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Invited Review

Snail defence responses to parasite infection: the *Lymnaea stagnalis-Trichobilharzia szidati* model

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Abstract

Lymnaea stagnalis is a common freshwater gastropod. Importantly, the snail serves as the intermediate host for more than one hundred species of digenetic trematodes, including the avian schistosome *Trichobilharzia szidati*, a causative agent of cercarial dermatitis in humans. Infection of *L. stagnalis* by *T. szidati* initiates a dynamic confrontation between the host and the parasite that culminates in immunocompatibility ensuring survival and development of larvae. Unfortunately, the molecular mechanisms determining this immunocompatibility remain poorly characterised. By employing a variety of immune elicitors, including chemical compounds, PAMPs and bacteria, research in the last two decades has elucidated some of the molecular processes that regulate the snail internal defence response such as haemocyte signalling pathways. These discoveries provide a framework for future studies of molecular interactions between *T. szidati* and *L. stagnalis* to help elucidate factors and mechanisms enabling transmission of schistosome parasites. Moreover, support from recently available next generation sequence data and CRISPR-enabled functional genomics should further enable *L. stagnalis* as an important model for comparative immunology and contribute to a more comprehensive understanding of immune function in gastropod molluscs.

Keywords: Lymnaea stagnalis; *Trichobilharzia szidati*; avian schistosome; haemocytes; phagocytosis; encapsulation

1. Introduction

The great pond snail, *Lymnaea stagnalis* (class: Gastropoda), is widely distributed in freshwater habitats over large parts of Europe, North America and Asia (Berrie, 1965; Faltýnková et al., 2007); it usually occupies shallow pond margins with dense vegetation where it feeds on algae or decaying plants (Bovbjerg, 1968). Although hermaphroditic, sexually mature *L. stagnalis* prefers cross-fertilisation and egg production may reach as many as 19 eggs per snail in natural conditions in 48 h (Boag and Pearlstone, 1979). The snail breeds for as long as it survives and the estimated survival is up to seven years (Boag and Pearlstone, 1979).

Naturally occurring pathogens such as viruses, bacteria, fungi, and digenetic trematodes infect freshwater gastropods (Loker, 2010), including *L. stagnalis*. Infections with digenetic trematodes have a substantial impact on the snail host; oviposition, snail growth or behaviour may dramatically be altered. Moreover, successful establishment of the infection results in the development of intramolluscan parasite stages (e.g. cercariae) that are infectious for a subsequent host(s). This is also the case for the avian schistosome *Trichobilharzia szidati* using *L. stagnalis* as its specific intermediate host (Horák et al., 2002). Other than waterfowl as suitable definitive hosts, *T. szidati* can penetrate accidental hosts such as humans, and cercarial dermatitis is the most prominent symptom of these infections (Horák et al., 2015) (Fig. 1).

Long-term laboratory maintenance of *T. szidati* and *L. stagnalis* has enabled experiments that have uncovered the intimate molecular interactions between both partners (de Jong-Brink et al., 2001; Haas, 2003; Horák et al., 2015) and elucidated, at least in part, the aspects of snail immune function and factors enabling transmission of the parasite. In addition, our knowledge on *L. stagnalis* defence responses has expanded through investigations with various other immune elicitors including chemicals, substances that represent pathogen-associated molecular patterns (PAMPs) and/or bacterial agents (van der Knaap et al., 1981a; van der Knaap et al., 1993; Walker and Plows, 2003; Walker et al., 2010). Herein, we discuss

the life cycle of *T. szidati* and snail host finding, immune-recognition and interplay and thereafter synthesise the current knowledge of molecular regulation of *L. stagnalis* cellular defence responses, particularly in the context of survival in the snail host. Furthermore, we anticipate that approaches such as next generation sequencing (NGS) (Dheilly et al., 2014; Schultz and Adema 2017) and CRISPR-enabled functional genomics (Abe and Kuroda, 2019) represent promising and powerfull tools to support in-depth investigations of *L. stagnalis*-*T. szidati* host-parasite interactions and immunocompatibility, and immune functions in gastropod molluscs.

2. *Trichobilharzia szidati* – a schistosome transmitted by *L. stagnalis* in temperate regions

Trichobilharzia szidati is a common European species of avian schistosome (Horák et al., 2015; Horák et al., 2019). Many publications originating from European laboratories concern *Trichobilharzia ocellata*, but this erroneously named parasite is identical to *T. szidati* (Rudolfová et al., 2005; Aldhoun et al., 2016) and thus the latter name is used here. If *T. ocellata* is explicitly mentioned, then it refers to non-European isolates.

Trichobilharzia szidati has a two-host life cycle (Fig. 1). In adulthood, the parasite resides in the blood system surrounding the intestinal wall of the definitive host (waterfowl) where the fertilised female produces eggs that pass (fully developed) via faeces into freshwater (Horák et al., 2002). Subsequently, miracidia hatch and seek an appropriate intermediate snail host (see below). Once inside the snail, parasite development (time interval ranging from 3-10 weeks) includes the transformation of a miracidium to a mother sporocyst, followed by asexual production of daughter sporocysts and cercariae (Fig. 1) (Horák et al., 2002). This latter stage is shed by *L. stagnalis* into water during the patent period of infection. Interestingly, almost 30,000 cercariae have been determined as the maximum daily emergence rate per snail (Soldánová et al., 2016). Swimming *T. szidati* cercariae exhibit a complex pattern of behaviour

to enhance their chances of finding a suitable definitive host (Feiler and Haas, 1988). Upon contact, cercariae actively invade the skin of the final host and transform to schistosomulae that migrate to the vasculature where, after maturation to adult worms, mating and egg laying ensue.

3. *Lymnaea stagnalis-T. szidati*: host finding and complex biological interplay

After hatching, the *T. szidati* miracidia actively seek and penetrate *L. stagnalis*. This process is time-limited since miracidia have restricted glycogen energy reserves and die within ~20 h at 20 °C (Neuhaus, 1952). In general, miracidia display negative geotaxis and positive phototaxis to help them navigate to water surface sites that are also preferred by *L. stagnalis* (Hertel et al., 2006). Subsequently, miracidia perceive and are attracted to chemical compounds emitted by the snail. These compounds are rich in mucin-type glycoproteins termed miracidium-attracting glycoproteins (MAGs) or miraxones and are important in determining species-specific recognition (Kalbe et al., 2000). The unique signal for the miracidium, which promotes parasite attachment and penetration, lies in the composition of carbohydrate moieties of these MAGs (Kalbe et al.,1997). Additionally, cytotoxic activity of mucus components secreted by glandular surface cells (mucocytes; Allam and Espinosa, 2015) has recently been suggested to determine, at least in part, larval trematode-snail compatibility (Coyne et al., 2015). So far, however, these components and their cytotoxicity towards trematodes, including *T. szidati*, have not been investigated in *L. stagnalis*.

Successful development of *T. szidati* in *L. stagnalis* is realised under conditions involving a vast array of parasite-induced alterations that allow survival of larvae, growth, and asexual multiplication (Joosse and van Elk, 1986; de Jong-Brink et al., 2001). To achieve this, components of the snail immune and neuroendocrine systems that maintain physiological homeostasis are modulated by the parasite, leading to, for example, reproductive system

112 impairment and castration. The mechanisms governing the dramatic parasite-induced effects on
113 *L. stagnalis* remain to be fully characterised.

114 Early phases of *T. szidati* infection (transition from miracidium to mother sporocyst)
115 are accompanied by the retarded development of reproductive organs in juvenile *L. stagnalis*
116 after one week. Thereafter, in the presence of daughter sporocysts with differentiating cercariae,
117 snail reproduction is completely inhibited after approximately three weeks, and egg laying does
118 not occur (Sluiters, 1981). Interestingly, mechanical damage to the affected tissues is not visible
119 (McClelland and Bourns, 1969). Older snails that become infected display increased or similar
120 rates of oviposition when compared to uninfected snails during the first four weeks, but then
121 activity drops during patency. Older infected snails also show a similar growth rate to
122 uninfected counterparts (Schallig et al., 1991) which is in contrast to juvenile snails exhibiting
123 giant growth, thought to provide sufficient space for larval development after two weeks of
124 infection (Sluiters et al., 1980). Apparently, the cessation of reproduction and stimulation of
125 giant growth are preceded by disruption of neuroendocrine control in infected snails.

126 The neuroendocrine system of *L. stagnalis* comprises at least ten cell types with
127 secretory activity (Roubos, 1976). Among them are caudodorsal cells (CDCs) of the cerebral
128 ganglia releasing neuropeptides such as calfluxin (CaFl) or caudodorsal cell hormone (CDCH)
129 (Dictus et al., 1987; Dictus and Ebberink, 1988; Li et al., 1992) and dorsal bodies (DB) that
130 produce dorsal body hormone (DBH) (Wijdenes et al., 1983) that controls egg laying. Together,
131 CaFl, CDCH, and DBH act as gonadotropic hormones for the snail reproductive system.
132 Growth control is principally attributed to other cells of the cerebral ganglia, light green cells
133 (LGCs), that release various molluscan insulin-related peptides (MIPs) as important metabolic
134 integrators (Smit et al., 1988; Geraerts, 1992).

135 In *L. stagnalis* infected by *T. szidati* for three weeks the albumen gland response to
136 CaFl is suppressed (de Jong-Brink et al., 1988a). An unknown parasite-derived substance(s)

(likely from the cercariae) induces cells of the connective tissue, teloglial cells, and haemocytes to release a factor, schistosomin, that is detectable in snail haemolymph after six weeks (de Jong-Brink et al., 1988a; Schallig et al., 1991; Schallig et al., 1992; de Jong-Brink, 1995). Schistosomin, comprised of 79 amino acids (8.7 kDa) (Hordijk et al., 1991a), acts as an antagonist of the neuroendocrine hormones regulating both reproduction and growth in *L. stagnalis* (de Jong-Brink et al., 1988a; de Jong-Brink et al., 1988b; de Jong-Brink and Bergamin-Sassen, 1989; de Jong-Brink et al., 2001; Hordijk et al., 1991b; Hordijk et al., 1992). For instance, it inhibits CaFl-regulated influx of Ca^{2+} ions into the cells of albumen gland (de Jong-Brink et al., 1988b) or adenylate cyclase activation by DBH in the follicle cells (de Jong-Brink and Bergamin-Sassen, 1989). Consequently, ovulation and egg laying are inhibited in the snail. Schistosomin also modulates the electrophysiological activity of neuroendocrine cells, as demonstrated by increased excitability of LGCs responsible for the growth in *L. stagnalis* (Hordijk et al., 1992; Geraerts, 1992). Schistosomin is also expressed in *B. glabrata* (Zhang et al., 2009). However, contrasting that seen with *L. stagnalis*, infection of *B. glabrata* with trematode parasites (*S. mansoni* or *Echinostoma paraensei*) does not cause altered expression of this neuropeptide; instead, schistosomin is thought to play a role in *B. glabrata* development rather than parasite-mediated castration (Zhang et al., 2009). Thus, schistosomin seems to possess distinct roles (e.g. developmental and reproductive) in different snail species and/or may be modulated differentially in response to certain parasitic infections (de Jong-Brink et al., 1991; de Jong-Brink, 1995; de Jong-Brink et al., 2001; Zhang et al., 2009). Parasitisation of *T. szidati* in *L. stagnalis* also alters gene expression of many proteins including immune molecules (see below), mitochondrial enzymes, and neuropeptide precursors, as revealed by differential screening of cDNA libraries of both infected and uninfected snails (Hoek et al., 1997; Hoek et al., 2005; de Jong-Brink et al., 2001). As for neuropeptides, the expression of the CDCH-encoding gene is down-regulated, an effect which contrasts upregulated transcription of

the MIP-III gene early in infection (Hoek et al., 1997). Other up-regulated genes in infected *L. stagnalis* encode FMRFamide-related peptides (Hoek et al., 2005). These peptides, via inhibition of LGCs and CDCs, suppress snail metabolism and reproduction. All these changes likely favour parasite development in *L. stagnalis* (de Jong-Brink et al., 2001).

Despite the interferences outlined above, the life-span of *T. szidati*-infected *L. stagnalis* is not thought to substantially differ from that of uninfected counterparts (Sluiters et al., 1980; Sluiters, 1981). In fact, in one study, the infected *L. stagnalis* survived longer under laboratory conditions with ~90% and ~50% of snails harbouring *T. ocellata* (North American isolate) surviving at 28 and 46 weeks, respectively (with three individuals shedding cercariae 19 months after infection), compared to control snails which all died after 28 weeks (McClelland and Bourns, 1969). *Trichobilharzia szidati*-infected snails have also been shown to prefer a cooler microhabitat that may prolong their survival rate (Zbikowska, 2005). The snails survive almost twice as long at 16 °C than at 25 °C and such life-span extensions increase the number of shed cercariae.

4. The internal defence system of uninfected *L. stagnalis* is only ineffective against compatible pathogens

In common with other gastropods (Loker, 2010), *L. stagnalis* has evolved an innate immune system capable of fending off pathogens, to maintain the integrity of the organism. *Lymnaea stagnalis* has an open circulatory system which contains "blood" called haemolymph; here (non-compatible) pathogens are usually recognised and eliminated by humoral and cellular arms of the snail internal defence system (IDS).

Humoral components of the *L. stagnalis* IDS include bacteriostatic substances that may directly exert antimicrobial activity (van der Knaap and Meuleman, 1986). Various soluble (or cell-bound) molecules called pattern recognition receptors (PRRs) such as lectins are also

present; these recognise pathogens and trigger cell-mediated defence responses (Horák and van der Knaap, 1997).

Cellular components of the *L. stagnalis* IDS encompass fixed defence cells that are dispersed through the connective tissue and entrap or phagocytose foreign particles, and mobile haemocytes considered to be more important for defence (Fig. 2A) (Sminia et al., 1979a; van der Knaap et al., 1993). Haemocytes circulate freely in the haemolymph and can be found in the connective tissue throughout the snail body (Sminia, 1972). They likely represent one cell type undergoing morphological changes during maturation (Sminia, 1972; Dikkeboom et al., 1984; Mohandas et al., 1992). Mature haemocytes are capable of employing multiple defence activities such as phagocytosis, encapsulation, and the production of reactive nitrogen (RNIs) or oxygen (ROIs) intermediates to eliminate pathogens (Fig. 2B-E, 3C-D) (Dikkeboom et al., 1987; Adema et al., 1993). Additionally, haemocytes of *L. stagnalis* (and those of *Planorbarius corneus* and *Radix lagotis* snails) have recently been shown to generate extracellular trap-like (ET-like) fibers *in vitro* (Skála et al., 2018). Despite the fact that the functional characterisation of the fibers in the snail defence is required, these structures produced by, for example, neutrophils (neutrophil extracellular traps) of vertebrates or haemocytes (ET-like fibers) of invertebrates including molluscs are considered to facilitate elimination of bacteria and eukaryotic unicellular/multicellular parasites in the extracellular milieu (Hermosilla et al., 2014; Robb et al., 2014; Poirier et al., 2014; Lange et al., 2017).

4.1. Recognition of foreignness and signalling cascades involved in *L. stagnalis* defence

Discrimination of non-self by haemocytes represents the first step in executing an appropriate immune response to invaders. Initial contact between haemocytes and foreign surfaces is influenced non-specifically by factors such as electrostatic forces or hydrophobicity while more rigorous discrimination requires the involvement of specific components. These

include receptors associated with the surface membrane of haemocytes and soluble (i.e. cell-free) recognition factors that activate haemocytes (van der Knaap et al., 1983b; Horák and van der Knaap, 1997; Plows et al., 2006a). Our knowledge of such receptor-ligand interactions, together with the downstream mechanisms that direct the signals to convey an appropriate immune response is currently poor (particularly considering *L. stagnalis*-*T. szidati* interactions) and most findings are derived from investigations where chemicals, PAMPs or bacteria have been used as immune elicitors.

In accordance with the view that widely distributed lectins serve as immunorecognition receptors in invertebrate taxa (Yeaton, 1981; Vasta et al., 2004), these carbohydrate binding proteins have been found in the *L. stagnalis* IDS and some of their properties and/or binding abilities have been characterised (van der Knaap, 1981; van der Knaap et al., 1982; van der Knaap et al., 1983a,b; Horák and van der Knaap, 1997; Horák and Deme, 1998; Horák et al., 1998). Lectins are produced by snail haemocytes as well as by connective tissue cells as demonstrated immunocytochemically (van der Knaap et al., 1981b), and they are either released to plasma or become associated with cell membranes. The presence of lectins on haemocytes has been illustrated by means of saccharide-dependent inhibition of phagocytosis (Horák and Deme, 1998; Horák et al., 1998), and some lectins have been found as reversibly bound cytophilic receptors on the cell surface (van der Knaap et al., 1983b). Saccharide moieties on haemocyte membranes that may participate in lectin-based immunorecognition have been, at least in part, also characterised (Fig. 3A) (Horák and Deme, 1998). In their soluble form, lectins act as opsonins, i.e. they bind to a foreign surface and create attractive epitopes that are recognised by haemocytes, or they function as agglutinins limiting the spread of microbes throughout the snail body (Sminia et al., 1979b; van der Knaap, 1981; van der Knaap et al., 1982; Horák and van der Knaap, 1997).

Currently, polymorphic lectin-like molecules, fibrinogen-related proteins (FREPs), have attracted considerable attention as PRRs of gastropods (Gordy et al., 2015). Gastropod FREPs possess a unique architecture since they are composed of a fibrinogen domain connected to one or two immunoglobulin superfamily domain(s) (Gordy et al., 2015). Importantly, FREPs are somatically diversified and thus exhibit functional specialisation against various pathogens; their central role is attributed to defence against trematode parasites, as comprehensively studied using the model *Biomphalaria glabrata-Schistosoma mansoni* (Adema et al., 1997; Gordy et al., 2015; Pila et al., 2017). In *L. stagnalis*, molluscan defence molecule (MDM), a different type of soluble snail non-self recognition factor, has been identified. This member of immunoglobulin superfamily consists of five tandemly arranged Ig domains but is otherwise not related to FREPs, and acts as an enhancer of haemocyte phagocytic activity (Hoek et al., 1996). While FREPs clearly play a vital role in immune recognition by *B. glabrata*, the presence of these factors in *L. stagnalis* and their possible immunoregulatory role(s) remains to be elucidated.

Another class of receptors, cell-surface bound integrins, has been found to be expressed on *L. stagnalis* haemocytes (Plows et al., 2006a). These receptors are of fundamental importance for cell adhesion (Ruoslahti, 1991). In *L. stagnalis* haemocytes, adhesion via integrins was found to be Ca^{2+}/Mg^{2+} dependent, and integrin blocking peptides significantly reduced haemocyte phagocytic activity and spreading, thus confirming the importance of integrins in cellular effector functions (Plows et al., 2006a). Furthermore, integrin engagement has also been linked to intracellular signalling events in *L. stagnalis* haemocytes (Plows et al., 2006a; Walker et al., 2010). Early stages of cell adhesion and spreading are accompanied by phosphorylation (activation) of protein kinase C (PKC) (Fig. 3B) and focal adhesion kinase (FAK); activated PKC also colocalised with focal adhesion sites (Walker et al., 2010). Furthermore, specific inhibitors of PKC and tyrosine kinase Src attenuated haemocyte

spreading. Association of PKC, FAK, and Src into complexes has been suggested to promote focal adhesion assembly in *L. stagnalis* haemocytes (Walker et al., 2010), and this may modulate haemocyte adhesion to pathogens.

Studies implicating the role of signalling pathways in defence activities of *L. stagnalis* haemocytes have also been performed with chemical compounds or PAMPs. Challenge of haemocytes with bacterial lipopolysaccharides (LPS) activates PKC (Walker and Plows, 2003) and extracellular signal-regulated kinase 1/2 (ERK1/2) that is a member of the mitogen-activated protein kinase (MAPK) family (Plows et al., 2004). MAPK/ERK kinase (MEK) inhibitors (PD98059/U016) suppress ERK1/2 activation that in turn attenuated phagocytosis of *E. coli* bioparticles; suppression of phagocytosis by FTase inhibitor I also implicates Ras in this process (Plows et al., 2004). Furthermore, when ERK1/2 activation was blocked together with PKC, phagocytic activity of *L. stagnalis* haemocytes was reduced to a greater extent than by ERK1/2 inhibition alone, suggesting that both kinases (PKC, ERK1/2) are important for effective haemocyte phagocytic activity (Plows et al., 2004). Additionally, other signalling cascades such as those involving phosphatidylinositol 3-kinase activation appear to control phagocytosis in *L. stagnalis* (Plows et al., 2006b).

PAMPs such as laminarin (a storage β-glucan of brown algae) and zymosan (cell wall β-glucan of fungi), or the chemical compound phorbol myristate acetate (PMA) acting as a reversible activator of PKC, have been found to stimulate *L. stagnalis* haemocyte nitric oxide (NO) production (Fig. 3C-D), with laminarin and PMA having the greatest effect (Wright et al., 2006). Furthermore, laminarin has been found to stimulate the release of the ROI hydrogen peroxide (H_2O_2) (Lacchini et al., 2006). At the molecular level, PKC and ERK1/2 signalling seem to play a pivotal role in nitric oxide synthase (NOS) activation and subsequent NO production, while PKC-mediated signalling has been linked to H_2O_2 release (Wright et al., 2006; Lacchini et al., 2006).

Together, PKC and ERK1/2 signalling have received particular attention with regard to the regulation of *L. stagnalis* haemocyte defence responses. However, while these pathways play important roles in such processes, a mosaic of yet to be discovered interactions of PKC and ERK1/2 with other pathways is expected to evoke an appropriate defence response to pathogens. Additionally, almost nothing is known about haemocyte membrane receptors *per se* as triggers of signalling cascades in *L. stagnalis* haemocyte defence processes. With the support of the recently published *B. glabrata* genome (Adema et al., 2017), there now exists fresh opportunity for molecular studies on gastropod defence processes employing models such as *L. stagnalis*. Importantly, transcriptomic data and a genome assembly are now publicly available for *L. stagnalis* (Davison et al., 2016; Liu et al., 2014; https://www.ncbi.nlm.nih.gov/assembly/GCA_900036025.1/).

4.2. Immune responses of *L. stagnalis* during *T. szidati* infection

It has long been observed (van der Knaap et al., 1987; Amen et al., 1991a) that *T. szidati* alters *L. stagnalis* defence responses, thus preventing its elimination. The parasite predominantly employs larval secretions and tegumental surface components to achieve both direct and indirect immunomodulation. However, the precise nature of the molecular interplay at the host-parasite interface that ultimately determines immunocompatibility between *L. stagnalis* and *T. szidati* warrants elucidation.

In the first few hours post infection (h p.i.) with *T. szidati*, the IDS of *L. stagnalis* is clearly activated as shown by enhanced phagocytosis of zymosan particles or *Aeromonas salmonicida* bacteria by haemocytes *in vitro* (Amen and de Jong-Brink 1992; Amen et al., 1992a; Nuñez et al., 1994). Such activation is likely triggered by the shed miracidial ciliated plates (Amen et al., 1992a) that are highly antigenic and have been observed within phagosomes of haemocytes of another lymnaeid snail species (*Radix lagotis*) after infection

with another species of avian schistosome, *Trichobilharzia regenti* (Skála et al., 2014). Excretory-secretory products (ESPs) of *T. szidati* may also be responsible, at least in part, for the induction of *L. stagnalis* haemocyte activity. A predominant low MW (<2 kDa) fraction of ESPs released by the parasite cultured *in vitro* for 33 h stimulates bacterial clearance by haemocytes (Nuñez et al., 1997). Intriguingly, this ESPs fraction operates in a rather non-specific manner, since it also activates haemocytes from the incompatible snail *Planorbarius corneus* (Nuñez and de Jong-Brink, 1997), a species of the family Planorbidae, different from the Lymnaeidae that incorporate *L. stagnalis*.

From 12-72 h p.i., *L. stagnalis* haemocytes display a reduced capacity to eliminate foreign particles (Amen and de Jong-Brink 1992; Amen et al., 1992a; Nuñez et al., 1994). *In vitro*, a high MW fraction of *T. szidati* ESPs obtained between 33-72 h of culture suppressed the phagocytosis of bacteria (Nuñez et al., 1997). Importantly, haemocytes from the incompatible planorbid snail (*P. corneus*) remained unaffected in terms of bacterial clearance; thus, this fraction appears to act specifically (contrary to the low MW ESPs fraction) (Nuñez and de Jong-Brink et al., 1997). Although the molecule(s) responsible for the attenuation of haemocyte activity in *L. stagnalis* is likely a glycoprotein(s) (Nuñez and de Jong-Brink, 1997), detailed characterisation has not been accomplished. Such analysis is at least in part complicated by the fact that the high MW ESP fraction is composed of around 20 components ranging from 10-100 kDa (Nuñez and de Jong-Brink, 1997). In addition, ESPs derived from the 72-96 h culture medium did not affect the phagocytic activity of the haemocytes (Nuñez et al., 1997).

The miracidium-to-mother sporocyst transformation also involves considerable changes in the composition of carbohydrate moieties on the surface of *T. szidati* as demonstrated by use of a wide range of lectin probes (Gerhardus et al., 1991; Horák, 1995). Although the functional significance of these moieties awaits elucidation, their role has been attributed to molecular mimicry, enabling the schistosome to evade recognition and thus

elimination by the host defence (Horák, 1995; Horák et al., 2002). The larval stages of *T. szidati* (miracidium, mother sporocyst, and cercaria) contain a polypeptide exhibiting N-acetylgalactosaminyltransferase activity that controls the synthesis of LacdiNAc-type glycans that are also common to *L. stagnalis* and might, therefore, contribute to molecular mimicry (Neeleman et al., 1994). Furthermore, solubilised carbohydrates (D-galactose, L-fucose) that are also present on the surfaces of schistosome larvae (Horák, 1995; Blažová and Horák, 2005; Chanová et al., 2009) down-regulated the activity of ERK1/2 and PKC in *L. stagnalis* haemocytes, a finding which suggests an immunosuppressive role (see above) (Plows et al., 2005; Walker, 2006). While the parasite surface moieties of cercariae bind snail-derived components that may serve a masking strategy to hide the non-self identity of the parasite (immune evasion), the binding of snail-derived components has not been shown for mother/daughter sporocyst stages of *T. szidati* (Roder et al., 1997; van der Knaap et al., 1985).

Although at least two factors (namely experimental design and seasonal variation in haemocyte activities; Amen and de Jong-Brink, 1992) challenge analysis, the existing literature indicates that the activity of the *L. stagnalis* IDS varies considerably from initial infection with *T. szidati* through long term patency. Parasite-mediated modulation of snail host immunity seems to differentially impact responses to different pathogens and it is not constant, potentially varying with the development of consecutive larval parasite stages. Haemocytes of infected snails eliminated live *Staphylococcus saprophyticus* bacteria less effectively at one and five weeks p.i., than thereafter (van der Knaap et al., 1987). In contrast, haemocytes efficiently phagocytosed rabbit red blood cells *in vitro* during the first three weeks of infection but the response declined from five weeks onwards (van der Knaap et al., 1987). Haemocyte phagocytic activity towards zymosan particles *in vitro*, and haemocyte peroxidase activity also increased in infected snails between two and four weeks p.i., but both activities were suppressed six weeks p.i. when parasite infection has developed such that daughter sporocysts with

differentiating cercariae were present (Fig. 4A-C) (Amen et al., 1991a). Contrary to these results, spreading of haemocytes was unaffected at the sixth week p.i. (Amen et al., 1991b). At five weeks p.i., an increased number of circulating haemocytes was detected (van der Knaap et al., 1987) which was also confirmed eight weeks p.i. when *T. szidati* cercariae began to escape the *L. stagnalis* host (Amen et al., 1991a). At the start of the patent period of infection (between weeks eight and ten p.i.), haemocyte peroxidase and phagocytic activities increased again (Amen et al., 1991a; Amen et al., 1992a), whereas Horák and Deme (1998) reported just minor differences between the infected snails and uninfected control *L. stagnalis* snails in terms of haemocyte phagocytic activity at ten weeks p.i. Contrary to these observations, phagocytic activity and capacity to generate hydrogen peroxide were suppressed in haemocytes from *R. lagotis* in the patent phase of infection with *T. regenti* (Skála et al., 2014). Moreover, these haemocytes displayed lower PKC and ERK activity when compared to those from uninfected snails. Considering the regulatory role of these pathways in haemocyte defence processes, this suggests how the parasite may achieve suppression of haemocyte defence activities in infected snails (Skála et al., 2014). In addition, the size distribution of circulating haemocytes in *L. stagnalis* was unaffected by *T. szidati* as revealed by flow cytometry at two, four, six, and eight weeks p.i. (Amen et al., 1992b).

Activities of *L. stagnalis* haemocytes are also indirectly altered by *T. szidati* infection, and several factors responsible for this effect are known, with some produced by snail connective tissue and neuroendocrine cells to favour progression of the infection. It is thought that parasite ESPs modulate the expression of genes for MDM (an enhancer of phagocytosis) and granularin (a suppressor of phagocytosis) in granular cells of connective tissue (Hoek et al., 1996; de Jong-Brink et al., 2001; Smit et al., 2004). While MDM expression (although initially up-regulated 5 h p.i.) is gradually down-regulated to 20% of the initial level during infection at eight weeks p.i. (Hoek et al., 1996), the gene encoding granularin is up-regulated from 1.5 h p.i.

onwards (Smitt et al., 2004). Interestingly, these data indicate that *T. szidati* can simultaneously induce (up- and down-regulate) two different genes within one cell type (de Jong-Brink et al., 2001). As for neuroendocrine factors in *T. szidati* infected *L. stagnalis*, up-regulation of genes for FMRFamide-related peptides likely plays an important immunosuppressive role since these substances inhibit haemocyte phagocytic activity (Hoek et al., 1997; de Jong-Brink et al., 2001). In addition, the escape glands of the intrasporocystic cercariae of *T. szidati* contain substances resembling these amides, as shown immunocytochemically (Solis-Soto and de Jong-Brink, 1994). These products are released by cercariae while migrating throughout the snail tissues, and likely alter *L. stagnalis* defence responses at this stage of infection. Overall, further elucidation of these complex interactions will likely reveal regulatory mechanisms that govern immune function in *L. stagnalis*, identifying the factors involved for characterisation toward better comprehensive understanding of gastropod immunobiology.

Perspectives

Lymnaea stagnalis naturally encounters various pathogens including digenetic trematodes. Given the diversity of trematodes, it is probable that diverse strategies are employed by particular species to achieve immunocompatibility with *L. stagnalis*. Therefore, immunological investigations of new models will lead to the discovery of novel mechanisms that enable such intimate relationship between the parasite and the host.

In the context of *L. stagnalis*-*T. szidati* host-parasite interactions, the parasite uses an array of diverse approaches to achieve immunocompatibility. Transition between larval stages is accompanied by changes in parasite morphology and surface components that, together with larval secretions, are considered to play a predominant and integrated role in dynamic immunosuppression of the *L. stagnalis* snail host. However, the molecular basis of this fascinating phenomenon requires further characterisation for which we propose three research

perspectives: (i) Investigation of snail mucous components and their activities in the presence of miracidia may help elucidate determinants of immunocompatibility at the snail surface. (ii) Functional evaluation of the non-self recognition factors like FREPs and other lectins, together with investigations of changes in their expression profiles during *T. szidati* infection, may help elucidate important processes preventing immunorecognition. (iii) Exploring the effect of intact larvae and their secretions on signalling pathways of haemocytes (particularly on those including PKC and ERK1/2) may help elucidate regulatory components of haemocyte activities that are affected by the parasite and, therefore, responsible for disruption of snail IDS function.

The increasing availability of NGS data (e.g. Dheilly et al., 2014; Schultz and Adema, 2017) providing unprecedented transcriptomic and genomic insights will facilitate an increasingly in-depth comparative analysis of general snail biology, including that focusing on snail immune function. Significantly, a recent report demonstrating the successful application of CRISPR-Cas-mediated genome editing (Abe and Kuroda, 2019) in *L. stagnalis* opens up significant opportunities for functional genomics to investigate the role of specific genes in snail immunobiology. Collectively, the further integration of molecular approaches to investigate the IDS of *L. stagnalis*, particularly in the context of parasitic disease transmission, will be crucial to help unravel the complexities of host-parasite compatibility and to help drive forward the study of gastropod immunology.

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774 **Figure captions:**

775 **Fig. 1.** The life cycle of *Trichobilharzia szidati*. **(A)** Eggs are released to freshwater with faeces
776 of the final host. **(B)** The miracidium leaves the egg, searches for and infects the specific
777 intermediate host, *Lymnaea stagnalis*. **(C)** Inside the intermediate host, the parasite reproduces
778 asexually and cercariae are formed and released **(D)** to find and invade the final host – a bird.
779 Inside the final host, the parasite migrates to the blood vessels of the intestine and sexually
780 matures; thereafter mating and egg laying occur. **(E)** Accidentally, humans may become
781 infected by cercariae. Here, the parasite does not complete the life cycle and dies. Cercarial
782 dermatitis (visible on the left forearm) is usually manifested in the places of cercarial
783 penetration (Author: Dr. J. Bulantová).

784 **Fig. 2.** Haemocyte defence responses of *Lymnaea stagnalis* against pathogens. **(A)** SEM image
785 of a spreading haemocyte, the most effective cell type in the snail defence system. **(B)** *In vitro*
786 phagocytosis of *Staphylococcus saprophyticus* bacteria (arrowheads) by haemocytes (arrows).
787 **(C)** Formation of the capsule (c) by haemocytes around incompatible *Trichobilharzia regenti*
788 miracidium (arrowheads) *in vitro* after 1.5 h (differential interference contrast microscopy).
789 Some haemocytes migrate (arrows) towards the parasite to participate in encapsulation. **(D)**
790 Complete encapsulation (arrows) of *T. regenti* miracidia (arrowheads) *in vitro* after 3 h. **(E)**
791 Cross-section through the snail head-foot region showing an encapsulated larva (l) of *T. regenti*
792 by snail haemocytes (arrows) within a few hours after penetration (toluidine blue staining).

793 **Fig. 3.** Lectin binding, signalling cascades and nitric oxide production in *Lymnaea stagnalis*
794 haemocytes observed by fluorescence microscopy. **(A)** Specific binding of wheat germ
795 agglutinin to the surface saccharide moieties of haemocytes. **(B)** Detection of phosphorylated
796 (activated) protein kinase C (arrows) in spreading haemocytes. **(C)** Basal and **(D)** phorbol
797 myristate acetate-induced nitric oxide production by snail haemocytes.

798 **Fig. 4.** Development of *Trichobilharzia szidati* within *Lymnaea stagnalis* hepatopancreas six
799 weeks post-infection (toluidine blue-stained sections). **(A)** Massive proliferation of *T. szidati*
800 larvae (arrowheads) within the snail tissue (st). **(B)** Developing germ balls (arrows) are visible
801 inside the body of daughter sporocyst (ds). **(C)** Cercaria (ce) is in the advanced stage of
802 development with visible penetration gland cells (arrows).

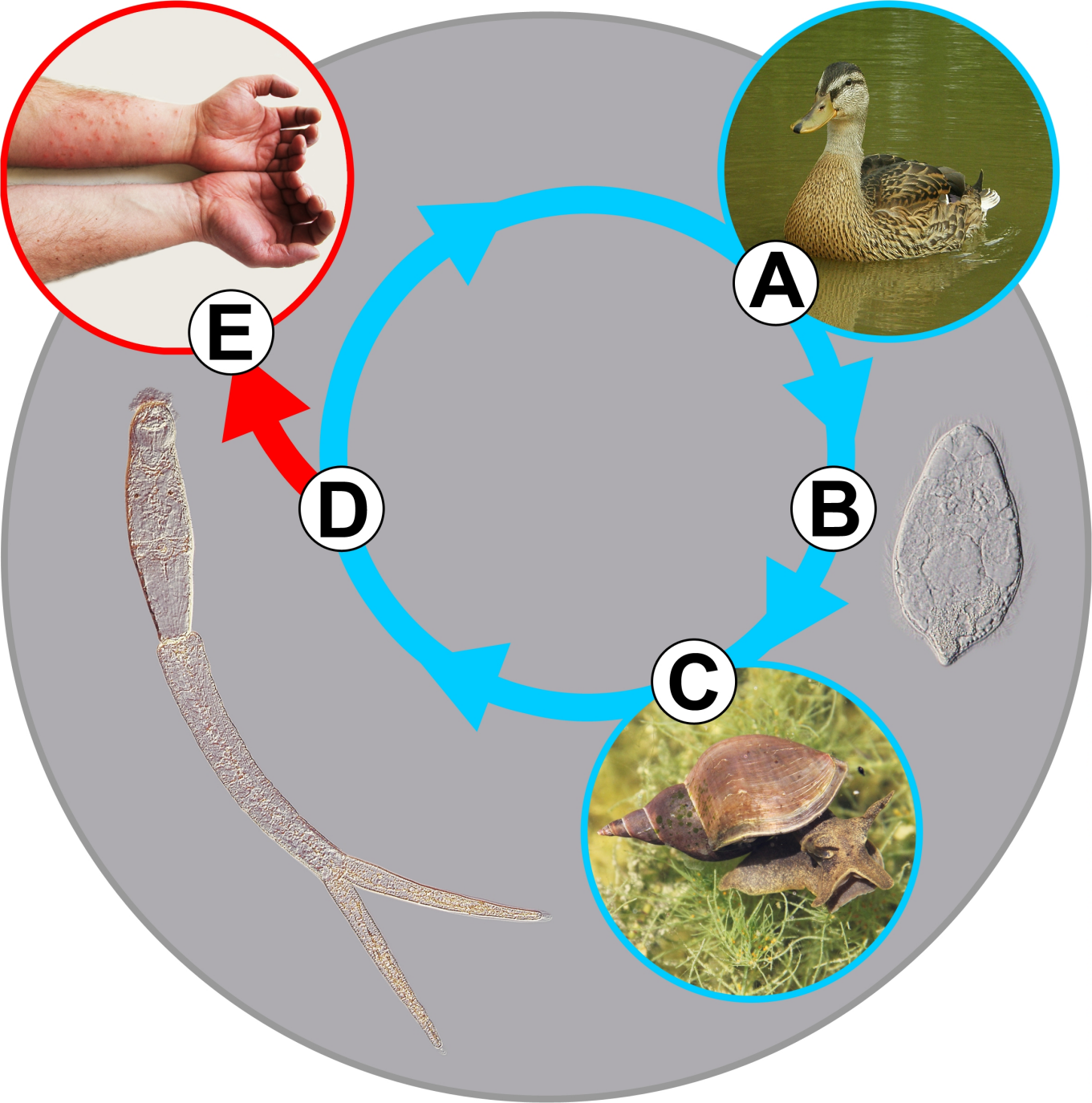
Highlights:

Lymnaea stagnalis is immunocompatible with *Trichobilharzia szidati*.

Trichobilharzia szidati alters *Lymnaea stagnalis* defence responses.

Secretions/surface components of *Trichobilharzia szidati* facilitate immunomodulation.

Molecular basis of *Lymnaea stagnalis*-*Trichobilharzia szidati* compatibility is unknown.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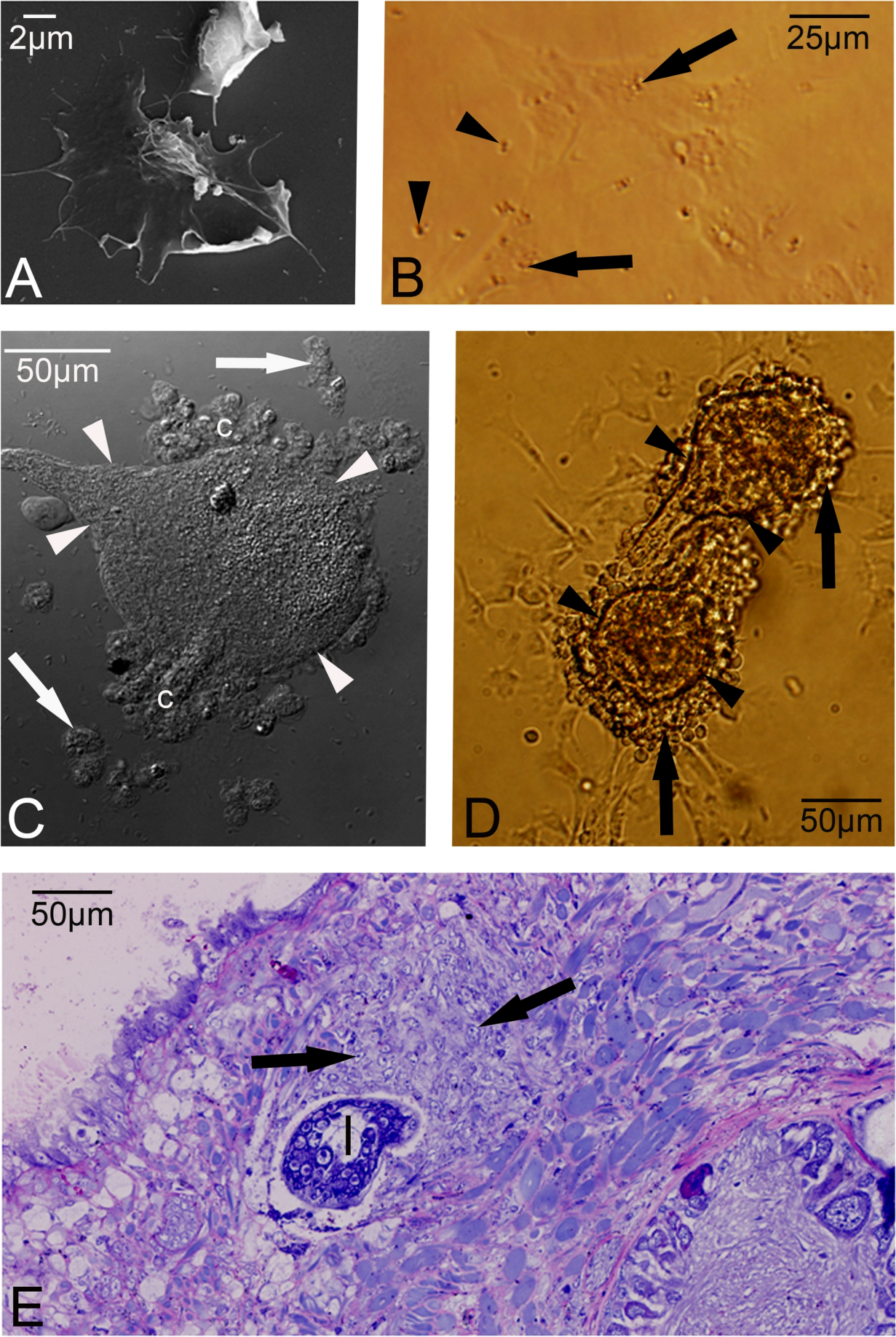
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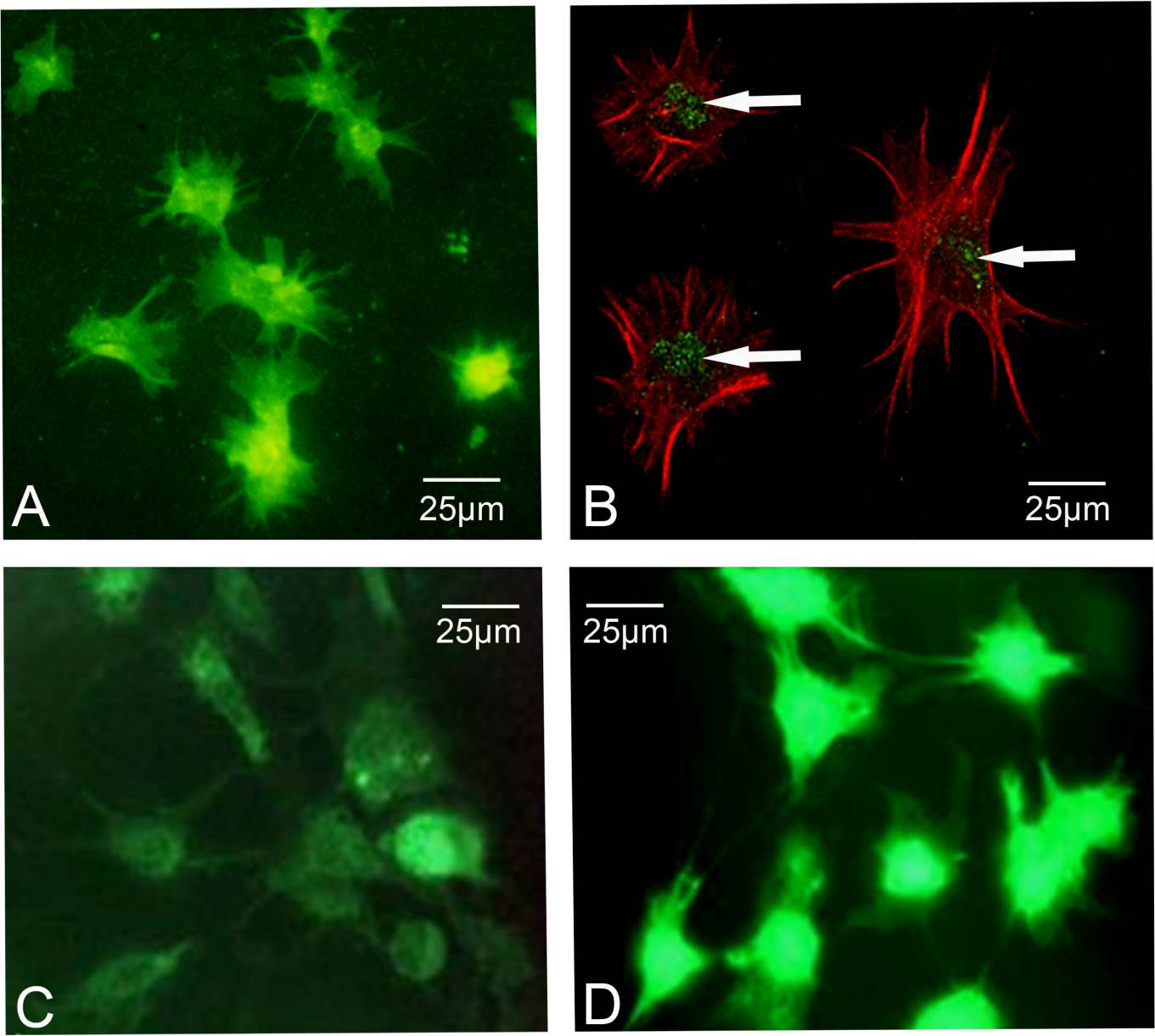
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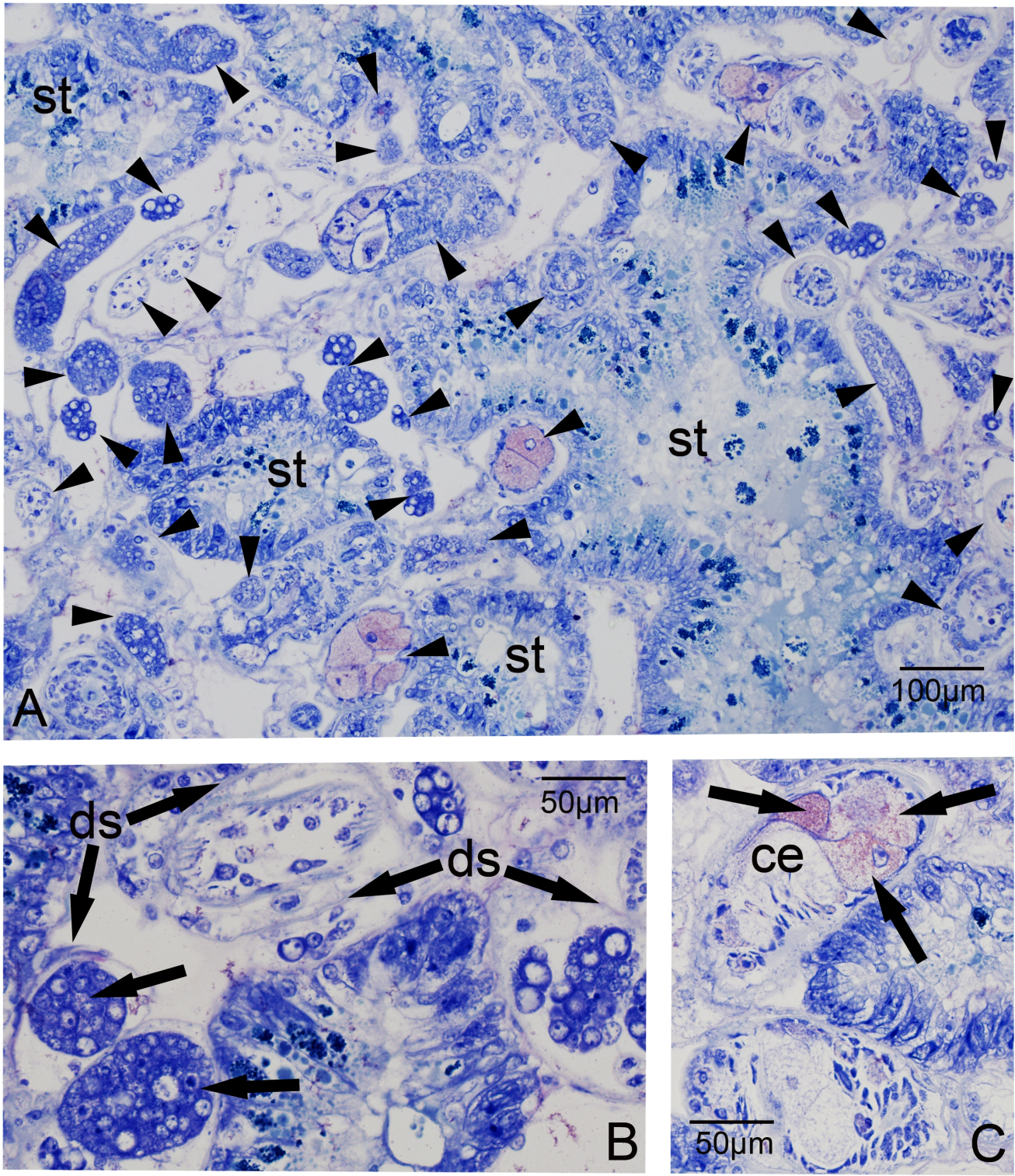
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