

**University of South Bohemia in České Budějovice**  
**Faculty of Science**

**Extracellular vesicles during the ontogeny  
of the tapeworm *Schistocephalus solidus***

Bachelor thesis

**Nikol Bušková**

Supervisor: Mgr. Hynek Mazanec

Consultant: doc. Roman Kuchta, PhD

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## **Abstract**

This bachelor thesis focuses on extracellular vesicles (EVs), which are small transport units derived from membranes and released by a variety of cells. Based on their biogenesis, two main classes were observed. First class, referred to as exosomes, are vesicles formed during an endosomal pathway and released by multivesicular bodies. The second class, microvesicles, are formed by a direct budding of the plasma membrane. We focused on EVs of different life stages of the tapeworm *Schistocephalus solidus* (Cestoda: Diphylobothriidae), a parasite of fish-eating birds with a three-host life cycle.

For our experiments, a full life cycle of *S. solidus* was established under laboratory conditions. EVs secretion was studied in procercooids from copepods, plerocercoids from fish and adults from the cultivation medium.

We have observed biogenesis of EVs on the surface of procercooid and plerocercoids of *S. solidus* using transmission electron microscopy and successfully purified EVs from the cultivation medium. Furthermore, we observed differences in secretory activity during the maturation of plerocercoids and a difference in releasing EVs from different parts of the the tapeworm surface.

## **Prohlášení**

Prohlašuji, že jsem autorem této kvalifikační práce a že jsem ji vypracovala pouze s použitím pramenů a literatury uvedených v seznamu použitých zdrojů.

V Českých Budějovicích,

dne .....

Podpis studenta .....

## **Poděkování**

Mé poděkování patří především vedoucímu této bakalářské práce Mgr. Hynku Mazancovi, který mi poskytl odborné vedení, cenné rady, připomínky a věnoval mi mnoho času ke konzultacím. Dále děkuji pracovníkům laboratoře helmintologie Parazitologického ústavu Biologického centra AV ČR, zejména prof. Tomáši Scholzovi za poskytnutí příjemného zázemí a doc. Romanu Kuchtovi za pomoc s prací v laboratoři. V neposlední řadě bych ráda poděkovala své rodině za podporu a trpělivost, kterou mi prokazovala nejen při psaní práce, ale při celém studiu.

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# 1 INTRODUCTION

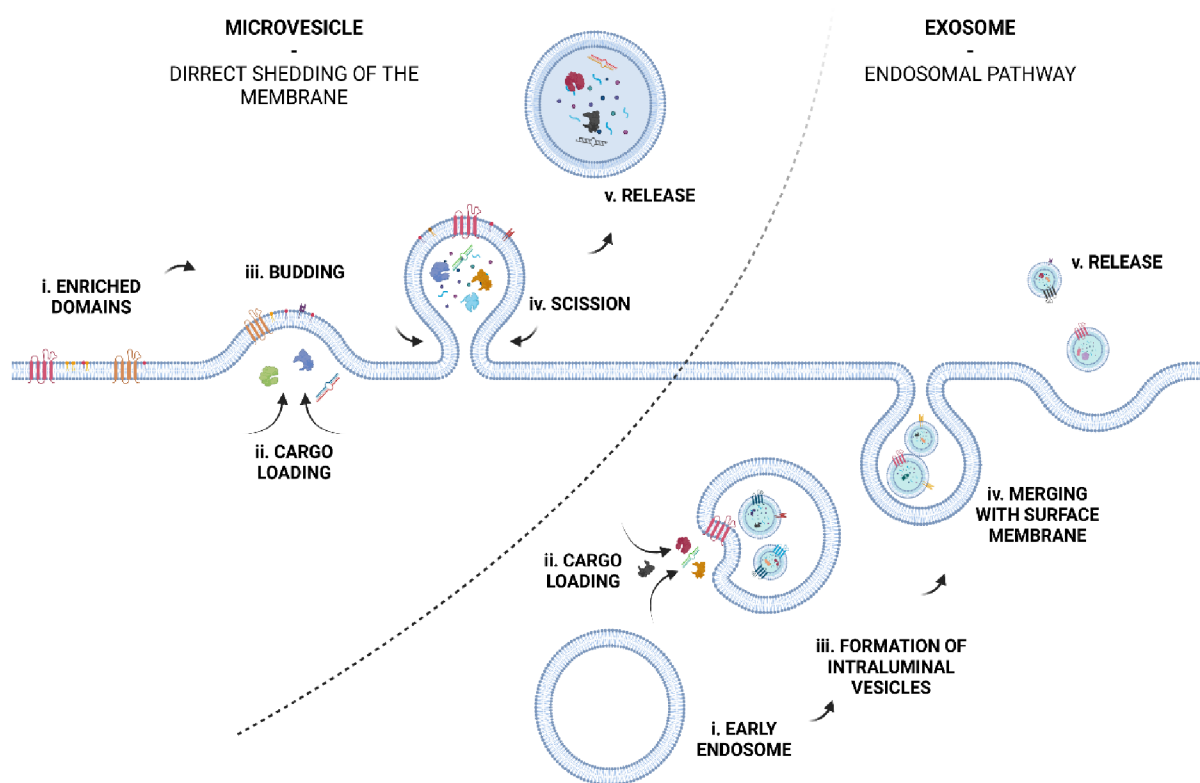
**Cell signaling** is a basic function of a cell to perceive and respond to its microenvironment. It is a crucial ability in cell development, maintaining homeostasis, defense from pathogens, immunity and tissue repair. There are many ways how cells can interact and communicate with their surroundings (endocrine, paracrine and autocrine) (Uings & Farrow, 2000; Hancock, 2017). Recently there has been an outbreak in discoveries of extracellular vesicles (EVs), as a means of cell-to-cell communication, across the kingdoms of life (Théry et al., 2018; Woith et al., 2019). EVs are small membrane-delimited structures that are released by a variety of cells but unlike these cells EVs cannot replicate. After their release from the plasma membrane, EVs can persist in the extracellular space or they get into the biological fluids like plasma, urine, milk or cerebrospinal fluid (Camussi et al., 2010).

The first mention of the existence of the **EV-like structures** comes from 1946 when they were mentioned in a study of human plasma and described as procoagulant platelet-derived particles (Chargaff & West, 1946). A few years later, EVs were observed again and referred to as “platelet dust” (Wolf, 1967). In more recent studies on the ultrastructure of the cell membrane, it was shown that multivesicular bodies (MVBs) release EVs after their fusion with the cell membrane (Pan & Johnstone, 1983; Harding et al., 1984; Johnstone et al., 1987). The first implication of the active role of EVs in cell signaling came with the discovery of EVs carrying MHC class II from B-lymphocytes (Raposso et al. 1996). Eventually, they were observed as carriers of proteins, RNA (mRNA, miRNA, lncRNA, etc.), DNAs (mtDNA, ssDNA and dsDNA), and lipids, and thus further indicating their importance in intercellular communication (Ratajczak et al., 2006; Valadi et al., 2007; Christianson et al., 2014; Colombo et al., 2014; Doyle et al., 2019). To date, EVs have been isolated from different cell types and biological fluids (see Table I).

**Table I.** Appearance of extracellular vesicles in human cell types and biological fluids.

	SOURCE	REFERENCE
CELL TYPE	B-lymphocytes	Raposo et al., 1996
	dendritic cells	Raposo et al., 1996
	hepatocytes	Conde-Vancells et al., 2008
	prostasomes	Aalberts et al., 2014
	oncosomes	van Niel et al., 2018
FLUID	urine	Pisitkun et al., 2004
	blood plasma	Caby et al., 2005
	seminal fluid	Poliakov et al., 2009
	breast milk	Lässer et al., 2011
	cerebral spinal fluid	Akers et al., 2013
	tears	Akers et al., 2013
	gastric acid	Kagota et al., 2019

Based on their biogenesis, EVs can be classified into two main types: i) **exosomes**, released via endosomal pathway; and ii) **microvesicles**, shed through budding of the outer membrane (Fig. 1). These classes can be further characterized by their size, density, biochemical composition, or cell of origin (van Niel et al., 2018).



**Fig. 1.** Scheme of a biogenesis of the extracellular vesicles (created with Biorender.com, edited by Hynek Mazanec).

The first type is formed during the endocytic pathway and is referred to as **exosomes** (ranging in size from 30 to 150 nm) (Matthews et al., 2004; Bobrie et al., 2011; Colombo et al., 2014). Prior to the release of the exosomes, at least three different stages of the endosomes can be observed: a) early endosomes, b) late endosomes, and c) recycling endosomes. The first stage, early endosome, absorbs endocytic, phagocytic or pinocytic vesicles and integrates their content (Akers et al., 2013). Early endosome has to undergo several transformations before maturing to a late endosome. The late endosomes then undergo two possible pathways: i) degradation through a fusion with lysosome (recycling endosome); or ii) intraluminal vesicles (ILV) are formed in the lumen, leading to a formation of multi-vesicular body (MVB) that can be subsequently transported to the cell membrane, where it fuses with the plasmatic membrane and releases the ILVs (now referred to as exosomes) into the surroundings (Matthews et al., 2004; Akers et al., 2013). Several types of machinery of ILV budding into the MVB lumen have been observed: i) endosomal sorting complex required for transport (ESCRT) dependent; ii) lipid-raft formation; iii) tetraspanin enriched domains; or iv) combination of all of the above (de Gassart et al., 2003; van Niel et al., 2006; Colombo et al., 2013; Skotland et al., 2017; Böker et al., 2018; Margolis et al., 2019).



The **ESCRTs machinery** is the most common pathway of exosome biogenesis. It is composed of four multimeric cytosolic complexes (ESCRT-0, -I, -II, and -III) and their associated proteins: vacuolar protein sorting-associated protein 4 (VPS4) and programmed cell death 6-interacting protein (PCD6IP or ALIX) (Colombo et al., 2013). They were first defined as a ubiquitin-dependent protein-sorting pathway in yeast (Henne et al., 2011). The ESCRT-0 is primarily responsible for recognizing and sequestering ubiquitylated proteins into the endosomal membrane (Hurley, 2010). The ESCRT-I and ESCRT-II then prime the initial budding of ILV and recruit the ESCRT-0-ubiquitin domains into the ILV matrix (Gill et al., 2007; Wollert & Hurley 2010). Furthermore, ESCRT-I is also responsible for binding the ESCRT-III which then causes the subsequent scission of ILV from the membrane (Wollert et al., 2009, Hurley, 2010). ESCRT-III is then usually dissociated from the membrane by VPS4 and recycled for future ILV biogenesis (Katzmann et al., 2001; Babst et al., 2002). A study of RNAi showed that not every ESCRT complex is essential in exosome biogenesis (Hurley, 2015). Several proteins cooperate with ESCRT complexes in all steps of exosome biogenesis, from endosomal budding to ILV's formation. For example, a knock-out of ESCRT-II leads to an alternate binding of ESCRT-III through an ALIX-syntenin-syndecan complex (Baietti et al., 2012).

The second subtype, **microvesicles (MVs)**, are vesicular bodies blebbing directly from the cell membrane (Mathieu et al., 2019). Their budding initiates through changes in cytoskeletal proteins (actin, myosin, microtubules and kinesins), lipid components (lipid rafts domains containing sphingomyelin molecules and cholesterol) and  $Ca^{2+}$  levels in the plasma membrane (Minciacchi et al., 2015). Membrane asymmetry regulated by phospholipid translocases (flipases, floppases) leads to the initial changes in the structure of the membrane which subsequently prime the membrane budding and MVs formatting (Akers et al., 2013; McMahon & Boucrot, 2015). Previously described ESCRT machinery can also take part in the budding and cargo loading (van Niel, 2018).

Waste disposal was originally considered to be the only **EVs function** (Woith et al., 2019). However, recent studies elucidated their role in a wide range of biological processes mainly mediated by the transmission of various effector molecules (proteins, lipids, miRNAs, etc.) (Torre-Escudero et al., 2016). EVs have been observed to be involved in the upkeep of regular physiology, for example, blood coagulation, immune surveillance, and tissue repair (Zhang et al., 2015; Qi et al., 2016). Nowadays, their natural role in living systems is being exploited in diagnostics and therapeutics (Corrado et al., 2013; Stremersch et al., 2016). For

example, microRNA signatures of tumor-derived exosomes (oncosomes) can serve as biomarkers of ovarian cancer (Taylor & Gercel-Taylor, 2008). Similarly, metastatic prostate cancer has been linked with the exosomal miR-141 and miR-357 (Li et al., 2016). Moreover, delivery potential of EVs is also considered for use in drug administration (Usman et al., 2018; Elsharkasy et al., 2020). Ever since their first discovery, EVs are observed to be participating in more and more processes in living organisms across different kingdoms (Woith et al., 2019).

**Parasites** are organisms forming a close relationship with other organisms (host) and living at the expense of their resources. This relationship is traditionally beneficial only for the parasite and can result in the death of the host. Helminths are a major group of metazoan parasites that included the Nematoda (roundworms) and Platyhelminthes (flatworms), with the latter subdividing into Cestoda, Trematoda, and Monogenea (Weinstock & Elliott, 2009; Drurey & Maizels, 2021). There are several species of agricultural and medical importance (Despommier et al., 2012). Economical losses in livestock husbandry due to the helminth infection reach annually up to several billion dollars (Charlier et al., 2020). Similarly, soil-transmitted helminthiasis affect more than 1.5 billion people worldwide (WHO, 2021).

Nonetheless, **helminth infections** may even have profitable or protective effects for their host (Elliott & Weinstock, 2012; McSorley & Maizels 2012). Mechanisms of parasite survival in the host is a precise manipulation of the immune system that has been developed during their coevolution (Jackson et al., 2009; McSorley et al., 2012). Parasitic helminths can suppress the immune system in many ways. Examples include initiation of apoptosis in immune cells, suppression of T-helper 1 and 2 (Th 1, Th 2) cells or affiliated cytokines, or increased proliferation of regulatory cells. (Oliveira et al., 2016; Zakeri, 2017). These mechanisms can actually have a protective role for the host when in contact with other pathogens or allergens, as was previously described in the hygiene hypothesis (Strachan, 1989). The hygiene hypothesis was based on the suggestion that any infection at an early age stimulated proper development of the immune system. Absence of infections predisposed towards increased and unbalanced immune response, which may lead to allergic reactions, autoimmune diseases (anaphylaxis, asthma) or inflammatory bowel disease (IBD) (Crohn's disease and ulcerative colitis) (Wilson et al., 2005; Croese et al., 2006; Smith et al., 2007; Bach, 2018). Therefore, helminths have been proposed for use in the experimental treatment of autoimmune disorders such as allergies, ulcerative colitis or Crohn's disease (Ryan et al., 2020).

However, despite their **therapeutic potential**, the usage of living worms in treatment faces many challenges that hinder it from faster development (Fleming & Weinstock, 2015; Maruszewska-Cheruiyot et al., 2018). Therefore, due to some of the harmful implications and side effects that whole worm experimental infections can cause during helminth-therapy, the helminth-derived products (HDPs) were researched as to their substitute (Robinson et al., 2013; Smallwood et al., 2017). They have been observed to interfere with essential signaling pathways, regulate the expression of important molecules of the host defense mechanism and build a beneficial niche for the parasite (Shepherd et al., 2015). Extracellular vesicles are an important subset of HDPs. Similar to HDPs, EVs have been observed to play role in the invasion, pathogenicity, and longevity of parasitic infections (Marcilla et al., 2012). So far, they have been observed to be released by representatives of each major helminth group (Buck et al., 2014; Ancarola et al., 2017; Gu et al., 2017; Samoil et al., 2018; Ding et al., 2019; Zhou et al., 2019; de la Torre-Escudero et al., 2019). EVs upon their release from the parasite have been observed to be internalized by the host immune cells and subsequently trigger an immune response (Eichenberger et al., 2018).

FM4-64-labeled vesicles of the trematode *Echinostoma caproni* have been observed to be internalized by intestinal rat cells in a time- and metabolically-dependent manner (Marcilla et al., 2012). Compared to their mammalian counterpart, the mechanism of EV release in parasitic worms is not yet fully understood. However, we can see conserved similarities in the biogenesis mechanism (Bennett et al., 2020; Sotillo et al., 2020). There are however differences in the sites of their secretion. The platyhelminthes, EVs have been observed to be released throughout the syncytial tegument with no apparent specific site or pore (Sánchez-López et al., 2020; Mazanec et al., 2021). On the contrary, nematodes have been observed to release EVs mostly through their anal and oral opening and associated secretory pores (Drurey et al., 2020).

In the case of metacestodes of *Echinococcus* spp., EVs can be seen near the germinal layer, engaging with the host in the early stages of development when the laminar layer is not complete yet or when the layer is breaking because of aging or chemotherapy (Ancarola et al., 2017). Similarly, encysted metacercariae of *Fasciola hepatica* have been observed to carry EVs pre-formed EVs that are released after the consumption by the host and associated excitement (Sánchez-López et al., 2020). More importantly, EVs of parasitic helminths have been observed to be participating in pathogen distribution and affecting the immune system of the host (Marcilla et al., 2014; Drurey et al., 2021). EV cargo of *F. hepatica* eases the

migration of parasites through the host tissue and prevents the host immune system to counteract (Cwiklinski et al., 2015). Furthermore, *F. hepatica*-derived EVs can ameliorate clinical symptoms of induced colitis in an experimental model (Roig et al., 2018).

EV interactions with the host immune cells have been observed also in other groups of parasitic worms. EVs released from protoscoleces of *Echinococcus granulosus* exhibit immunomodulatory potential and are internalized by dendritic cells (Nicolao et al., 2019). Moreover, recent studies start to show potential use of parasite-derived EVs in treatment and prevention (Drurey et al., 2020). EVs of the nematode *Teladorsagia circumcincta* carry immunomodulatory cathepsin F (Tci-CF-1) which has also been used in vaccination trials based on its antigenic and immunogenic properties (Tzelos et al., 2016).

The **avian tapeworm** *Schistocephalus solidus* (Müller, 1776) (Cestoda: Diphylobothriidea) has been established as an important model organism in the past (Barber & Scharsack, 2010). The life cycle of *S. solidus* consists of three-hosts (Smyth, 1946). Adults of *S. solidus* reside in the intestine of fish-eating birds, where they produce eggs that enter the water with bird's feces. Released eggs then hatch into free-swimming coracidia in a fresh-water environment. This free-swimming larvae is subsequently consumed by the first intermediate host, cyclopoid copepod, where the coracidium develops into procercoid, the second larval stage. In copepods, procercoids develop into infectious form within 3 weeks, depending mainly on water temperature (Benesh & Hafer, 2012). Infected copepods should be consumed by the second intermediate host, three-spined stickleback (*Gasterosteus aculeatus*). The procercoid transforms in the fish's body cavity to plerocercoid, the third larval stage. The whole life cycle is completed when infected *G. aculeatus* is eaten by the definitive host, a fish-eating bird (Smyth, 1946).

The egg production in the final host begins shortly after the infection as the worm reaches final size in the fish body cavity and its reproductive system is well-developed, except for egg production (Wedekind et al., 1998). In combination with the maturation of plerocercoids in the fish host, use of this suitable experimental model has contributed to a significant increase in knowledge of tapeworm physiology (Barber & Scharsack, 2010). Furthermore, it significantly contributed to our understanding of host-parasite interactions and helminth mating behavior (Barber & Huntingford, 1995; Weinreich et al., 2014). The behavioral changes observed in stickleback can be lethargy or reduction in maintenance behaviors (Barber & Huntingford, 1995; Barber & Scharsack, 2010; Nordeide & Matos, 2016). Moreover, the reduced liver size indicates high-energy consumption due to parasitic infection (Walkey &

Meakins, 1970). The copepods infected by procercooids have also shown different behavior when compared to non-infected individuals (Urdal et al., 1995; Jakobsen & Wedekind, 1998). Furthermore, *S. solidus* can regulate the stickleback's immune system through excretory-secretory products (ESP) (Scharsack et al., 2013). The ESPs have been observed to play a role in the maintenance of an effective and lasting infection (Kochneva et al., 2021). Moreover, they can cause an increase in the respiratory burst activity in head kidney leukocytes of uninfected sticklebacks (Scharsack et al., 2013). Research done on plerocercoids of closely related species (*Dibothriocephalus dendriticus* and *Ligula interrupta*) hypothesize secretion of the immunomodulatory ESP from the tegument (Kutyrev et al., 2021). The syncytial tegument is formed by many vesicular bodies, such as electron-dense bodies (DB), pinocytic vesicles and other vesicular bodies of unknown origin or function (Charles & Orr, 1968). DBs have been observed to be transported to the apical cytoplasm and subsequently released to the surrounding surface (Kuperman, 1988; Yoneva et al., 2017). Furthermore, the function of DBs might be associated with nourishment and protection due to the allowance of integration of nutrients from the environment and interactions with immune system components (Hopkins et al., 1978). Their role has also been implicated in the biogenesis of EVs (Mazanec et al., 2021). However, in general, very little has been done in research on vesicular bodies of the tegument in tapeworms and their subsequent release. To the author's knowledge, extracellular vesicles and their secretion have not been characterised in any of the stages of *S. solidus*.

## **2 AIMS OF STUDY**

1. To establish the life cycle of *Schistocephalus solidus* in laboratory conditions.
2. To observe the production of extracellular vesicles in the model organism in as many stages as possible (eggs, coracidium, proceroid, plerocercoid, adult).
3. To compare differences in the production of EVs across the different life cycle stages and during the maturation of fully formed plerocercoids.
4. To search for specific sites of EVs release on the surface of the adult *S. solidus*.

## 3 MATERIALS AND METHODS

### 3.1 Animals and parasites

Parasites studied in this study were obtained from experimentally infected copepods (*Macrocyclus albidus*) and three-spined sticklebacks (*Gasterosteus aculeatus*). Eggs of *Schistocephalus solidus* and initial culture of copepods were kindly provided by Dr. Tobias Lenz (Max Planck Institute for Evolutionary Biology, Plön, Germany). Three-spined sticklebacks were provided by a private breeder (Postoloprty, Czech Republic) and transferred to the Laboratory of Helminthology (Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic). Cultures of copepods and three-spined sticklebacks were kept and bred in separate tanks in a controlled environment of a dark, cooled room.

### 3.2 Experimental life cycle

Previously obtained eggs of *Schistocephalus solidus* were placed in a Petri dish for two days to hatch either at 17°C or at room temperature around 22°C. After two days, eggs were already hatched and 1 or 2 free-living coracidia were transferred to each of 24-well plate and kept with copepods (*M. albidus*). The well plate was subsequently put to 17 °C overnight and checked after 24 hours for the coracidium consumption, after which the copepods were kept in a separate culture for infection to establish. Three weeks after infection, copepods were checked for procercoid development. Prior to their infection, each fish was placed in a separate container and left to acclimatize for 24 hours. Thereafter, 1 or 2 copepods containing procercoids were transferred to the previously prepared containers with fish. One day post-co-cultivation, fish were placed into aquaria, and water from containers was checked for copepod presence, thus indicating their successful consumption if no copepods were found. Three months later, sticklebacks were dissected and checked for infection. Obtained adults of *S. solidus* were washed with PBS, weighed and transferred into sterile filtered RPMI-1640 medium supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin for one week at 37 °C. The medium was collected and changed every day. It was either used immediately in subsequent purification of extracellular vesicles or stored at -80°C. After the incubation period, adult worms were removed and immediately fixed for electron microscopical studies (described below). Eggs produced by the adults of *S. solidus* were collected, washed in PBS, placed in tubes and stored in 4°C and dark for other experiments.

### 3.3 EV purification

The collected medium was concentrated using a stirred cell (Amicon®, USA) with 10 kDa ultrafiltration discs (Milipore, USA) to a final volume of 2–3 ml. The concentrated medium was then used to isolate EVs. Two different techniques were applied: i) **ultracentrifugation** (UC); and ii) commercial **size exclusion chromatography columns** (SEC). For ultracentrifugation, an isolated medium was centrifuged at 120,000g for 1 hour at 4°C (XPN-90 Beckman Coulter, USA). Obtained pellet (120k pellet) was gently resuspended in 200 µl and used in subsequent analyses. SEC qEVoriginal/70 nm (IZON, New Zealand) were used according to the manufacturer's protocol. Briefly, SEC column was loaded with 500 µl of the concentrated medium, after which different fractions were continuously collected: 2× 1,500 µl of void fraction, 4× 500 µl of small-EV fraction and 2× 1,500 µl of soluble protein fraction. Qubit™ Protein Assay Kit (Invitrogen, USA) was used to determine the protein concentration of isolated EVs and collected medium.

### 3.4 Electron microscopy

Anterior, middle and posterior regions of 2 adults were cut into small  $\pm 2$  mm fragments and 2 infected copepods were fixed by a high-pressure freeze and freeze-substitution method (HPF/FS). The sample carrier was inserted into the rapid transfer system of the EM PACT2 (Leica Microsystems, Germany) and frozen at a pressure of 2.1 kbar. The frozen samples were subsequently transferred under liquid nitrogen to cryovials with anhydrous acetone containing 2% osmium tetroxide and stored in liquid nitrogen. For freeze substitution, cryovials were put in the chamber of a Leica AFS FS system/device (Leica EM AFS) pre-cooled to -90 °C. Samples were maintained for 24 h at -90 °C. FS samples were then warmed to room temperature at a rate of 5 °C/h. At room temperature, specimens were washed three times for 1 h in fresh anhydrous acetone. Subsequently, samples were washed with 0.1 M HEPES (pH 7.4), post-fixed in cold (4 °C) 1% osmium tetroxide (OsO<sub>4</sub>) in the same buffer for 1 h, dehydrated in a graded series of acetone, embedded in Spurr's epoxy resin and polymerized at 62 °C for 48 h. Afterward, ultrathin slices (60–90 nm in thickness) were cut on a Leica Ultracut ultramicrotome (Leica Microsystems, Germany), placed on formvar-coated copper grids, and stained afterward with uranyl acetate and lead citrate according to standard protocols (see Conn & Rocco, 1989). Sections were examined in a JEM 1010 (Jeol, Japan) transmission



electron microscope operated at an accelerating voltage of 80 kV. Images were taken with a CCD Sis MegaView III digital camera and image acquisition software Analysis 3.2.

For the room temperature electron microscopy analysis of the purified EVs, the samples were prepared with a negative staining method. Purified extracellular vesicles were placed on glow-discharged carbon-coated copper grids, stained with 1.5% uranyl acetate, and visualized using a JEOL JEM-2100F transmission electron microscope (Jeol, Japan) operated at an accelerating voltage of 200 kV. TEM images were captured using a bottom-mounted Gatan CCD Orius SC1000 camera (Gatan, USA).

For Cryo-TEM, the purified EVs were laden on holey carbon grids and rapidly frozen with plunge freezer LEICA EM GP2 (Leica Microsystems, Germany). For visualizing samples, a 200 kV JEOL JEM-2100F microscope equipped with a Gatan ELSA cryo transfer holder was used. Images were taken with a Gatan K2 Summit direct electron detector.

Obtained micrographs from TEM were analyzed using ImageJ software (NIH, USA) and TEM Exosome Analyzer (CBIA, Czech Republic).

All the captured micrographs from TEM were taken in the Laboratory of Electron Microscopy, Biology Centre of the Czech Academy of Sciences, Czech Republic.

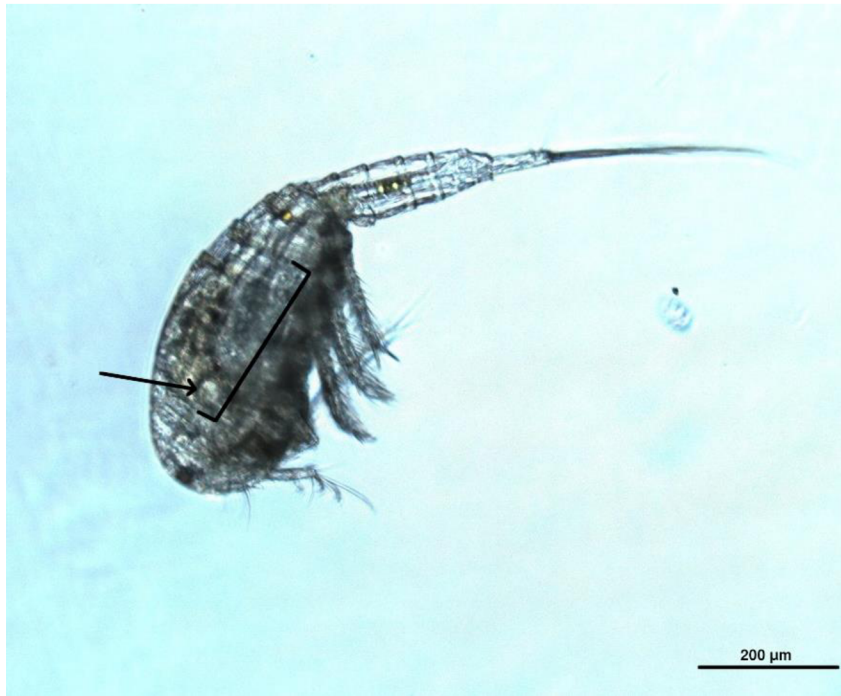
## 4 RESULTS

### 4.1 Experimental life cycle

We have successfully established an experimental life cycle of a diphylobothriidean tapeworm *Schistocephalus solidus* in laboratory conditions. Eggs (Fig. 2) after one month of development in dark and 4°C were exposed to light and two different temperatures (17°C and room temperature 22°C). After two days of incubation, the eggs began to hatch. Different temperatures did not have a significant effect on the egg hatching. Coracidia, acquired from the hatched eggs, were subsequently used to infect copepods (*Macrocyclus albidus*). Three weeks later, procercooids with fully developed cercomer (Fig. 3) were observed in the body cavity (thoracal segments) of experimentally infected *M. albidus*. Twenty-four hours post-infection, infected copepods were no longer detected in the container with stickleback, which indicated their consumption. After 90 days, sticklebacks were dissected and recovered plerocercoids (Fig. 4) were cultivated in a RPMI medium for one week. No differences in the size of the abdomen or behavior of the infected and non-infected fish were observed. The cultivation medium was exchanged and collected on a daily basis. The beginning of egg production was observed on the second day and lasted until the fourth day. Collected eggs were then placed in the dark at 4°C before further experiments. These eggs successfully developed and hatched into infectious coracidium and thus confirming successful completing the established life cycle.



**Fig. 2.** Eggs of the tapeworm *Schistocephalus solidus*.



**Fig. 3.** Proceroid of the tapeworm *Schistocephalus solidus* with fully developed cercomer (arrow) in the body cavity of the copepod *Macrocyclus albidus*. Line indicates the full length of the body.



**Fig. 4.** Plerocercoids of the tapeworm *Schistocephalus solidus* after dissection from three spined sticklebacks.

## 4.2 Description of the ultrastructure of different life cycle stages

Two infected procercooids and two adults from cultivation medium were fixed for transmission-electron microscopy to observe the ultrastructure of their tegument and their secretory activity. Extracellular vesicles were observed to be secreted in both stages.

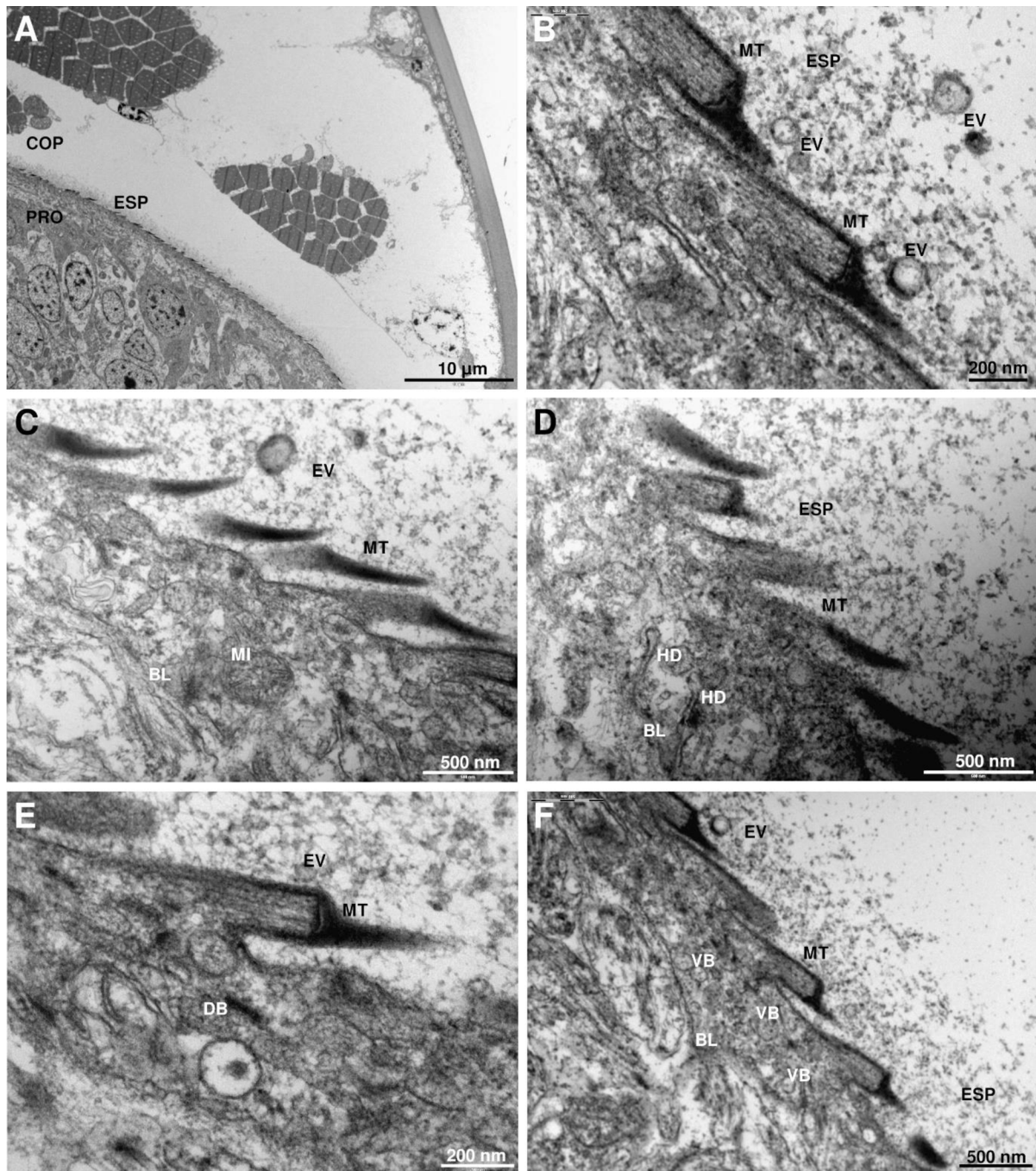
### 4.2.1 Procercooid

TEM micrographs of sections of infected copepods reveal procercooids in the thorax region similar to observations in light microscopy. A dense layer ( $\pm 1,700$  nm thick) of excretory secretory products is observed surrounding the surface of the procercooid (Fig. 5A). No copepod organelles have been observed in direct contact with the ESP layer. Furthermore, EV-like bodies of different sizes (45–90 nm) are frequently observed in the ESP layer (Fig. 5B). Moreover, the surface of the procercooid consists of a uniform layer of microtriches with typical electron-dense caps (Fig. 5B). Underlying the microtrich layer is a thin syncytial tegument ( $\pm 90$  nm). A distal cytoplasm of the syncytial tegument contains mitochondria (Fig. 5C) neighboring the basal lamina as well as anchored hemidesmosomes (Fig. 5D). A small amount of dense bodies is observed in the tegument of no clear origin (Fig. 5E). Vesicular bodies of similar structure and size (45–67 nm) to the EVs are present in ESP layer (Fig. 5F). No clear evidence of their release into the surroundings has been observed.

### 4.2.2 Adult

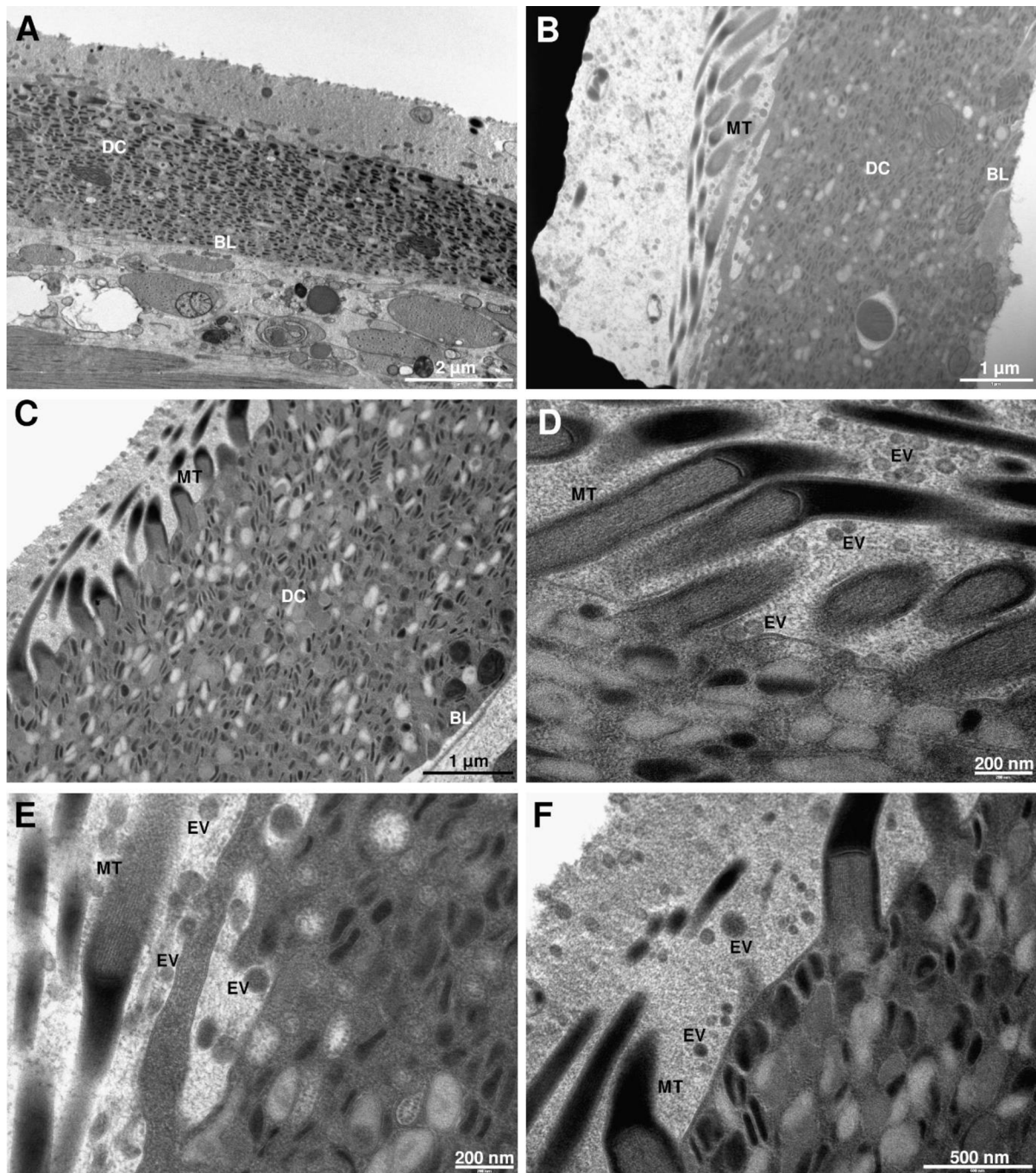
TEM micrographs of section of adult *S. solidus* showed typical ultrastructure as previously described (Charles & Orr, 1968). The surface of the adult consists of a layer of microtriches with typical electron-dense caps (Fig. 6A–C). Anterior region of the adult strobila exhibited more disturbance in the surface stability (e.g. uniform microtrich distribution, phagosome formation, non-specified tegument structures) contrary to the other regions (Fig. 6A–C). EV-like bodies of different sizes (35–140 nm) are frequently observed between the microtriches across the whole surface. Anterior section showed higher abundancy of vesicles secreted on its surface in comparison to the middle and posterior regions (Figs. 6D–F). Beneath the surface membrane is a syncytial tegument with thickness varying based on the sections observed (anterior part 2.0–2.9  $\mu\text{m}$  thick; middle 3.4–3.7  $\mu\text{m}$ ; posterior about 3.9  $\mu\text{m}$ ). The distal cytoplasm of the syncytial tegument contains similar organelles in all observed sections.

The basal lamina is anchored to the syncytial tegument through hemidesmosomes (Fig. 6C). Neighboring basal lamina is mitochondria (Fig. 7A, B) of different densities and sizes (170–630 nm). A large number of dense bodies, presumably originating from mitochondria, is observed throughout the whole tegument (Fig. 7B). MVBs of different densities carrying vesicles similar to those observed on the surface were also seen (Fig. 7C, D). A process similar to shedding of MVs into the surroundings have been detected (Fig. 7E, F).



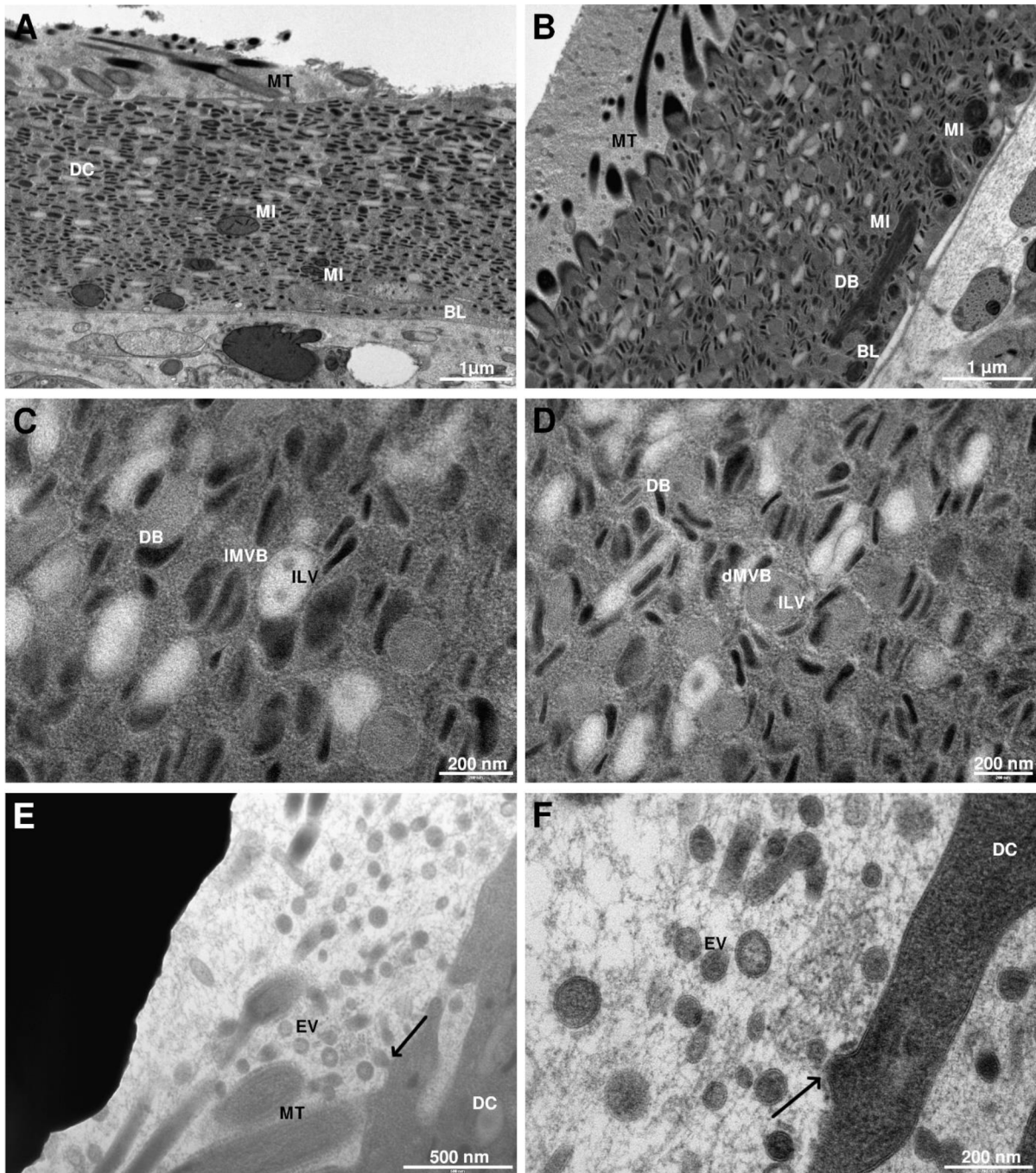
**Fig. 5.** Proceroid of the tapeworm *Schistocephalus solidus*. Ultrastructure of the proceroid in the copepod body cavity (A). Detailed view of the surface with extracellular vesicles-like

bodies in the excretory/secretory products layer (B). Distal cytoplasm of the syncytial tegument with mitochondria (MI) (C). Basal lamina with anchored hemidesmosomes (HD) (D). Dense bodies in the tegument (DB) (E). Vesicular-like bodies in the tegument (F). BL, basal lamina; COP, copepod; DB, dense body; EV, extracellular vesicle; ESP, excretory/secretory product; HD, hemidesmosome; MI, mitochondrion; MT, microtrix; PRO, proceroid; VB, vesicular-like body.



**Fig. 6.** Different regions of an adult *Schistocephalus solidus*. Surface of different regions: anterior (A), middle (B), posterior (C). EVs secreted on different regions of the body: anterior

(D), middle (E), posterior (F). BL, basal lamina; DC, distal cytoplasm; EV, extracellular vesicle; MT, microthrix.



**Fig. 7.** Detailed view of the tegument of the adults of *Schistocephalus solidus* and its organelles. Various sizes of mitochondria (A) and their related dense body secretion (B). Differences between lucent – (C) and dense – (D) multivesicular body (MVB). Shedding of microvesicles (MV) (E, F). BL, basal lamina; DB, dense body; DC, distal cytoplasm; EV, extracellular vesicle; ILV, intraluminal vesicle; MI, mitochondria; MT, microthrix; dMVB, dense multivesicular body; IMVB, lucent multivesicular body.

### 4.3 ESP products of plerocercoids

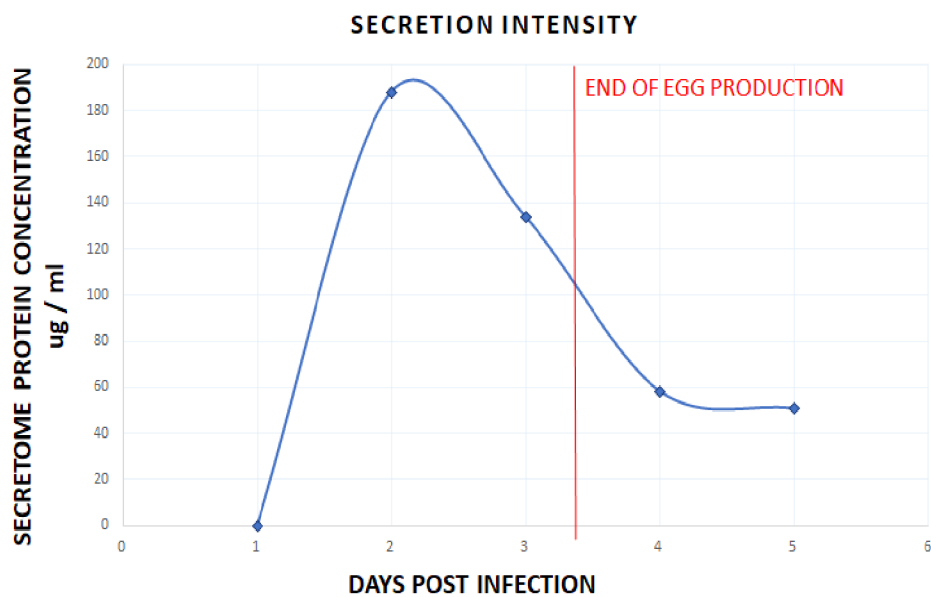
#### 4.3.1 Secretory activity

The secretory activity was accessed from the collected medium after every 2 days. Four subsequent time points were checked for protein concentration (Fig. 8). Similar to egg production, protein secretion significantly decreased on the fifth day.

#### 4.3.2 Isolation of extracellular vesicles

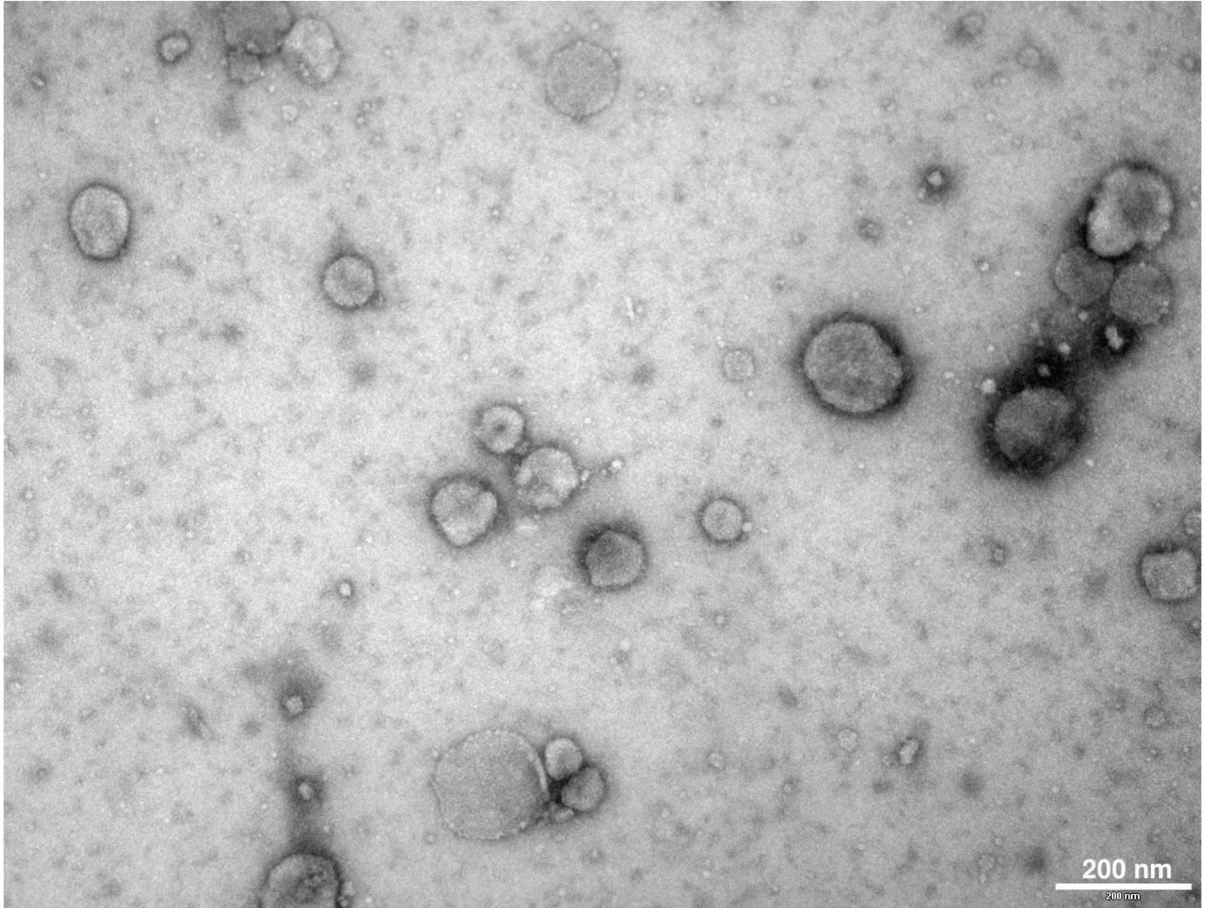
Due to the relatively small protein concentration in collected excretory/secretory products (ESP), samples were pooled together and treated as one. EVs were observed in a 120k pellet after ultracentrifugation (Fig. 9). Their structure and size correspond (30–170 nm) well with some of the EVs observed on the surface of the adult's tegument. However, several EVs exhibiting collapsed membranes have been detected. Nanosight data further confirmed our observations from TEM (Fig. 10). Three major peaks revealed at 77, 113 and 256 nm show the highest particle concentrations (Fig. 10A). Particle volume comparison shows significant peaks at 121, 183, and 266 nm<sup>3</sup>/ml (Fig. 10B).

SEC-based isolation of EVs of the pooled samples yielded low concentrations and was not therefore used in subsequent analyses.

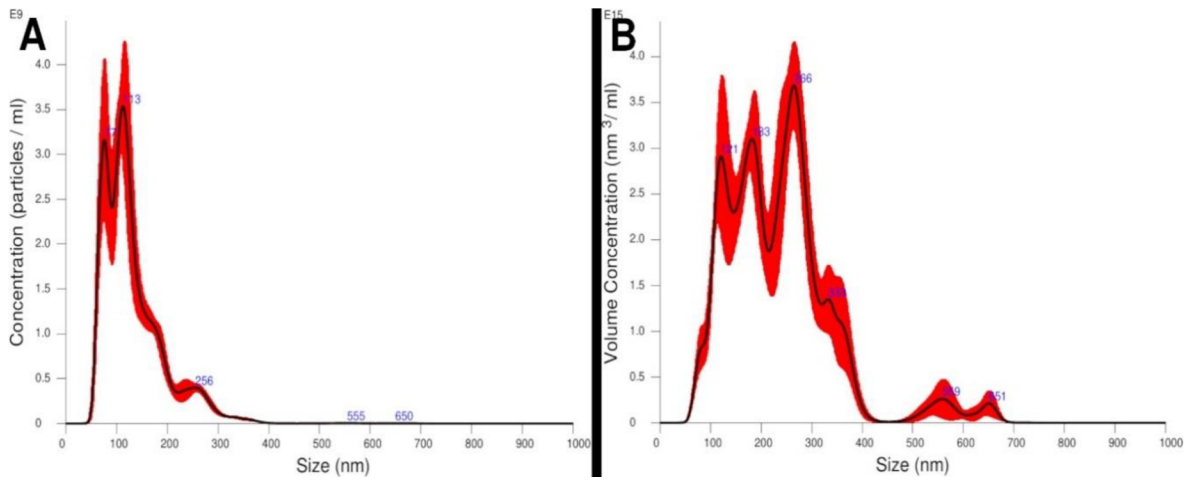


**Fig. 8.** Secretion activity of *Schistocephalus solidus* in relation to eggs production.





**Fig. 9.** Isolated extracellular vesicles from a cultivation medium with *Schistocephalus solidus*.



**Fig. 10:** Different peaks of extracellular vesicles subpopulations according to size (A) and volume (B) from Nanosight.

## 5 DISCUSSION

In the present study, secretion of EVs from the surface of the proceroid and adult of the tapeworm *Schistocephalus solidus* was successfully observed. Moreover, an experimental life cycle was established to obtain a fresh material from a controlled environment. Presence of several different subpopulations of EVs was hinted as well as two different classical biogenesis pathways: a) direct membrane shedding; b) endosomal origin. Obtained data correspond well with the recent discoveries in the field of helminth-derived EVs (Drurey & Maizels, 2021; Sánchez-López et al., 2021).

*Schistocephalus solidus* is a diphylobothriidean tapeworm and the first tapeworm with partial aquatic life cycle where EV secretion was observed. It has been in the past established as an important model organism (Barber & Scharsack, 2010, Hébert et al., 2016). The life cycle as well as tegumental dynamics (pinocytosis/phagocytosis, secretion, etc.) were intensively studied, which provided a solid ground for novel molecular studies such as transcriptomics or secretome studies (Smyth, 1946; Berger & Aubin-Horth, 2020).

In the present study, life cycle of *S. solidus* has also been maintained in the laboratory. However, amount of EVs available was rather low, which limited the present study in some aspects. Obtaining just few plerocercoids from experimental fish represented a serious methodological obstacle. For this reason, two different isolation techniques (UC and SEC) were implemented. SEC was initially planned as the sole isolation technique to be used in the experiments, it had to be replaced as an insufficient amount of EVs was collected (data not shown). This subsequently led to the use of UC, which provided better results in the terms of particle concentration (Figs. 9–10). However, this method may lead to isolation of a higher amount of soluble particles and protein aggregates, which would hinder possible molecular characterization of isolated EVs or eventually internalization studies, as was previously reported (Mol et al., 2017). Moreover, we could observe that high speeds of UC led to deformation of isolated EVs, when compared to those observed on the tegumental surface. Furthermore, SEC-based protocols would allow for a partial separation of different subpopulations of EVs.

Despite the initial difficulties in the methodology, EVs isolated from the cultivation medium of adults of *S. solidus* corresponded well to those observed in other tapeworm species (Sotillo et al., 2020). Moreover, studies of the tegument of proceroids and adults showed EV-

like structures released on their surface (Figs. 5B, 6D–F). Regarding their biogenesis, no solid evidence of their secretion pathways has been observed. Only a single case of direct shedding of MV from the plasmatic membrane was observed (Fig. 7E, F). It is likely that their secretion is dependent on direct contact with the host environment as previously suggested (Angelot et al., 2009). It can be thus expected that if plerocercoids were fixed immediately after dissection of sticklebacks, MV abundance would be significantly higher. Apart from this, when observing micrographs of the syncytial tegument for the signs of endosomal pathway, multiple MVB-like bodies were detected (Figs. 5F, 7C, D). Without implementing more advanced techniques such as specific immunolabelling, these different vesicle types cannot be classified with certainty. Based on the density of their content, we can theoretically differentiate between pinosome/phagosomes and multivesicular bodies carrying ILV. We believe that the lucent-MVB content (Fig. 7C) indicates absorption of the vesicle from the extracellular environment into the tegument, and thus could be considered to be pinosome/phagosome. In contrast, the dense MVB (Fig. 7D) content indicates a possible origin of the vesicle in the tegument. Moreover, we can see that some of the presumed pinosomes/phagosomes carry EV-like bodies, indicating a possible autophagy of own EVs. Further experiments should be done to confirm or deny our hypothesis. In order to do so, proteomic data should be obtained and machineries involved in the endosomal pathways should be detected. Furthermore, it is still not clear whether or not the EV biogenesis follows the same mechanism as that described in mammalian cells. However, a recent study on conserved domains of protein of the biogenesis machineries shows a relatively high similarity with mammalian counterparts (Bennett et al., 2020). Moreover, differences between segments (anterior, middle and posterior) of the adult strobila indicate that EVs are released across the whole length. It seems that the youngest (anterior) proglottids release more EVs than the remaining part of the strobila (no statistical data provided). As another mechanism of EV or ESP secretion could be considered dense bodies that have been for long considered as the main mechanism of microthrix formation (Bråten, 1968). It has been revealed that they can play a role in EV secretion as well (Mazanec et al., 2021).

Similar to the observations of adults, EV production by proceroids in the copepod body cavity was detected (Fig. 5B). In contrast to the adults, larval stages of tapeworms have been more studied in the term of their EV release, most likely due to their easier acquisition (Santos et al., 2016; Ancarola et al., 2017; Siles-Lucas et al., 2017; Zheng et al., 2017; Wang et al., 2020). Compared to the observations in larval stages of cyclophyllidean tapeworms,

procercoid of *S. solidus* is not protected by any form of laminated layer that would protect the plasmatic membrane and released EVs. Moreover, EVs of the larval stages of the cyclophyllidean tapeworms are mostly detected in the interface of the germinal and laminated layer, implying their role after the disintegration of laminated layer upon their ingestion (Ancarola et al., 2017). In the case of procercoids observed in this study, we saw ESP layer and associated EVs unprotected from the host environment. Interestingly, we saw that ESPs create a dense layer close to the procercoid surface and no EV-like bodies were observed further away in the copepod body cavity. This would imply that there is no intense interaction of ESPs with the host environment. This observation can however be simply an artifact of the fixation method used.

Although it was not a subject of the present study to reveal a role of EVs in the life cycle of *S. solidus*, available data indicate a pattern in the ESP secretion of adults. The secretory activity mirrored that of the egg production during the cultivation of plerocercoids (Fig. 8). Data show that the peak and the end of the increased secretory activity is on the same days as was the peak and end of egg production. Therefore, it can be assumed that: i) ESP may have a protective role for the eggs during their release into the intestine, which would increase the chances of release of healthy eggs into the environment; ii) ESPs have a protective role for the adult during the egg production; or iii) ESPs are simply a byproduct of the egg production. Interestingly, even after the ceased egg release, the adults continued to show motility for the next two days. Nevertheless, this can be simply due to a lack of natural environment that would normally lead to their disintegration. To find a better explanation for the parasite-host interaction and the pattern, we should focus more on the molecular characterization of their protein or RNA content. Since we do not have any of these data, we are not able to say more or confirm hypotheses on the role of EVs/ESP during the maturation of plerocercoids in cultivation medium.

## 6 CONCLUSION

1. The life cycle of the tapeworm *Schistocephalus solidus* was successfully established and maintained in the laboratory conditions.
2. The present study confirmed for the first time that *S. solidus*, which is an important experimental model in behavioural and evolutionary parasitology, produces extracellular vesicles.
3. EVs secretion was observed in different parts of the surface of the proceroid and adult of *S. solidus*.
4. Secreted EVs were isolated from the cultivation medium collected during the maturation of plerocercoids.
5. Based on the observed secretory activity, we hypothesized several functions of EVs during the maturation of plerocercoids of *S. solidus*.

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