ultracut UCT ultramicrotome and, after staining, examined in JEOL 1011 TEM in Centre of Electron Microscopy, I.D. Papanin Institute for the Biology of Inland Waters, Russian Academy of Sciences, Borok, Russia.

High pressure freezing, freeze substitution and infiltration with resin

Live specimens of *Mediogonimus jourdanei* were cut open and pieces of uterus were selected in small Petri dishes under a stereomicroscope in PBS with 20% BSA. The sections of uterus were transferred to the cavity of a 200 μm-deep flat specimen carrier. The specimen holder was then inserted into the rapid transfer system, high pressure frozen using a Leica EM PACT and stored in liquid nitrogen.

For freeze substitution in preparation of the *M. jourdanei* specimens, sample holders were transferred to precooled cryovials (–120°C) and freeze substitution was per-formed in anhydrous acetone containing 2% osmium tetroxide. Using a Leica EM AFS, samples were maintained for 24 h at –90°C. Hereafter, the temperature was raised at a rate of 2°C/h to –60°C and then to –30° C. Samples were kept at each level for 9 h in the original substitution medium. Specimens were then washed three times for 10 min in fresh anhydrous acetone. After the washes, the temperature was gradually raised to room temperature and the specimens were infiltrated with Spurr resin (one part resin/three parts acetone) overnight; 1:1 for 4 h; 3:1 for 4 h and 100% resin for 4 h and then overnight. Polymerization was carried out by heat at 60°C for 72 h. Ultrathin sections were cut using a Reichert-Jung Ultracut E ultramicrotome, placed on copper grids and

poststained with uranyl acetate (2%) in methanol for 5 min and lead citrate for 4 min. Finally, ultrathin sections were examined using a JEOL 1010 TEM operated at an accelerating voltage of 80 kV in the CCiTUB.

Results

Side-by-side comparison of transmission electron micrographs (TEM) the in utero eggs within the intact parent of all four microphalloid species demonstrated significant similarities in the basic patterns of embryogenesis and ultimate structure of the egg shell and embryonic envelopes. However, substantial variation occurred among the four species, especially relating to the timing of postembryonic development into the fully formed miracidium (i.e., larvigenesis), and in the presence of unique structures enclosing each egg outside the egg shell. Most details have been reported previously for each individual species (Świderski et al. 2010, 2013a, 2013b, 2014, 2015a). To expand on these individual studies, our descriptions presented here provide new comparative data on these four representatives of the superfamily Microphalloidea. Essential new data in the form of TEM micrographs for each species are shown in Figs. 1-5. General observations on comparative aspects are presented below and in Table 1.

The eggs of all four species followed general aspects of the pattern that we and others have described previously for all trematodes and cestodes; however, there are some variations among species in details as well as in some more generalized features described here.

Embryogenesis and embryonic envelopes

In all four species, the early embryo (*M. feliui* and *P. confusus*) or the fully formed miracidium (*M. jourdanei* and *B. turgida*), is surrounded by two syncytial embryonic envelopes: 1) an inner embryonic envelope formed from mesomeres; 2) an outer embryonic envelope formed from macromeres and vitellocyte remnants. The maternally derived egg shell covers these and constitutes the outer protective layer of the enclosed embryo, and fully formed larva in the case of *M. jourdanei* and *B. turgida*.

The macromeres forming the outer envelope initially have well-developed nuclei and complex perinuclear cytoplasm containing ribosomes and endomembrane elements. These cells deteriorate very quickly in *M. jourdanei*, *B. turgida*, and *P. confusus*, but persist for longer in *M. feliui*, in which they undergo migration to the poles of the egg prior to forming a syncytium (Fig. 2). In all species, the embryo proper and ultimately the miracidium forms from micromeres. Some of these undergo apoptosis during early embryogenesis, but others persist through embryonic development, and ultimately their derivative cells form the completed miracidium larva, which develops completely within the in-utero egg in *B. turgida* (Fig. 1), and *M. jourdanei* (Fig. 4), in which fully developed cilia are easily visible.

Larval development

The micrographs presented here confirm the complete development of ciliated miracidia larvae within the intrauterine eggs of both *B. turgida* (Figs. 1, 2) and *M. jourdanei* (Fig. 4), thus

consitituting very late-stage ovoviviparity in these two species. These do not attain true viviparity, as the miracidia never are released within the parent uterus. In contrast to these two ovoviviparous species, the two other species, *M. feliui* and *P. confusus*, possess eggs containing only early embryos (Figs. 3, 5), thus constituting either oviparity or very early-stage ovoviviparity.

Egg shell formation and structure

All four species examined comparatively here possess an egg shell formed from secreted components of the parent worm, including vitellocytes and Mehlis‘ gland. In all four species, the egg shell is thick and homogenous in composition within its primary layer. A single highly electron-dense layer constitutes the egg shell of *P. confusus* (Fig. 5), but the other three species have outer or inner layers that are more electron-dense than the central primary layer (Figs. 1-4). In *M. jourdanei* and *M. feliui* , the outermost layer appears membranous (Figs. 3, 4), and possibly constitutes a fixation artefact. An operculum is clearly visible in both ovoviviparous *B. turgida* (Fig. 1, 2), as well as oviparous *M. feliui* (Fig. 3) presented here, and was present in all four species.

Cocoons and extra-egg shell layers

In addition to the egg shell, the eggs of *B. turgida* and *P. confusus* were enclosed by a thick layer external to the egg shell (Figs. 2, 5). This layer consisted of a thick layer of electron-lucent material proximally and an irregular series of electron-dense islands distally, attached to a bounding membrane. For this unique structure, which has not been described in any other trematode, we have coined the term „cocoon“. Our examination did not reveal any information regarding the origin, composition, or function of this unique layer. The uterine lumen outside this cocoon, and outside the egg shells of the *M. feliui* and *M. jourdanei*, contained amorphous material that appeared unassociated with the eggs or with the surrounding uterine epithelium (Figs. 1-5).

Discussion

Few detailed studies have been done on ultrastructural aspects of trematode eggs, probably because the technical difficulties encountered in processing the highly resistant eggs require advanced methodological experience and skill. Despite the paucity of detailed ultrastructural studies, scanning (SEM) and/or transmission electron microscopy (TEM) have been applied to very generalized reports of many species. Thus, the literature contains many very superficial descriptions, primarily of the fully formed egg shells of trematodes that are of some medical or veterinary importance.

General pattern for all trematode eggs

A general overview of the published research on trematode egg structure, including the new data included in this present review, shows clear patterns of common origin and development among all the species examined, in both the Digenea and the Aspidogastrea (Świderski 2011, 2012). The common developmental pattern for each egg of each species, regardless of trematode taxon, consists of an embryo surrounded by an egg shell, with shell material deriving from vitellocyte secretions. This common pattern is very similar to that which occurs in the polylecithal eggs of pseudophyllidean (Korneva 2001), bothriocephalidean (Świderski 1993, 1994b; Mlocicki et al. 2010a), caryophyllidean (Mlocicki et al. 2010b), spathebothriidean (Poddubnaya et al. 2005), and other cestodes (see reviews by Świderski 1994c; Świderski and Mackiewicz 2007; Conn and Świderski 2008), as well as in the phylogenetically more basal gyrocotylideans (Levron et al. 2016), which may be more closely related to trematodes. A similar pattern occurs in least some neoophoran turbellarians (Shinn 1993), though critical comparisons between eggs of neodermatans and more basal Platyhelminthes are still lacking and need further study.

Diversity in trematode egg shell surface ultrastructure

As a technicality, ultrastructural studies are often as regarded as encompassing investigations that employ scanning (SEM) as well as transmission (TEM) electron microscopy. However, true cellular ultrastructure requires either TEM or very specialized modified SEM that is coupled with cryofracturing or other methods to show internal structure. Our comparative study presented here, as well as our earlier related work, employs TEM exclusively or primarily, as this is necessary to discern cellular and subcellular details. Nevertheless, while SEM reveals few if any internal structures, and is not adequate for demonstrating embryogenetic details, it is a much easier and less expensive technique to use for general descriptive studies. Thus many authors have published generalized SEM micrographs of various trematode eggs, so that some information is available on variations in egg shell surface features; in some cases this has been supplemented with TEM of only the egg shell, with no internal cellular features of the embryo or larva available, presumably due to inadequate penetration of the egg shell with fixative and embedding medium. This generalized literature is far too voluminous to cover in this review, but some examples are presented here to demonstrate the variety of digenean egg shell surfaces. Krupa (1974) used SEM and TEM to show shallow ridges covering the surface of *Cryptocotyle lingua* eggs. In an early comparative SEM study of eggs from the three primary human schistosome species, *Schistosoma haematobium*, *Schistosoma japonicum*, and *Schistosoma mansoni*, Ford and Blankespoor (1979) showed interspecific variations in surface microspines. Bundy (1981) presented SEM and some TEM data showing filamentous extensions of the egg shell of *Transverotrema patielense*. Fujino et al. (1989) used SEM and TEM to show complex ridges and folds in the eggs of two species each of the genera *Haplorchis* and *Metagonimus*. Their results were corroborated for these species, as well as other trematode parasites of humans in Thailand (Tesana et al., 1991). Ditrich et al. (1992) used extensive SEM and a single TEM to reveal extensive variation in the surface sculpturing of several medically important heterophyid and opisthorchiid flukes, including some with extensive and complex folding surface extensions; this was corroborated for *Opishorchis viverrini* by Scholz et al. (1992). Krejci and Fried (1994) and Fujino et al. (2000) reported relatively smooth surfaces for the eggs of several

echinostomatid species. Similarly, *Eurytrema coelomatica* eggs seem to have a nearly smooth or only slightly sculptured surface (Pinheiro et al. 2015). Shell sculpturing seems to be a consistent character for any given species, and its distinctive characteristics are identifiable even after thousands of years mummified within their deceases hosts (Shin et al. 2009). This present review is not intended to review all of the known cases of surface SEM studies, as many are parts of cursory case studies in which ultrastructural examination was not the intended goal. Nevertheless, the literature contains much that has not yet been reviewed and synthesized, though the available material is primarily related to trematodes of human and veterinary significnce. Analysis and synthesis of diverse egg shell surface form should be the main objective of a future literature review, of more original research on a broad range of trematode species.

Diversity of trematode intrauterine structures outside the egg shell

This report corroborates and extends information from recent individual reports (Świderski et al. 2013b, 2015a) that *B. turgida* and *P. confusus* possess a unique bilayered structure outside the egg shell, separating it from the uterine lumen contents. This distinctive structure, which we designate as a "cocoon", is of undetermined function, though we suggest that it is protective. No other extra-egg-shell structure has been described from any other trematode.

To the contrary, such structures have been described from many cestodes, although none has the same appearance as those described for the two trematodes. Outside the vitelline capsule and egg shell, or uterine-derived capsule in the case of oligolecithal cestodes (Conn and Świderski 2008), some cestode eggs are further surrounded by diverse uterine, parenchymal, or utero-parenchymal structures (Świderski et al. 2016b). Such complex parental structures appear to be confined to certain members of the cestode order Cyclophyllidea. No trematode to date has been demonstrated to possess such parental structures outside the basic reproductive system. The cocoons reported here for *B. turgida* and *P. confusus* are clearly within the uterus, and thus unlike the somatic protective structures described for cestodes such as *Mesocestoides* spp., nematotaeniids, and davaineids (Conn et al. 1984; Conn 1999; Świderski and Conn 2004; Świderski et al. 2015b). However, they are somewhat similar to the intrauterine capsules present in *Oochoristica anolis* (Conn and Etges 1984; Conn 1985) The two species described in this report are the only trematode species for which any additional layer outside the egg shell has been described. Both of these trematode species belong to the microphalloid family Pleurogenidae, and this cocoon structure may be a feature specific to this family. However, detailed ultrastructure of potential extra-egg intra-uterine structures has been examined for very few trematode families, even in comparison to the number of such studies among the cestodes (Conn 1985; Świderski and Conn 2004). Wittrock (1982) used TEM and SEM to demonstrate a double-layered egg shell for *Quinqueserialis quinqueserialis*, a notocotylid digenean, but the exact nature of the outer layer was not elucidated in that study, and may have been extraneous uterine secretion on the surface of the vitelline-derived egg shell. Detailed ultrastructural studies of the eggs, uterine epithelium, and uterine contents of a wider taxonomic variety of trematodes should be a high priority in the future to understand both phylogenetic and functional morphological aspects of these and similar structures.

Diversity in trematode vitellocyte number and contribution

By far the majority of trematode eggs conform to the polylecithal pattern, in which very many vitellocytes accompany the oocyte and sperm into the ootype, and are subsequently enclosed by the vitelline membrane and finally the egg shell. Indeed, in terms of organelle volume, the primary function of vitellocytes seems to contribution of egg shell precursor materials by secretion from numerous egg shell vesicles (Björkman and Thorsell 1963; Sato et al. 1966; Irwin and Threadgold 1970, 1972; Justine and Mattei 1984; Conn 2000; Meepool et al. 2006). In the plagiorchiid trematode, *Plagitura salamandra*, it was shown that a malformed worm lacking connections between the vitelline ducts and the ootype resulted in normal vitellocytes and normal oocytes and embryos, but no secretion of egg shell materials and thus no formation of egg shells (Conn and Etges 1983). Thus, the reference to "lecithality" among trematodes, and even the use of the term "vitellocyte", are perhaps misnomers, as both terms imply the typically nutritive function of yolk in other animals. Conversely, ectolecithal animals such as the trematodes, cestodes, and all neoophoran Platyhelminthes (see review by Conn 2000), appear to supply nutrients to the developing embryo primarily through the autolysis of blastomeres and their later resorption by differentiating embryonic cells (Świderski et al. 2012). In trematode eggs, the nutritive role is minor, being secondary to egg shell production and perhaps to other functions surrounding fertilization and embryogenesis (Wittrock 1982; Conn and Etges 1983; Holy and Wittrock 1986; Orido 1988; Colhoun et al. 1998; Khampoosa et al. 2011). It has been proposed, with some ultrastructural evidence, that vitelline secretions of at least some oligolecithal cestodes may function in polyspermy prevention (Conn 1988); however, this has been scarcely studied for cestodes, and not at all for trematodes, and thus is a prime subject in need of new research.

Diversity in trematode embryogenesis and larvigenesis

The basic events of cleavage and embryonic development are remarkable uniform in all species of neodermatans that have been studied. In all, the early macromeres break away from the blastomere mass to form the syncytial outer envelope. This is followed by a similar separation of mesomeres from the remaining mass to form the syncytial inner envelope in all groups studied thus far except the aspidogastreans, which form a discrete inner vitelline syncytium and no inner embryonic envelope; this unusual pattern has been described for *Aspidogaster limacoides*, only one aspidogastrean thus far studied in detail (Świderski et al. 2011, 2012). Possibly similar to *A. limacoides*, Levron et al. (2016) described the cestodarian *Gyrocotyle urna* as having only a single embryonic envelope, at least in early development, but cautioned that two envelopes may differentiate in later stages than those examined in their study. This point of variation among embryonic envelopes of neodermatan taxa needs further study of more species and at more developmental stages.

For all neodermatan taxa studied to date, the embryo proper is thus formed exclusively from the remaining micromeres, and develops into the hexacanth (Cestoda), decacanth (Cestodaria), miracidium (Digenea), oncomiracidium (Monogenea), or cotylocidium (Aspidogastrea) larva (Burt 1987; Conn 2000; Conn et al. 2007; Levron et al. 2016). The blastomere mass and later embryonic stages differentiate into the larva at a rate and in a location that varies among different taxa. In cestodes, the usual pattern results in a fully formed

hexacanth larva within the parent worm's gravid uterus, thus constituting ovoviviparous development (Conn and Świderski 2008). Similarly, monogenean oncomiracidia frequently are ovoviviparous (Tinsley 1993; Cable and Tinsley 1991; Cable et al. 1997), as is the aspidogastrean *A. limacoides* (Świderski et al. 2012). However, among neodermatan flatworms, viviparity has been confirmed only for a few gyrodactylid monogeneans (Cable et al. 1996), and never for any digenean. Among digeneans, some are ovoviviparous, including *B. turgida* and *M. jourdanei* described here, along with a few others studied recently (Swiderski et al. 2017a, 2017b). However, many others, like *P. confusus* and *M. feliui* described here, are oviparous. Ovoviparity may be the most common pattern among digeneans, whether the eggs develop in the external environment (Born-Torrijos et al. 2017), or within the body of the definitive hosts, such as the extensively studied schistosomatids (Eklu-Natey et al. 1982; Neill et al. 1988; Ashton et al. 2001; Jones et al. 2008; Jurberg et al. 2009; Świderski 1984, 1985, 1986, 1988, 1994a; Świderski et al. 1980).

Conclusions

Our new data have shown remarkable diversity among the eggs of four closely related microphalloidean trematodes, from similar habitats, and all native to the Eurasian contiguous land mass (see Table 1). In this case, variation in definitive host (amphibian vs. mammal), host habitat (freshwater vs. terrestrial) and trematode family are not the apparent bases of these variations. These new data reflect the growing understanding of trematode eggs as being very diverse in structure and developmental timing of larvigenesis, while conserving much basic similarity in terms of fundamental embryogenetic patterns and essential contributions from the female reproductive system of the parent worm. Clearly, a broad taxonomic range of trematode eggs is in need of much more detailed study that can only be accomplished through electron microscopy of cellular and subcellular characteristics. As we have observed in the past two decades with cestodes (reviewed by Conn and Świderski 2008), further studies of trematode eggs are likely to reveal that, contrary to past assumption, the microscopic but highly complex and varied eggs are likely to provide much greater insight into the population and community dynamics of the trematodes and their gastropod and vertebrate hosts.

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661 **Table 1.** Comparative host-parasite data and ultrastructure of the intrauterine eggs in some
662 European Microphalloidea.

PARASITE AND HOST DATA				
DIGENEAN SPECIES	***Maritrema feliui***	***Mediogonimus jourdanei***	***Brandesia turgida***	***Prosotocus confusus***
DIGENEAN FAMILY	Microphallidae	Prosthogonimidae	Pleurogenidae	Pleurogenidae
DEFINITIVE HOST	*Crocidura russula*	*Myodes glareolus*	*Pelophylax ridibundus*	*Pelophylax lessonae*
HOST SYSTEMATICS	Mammalia: Soricimorpha	Mammalia: Rodentia	Amphibia: Anura	Amphibia: Anura
HOST HABITAT	Terrestrial	Terrestrial	Aquatic	Aquatic
HOST LOCALITY	La Ricarda, Barcelona (Spain)	Nature Reserve of Py (France)	Rybinsk Reservoir (Russia)	Bugskiy landscape reserve (Belarus)
ULTRASTRUCTURAL DATA				
EGG SHELL TYPE	Oligolecithal	Polylecithal	Polylecithal	Polylecithal
EGG SHELL ORIGIN	Vitellocytes and Mehlis	Vitellocytes and Mehlis	Vitellocytes and Mehlis	Vitellocytes and Mehlis
EXTRA-EGG SHELL "COCOON"	No	No	Yes	Yes
"COCOON" ORIGIN	N/A	N/A	Undetermined	Undetermined
DEVELOPMENTAL STAGE	Early embryo	Fully formed miracidium	Fully formed miracidium	Early embryo
OUTER ENVELOPE	Macromeres persist into later development	Macromere nuclei degenerate early	Macromere nuclei degenerate early	Macromere nuclei degenerate early
INNER ENVELOPE	Mesomere syncytium?	Mesomere syncytium	Persistent mesomere syncytium	Mesomere syncytium?

663 N/A not applicable

664

Abbreviations to all figures: AG – apical gland, Bl – blastomere, C – cilia, DI – dense islands of electron-dense material at peripheral membrane of external, electron lucent cocoon, DB – dense bodies, DM – degenerating micromeres, ES – egg shell, FA – possible fixation artefacts, FCD - areas of focal cytoplasmic degradation, GER-B – granular endoplasmic reticulum body, gl – glycogen, α-gl – alpha-glycogen rosettes, β-gl – beta-glycogen particles, HCh – heterochromatin islands, L – lipid droplets, LG – lateral gland, m – mitochondria, MaN – macromere nucleus, Mi – miracidium, MiG – miracidial gland, N – nucleus, np – nuclear pore, Op – operculum, SG – secretory granules, Sp – spermatozoa, SR – striated rootlets, TL – transparent layer of external electron-lucent cocoon.

Fig. 1A and B. TEM micrographs of differentiating eggs of *Brandesia turgida.* **A** – Low power TEM micrograph illustrating the general topography of a mature intrauterine egg in the distal part of the uterus. Note: (1) an outer anucleate layer, situated externally to the egg shell and forming a thick cocoon composed of a transparent, electron-lucent substance; and (2) numerous small, electron dense islands irregularly dispersed around the egg surface and attached to its peripheral membrane. **B** – Operculated pole of a mature, intrauterine egg showing details of the three egg envelopes, the operculum and the apical part of a ciliated miracidium. Note: (1) the very close contact between the operculum and the flat discs of the peripheral islands of electron dense material situated at the surface of the transparent, electron-lucent cocoon.

Fig. 2A and B. TEM micrographs of the apical region of differentiating eggs of *Brandesia turgida.* **A** – Anterior part of a mature egg containing a fully formed, ciliated miracidium. Note: (1) the characteristic apical gland with large nucleus containing heterochromatin islands and (2) numerous cilia and their striated rootlets embedded in the peripheral layer of the miracidium providing an evidence for the miracidial maturity in the intrauterine eggs. **B** – High power micrograph showing details of the apical part of ciliated miracidium. Note numerous secretory granules and elongated mitochondria in the apical gland cytoplasm and the several cross section of cilia and oblique section of their striated ciliary rootlets.

Fig. 3A, B, C and D. TEM micrographs of differentiating eggs of *Maritrema feliui.* **A and B** – Low-power TEM micrographs of two eggs with early embryos comprising only a very few blastomeres but already exhibiting the degenerating, pycnotic nuclei of micromeres undergoing apoptosis. **C and D** – TEM micrographs showing details of the apical pole of differentiating eggs. Note: (1) a well-defined operculum in the shell of each egg, (2) numerous spermatozoa in the uterine lumen, frequently surrounding the egg shell surfaces, and (3) a peeling of the egg shell surface, possibly a fixation artefact.

Fig. 4A, B, C and D. TEM micrographs of mature eggs of *Mediogonimus jourdanei*. **A** – The general topography of the mature egg. Note: (1) peeling of the outer surface of the egg shell,

possibly representing fixation artefacts, (2) flattened nucleus of the mesomere and several spherical lipid droplets in the inner envelope cytoplasm, and (3) a great number of cilia which occupy all the space between the egg envelopes and miracidium. **B** – Part of the egg showing the miracidium surrounded by numerous cilia and miracidial gland with numerous electron-dense secretory granules in the central part of the micrograph. **C** – High-power TEM micrograph showing details of the miracidial gland nucleus. Note: (1) numerous large heterochromatin islands situated around the nuclear membrane and in the central part of the nucleoplasm, and (2) numerous nuclear pores around the nuclear membrane. **D** – Peripheral cytoplasm of the miracidium. Note: (1) several electron-dense secretory granules of different sizes, (2) numerous cross-sectioned at different levels miracidial cilia, and (3) heavy accumulation of alpha-glycogen rosettes and beta-glycogen particles.

Fig. 5 A and B. TEM micrographs of differentiating eggs of *Prosotocus confusus*. **A** – Low-magnification micrograph illustrating the general topography of three differentiating eggs in the proximal part of the uterus. Note: (1) an outer anucleate layer situated externally to the egg shell of each egg, forming a thin layer of cocoon composed of a transparent, electron-lucent substance; (2) numerous small, electron-dense islands irregularly dispersed around the egg surface, all attached to its peripheral membrane; and (3) dense bodies representing mainly degenerating early blastomeres and/or their nuclei undergoing apoptosis. **B** – Enlarged micrograph showing entire egg in early stage of embryonic development showing already three large areas of focal degradation and adjacent to a few small GER bodies, representing evident signs of cellular apoptosis.

Table 1. Comparative host-parasite data and ultrastructure of the intrauterine eggs in some European Microphalloidea.

PARASITE AND HOST DATA				
DIGENEAN SPECIES	***Maritrema feliui***	***Mediogonimus jourdanei***	***Brandesia turgida***	***Prosotocus confusus***
DIGENEAN FAMILY	Microphallidae	Prosthogonimidae	Pleurogenidae	Pleurogenidae
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HOST SYSTEMATICS	Mammalia: Soricimorpha	Mammalia: Rodentia	Amphibia: Anura	Amphibia: Anura
HOST HABITAT	Terrestrial	Terrestrial	Aquatic	Aquatic
HOST LOCALITY	La Ricarda, Barcelona (Spain)	Nature Reserve of Py (France)	Rybinsk Reservoir (Russia)	Bugskiy landscape reserve (Belarus)
ULTRASTRUCTURAL DATA				
EGGSHELL TYPE	Oligolecithal	Polylecithal	Polylecithal	Polylecithal
EGGSHELL ORIGIN	Vitellocytes and Mehlis	Vitellocytes and Mehlis	Vitellocytes and Mehlis	Vitellocytes and Mehlis
EXTRA-EGGSHELL "COCOON"	No	No	Yes	Yes
"COCOON" ORIGIN	N/A	N/A	Undetermined	Undetermined
DEVELOPMENTAL STAGE	Early embryo	Fully formed miracidium	Fully formed miracidium	Early embryo
OUTER ENVELOPE	Macromeres persist into later development	Macromere nuclei degenerate early	Macromere nuclei degenerate early	Macromere nuclei degenerate early
INNER ENVELOPE	Mesomere syncytium?	Mesomere syncytium	Persistent mesomere syncytium	Mesomere syncytium?

N/A not applicable

Figure 1

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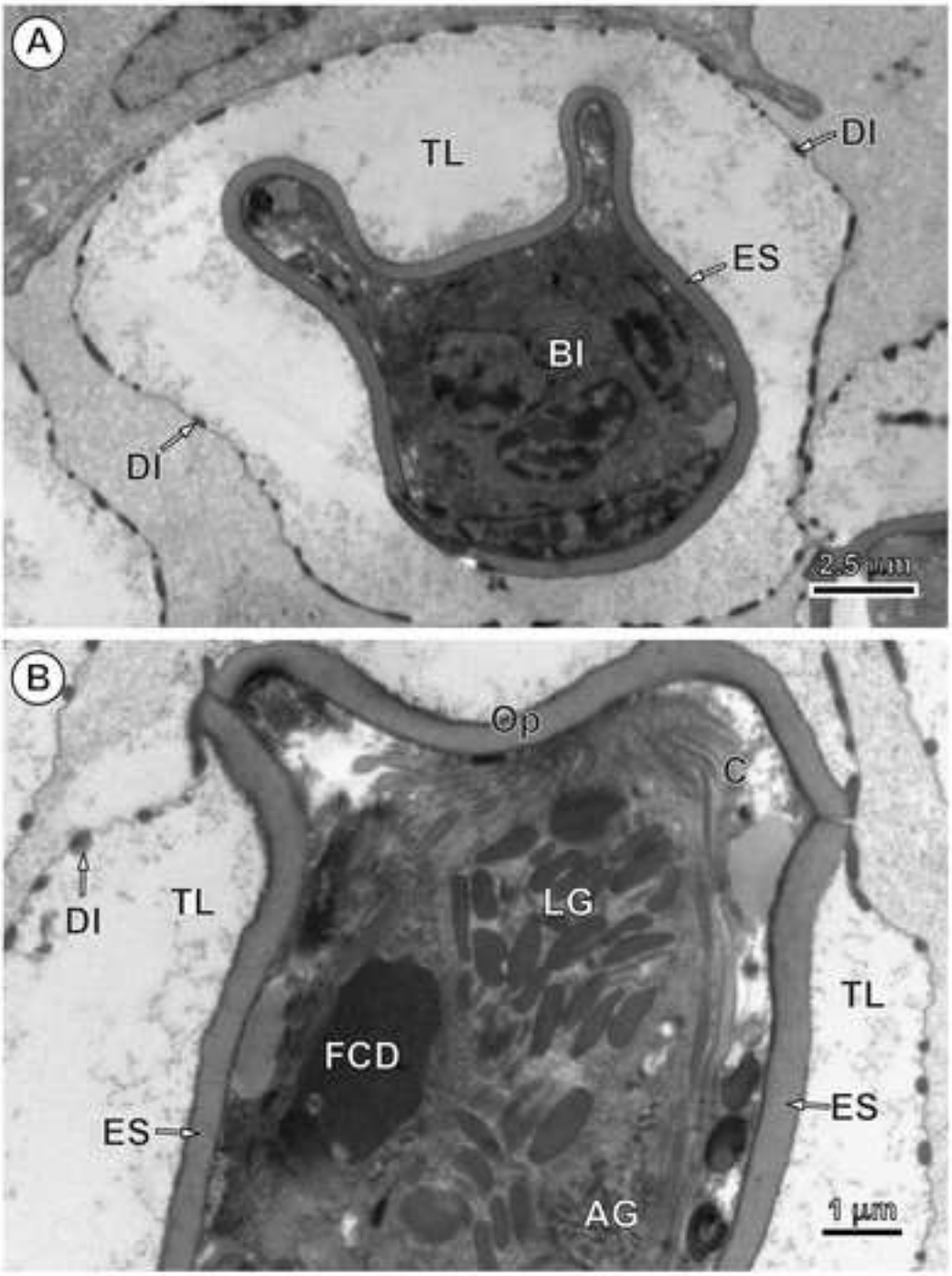

Figure 2

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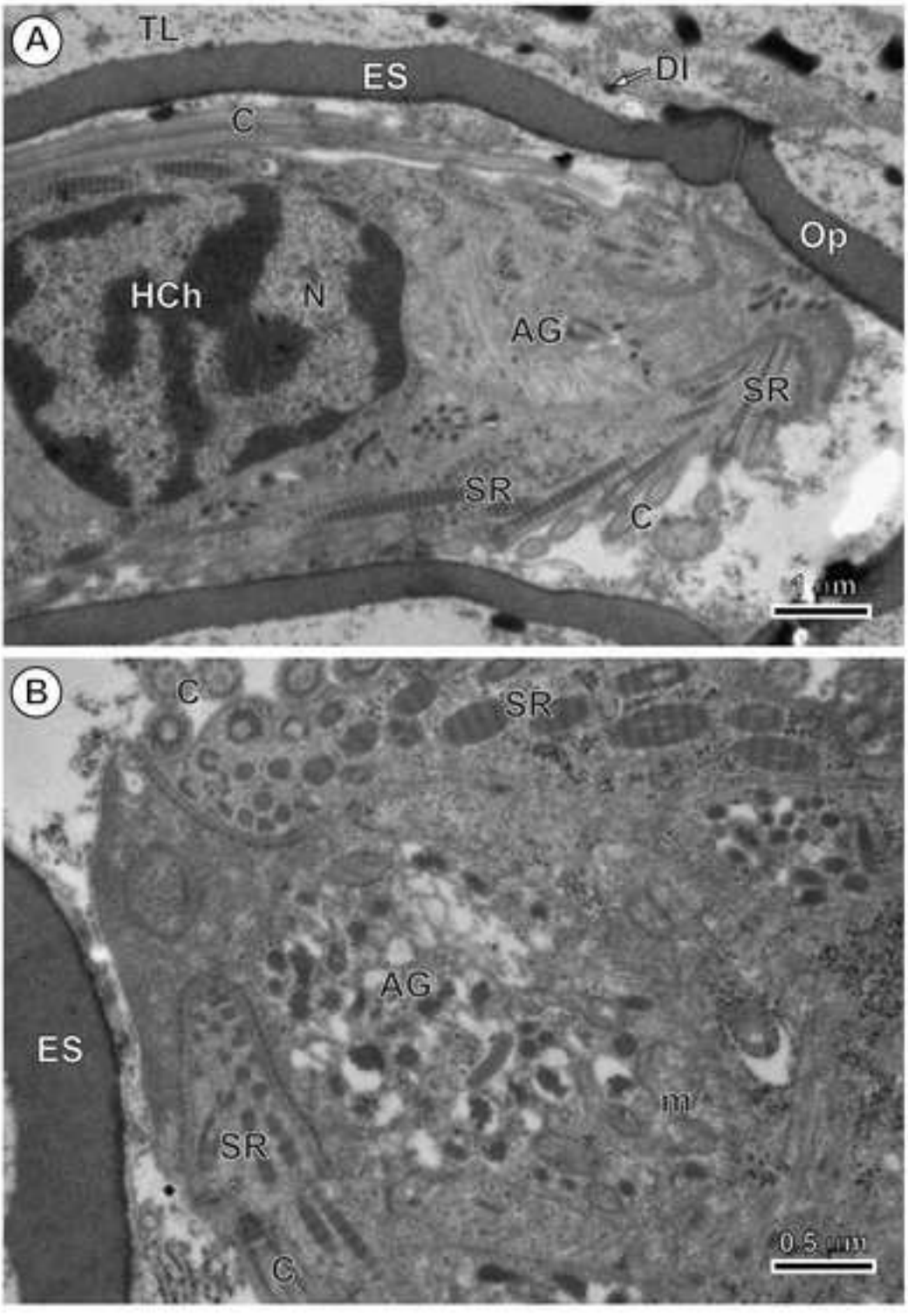

Figure 3

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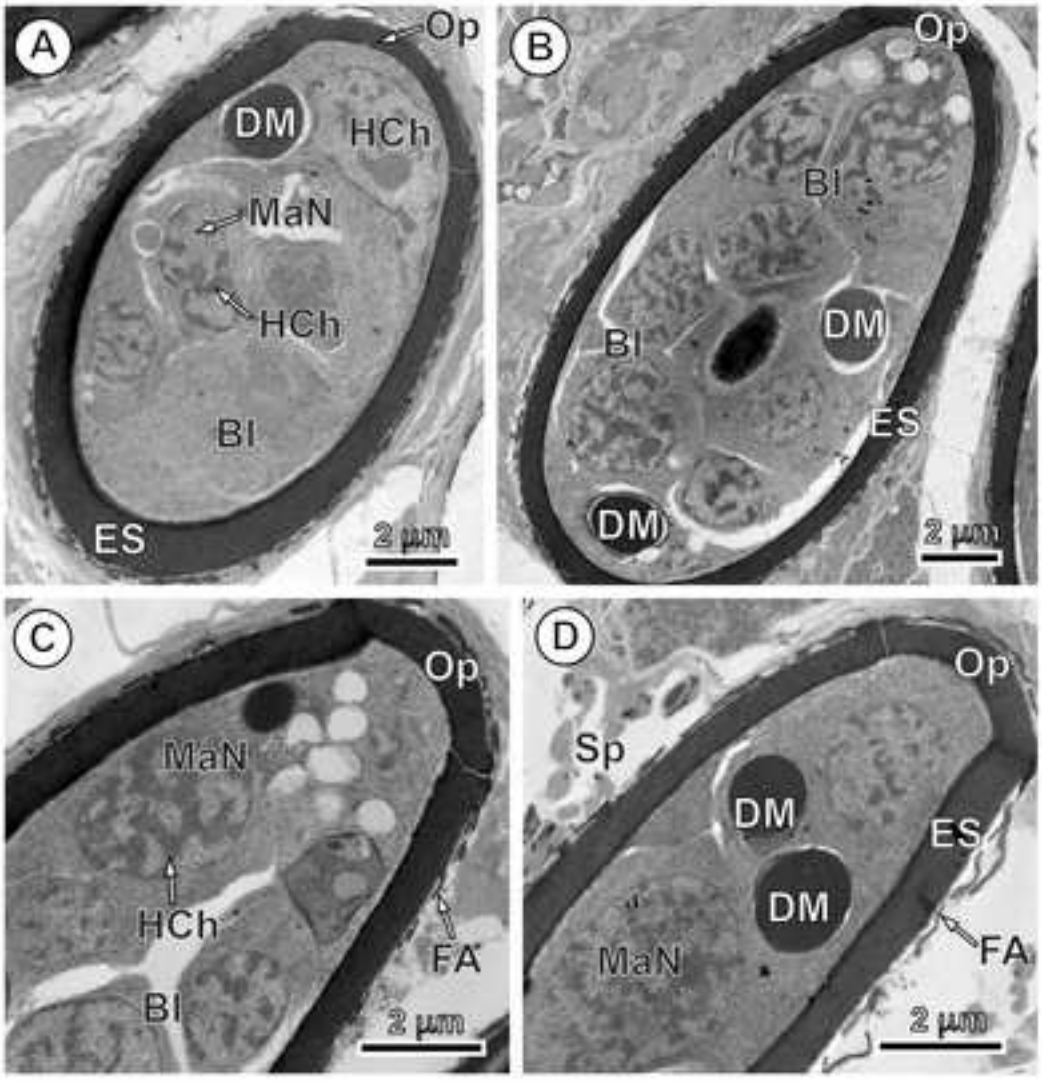

Figure 4

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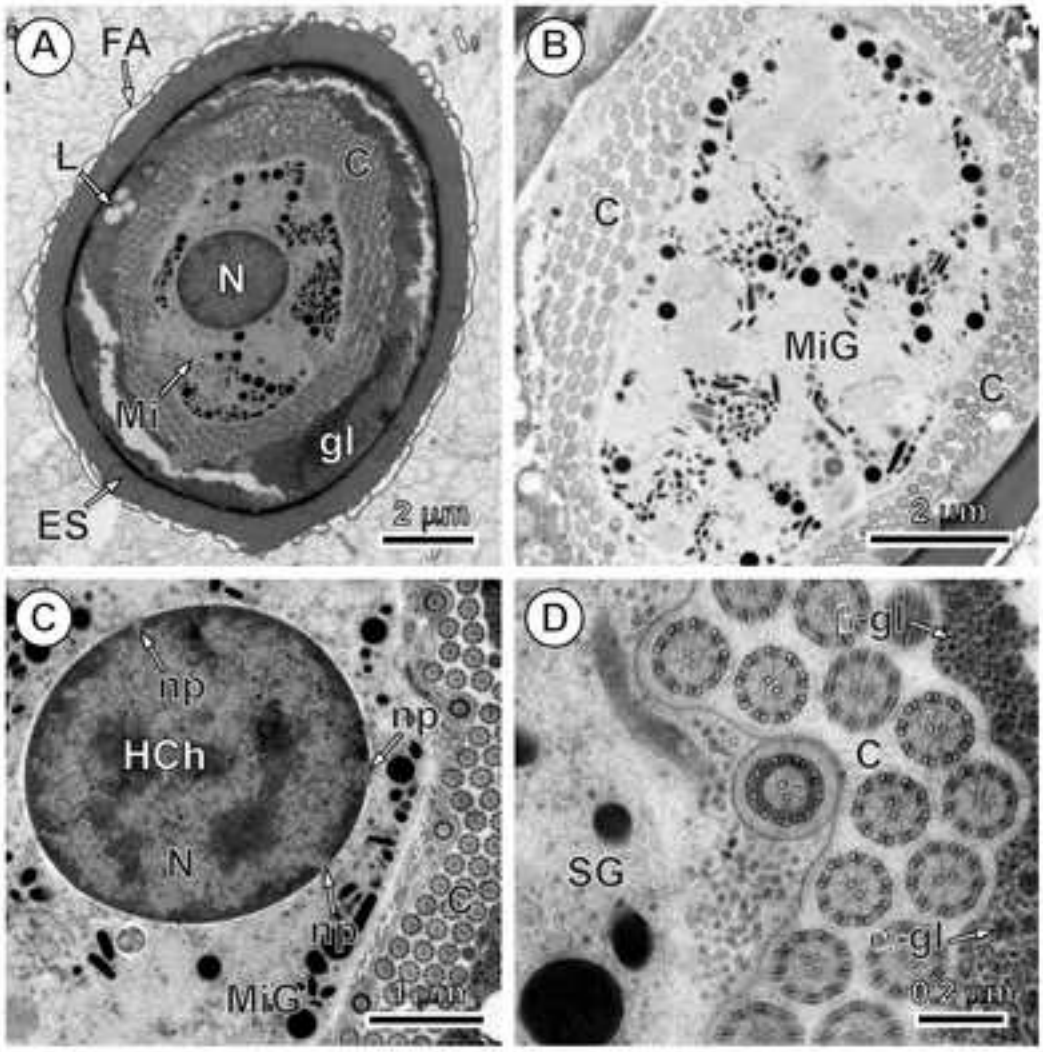

Figure 5

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