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Secretion of extracellular vesicles during ontogeny of the tapeworm *Schistocephalus solidus*

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Abstract: We provide the first ultrastructural evidence of the secretion of extracellular vesicles (EVs) across all parasitic stages of the tapeworm *Schistocephalus solidus* (Müller, 1776) (Cestoda: Diphyllobothriidea) using a laboratory life cycle model. We confirmed the presence of EV-like bodies in all stages examined, including the hexacanth, procercoids in the copepod, *Macrocyclops albidus* (Jurine, 1820), plerocercoids from the body cavity of the three-spined stickleback, *Gasterosteus aculeatus* Linnaeus, and adults cultivated in artificial medium. In addition, we provide description of novel tegumental structures potentially involved in EV biogenesis and the presence of unique elongated EVs similar to those previously described only in *Fasciola hepatica* Linnaeus, 1758 (Trematoda), *Hymenolepis diminuta* (Rudolphi, 1819) (Cestoda), and *Trypanosoma brucei* Plimmer et Bradford, 1899 (Kinetoplastida).

Keywords: EVs, Cestoda, ESP, TEM, ultrastructure, novel tegumental structures

This article contains supporting material (**Supplementary Fig. 1**) online at http://folia.paru.cas.cz/suppl/2023-70-003.pdf

Parasitic helminths are known to be important pathogens of vertebrates, including humans, and some of them are even capable of manipulating their host through excretory-secretory products (ESPs) (Robinson et al. 2010, McSorley et al. 2013). Moreover, extracellular vesicles (EVs) have recently been shown to be essential components of the ESPs of various cells and organisms, including parasitic helminths (Yáñez-Mó et al. 2015). Previously, EVs were considered exclusively waste expulsion, but recently they have been observed to also contain active proteins, nucleic acids and lipids that facilitate parasite survival and proliferation in the host (Marcilla et al. 2014, Hessvik et al. 2018).

To date, the production of EVs by helminths has been observed mainly in helminths of mammals (cestodes, trematodes, and nematodes) (Bennett et al. 2020, Boysen 2020, Cavallero et al. 2022). Moreover, the available data are almost exclusively limited to a single developmental stage, mainly adult or one type of larva or metacestode, and there are practically no data on the different ontogenetic stages of helminths with complex life cycles. An exception are studies on the trematode *Fasciola hepatica* Linnaeus, 1758 that compared EVs production in three developmental stages (embryonated egg, excysted metacercariae and adult) (Sánchez-López et al. 2020, Trelis et al. 2022).

However, to date, no single study has been able to observe EVs secretion in all developmental stages of a given parasite. Furthermore, there are no records of EVs production in non-vertebrate intermediate host. Moreover, a detailed understanding of EVs excretion throughout the whole helminth life cycles could lead to a better control of parasite infections and elucidation of the mechanisms of parasite-host interactions and life-cycle evolution.

Stage specificity of EVs populations has already been observed in the filarial nematode *Brugia malayi* (Brug, 1927), where EVs secretion and composition appear to be sex- and stage-specific (Harischandra et al. 2018). However, a comparison of EVs production in different developmental stages of tapeworms is still missing. Moreover, in most cases, only the species of medical or veterinary importance have been studied, while there has not been a single study on non-mammalian species (Fratini et al. 2020, Wititkornkul et al. 2021).

One of the iconic and best known non-mammalian model tapeworm is *Schistocephalus solidus* (Müller, 1776) (Diphyllobothriidea), which uses fish-eating birds as a definitive host and two intermediate hosts, namely freshwater copepods and three-spined sticklebacks (Barber 2013). The procercoid in copepods and the plerocercoid in sticklebacks are known to manipulate the behaviour of their intermediate hosts to increase the probability of transmission to the next host, but the mechanism of manipulation remains unknown (Urdal et al. 1995, Jakobsen and Wedekind 1998, Barber 2013). Moreover, the life cycle of this tapeworm

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has been extensively studied in detail and protocols have been established for their *in vivo* and *in vitro* cultivation (Jakobsen et al. 2012, Kalbe et al. 2016).

While the ultrastructure of plerocercoids and adult *S. solidus* has been studied extensively, information on their EVs production, similar to other developmental stages or other non-mammalian tapeworm, is unknown (Charles and Orr 1968, Hopkins et al. 1978, Threadgold and Hopkins 1981, Kuperman 1988, Levron et al. 2013, Yoneva et al. 2018). The presence of vesicle-like structures described in processes of pinocytosis and exocytosis was observed in plerocercoids and adults stages of *S. solidus* by Hopkins et al. (1978) and Threadgold and Hopkins (1981). Scharsack et al. (2013) demonstrated a significant immunomodulatory effect of *S. solidus* ESP on the stickleback immune system. In addition, proteomic studies of *S. solidus* ESP have revealed proteins that are thought to play a role in influencing host behaviour (Berger et al. 2021). However, the EVs production and their biogenesis have never been studied in any non-mammalian helminth, including this important model species.

Herein, we confirmed for the first time the secretion of EVs in four developmental stages of the *S. solidus* tapeworm, including the hexacanths in eggs, procercoids in copepods, plerocercoids from three-spined sticklebacks, and adults after cultivation in artificial medium. In addition, we compared EVs secretion between different mature proglottids of adult *S. solidus*.

MATERIALS AND METHODS

Experimental life cycle

All the studied developmental stages of *Schistocephalus solidus* were obtained from experimentally infected copepods *Macrocyclops albidus* (Jurine) and three-spined sticklebacks *Gasterosteus aculeatus* Linnaeus under laboratory conditions. Eggs of *S. solidus* and the initial culture of copepods were kindly provided by Tobias Lenz (Max Planck Institute for Evolutionary Biology, Plön, Germany). Three-spined sticklebacks were provided by a private breeder (Postoloprty, Czech Republic). Cultures of copepods and three-spined stickleback were kept and bred in separate tanks in a controlled environment (stale water, 18°C, day/night light cycle) at the Laboratory of Helminthology (Institute of Parasitology, Biology Centre, Czech Academy of Sciences, Czech Republic).

The cultivation protocol was modified based on Wedekind et al. (1998) and Weinreich et al. (2014). Freshly collected eggs were incubated for a minimum of three weeks in the dark at 4°C. Thereafter, eggs were placed in a Petri dish with tap water and incubated in the daylight at a stable temperature (17 or 22 °C). After approximately three days free-swimming coracidia hatched from eggs. Uninfected cultured copepods were transferred to 24-well plates with tap water and co-cultivated with one or two newly hatched coracidia for 24 hours, after which the copepods were kept in a separate culture to establish infection.

Three weeks post-infection, fully developed procercoids were observed in the body cavity of alive copepods under a light microscope and subsequently used for infection of three-spined sticklebacks. Thereafter, 1–2 copepods containing 1–2 procercoids were transferred to the 1l containers with tap water and one stickleback each for 24 hours of cultivation. One day post-cocultivation, fish were placed into aquaria and water of each cultivation container was carefully checked to determine if all copepods were eaten by fish.

After three months, the fish were dissected, recovered plerocercoids were carefully washed several times in PBS and RPMI-1640, paired up and finally transferred to 50 ml falcon tubes with RPMI-1640 cultivation medium pre-warmed to 37°C and supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin. The cultivation medium was collected daily and substituted with a fresh one for a week. The recovered medium was then centrifuged (2,000 g for 5 min) to clear the medium of eggs and cell debris. Pelleted eggs were washed with stale tap water, stored as described above and used in later infections.

Purification of extracellular vesicles

In total eight adults (duplicates of four) were used for EVs collection. The centrifuged cultivation medium obtained from the culture of freshly obtained plerocercoids as well as gravid worms, cleared of eggs and debris, was concentrated using a stirred cell (Amicon®, Miami, USA) with 10 kDa ultrafiltration discs (Millipore, Burlington, USA) to the final volume of 4 ml. Protein concentration was determined using Qubit 4 fluorometer (Invitrogen™, USA). Excretory-secretory product positive daily yields were pooled together and treated as a single sample. The collected ESPs were then ultracentrifuged at 120,000 g for 1 hour. Pelleted EVs were then washed two times with PBS at the same speed to further remove contaminants and clear aggregates. Finally, washed EVs were resuspended in 200 µl of PBS. The size and frequency distribution of individual vesicles were determined using a Nanosight NS300 (Malvern, UK). For Nanosight, samples were diluted (1 : 1000) in PBS, observed with sCMOS camera under room temperature. Five replicates were obtained and analysed using NTA software version 3.4.

Transmission electron microscopy (TEM)

Unembryonated eggs, infected copepods, freshly recovered plerocercoids (middle region) and different regions (anterior, middle and posterior mature proglottids) of adults were fixed for ultrastructural studies and visualised following the protocol of Mazanec et al. (2021). The eggs were isolated in cellulose capillary tubes using the method of Müller-Reichert et al. (2007) and then subjected to high-pressure freezing followed by freeze substitution (HPF/FS) using the rapid transfer system of the EM PACT2 (Leica Microsystems, Wetzlar, Germany) and frozen at a pressure of 2.1 kbar. Infected copepods were fixed in cold (4 °C) 1.5% glutaraldehyde and 1.5% paraformaldehyde solutions in 0.1M Hepes (pH 7.4), then stored for four weeks at 4 °C and processed for standard TEM following the protocol of Yoneva et al. (2015). Selected sections of plerocercoids and adults were fixed by HPF/FS. All samples were then visualised using a JEOL JEM-1400F transmission electron microscope (Jeol, Tokyo, Japan) operated at an accelerating voltage of 120 kV. TEM images were recorded using a bottom-mounted CMOS camera XAROSA (EMSIS, Münster, Germany).

The purified EVs were visualised by the classical TEM using methods of negative staining (NS) and Cryo-TEM following the protocol of Mazanec et al. (2021). For NS vesicles were deposited on glow-discharged carbon-coated copper grids, stained with

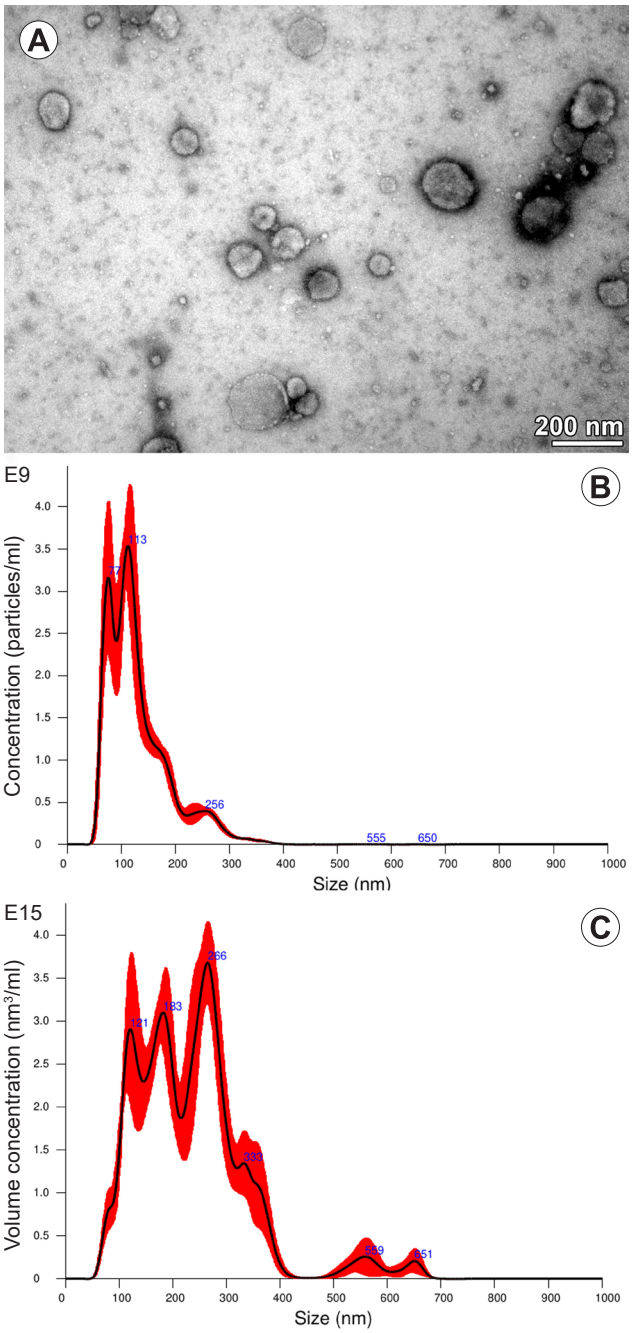

Fig. 1. Evidence of secretion of extracellular vesicles (EVs) during the sexual maturation of *Schistocephalus solidus* (Müller, 1776). **A** – micrographs from the Cryo-TEM of EVs isolated by ultracentrifugation from the cultivation medium with adults; **B**, **C** – Nanosight-based measurements of abundance (B) and volume (C) of different EV subpopulations present in the isolated EVs.

1.5% uranyl acetate and visualised using a JEOL JEM–2100F. For Cryo-TEM, the purified EVs were loaded on holey carbon grids and rapidly frozen with plunge freezer LEICA EM GP2 (Leica Microsystems) and visualised by JEOL JEM-2100F. Images were taken with a Gatan K2 Summit direct electron detector. Obtained micrographs were analysed using ImageJ software (NIH, http://imagej.nih.gov/ij/) and TEM Exosome Analyser (CBIA, https://cbia.fi.muni.cz/software/exosomeanalyzer.html).

RESULTS

Using TEM, EV-like bodies were observed on the surface and also in the tegument of all observed developmental stages of *Schistocephalus solidus* (Figs. 2D,F, 3C,D). In addition, the cultivation medium containing the developing adults contained ESP from the second to the fifth day of cultivation, which correlates with the period of egg production. Both Nanosight and TEM micrographs of the purified medium confirmed multiple size subpopulations of EVs from cultivated adults (Fig. 1). The data obtained correlate well with those from the TEM study of EVs on the surface of cultivated adults.

Hexacanth

The TEM of unembryonated *S. solidus* eggs fixed by the HPF/FS method (Supplementary Fig. 1A) provides evidence for the presence of EV-like bodies (diameter 100–160 nm) under the egg shell in the outer envelope (Fig. 2A, B). Moreover, endosomal multivesicular bodies (MVBs) bearing intraluminal vesicles (ILVs) of the same size and structure as the EV-like bodies in the lumen and under the egg shell were observed in the tissue of the forming hexacanth (Fig. 2C,D). No EVs were observed to pass through the outer shell.

Procercoid

The procercoids were observed by TEM in the thoracic region of infected copepods (Supplementary Fig. 1B). A dense ESP-like layer (1000–1500 nm thick) was observed surrounding the surface of the studied procercoid, covered by numerous acicular filitriches (Fig. 2E). EV-like bodies of different sizes (70–150 nm) were rarely observed in the ESP layer and among the filitriches (Fig. 2F). A thin syncytial tegument (± 650 nm) was observed underlying the surface. A small number of dense bodies (DB) were observed in the tegument, but their origin is unclear (Fig. 2G). Furthermore, MVBs bearing a single ILV were also occasionally found (Fig. 2G). Vesicular bodies of similar structure and size (100–160 nm) to the vesicles on the surface were present in the distal cytoplasm (Fig. 2F, G). No clear evidence of their release into the surroundings was observed.

Plerocercoid

The surface of plerocercoids was covered by a dense layer of capilliform filitriches that were surrounded by EVs of different sizes (50–70 nm) (Fig. 3A). Several unique elongated forms of EVs (± 40 by 240 nm) were observed (Fig. 3C). The distal cytoplasm of the tegument was filled with numerous vesicle-like bodies of unclear origin (Fig. 3A). Multiple MVBs bearing a single ILV have been frequently detected throughout the cytoplasm (Fig. 3E). Furthermore, the size of the observed ILVs (± 60 nm in diameter) corresponds to that of the EVs on the surface. Moreover, DBs appear to originate in mitochondria and being distributed throughout the rest of the tegument (Supplementary Fig. 1C). Some of these DBs were shown to fuse with the surface membrane and possibly discharge into the surround-

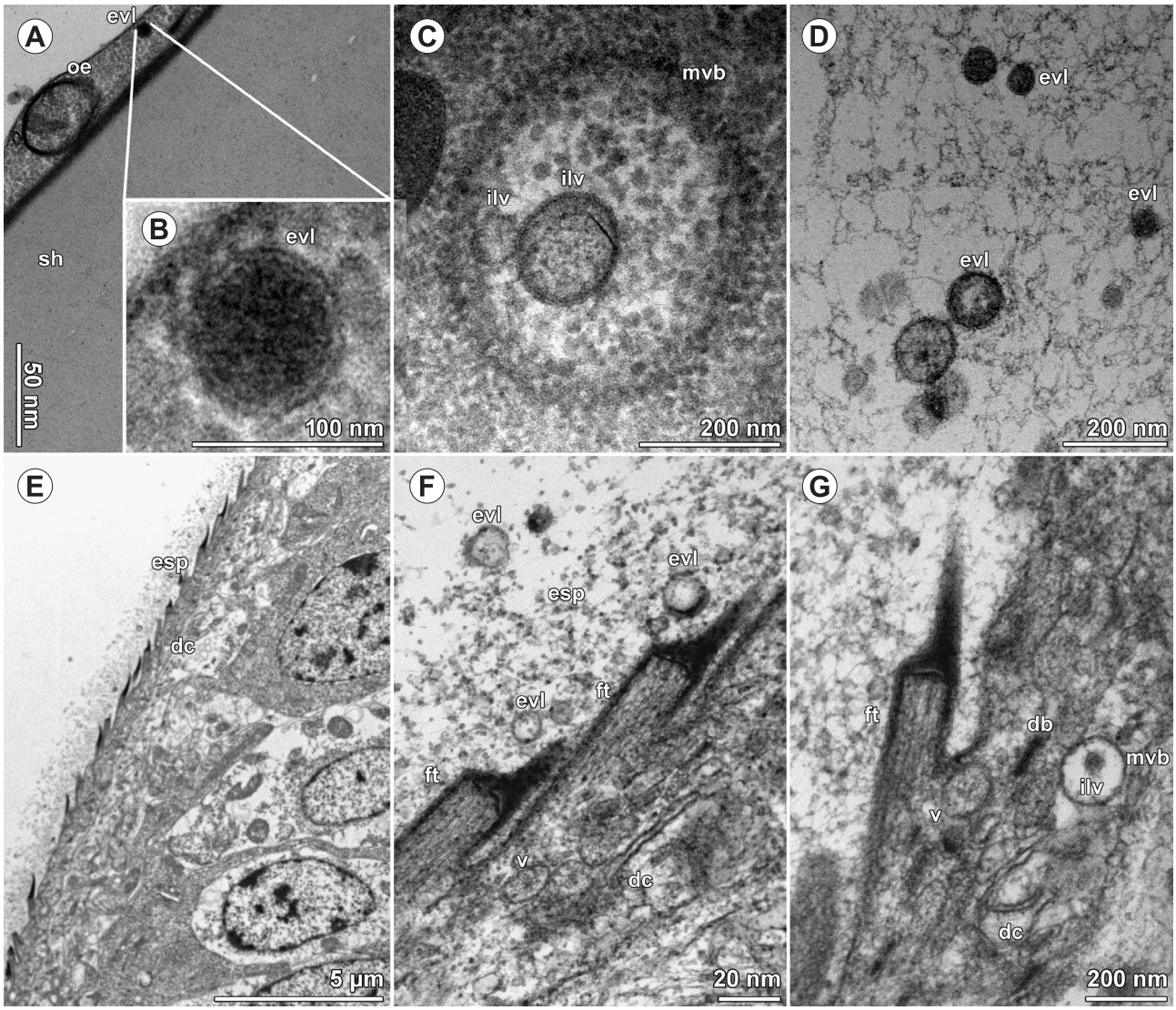

Fig. 2. Transmission electron microscopy of the egg and procercoid stages of *Schistocephalus solidus* (Müller, 1776). **A** – solid structure of the egg shell; **B** – detail of an extracellular vesicle (EV)-like body in the underlying outer envelope; **C** – multivesicular bodies (MVB)-like structure with two distinct intraluminal vesicles (ILVs) of different sizes observed in the tissue of the developing embryo; **D** – EV-like bodies of the same structure and size as the observed ILVs in the lumen of the developing egg; **E** – procercoid of *S. solidus* in the infected copepod with a distinct layer of excretory-secretory products (ESP) surrounding the surface layer; **F** – detail of the EV-like bodies in the ESP-layer; **G** – vesicles/MVB-like structures in the underlying distal cytoplasm. *Abbreviations*: db – dense body; dc – distal cytoplasm; esp – excretory-secretory products; evl – extracellular vesicle-like body; ilv – intraluminal vesicle; mvb – multivesicular body; ft – filithrix; oe – outer envelope; sh – shell; v – vesicle.

ings (Fig. 3C). Electro-lucent bodies (LB) had a similar shape to DB, with no clear origin (Fig. 3A).

Adult

The tegument ultrastructure of adults was similar to that of plerocercoids (Fig. 3B). EVs of varying sizes (40–120 nm) and shape were frequently observed among capilliform filitriches throughout the whole surface (Fig. 3D). As in plerocercoids, a small number of unique elongated EVs have been observed (Fig. 3D). The anterior and middle regions of the adult strobila, in contrast to the posterior region, exhibited more disturbances in surface structure (e.g., filitriches length, surface protuberances, phagosome formation, unspecific tegument crypts and infolds, etc). Some of these elements suggest a role in the EV secretion/absorption (Supplementary Fig. 1D,E). Furthermore, surface protuberances were observed to form chains of EV-like structures that break up into separate EVs (Fig. 3F). The distal cytoplasm of the syncytial tegument contains the same organelle composition in all sections. MVBs of different density bearing ILVs similar to the EVs observed on the surface were also detected (Fig. 3B,G). Furthermore, a small number of MVB subtypes bearing elongated EVs was detected (Supplementary Fig. 1F).

DISCUSSION

The tegumental vesicles of *Schistocephalus solidus* have been described superficially several times (Hopkins et al. 1978, Threadgold and Hopkins 1981, Yoneva et al. 2018). However, a clear description and confirmation of

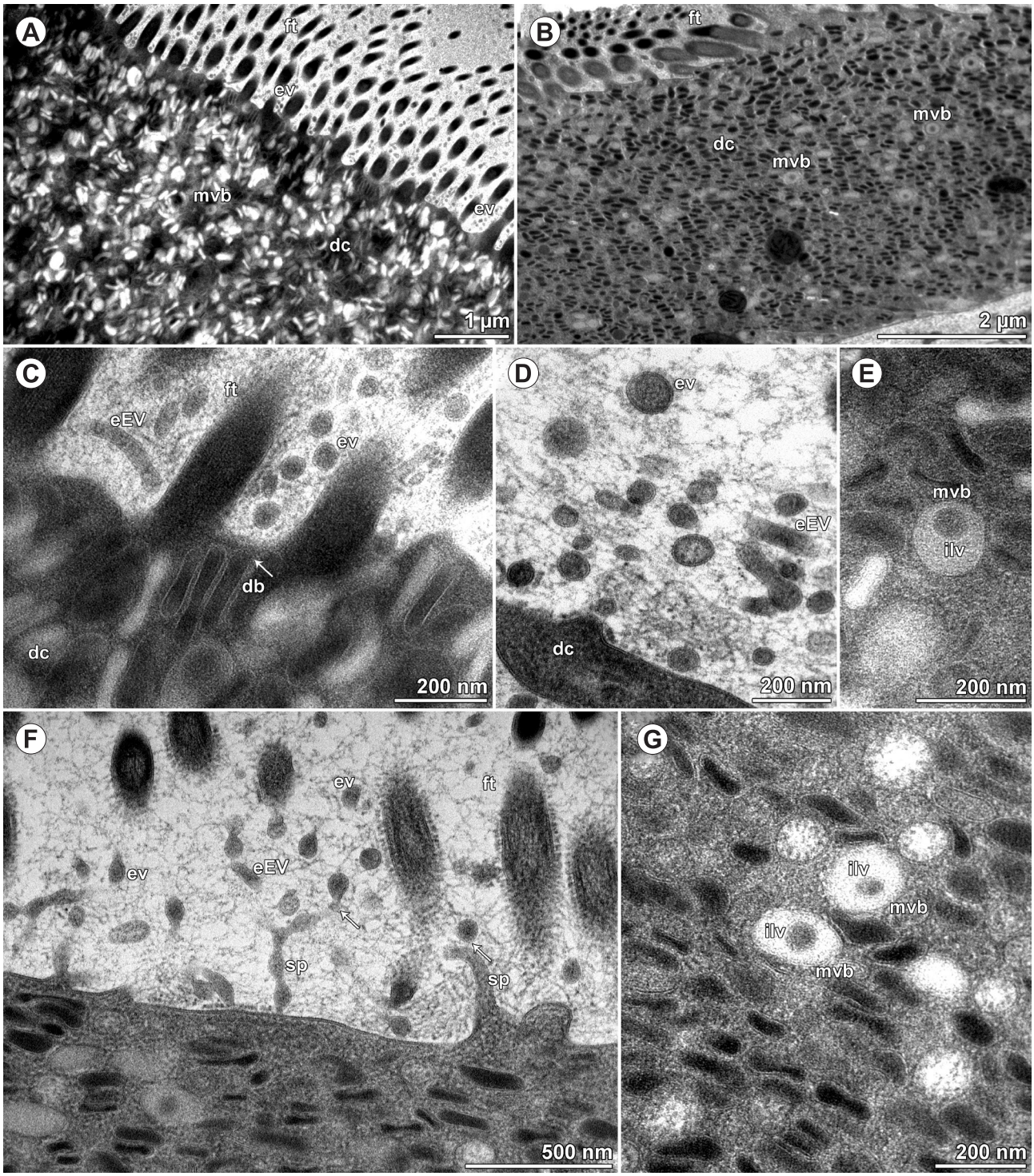

Fig. 3. Transmission electron microscopy of the plerocercoid and adult stages of *Schistocephalus solidus* (Müller, 1776). **A**, **B** – overview of the internal structure of the syncitial tegument in plerocercoid (A) and adult (B); **C**, **D** – extracellular vesicles (EVs) of classical and elongated form (eEV) secreted on the surface of plerocercoids (C) and adults (D). Moreover, fusion of a dense body with the surface membrane is detected (arrow) (C); **E**, **G** – detail of multivesicular body (MVB)-like structures and their intraluminal vesicles (ILVs) in the syncitial tegument of plerocercoids (E) and adults (G); **F** – surface protuberances forming chains of EV-like structures that break (arrow) into separate EVs (F). *Abbreviations*: db – dense body; dc – distal cytoplasm; eEV – elongated extracellular vesicle; ev – extracellular vesicle; ft – filithrix; ilv – intraluminal vesicle; mvb – multivesicular body; sp – surface protuberance.

their secretion into the environment, as presented here, has been lacking. Similarly, their role in parasite biology and host-parasite interactions remains unclear. There is only some suggestion of their involvement in microthrix formation, glycocalyx renewal and pinocytosis (Lumsden 1975). In contrast, their association with the secretion of EVs in flatworms is generally lacking. There are few data on the production of EVs by mammalian tapeworms (Ancarola et al. 2017, Liang et al. 2019, Mazanec et al. 2021). This may be caused by classical chemical fixation methods for TEM,

resulting in most of the secreted EVs being washed out and thus ultimately absent. However, modern high-pressure freezing fixation and freeze substitution (HPF/FS) of live samples for TEM provide a higher chance of preserving all biological processes, including secretion of EVs and preservation of membraneous structures, while limiting artefact formation (Huang and Yeung 2015, Mazanec et al. 2021).

Here we performed an ultrastructural study of the tegument and associated EVs of several ontogenetic stages (hexacanth, procercoid, plerocercoid and adult) of the non-mammalian tapeworm *S. solidus* and confirmed for the first time the secretion of EVs at all parasitic stages of a metazoan parasite with a complex life cycle. The ultrastructure of the hexacanth and procercoid of *S. solidus* was studied for the first time. There are very few and relatively old studies on the ultrastructure of the hexacanths and procercoids of diphyllobothriid tapeworms, covering only three species, namely *Dibothriocephalus latus* (Linnaeus, 1758), *D. dendriticus* (Nitzsch, 1824) and *Spirometra mansoni* (Cobbold, 1883) (Bråten 1968, Grammeltvedt 1973, Lumsden et al. 1974, Kuperman 1988). In addition, there is a brief description of EV-like spheroid structures secreted from the oncospheres of *Eubothrium salvelini* (Schrank, 1790), a parasite of salmonid fishes (Młocicki et al. 2010). The secreted EVs had similar size and morphology at all studied life stages, but they were much less abundant in hexacanths and procercoids.

Given the internal structure of the unembryonated egg (Supplementary Fig. 1A), it is almost impossible to distinguish precisely between the different vesicles. However, no EV-like bodies were observed to penetrate the outer shell (Fig. 2A). While the ultrastructure of the eggs of *S. solidus* or other diphyllobothriid tapeworms has never been studied, the secretion of EVs has been demonstrated in eggs of trematodes (namely *Fasciola hepatica* and *Schistosoma mansoni* Sambon, 1907) (Sánchez-López et al. 2020, Mossallam et al. 2021). However, the process of their release into the environment remains enigmatic as no form of secretion has been observed.

Because free-living coracidia hatch from *S. solidus* eggs, we hypothesise that the EV-like bodies detected in the lumen play an important role in communication between the embryo and vitelline cells of the flatworm eggs. Furthermore, they may simply serve as release vehicles for metabolites produced during coracidium development. In the case of procercoid, the secreted EV-like bodies were detected only near the procercoid tegument/glycocalyx and were not distributed throughout the rest of the host body cavity. However, this could simply be due to sample preparation (chemical fixation).

Furthermore, unlike plerocercoids and adults, we did not see many tegument vesicles that might indicate participation in EV biogenesis, and thus the mechanism of their secretion remains unclear. This is not true for plerocercoids and adult stages, where the tegument increases significantly in complexity and multiple vesicle types can be observed throughout the tegument. Moreover, the tegumental organisation of plerocercoids and adults remains similar, as plerocercoids undergo almost no further morphological changes after transfer to the definitive host (McCaig and Hopkins 1963, Barber and Scharsack 2010).

Although we have noted surface disturbances in the anterior region of adult strobila, we believe that some of these tegumental structures are involved in EV biogenesis. Several crypts have been observed to be associated with EVs. However, it is not clear whether these are phagosomes or MVBs that are in the process of fusing with the surface membrane. The same is true for the MVBs observed throughout the tegument. Similar structures have been previously observed in adults of *Hymenolepis diminuta* (Rudolphi, 1819) and juveniles of *F. hepatica* (see Sánchez-López et al. 2020, Mazanec et al. 2021). Moreover, we discovered unique elongated EVs (eEVs) similar to those described only in *F. hepatica* and *H. diminuta* (Sánchez-López et al. 2020, Mazanec et al. 2021).

Regarding their biogenesis, we observed surface protuberances forming chains of EV-like structures that break into separate EVs (Fig. 3F). We hypothesise that the elongated EV-like structures are a product of imperfect splitting from these surface protuberances. A similar mechanism of EVs chains has also been described in the kinetoplastid *Trypanosoma brucei* Plimmer et Bradford, 1899 (see Szempruch et al. 2016). Some of these EVs were also observed within tegumental MVBs (Supplementary Fig. 1F). However, it is not clear whether these structures are a mechanism of EV secretion or a result of their autophagy. Considering that secretion of ESP was highest during the egg production phase of the adult animal, we assume that most of the secreted vesicle-like bodies observed are not a product of tissue decay or apoptosis.

In recent years, the number of studies on EVs in parasitic worms and their potential role in host-parasite interactions has increased significantly, but they focus almost exclusively on species infecting mammals (Drurey and Maizels 2021). However, there are still gaps in our knowledge of their biogenesis and their role during ontogeny of a given species. Here, we confirm for the first time the secretion of EVs at all parasitic stages (hexacanth, procercoid, plerocercoid, and adult) of a metazoan parasite with a complex life-cycle. Different mechanisms of biogenesis were suggested by tegumental perturbations and different shapes and sizes of EVs. We believe that by confirming EVs secretion in this species, we provide a springboard for future biochemical and immunological analyses, which were not possible in this study due to technical difficulties, to find a new agent that may be responsible for host manipulations.

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