**Charles University, Faculty of Science**

**Univerzita Karlova, Přírodovědecká fakulta**

Ph.D. study programme: Parasitology

Doktorský studijní program: Parazitologie

**Mgr. Vladimír Skála**

# Influence of bird schistosome *Trichobilharzia regenti* on haemocyte activity of lymnaeid snails

# Vliv ptačí schistosomy *Trichobilharzia regenti* na aktivitu hemocytů plovatkovitých plžů

Ph.D. thesis/Dizertační práce

**Supervisor/Školitel: Prof. RNDr. Petr Horák, Ph.D.**

**Advisor/Konzultant: Prof. Anthony J. Walker, Ph.D.**

Prague, 2018

**Declaration:**

I declare that the Ph.D. thesis is an original report of my research, has been written by me and all the literary sources have been cited properly. I also declare that neither the thesis nor its substantial part has been used to obtain the same or any other academic degree.

Prohlašuji, že tato dizertační práce je souhrnem mého výzkumu, byla sepsána samostatně a všechny literární zdroje byly řádně uvedeny. Dále prohlašuji, že práce ani její podstatná část nebyla předložena k získání stejného či jiného akademického titulu.

In Prague, 12th April 2018 Mgr. Vladimír Skála

V Praze, 12. dubna 2018

I declare that Vladimír Skála played a major role in the preparation and execution of the experiments, and he substantially contributed to the data analysis, interpretation as well as to the writing of the manuscripts.

Prohlašuji, že Vladimír Skála se podílel na plánování i provedení většiny experimentů a rovněž podstatně přispěl k analýze získaných dat, jejich interpretaci i sepsání přiložených publikací.

In Prague, 20th April 2018 Prof. RNDr. Petr Horák, Ph.D.

V Praze, 20. dubna 2018

**Acknowledgement**

Foremost, I would like to express my sincere thanks to Petr Horák and Tony Walker for the infinite support during my Ph.D. study. I also thank to my colleagues for the stimulating atmosphere in the laboratory, and for all the fun we had.

My special thanks belong to my wife and son.

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**Abstract:**

Gastropod molluscs are naturally exposed to various pathogens such as bacteria, or multicellular parasites that include digenetic trematodes (digeneans) which develop in snails. To combat these pathogens gastropods have evolved a sophisticated internal defence system that is composed of humoral and cellular arms. Lectins are probably the most important humoral components, whereas haemocytes represent the main effector cells. Immunity is one of the important factors determining compatibility/non-compatibility of gastropods and pathogens (particularly snails and trematodes).

The introductory part of this thesis includes a review of literature focused on the components of the gastropod immune system and their reactions against pathogens represented by bacteria and digeneans. Additionally, selected immunomodulations caused by compatible digenean species are reviewed. Experimental work (presented in publications) focused mainly on the influence of the bird schistosome *Trichobilharzia regenti* on haemocyte activities of two lymnaeid snail species, *Radix lagotis* and *Lymnaea stagnalis* that are susceptible or refractory to the parasite, respectively. This schistosome parasite causes neuromotor disorders in specific definitive hosts (waterfowl), but it also causes cercarial dermatitis in accidental hosts such as humans.

The original papers include a review that in part concentrates on intramolluscan development of bird schistosomes, and immune interactions between the parasites and the snail hosts. The publication that focused on *R. lagotis* describes haemocyte defence responses related to the initial phase of *T. regenti* infection, and their modulations during the patent phase of infection. The publication concerning *L. stagnalis* summarises investigations on extracellular trap-like (ET-like) fiber production by snail haemocytes against *T. regenti* and other components as a novel defence response. Furthermore, this phenomenon was studied in two other snail species (*R. lagotis* and *Planorbarius corneus*) for comparative purposes.

The results showed that *R. lagotis* haemocytes aggregate near invading *T. regenti*, however, the parasite appears undamaged. During the patent phase of infection, snail defence activities are modulated as shown for phagocytosis and hydrogen peroxide production. Importantly, such modulations likely occur via interference with cell signalling pathways and such changes may be important for sustained *T. regenti* survival and propagation within *R. lagotis*. The ability of haemocytes from several snail species to produce ET-like fibers is low and, therefore, their role in defence against pathogens is likely marginal. Together, the obtained data provide the first insights into the immune reactions of snails against *T. regenti* allowing us to better comprehend compatibility/incompatibility in snail-schistosome interactions.

**Abstrakt:**

Plži (Gastropoda) jsou ve svém přirozeném prostředí exponováni různým patogenům, a to například bakteriím nebo mnohobuněčným parazitům (digenetickým motolicím), které se v plžích vyvíjejí. V boji proti těmto patogenům využívají plži sofistikovaný vnitřní obranný systém, který je tvořen humorální a buněčnou složkou. Lektiny jsou považovány za nejdůležitější humorální komponenty, zatímco hemocyty představují nejvýznamnější efektorové buňky. Imunita je jeden z důležitých faktorů podmiňujících kompatibilitu/nekompatibilitu plžů a patogenů (zejména plžů a motolic).

Úvod této dizertační práce zahrnuje přehled literatury o imunitním systému plžů a jeho reakcích proti patogenům, a to bakteriím a motolicím. Zároveň jsou v této části shrnuty i poznatky o imunomodulacích způsobených kompatibilními motolicemi. Experimentální práce (prezentována v přiložených publikacích) se zaměřila zejména na vliv ptačí schistosomy *Trichobilharzia regenti* na aktivitu hemocytů dvou druhů plovatkovitých plžů: (i) *Radix lagotis*, v němž se *T. regenti* vyvíjí a (ii) *Lymnaea stagnalis,* který je k infekci rezistentní. Tento parazit způsobuje neuromotorické poruchy u specifických defnitivních hostitelů (vodních ptáků), ale náhodně může infikovat i člověka a způsobovat tzv. cerkáriovou dermatitidu.

Originální publikace zahrnují review, které se v jedné části soustřeďuje na vývoj ptačích schistosom v plžích a jejich imunitní interakce. Publikace zaměřená na plže *R. lagotis* popisuje obranné reakce hemocytů v prepatentní periodě infekce *T. regenti* a jejich modulace v patentí periodě infekce. Publikace týkající se *L. stagnalis* shrnuje výsledky studia produkce extracelulárních chromatinových vláken hemocyty plže proti *T. regenti* a jiným komponentám jako nového typu obranné reakce. Tento fenomén byl navíc pro srovnání studován u dvou dalších druhů plžů, a to *R. lagotis* a *Planorbarius corneus*.

Výsledky prokázaly, že hemocyty *R. lagotis* jsou sice schopny agregace u invadující *T. regenti*, ale parazita nijak nepoškozují. Během patentní periody infekce jsou obranné reakce plžů modulovány, což potvrdilo testování fagocytární aktivity hemocytů a sledování produkce peroxidu vodíku. Tyto modulace mají zřejmě význam pro přežívání *T. regenti* v *R. lagotis* a pravděpodobně k nim dochází ovlivněním buněčných signálních drah. Hemocyty studovaných druhů plžů produkují malé množství extracelulárních chromatinových vláken, což naznačuje, že se v obraně proti patogenům významně neuplatňují. Získaná data představují unikátní pohled na imunitní reakce plžů proti *T. regenti*, který nám umožňuje lépe pochopit kompatibilitu/nekompatibilitu plžů s touto ptačí schistosomou.

**Introduction:**

Gastropod molluscs possess a potent innate immune system that can coordinately eliminate pathogens including bacteria or eukaryotic multicellular parasites such as digenetic trematodes (digeneans). There are about 18,000 digenean species recorded worldwide (Bray et al., 2008) and many of them exclusively rely on gastropods (snails) to complete a part of their life cycle - intramolluscan larval development.

Digenean infections of snails are characterised by a high degree of specificity and compatibility between both partners which is influenced, at least in part, by immune interactions. In an incompatible snail-digenean combination, the host mounts humoral and cellular defence responses that are rapidly activated to eliminate the invaders. On the other hand, a compatible digenean species attempts to suppress snail immune responses and thus ensure its own survival and proliferation.

Among the wide range of parasitic infections caused by digeneans, human schistosomiasis caused by species of the genus *Schistosoma* is the most important tropical disease transmitted by snails, affecting more than 200 million people in approximately 76 countries (Chitsulo et al., 2004). Becuase of the enormous effect of this disease on human health, most immunological studies of snails have focused on snail hosts of *Schistosoma* spp., particularly on *Biomphalaria glabrata* which transmits *S. mansoni*. Recent knowledge of this model provides an advanced view on the factors influencing compatibility between *B. glabrata* and *S. mansoni* (Pila et al., 2017). Such understanding also serves as a basis to design snail control strategies that aim to disrupt the transmission of human schistosomiasis between hosts.

Besides *Schistosoma* spp., many other trematodes (e.g. *Fasciola* spp., *Opisthorchis* spp.) have a significant impact on human and/or animal health. However, the immunobiology of the snail hosts that transmit such parasites is poorly understood. This is also the case for the

lymnaeid snail species *Radix lagotis* which serves as a compatible host for the bird schistosome *Trichobilharzia regenti*. Previous studies have been mainly focused on *T. regenti* development within vertebrate hosts and related pathological consequences (Horák et al., 1999; Blažová and Horák, 2005; Kolářová et al., 2001). Importantly, the cercarial stage of *T. regenti* is responsible for development of local skin inflammatory immune reaction in humans known as cercarial dermatitis or swimmer´s itch which is currently considered as an emerging infectious disease (Kolářová et al., 2013).

The research in the thesis on *R. lagotis* haemocyte immune interactions with *T. regenti* attempted to uncover, at least in part, the mechanisms allowing compatibility between both partners. Another part of the research focused on *Lymnaea stagnalis* haemocytes that were utilised to explore extracellular trap-like fiber formation as a novel snail defence response against incompatible *T. regenti*. For comparative purposes, this phenomenon was also investigated in *R. lagotis* and *Planorbarius corneus* snails using synthetic and bacterial components.

## 1. The distinctive architecture of the gastropod immune system

Gastropod molluscs have evolved an innate immune system capable of fending off pathogenic agents. The first barrier against infection is provided by a surface epithelium that contains glandular cells (i.e. mucocytes) that produce and secrete mucus (Allam and Espinosa, 2015). Mucus prevents dessication of the gastropods, especially for those inhabiting terrestrial environments, and protects their soft bodies against physical injury. Furthermore, mucosal components act as a chemical barrier to prevent colonisation by pathogens (Ehara et al., 2002; Loker, 2010; Zhong et al., 2013). The internal milieu of gastropods includes an open circulatory system with "blood" called haemolymph. Molluscan haemolymph consists of water, ions (e.g. $Na^{+}$, $K^{+}$, $Mg^{2+}$), amino acids and, importantly, components of the internal defence system (IDS) that form two arms – humoral and cellular. Humoral factors (e.g. lectins) play an essential role in the recognition of foreigners (Adema et al., 1997) while immune cells (e.g. haemocytes) employ multiple defence activities for their elimination (van der Knaap et al., 1993; Loker et al., 1982; Hahn et al., 2001). A precise cooperation of humoral and cellular elements is required to deliver an effective defence response towards a pathogen.

### 1.1. Cellular arm of the gastropod IDS

The cellular arm of the gastropod IDS is represented by several types of cells with haemocytes being considered as the most important type. Haemocytes can be found floating free in the haemolymph as well as embedded within connective tissue. These defence cells are considered as the equivalent to mammalian macrophages and monocytes. Haemocytes are produced by a haemopoietic organ called amoebocyte producing organ (APO) in some gastropod species such as *Biomphalaria glabrata* or *Lymnaea truncatula* (Lie et al., 1975;

Rondelaud and Barthe, 1981), while haemocyte origin remains unknown for other representatives such as *Lymnaea stagnalis* (van der Knaap et al., 1993).

There have been numerous studies attempting to identify haemocyte (sub)population(s) in gastropods, however, opinions differ regarding characterisation/nomenclature of such cells. As an example, two haemocyte subpopulations were characterised in *Oncomelania hupensis* snails (Pengsakul et al., 2013) whereas three cell lines were identified in *B. glabrata* (Matricon-Gondran and Letocart, 1999). In *L. stagnalis*, one type of haemocytes was initially described (Sminia, 1972), however, two haemocyte subpopulations were later observed/defined (Dikkeboom et al., 1984). In this case, there are probably differentially developed cells of one cell line that fullfill multiple defence strategies (Sminia, 1972).

### 1.1.1. Haemocyte defence activities

Haemocytes of gastropods are potent mediators of multiple defence activities. They can produce the short-lived reactive nitrogen or oxygen species (RNS/ROS) that cause lipid peroxidation and thus loss of cell membrane integrity, or induce protein/nucleic acid denaturation (Gornowicz et al., 2013). RNS is represented by nitric oxide (NO) generated by nitric oxide synthase (NOS) isoforms which oxidise L-arginine to L-citrulline and NO (Nathan and Xie, 1994). The oxidative burst of haemocytes leads to ROS production. Initial ROS is represented by the superoxide anion ($O_2^-$) that is released by plasma membrane-associated enzyme NADPH oxidase (Adema et al., 1993). The superoxide anion is unstable and it is readily transformed to subsequent ROS including hydrogen peroxide ($H_2O_2$), hypochlorous acid (HOCl) or hydroxyl radical ($OH^-$) (Adema et al., 1993; Adema et al., 1994; Bayne et al., 2001). The capacity of snail haemocytes to generate ROS is influenced by

several factors such as trematode infection (Gornowicz et al., 2013) or snail age (Dikkeboom et al., 1985).

Haemocyte RNS/ROS production is usually linked to phagocytosis or encapsulation responses. Phagocytosis is a process whereby single cells internalise objects that they encounter and recognise as foreigners (Stossel, 1974). Immature haemocytes of juvenile snails usually possess lower phagocytic capacity than those of adult ones (Dikkeboom et al., 1985). Furthermore, the phagocytic activity of haemocytes differs between species of snails and it is lower in *Planorbarius corneus*, *Helix aspersa* and *B. glabrata* in comparison to *L. stagnalis* (Dikkeboom et al., 1988). In addition, phagocytosis also participates in homeostasis of the body by self-cell clearance (autophagy).

Encapsulation involves the formation of a multilayered structure of closely attached and spread haemocytes around a pathogenic agent that is large and thus not suitable for phagocytosis (Sminia et al., 1974). The thickness of the structure may vary between 10 and 40 μm (Harris, 1975) and the internal layer of haemocytes is in direct contact with the pathogen (Loker et al., 1982).

Recently, a novel defence response of haemocytes called ETosis was discovered in different gastropod species (*Limax maximus*, *Arion lusitanicus* and *Achatina fulica*) (Lange et al., 2017). During such response, extracellular trap-like (ET-like) fibers consisting of DNA, histones and myeloperoxidase are released by haemocytes to contact/ensnare larvae of parasites.

### 1.1.2. Regulation of haemocyte defence activities

Haemocyte effector activities are governed by signal transduction pathways that are activated by exposure of haemocytes to exogenous stimuli. Current knowledge of these pathways in molluscan defence is fragmentary although some of the key molecules have been

defined. These include protein kinase C (PKC) and extracellular signal-regulated kinase 1/2 (ERK1/2), both of which are ubiquitously present in animal cells across various taxa (Kruse et al., 1996; Johnson and Lapadat, 2002; Manning et al., 2002). PKC is a family of protein kinase enzymes that can be activated by signals such as increases in the concentration of diacylglycerol (DAG) and calcium ions ($Ca^{2+}$). ERK1/2 belong to the mitogen-activated protein kinase (MAPK) family that are activated by a range of extracellular signals such as growth factors that bind growth factor receptors.

A multiplicity of functions have been ascribed to activated PKC and ERK1/2, such as regulation of cell growth, cell cycle progression, gene expression and, importantly, mediation of the immune response (Newton, 1995; Marshall, 1995). In gastropods, activated PKC, initially described in *L. stagnalis* haemocytes (Walker and Plows, 2003), coordinates $H_2O_2$/NO production or spreading of these cells (Lacchini et al., 2006; Wright et al., 2006; Walker et al., 2010), and it is also essential for $H_2O_2$ release in *B. glabrata* (Bender et al., 2005; Humphries and Yoshino, 2008) or $O_2^-$ release in *Littorina littorea* (Gorbushin and Iakovleva, 2007). PKC also acts upstream of ERK1/2 activation; the latter molecule consequently plays an important role in controlling phagocytic activity or $H_2O_2$/NO release in *L. stagnalis* haemocytes (Lacchini et al., 2006; Wright et al., 2006; Plows et al., 2004; Zahoor et al., 2009). Activated ERK1/2 is also required for $H_2O_2$ production in *B. glabrata* cells (Humphries and Yoshino, 2008). Additional to PKC and ERK1/2, a regulatory role of other signalling molecules in molluscan defence has also been suggested. As an example, activated phosphatidylinositol 3-kinase is important for phagocytosis in *L. stagnalis* haemocytes (Plows et al., 2006) while p38 MAPK promotes $H_2O_2$ generation by *B. glabrata* haemocytes (Humphries and Yoshino, 2008). Together, although the signalling pathways leading to activation of PKC and ERK1/2 seem to be central to haemocyte immune responses in gastropods, our knowledge of activation/inhibitory signals upon infection of gastropods with

compatible/incompatible pathogens is limited and their interplay with other pathways such as those linked to stress signalling (e.g. p38 MAPK) is not well understood. All this is worthy of more study.

### 1.1.3 Cellular activities of fixed phagocytes and rhogocytes

Additionally to haemocytes, gastropods possess fixed phagocytes that are dispersed throughout the connective tissue and trap and/or phagocyte foreign particles as described in *L. stagnalis* (Sminia et al., 1979) and *B. glabrata* (Matricon-Gondran and Letocart, 1999). Overall, how fixed phagocytes participate in elimination of foreigners within gastropods is not well understood (Loker, 2010). Another cell type, embedded in the conncetive tissue or floating in the haemolymph, are rhogocytes (also known as pore cells) that are involved in protein uptake and degradation, heavy metal detoxification, and synthesis and secretion of respiratory proteins such as haemocyanin in *Haliotis tuberculata* (Albrecht et al., 2001) or haemoglobin in *B. glabrata* (Kokkinopoulou et al., 2015). Molecular processes underpinning defence activities of these cell types remain largely unknown.

## 1.2. Humoral arm of gastropod IDS

The humoral arm of the gastropod IDS is represented by soluble (or cell bound) immune effector molecules such as pattern recognition receptors (PRRs), cytotoxins and variable molecules containing immunoglobulin and/or lectin domain(s). These molecules are involved in recognition/killing of pathogens and they cooperate with the cellular arm through activation of haemocyte-mediated defence responses.

A pivotal role in gastropod defence is attributed to lectins that are synthesised and released by haemocytes as well as by connective tissue cells; they are also produced by the albumen gland (van der Knaap et al., 1981; Horák and van der Knaap, 1997; Gerlach et al.,

2005). Lectins are (glyco)proteins that specifically recognise and reversibly bind carbohydrate moieties on the surface of pathogens that subsequently triggers cellular defence (Barondes, 1988; Horák and van der Knaap, 1997). Another class of well studied molecules are the fibrinogen-related proteins (FREPs) that are polymorphic lectin-like molecules (Gordy et al., 2015; Pila et al., 2017). Perhaps, the most well characterised FREPs are in *B. glabrata* snails (Pila et al. 2017), although they were also investigated in other gastropod species (Adema et al., 1997; Gorbushin and Borisova, 2015). FREPs of gastropods possess a unique architecture since they are composed of fibrinogen domain connected to one or two immunoglobulin superfamily domain(s) (Gordy et al., 2015). Importantly, FREPs are somatically diversified and thus they exhibit functional specialisation against various pathogens; their central role is attributed to the defence against digenean trematodes (Adema et al., 2017; Gordy et al., 2015; Pila et al., 2017). Molluscan defence molecule (MDM) is another member of the immunoglobulin superfamily that is expressed by granular cells of connective tissue in *L. stagnalis* and enhances the phagocytic activity of haemocytes (Hoek et al., 1996). Granularin is similarly secreted by granular cells of *L. stagnalis* and it plays a role in phagocytosis (Smit et al., 2004). Granularin has two actions on phagocytic activity of haemocytes: (i) it enhances phagocytosis when treated with particles that are then exposed to haemocytes, (ii) it reduces phagocytosis when treated with haemocytes before contact with target particles (Smit et al., 2004).

More recently, a putative cytolytic protein called biomphalysin belonging to the ß pore-forming toxin (ß-PFT) superfamily was identified in the plasma of *B. glabrata* (Galinier et al., 2013). Structural analysis revealed that, unlike to known ß-PFTs, biomphalysin lacks a lectin-like domain and probably does not bind to carbohydrates (Galinier et al., 2013; Pila et al., 2017). However, pathogen specific molecules that are recognised by *B. glabrata* biomphalysin remain to be elucidated. In addition, humoral factors

that directly exert cytotoxicity towards target cells have also been described in the plasma of *L. stagnalis* (Mohandas et al., 1992).

## 2. Infections of gastropods and pathogen elimination

Gastropods live in a microorganism replete environment are exposed to pathogens such as viruses (Prince, 2003; Savin et al., 2010; De Vico et al., 2017) or bacteria (Nicolas et al., 2002; Raut, 2004; Duval et al., 2015) continuosly. They may also encounter metazoan parasites such as nematodes or digenetic trematodes (Bayne, 2009; Loker et al., 2010; Cowie, 2017). Incompatible pathogens and parasites are recognised and eliminated by effectors of gastropod IDS. Currently, the anti-viral responses of gastropods are almost unknown in contrast to anti-bacterial immune strategies; however, the most immunological studies focused on interactions between gastropods (snails) and larval stages of digeneans (Loker, 2010).

### 2.1. Immune reactions at the outer surface of gastropods

Components of the surface mucus provide the first line of defence of gastropods against invaders. At least two antimicrobial peptides (AMPs) (achacin and mytimacin-AF) were characterised in the mucus of *Achatina fulica* snail (Ehara et al., 2002; Zhong et al., 2013). Achacin is a glycoprotein (L-amino oxidase) that generates cytotoxic $H_2O_2$ that preferentially recognises and binds to bacteria at growth phase as shown for *E. coli* and *Staphylococcus aureus* (Ehara et al., 2002). Mytimacin-AF is a cysteine-rich polypeptide that exhibits antibacterial activity against various Gram-negative and Gram-positive bacteria, and the yeast *Candida albicans* (Zhong et al. 2013). Additionally, a lectin that is able to agglutinate *E. coli* and *S. saprophyticus* has been isolated form the mucus of *A. fulica* (Ito et al., 2011).

Recently, the effect of mucus produced by freshwater snails (*Helisoma trivolvis* and *Lymnaea elodes*) on the survival rate of miracidia of the giant liver fluke *Fascioloides magna* was investigated (Coyne et al., 2015). While all larvae (miracidia) died in the mucus derived from *H. trivolvis* that is incompatible with *F. magna* development, no miracidia were killed in the compatible *L. elodes* mucus. This suggests that cytotoxic activity of mucus components is one determinant of larval trematode-snail compatibility. However, these components remain to be characterised and the relative contribution of mucus to prevent penetration of *F. magna* miracidia into incompatible snail species is worth of elucidation.

Importantly, haemocyte defence responses were currently described in the mucus of the slug *L. maximus* against invading nematode larvae *Angiostrongylus vasorum* (Lange et al., 2017). Haemocytes were observed to be firmly attached to the parasite cuticle and some of the haemocytes expelled ET-like fibers that caused *A. vasorum* entrapment (Lange et al., 2017). Such responses likely prevented invasion of the larvae into the slug body, however, detailed functional characterisation of ET-like fibers is required.

## 2.2. Recognition and elimination of pathogens by the IDS

Implementation of an efficient immune response requires specific recognition of pathogens that is mediated by different soluble and membrane-bound immune receptors (i.e. pathogen recognition receptors, PRRs). These PRRs display ability to bind various pathogen-associated molecular patterns (PAMPs) and thereby trigger immune signalling pathways. Immune mechanisms in pathogen-gastropod interactions are described in several comprehensive reviews (van der Knaap and Loker, 1990; Fryer and Bayne, 1996; Loker and Adema, 1995; Loker, 2010; Mitta et al., 2012; Adema and Loker, 2015; Coustou et al., 2015; Pila et al., 2017).

In the first studies, discrimination and elimination of bacteria or foreign cells by the IDS was documented in *Helix pomatia* (Renwrantz, 1981). These nonself components were initially bound to the membrane of cells lining haemolymph sinuses and then they were phagocytosed by circulating haemocytes. Clearance of bacteria introduced into *L. stagnalis* was also investigated with pore cells, fixed phagocytes and haemocytes participating in the process (van der Knaap, 1981). Furthermore, the oxygen transporting protein haemocyanin was suggested as an enhancer of haemocyte mediated phagocytic response (van der Knaap, 1981). In *L. stagnalis,* haemocytes exhibited increased clearance capacity towards *S. saprophyticus* and *E. coli* when the snails were first injected with dead bacteria of both species (van der Knaap et al., 1983). Thus, a certain level of specificity and immune memory was suggested to exist in the internal defence mechanisms of *L. stagnalis* (van der Knaap et al., 1983).

Involvement of carbohydrate-binding specific molecules (lectins) in recognition of pathogens is considered an important and evolutionarily ancient binding principle (Renwrantz, 1983; Jacobson and Doyle, 1996; Horák and van der Knaap, 1997). Carbohydrate-binding proteins have been revealed in the plasma of *B. glabrata* following exposure to larvae of the trematodes *Echinostoma paraensei* and *S. mansoni* (Monroy and Loker, 1993). Subsequently, their binding to Gram-positive bacteria (e.g. *Bacillus subtilis*), Gram-negative bacteria (e.g. *Serratia marcescens*), and to sporocysts and rediae of *E. paraensei* was reported (Hertel et al., 1994). Furthermore, an opsonic role of such binding probably facilitated destruction of these pathogens by haemocytes. Haemocyte membrane-bound lectins have also been implicated in detection of pathogens. As an example, a galectin of *B. glabrata* haemocytes was characterised and its binding to the surface of *S. mansoni* sporocysts was confirmed (Yoshino et al., 2008). Besides recognition, lectins of gastropods may also exert other activities towards intruders, such as agglutination

demonstrated for C-type lectin of *Haliotis discus discus* in the presence of *Vibrio alginolyticus* (Wang et al., 2008).

As stated above **(Section 1.2.)**, lectin-like molecules, FREPs, have attracted considerable attention as PRRs of gastropods, and their involvement in immune responses has been comprehensively studied using the model *B. glabrata-S. mansoni* (Coustau et al., 2015; Gordy et al., 2015; Mitta et al. 2012; Pila et al., 2017). Penetration of the parasite into the snail host initiates miracidium-mother sporocyst transformation on the one hand, and proliferation and differentiation of haemocytes that produce FREPs (BgFREPs) on the other hand. Among BgFREPs, BgFREP2 recognises and binds to the surface glycosylated proteins of mother sporocysts called *S. mansoni* polymorphic mucins (SmPoMucs) (Roger et al., 2008; Mitta et al., 2012; Gordy et al., 2015). Furthermore, it has been suggested that BgFREP2 bound to SmPoMucs forms an immune complex with thioester-containing protein (TEP) prior to recruitment of haemocytes (towards the parsite) and subsequent encapsulation (Gordy et al., 2015; Pila et al., 2017). At least one pro-inflammatory cytokine named macrophage migration inhibitory factor (MIF) has been shown to enhance the encapsulation response (Garcia et al., 2010), while identity of others remains unknown. After encapsulation, haemocytes release ROS with $H_2O_2$ being considered as the most important metabolite that facilitates killing of *S. mansoni* sporocyst (Hanh et al., 2001). Pieces of damaged parasite body were then observed to be actively phagocytosed by snail haemocytes (Loker et al., 1982). While newly penetrated parasites were contacted by host haemocytes as early as 1 h post-infection (p.i.), the entire encapsulation and elimination of *S. mansoni* occurred within 4-48 h p.i. (Loker et al., 1982; Mitta et al., 2012).

Importantly, a shift from cellular to humoral response probably contributes to the development of innate memory and ensures complete protection of *B. glabrata* against a secondary challenge with *S. mansoni* (Pinaud et al., 2016; Coustau et al., 2016). Detailed

analysis of whole-snail transcriptomes revealed that transcripts for BgFREPs, biomphalysin and other bioactive molecules were overexpressed in snails after the secondary exposure to the parasite when compared to initial infection (Pinaud et al., 2016). Functional tests further indicated that BgFREP2, 3, and 4 are likely involved in *B. glabrata* innate immune memory (Pinaud et al., 2016). However, given that small interfering RNA-mediated knock-down of these BgFREPs reduced the innate immune memory phenotype by only 15%, other BgFREP variants and/or molecules certainly participate in this phenomenon (Pinaud et al., 2016). Increasing evidence for novel molecules in *B. glabrata* likely playing an immune role (Adema et al., 2017; Dheilly et al., 2015; Tetreau et al., 2017) represents a perspective for their identification.

Except for *S. mansoni* infection, expression profiles of various BgFREPs were also examined during other snail immunological challenges, and recognition and binding of BgFREPs to the respective pathogens have been evaluated. As an example, BgFREP4 was significantly up-regulated following infection of *B. glabrata* with *E. paraensei* whereas BgFREP8 was down-regulated (Zhang et al., 2008; Adema et al., 2010). Furthermore, it was demonstrated that BgFREP4 binds to *E. paraensei* sporocysts and their excretory-secretory products (ESPs) (Zhang et al., 2008). When bacteria were used as a challenge, BgFREP7 was shown to increase in abundance after snail exposure to *Micrococcus luteus* while it decreased after injections of *B. glabrata* with *E. coli* (Adema et al., 2010). In addition, BgFREP3 has been evaluated for its binding capability to *E. coli*, but also to *S. aureus* or *Saccharomyces cerevisiae* (Zhang et al., 2008). Although the above described evidence strongly supports the role of BgFREPs in pathogen recognition, targets recognised by these molecules and subsequent molecular interplay leading to activation of haemocytes remain largely unknown.

Histological and ultrastructural studies have shown encapsulated sporocysts of *E. paraensei*, *Echinostoma lindoense* and/or *Echinostoma caproni* in the ventricle of

*B. glabrata* (Ataev and Coustau, 1999; Jeong et al., 1984; Loker et al., 1987). Furthermore, parts of *E. paraensei*/*E. lindoense* tegument actively engulfed by snail haemocytes were also observed (Jeong et al., 1984). Similarly, encapsulation responses were observed in other models of trematode-gastropod interactions such as *Bulinus guernei-Schistosoma haematobium* or *L. stagnalis-Trichobilharzia regenti* (Krupa et al., 1997; L. Trefil, Charles University, Prague, Czechia). Advanced understanding of these interactions at the molecular level might likely be achieved through studies of FREPs molecules representing an important perspective.

## 3. Immunomodulation of gastropod IDS by compatible pathogens

Compatible pathogens, upon entering the susceptible gastropod host, have to avoid attack by the immune system. They employ both passive and active strategies to modulate and down-regulate specific immune responses to ensure parasite survival and replication. Alterations in gastropod IDS by pathogens are most comprehensively described for snails infected by trematodes.

In the snail host, trematode larvae interfere with both cellular and humoral components of the IDS. Disruption of haemocyte effector functions has been described in parasitised snails at various times during the course of trematode infection, and such evidence is also available from *in vitro* experiments. In *L. stagnalis* infected with *Trichobilharzia szidati*, an activation of both IDS arms likely coinciding with phagocytosis of miracidial ciliated plates was found early after parasite penetration, however, suppression of haemocyte phagocytic activity later occurred (Amen et al., 1992). *In vitro*, haemocytes failed to encapsulate and destroy *T. szidati* sporocysts (Adema et al., 1994). The inability of *B. glabrata* haemocytes to form capsules around *E. paraensei* sporocysts and daughter rediae was also shown *in vitro* (Adema et al., 1994). Haemocytes from *B. glabrata* infected with

*E. paraensei* also displayed decreased phagocytic activity (Noda and Loker, 1989). Alteration in ROS production has been also reported such as in *B. glabrata-S. mansoni* (Connors et al., 1991) or *Himasthla elongata-Littorina littorea* associations (Gorbushin and Yakovleva, 2008).

Although modulation of haemocyte immune reactions has been demonstrated for many trematode-snail combinations, the mechanisms leading to immunosupression and key molecules involved in the process are unknown for most of them. It has been revealed that parasite ESPs participate in the modulation of haemocyte activities. As an example, a 100 kDa fraction of ESPs of *E. paraensei* sporocysts has been proven to affect larval encapsulation by *B. glabrata* haemocytes (Loker et al., 1992). Disruption of ERK1/2 signalling in susceptible *B. glabrata* haemocytes by ESPs of *S. mansoni* sporocyst (and whole larvae) was also proposed as a mechanism facilitating parasite survival within the snail host (Zahoor et al., 2008). Furthermore, carbohydrate moieties (D-galactose, L-fucose) mimicking those present on the surface of trematode larvae, such as in the bird schistosome *T. regenti* (Blažová and Horák, 2005; Chanová et al., 2009), down-regulated the activity of ERK1/2 and PKC in *L. stagnalis* haemocytes, which, given the role of these pathways in defence responses, suggests an immunosupressive role (Plows et al., 2005; Walker, 2006).

As far as alterations in humoral components are concerned, it has been shown that infection of susceptible *B. glabrata* with *E. paraensei* provoked a substantial increase in soluble plasma polypeptides while a little change occurred in resistant snails (Loker and Hertel, 1987). In concordance, infection by *E. paraensei* or *S. mansoni* in *B. glabrata* also resulted in increased concentration of carbohydrate-binding proteins and, moreover, profiles of these proteins differed according to the two parasite species used indicating specific snail responses (Monroy et al., 1992). Unfortunately, the functional significance of such alterations observed in *B. glabrata* remains unclear. In *L. stagnalis* infected with *T. szidati*, expression of

MDM (an enhancer of phagocytosis) was down-regulated (Hoek et al., 1996; de Jong Brink et al., 2001) whereas the encoding gene for granularin (reducer of phagocytosis) was up-regulated in parasitised snails (Smit et al., 2004). Both these modulations likely favoured *T. szidati* infection in *L. stagnalis*.

Snail FREPs may also be important targets in immunosupressive processes as demonstrated in *B. glabrata*. RNA interference (RNAi) mediated knock-down of BgFREP3 in snails resistant to *E. paraensei* resulted in successful establishment of the infection in 28-33% of individuals (Hanington et al., 2010). Similarly, decreased BgFREP3 also altered the resistance phenotype in *B. glabrata* towards *S. mansoni* with 21% of snails progressing to patent phase of infection (Hanington et al., 2012). BgFREP3 expression was also attenuated in snails exposed to irradiated *E. paraensei* that subsequently increased susceptibility of *B. glabrata* to *S. mansoni* by 46% (Hanington et al., 2012). Together, these observations suggest that BgFREPs are not the only factors responsible for snail resistance to infection by trematodes (Gordy et al., 2015), and therefore, evaluation of other molecules (mechanisms) is desired to explore this unique phenomenon.

## 4. Concluding remarks

Current knowledge of gastropod immunology is based on a few species, mostly in the context of infections by digenetic trematodes. Given the diversity of gastropods and immune stimuli in different habitats, it is probable that diverse modes of defence strategies are employed in particular gastropod-pathogen combinations. Therefore, it can be assumed that immunological investigations of new models will lead to the discovery of novel defence mechanisms, effector molecules, etc. Last but not least, a potential control agent for medically important snails might also emerge from such investigations.

It is anticipated that medically important snails (and also commercially used gastropod species) will further be the main subject of invertebrate immunology. Despite the increasing evidence of novel immune molecules in some investigated species, the functional relevance is known for a minority of them. Approaches such as RNAi should enable more comprehensive insight into their role in immunity. Completing the mosaic of cellular and humoral immune factors will contribute to our understanding of molecular mechanisms underpinning transmission of pathogens via gastropod molluscs.

**Aims of the thesis**

The thesis aimed to explore the defence activities of haemocytes of two lymnaeid snail species (*Radix lagotis* and *Lymnaea stagnalis*) that transmit bird schistosomes, and study the immunomodulation caused by trematodes in their snail hosts. The data obtained can contribute to our knowledge of mechanisms allowing compatibility/incompatibility between the parasite and the intermediate snail host.

The specific aims were to:

1) Summarise the current knowledge of intramolluscan development of bird schistosomes, and immune interactions between the parasite and the snail host.
2) Examine haemocyte defence activities of *R. lagotis* against *Trichobilharzia regenti* during the initial phase of infection, and immunomodulation during the patent phase of infection.
3) Investigate the ability of *L. stagnalis* haemocytes to produce extracellular trap-like fibers against incompatible *T. regenti* and other stimulants, and to compare fiber formation in *R. lagotis* and *Planorbarius corneus* snails.

## Original papers and author contribution

Horák P., Mikeš L., Lichtenbergová L., **Skála V.**, Soldánová M., Brant S.V., 2015: Avian schistosomes and outbreaks of cercarial dermatitis. *Clinical Microbiology Reviews*, https://doi.org/10.1128/CMR.00043-14

*author contribution: writing responsibility for the section "Intramolluscan development of avian schistosomes"*

**Skála V.**, Černíková A., Jindrová Z., Kašný M., Vostrý M., Walker A.J., Horák P., 2014: Influence of *Trichobilharzia regenti* (Digenea: Schistosomatidae) on the defence activity of *Radix lagotis* (Lymnaeidae) haemocytes. *PLoS ONE*, https://doi.org/10.1371/journal.pone.0111696

*author contribution: performing all the experiments except histological examinations; analysing the data; writing the manuscript*

**Skála V.**, Walker A.J., Horák P., 2018: Extracellular trap-like fiber release may not be a prominent defence response in snails: evidence from three species of freshwater gastropod molluscs. *Developmental and Comparative Immunology*, https://doi.org/10.1016/j.dci.2017.10.011

*author contribution: performing all the experiments; analysing the data; writing the manuscript*

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# Avian Schistosomes and Outbreaks of Cercarial Dermatitis

Petr Horák,[a] Libor Mikeš,[a] Lucie Lichtenbergová,[a] Vladimír Skála,[a] Miroslava Soldánová,[b] Sara Vanessa Brant[c]

Department of Parasitology, Faculty of Science, Charles University in Prague, Prague, Czech Republic[a]; Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic[b]; Museum Southwestern Biology, Department of Biology, University of New Mexico, Albuquerque, New Mexico, USA[c]

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## SUMMARY

Cercarial dermatitis (swimmer's itch) is a condition caused by infective larvae (cercariae) of a species-rich group of mammalian and avian schistosomes. Over the last decade, it has been reported in areas that previously had few or no cases of dermatitis and is thus considered an emerging disease. It is obvious that avian schistosomes are responsible for the majority of reported dermatitis outbreaks around the world, and thus they are the primary focus of this review. Although they infect humans, they do not mature and usually die in the skin. Experimental infections of avian schistosomes in mice show that in previously exposed hosts, there is a strong skin immune reaction that kills the schistosome. However, penetration of larvae into naive mice can result in temporary migration from the skin. This is of particular interest because the worms are able to migrate to different organs, for example, the lungs in the case of visceral schistosomes and the central nervous system in the case of nasal schistosomes. The risk of such migration and accompanying disorders needs to be clarified for humans and animals of interest (e.g., dogs). Herein we compiled the most comprehensive review of the diversity, immunology, and epidemiology of avian schistosomes causing cercarial dermatitis.

## INTRODUCTION

Cercarial dermatitis is a condition caused by both mammalian and avian schistosomes (Trematoda: Schistosomatidae). Which of those species is more prevalent in a dermatitis outbreak depends on where you are in the world and how humans and birds/mammals (and, by association, snails) come into contact with a particular type of aquatic environment. The name "cercarial dermatitis" is derived from the term "cercaria," the last larval stage developing in an aquatic snail. Cercaria is the infective stage that, after leaving the snail, searches for and invades a warm-blooded vertebrate host via skin penetration. Besides the official name, "cercarial dermatitis," many local terms are used ("sawah itch," "koganbyo," etc.), with the most widely used name being "swimmer's itch."

Schistosome cercariae were disclosed as the causative agent of cercarial dermatitis in the United States in 1928 (1). Since that time, numerous reports of cercarial dermatitis have been documented from different parts of the world. Global economic losses due to outbreaks of cercarial dermatitis are not known, as there is no systematic method of reporting either the number of cases or incurred economic losses in terms of recreation or person work hours. Furthermore, what data do exist that estimate local costs are usually not available to the public domain, but it is accepted that outbreaks can have considerable impacts on local, tourism-based economies in the areas of recreational lakes (2). For example, in the recreational area of Naroch Lake (Belarus), 4,737 cases of cercarial dermatitis were recorded between 1995 and 2006 (3). In addition, cercarial dermatitis may represent a debilitating oc-

Citation Horák P, Mikeš L, Lichtenbergová L, Skála V, Soldánová M, Brant SV. 2015. Avian schistosomes and outbreaks of cercarial dermatitis. Clin Microbiol Rev 28:165–190. doi:10.1128/CMR.00043-14.

Address correspondence to Petr Horák, petrhorak@petrhorak.eu.

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doi:10.1128/CMR.00043-14

cupational disease among rice farmers (4) and may incur costs in terms of lost person work hours. Older reports refer to 75% or more of the population experiencing the characteristic symptoms of "koganbyo" in the areas of Japan where the disease is most highly endemic (5). Recent reviews (6–9) agree that in some regions cercarial dermatitis has appeared as a new problem, either because the dermatitis was previously unknown (e.g., the U.S. Southwest and Chile) or because the number of reports of outbreaks increased (8, 10, 11). Consequently, cercarial dermatitis is now regarded as an emerging disease. Besides human schistosomes (*Schistosoma* spp.), no animal (e.g., avian) schistosomes have any other presently known pathogenic effects on humans. Thus, the use of animal models to study the potential risk of animal (avian) schistosomes to human health is invaluable.

The last decade has revealed diverse avian schistosome species and biology, as well as the snails that host them. These discoveries have outpaced the equally essential host-parasite biological, immunological, pathological, and epidemiological studies of species diversity in terms of incorporating the results of such studies into the current known diversity of schistosomes. Such studies are difficult and time-consuming, and consequently, only a few species have been adapted to experimental conditions. Nevertheless, such studies are crucial to understanding the current and future roles that these species might play in the frequency and distribution of cercarial dermatitis, as well as understanding how to break the life cycle to prevent outbreaks. What has been documented, and is detailed in the following sections, points to an understanding of the avian schistosome-host relationships and thus offers the foundation on which future studies will be modeled.

## DIVERSITY OF SCHISTOSOMES CAUSING DERMATITIS

Considering only the named species in the literature, there are 4 schistosome genera from mammals and 10 from birds, with about 30 described species from mammals and about 67 from birds (12). The total is close to 100 species, with ~70% of them being avian schistosomes distributed around the world that may initiate cercarial dermatitis. The role of some of the species of avian schistosomes as dermatitis agents has not been studied sufficiently, as they are not often found in areas where people most commonly are in contact with water and snails. To discuss the distribution and diversity of schistosomes causing dermatitis within the phylogenetic framework of the family Schistosomatidae, we refer to Fig. 1 (12–14).

The basal clade of the family tree (Fig. 1, clade A) comprises the exclusively marine avian schistosomes *Austrobilharzia* (4 species) and *Ornithobilharzia* (2 species); the species shown in the tree are those for which there are genetic data. Species of these two genera are associated with outbreaks of dermatitis in shallow marine environments (15, 16). Infection often occurs in people who are swimming, playing in tidal pools, or working, for example, collecting tidal invertebrates in the sand (15, 17–27). Both of these genera have robust, large worms as adults and are common schistosomes of marine birds, particularly gulls. Species of *Austrobilharzia* are more often implicated as a cause of dermatitis outbreaks (25).

The next main clade includes the remaining schistosomes (Fig. 1, clades B, C, and D). Clades B and C are exclusively freshwater mammalian schistosomes. The largest clade of mammalian schistosomes includes the genus *Schistosoma*, with ~25 species (clade B). In particular, three of these species (*Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum*) cause one of the most devastating helminth diseases in humans, schistosomiasis, affecting about 220 million people, mainly in the tropical and subtropical latitudes around the world (WHO). All but one species (*S. mansoni*) occur exclusively in the Eastern Hemisphere. These species are not typically implicated in dermatitis outbreaks, yet there is a mild eruption of dermatitis following penetration by all schistosomes (28). Most reported cases of dermatitis caused by the genus *Schistosoma* are from parasites that infect domesticated work animals, such as cattle and buffalo, mainly in Asia. For example, in countries such as India and Nepal, the species *Schistosoma turkestanicum*, *Schistosoma nasale*, *Schistosoma indicum*, and *Schistosoma spindale* are often implicated in outbreaks of dermatitis (29–38). This relationship may not be a surprise, as bovids are the definitive host, and the people in these areas depend upon these animals for their livelihood in farming. Additionally, the snail host for the major species causing dermatitis (*S. nasale*, *S. indicum*, and *S. spindale*) is *Indoplanorbis exustus*, a widespread and abundant snail that is found mainly in Nepal and India, to the exclusion of *Biomphalaria* and *Bulinus*, snail hosts for a majority of the African transmitted species of *Schistosoma*.

The genus *Bivitellobilharzia* is considered a schistosome of elephants, but it has also been reported from wild rhinoceroses in Nepal (38–41). There are no known reports of cercarial dermatitis in humans from areas inhabited by African elephants (with the *Bivitellobilharzia loxodontae* schistosome), but in areas where domesticated Asian elephants are used, there have been cases of dermatitis in the mahouts, or elephant handlers, when the elephants are taken for bathing (e.g., in Sri Lanka [40]). In Nepal, *Bivitellobilharzia nairi* has thus far been found in wild, not domesticated, elephants (38). The snail host remains unknown but is likely a pulmonate snail (42). At least two species of *Schistosoma* from *Biomphalaria* snails infect the African hippopotamus, but these species have not been implicated directly in dermatitis outbreaks, despite the presence of humans working on lakeshores where there are hippopotamuses (43–45). Given the prevalence of human schistosomiasis in these areas, however, dermatitis caused by hippopotamus schistosomes may easily go undetected.

The small clade C (Fig. 1) has two species of mammalian schistosomes that, as far as we know, are found only in North America and are not frequently associated with dermatitis outbreaks, though they both produce a skin reaction (46–50). These two species are parasites of lymnaeid snails (often *Stagnicola elodes*), usually with raccoons and muskrats as mammalian hosts. *Schistosomatium douthitti* adults inhabit aquatic and semiaquatic rodents in more northern latitudes or at high elevations (48, 51, 52). *Heterobilharzia americana* has been reported from a wide range of mammalian hosts (rivaling *Schistosoma japonicum*), including horses, in the southern regions of North America (49, 53, 54).

Perhaps the most remarkable clade of schistosomes responsible for dermatitis is clade D, a large clade of avian schistosomes whose adults are long and threadlike (except *Dendritobilharzia* and *Bilharziella*) and that includes both freshwater and marine species. In particular, the genus *Trichobilharzia* has achieved notoriety as the primary etiological agent for dermatitis outbreaks around the world. The diversity of aquatic environments, host use, morphology, definitive host habitat, and cercarial behavior is unparalleled in any other group of schistosomes, and probably most other groups of trematodes (12, 14). Figure 1 includes a molecular phylogeny of all the known genera of schistosomes ex-

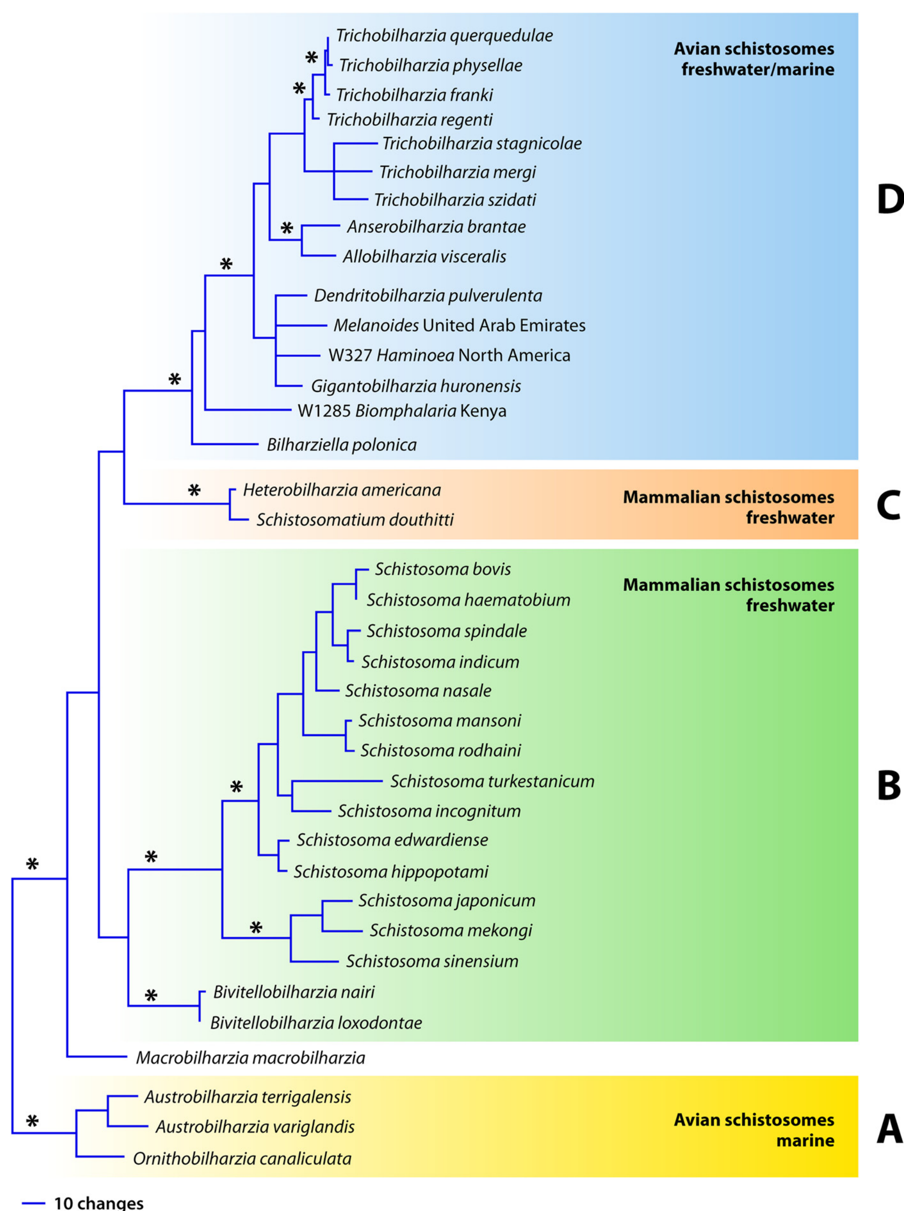


**FIG 1** Phylogenetic tree showing generic and species positions based on Bayesian analysis of the nuclear ribosomal DNA 28S region (1,200 bp) of Schistosomatidae. Panels A to D refer to the clades discussed in the text. This tree is based on genetic data, not morphological data, and as such, there are more species that have been described morphologically than genetically. Asterisks denote significant posterior probabilities (>0.95).

cept one. *Jilinobilharzia* has not been reported since the original paper reporting it from the duck *Anas crecca* in northeastern China; its snail host remains unknown (55). Morphological characteristics and host use suggest that *Jilinobilharzia* belongs in the large clade of avian schistosomes (Fig. 1, clade D), perhaps even to *Trichobilharzia*.

At the base of clade D is an unresolved group of avian schistosomes, most of which have been implicated in dermatitis outbreaks and comprise the most diverse range of both bird and snail (9 families) host use (13, 40, 56–66). Current results based on all of the available sequence data in GenBank for the internal transcribed spacer (ITS) region indicate that there are about nine distinct lineages, only two of which are described: *Gigantobilharzia* and *Dendritobilharzia* (40, 64–69). Most of the lineages in this part of clade D have one to a few species and have been seen in only a few cases, many related to dermatitis outbreaks (12) (Table 1). Thus far, the literature suggests that species in this clade cause dermatitis in more local areas, whereas *Trichobilharzia* causes cercarial dermatitis globally. For example, in the San Francisco Bay area (California), one beach in particular has annual cases of dermatitis (64). The prevalence of dermatitis caused by schistosomes from *Valvata* or *Melanoides* snails (63, 65, 66) depends on how often people use areas where these snails release cercariae.

Species of *Trichobilharzia* (Fig. 1, clade D) are globally distributed and cause the majority of recreational and occupational reports of dermatitis found in the literature, especially in the temperate latitudes. In North America and Europe, where most of the research has been focused, outbreaks occur in recreational ponds

**TABLE 1** Summary of general host use of known genera of schistosomes, reflecting current knowledge, habitat in the definitive host, and broad geographic locality

| Genus | Snail host | Mammalian/avian host | Definitive host habitat | Locality | Aquatic habitat | Major areas for outbreaks |
|---|---|---|---|---|---|---|
| *Austrobilharzia* | Nassariidae, Batillaridae, Littoriniidae, Potamididae | Charadriiformes | Visceral | Global | Marine | Shallow marine areas, tidal pools |
| *Ornithobilharzia* | Batillaridae | Charadriiformes | Visceral | Global | Marine | Shallow marine areas, tidal pools |
| *Macrobilharzia* | Unknown | Suliformes (*Anhinga*) | Visceral | North America, Africa | Unknown | Unknown if causes dermatitis |
| *Bivitellobilharzia* | Unknown | Elephantidae, Rhinocerotidae | Visceral | Africa, Asia | Freshwater | Probably freshwater rivers |
| *Schistosoma* | Planorbidae, Lymnaeidae, Pomatiopsidae | Mammalia | Visceral, Nasal | Eurasia, Africa, South America | Freshwater | Mostly eutrophic ponds |
| *Heterobilharzia* | Lymnaeidae | Mammalia | Visceral | North America | Freshwater | Marshy areas |
| *Schistosomatium* | Lymnaeidae | Rodentia | Visceral | North America | Freshwater | Marshy areas |
| *Bilharziella* | Planorbidae | Anseriformes, Gruiformes, Ciconiformes, Podicipediformes | Visceral | Europe | Freshwater | Eutrophic ponds |
| Species isolated from *Haminoea* | Haminoeidae | Charadriiformes, Pelicaniformes | Visceral | North America | Marine | Shallow marine areas, tidal pools |
| *Gigantobilharzia*[a] | Physidae | Passeriformes | Visceral | North America | Freshwater | Marshy areas, usually with cattails |
| *Dendritobilharzia* | Planorbidae | Anseriformes, Gruiformes, Pelicaniformes, Gaviiformes | Visceral | Global | Freshwater | Unknown if reports of dermatitis |
| *Jilinobilharzia* | Unknown | Anseriformes (Anatidae) | Visceral | China | Unknown | Unknown if reports of dermatitis |
| *Allobilharzia* | Unknown | Anseriformes (swans) | Visceral | Northern Hemisphere | Unknown | Unknown if causes dermatitis |
| *Anserobilharzia* | Planorbidae | Anseriformes (geese) | Visceral | Northern Hemisphere | Freshwater | Eutrophic ponds, reservoirs |
| *Trichobilharzia* | Lymnaeidae, Physidae | Anseriformes (Anatidae) | Visceral, nasal | Global | Freshwater | Eutrophic ponds, glacial lakes, reservoirs |

[a] Since *Gigantobilharzia* is not a monophyletic genus, the information listed here is for *G. huronensis* only.

and reservoirs. These outbreaks have been reviewed extensively (7, 8, 70, 71). Species of *Trichobilharzia* have been reported to cause dermatitis from other areas as well, such as Rwanda-Burundi (72), South Africa (73, 74), New Zealand and Australia (75–79), Malaysia/Indonesia (80–82), Iran (65, 83–86), United Arab Emirates (66), Thailand (87), and China (88, 89) in the Eastern Hemisphere and Argentina (57, 90, 91), Chile (11), and El Salvador (92) in the Western Hemisphere. We are just beginning to better understand the significant disease components for dermatitis as a global problem.

Cercarial dermatitis is also recognized as an occupational hazard in many areas of the world, especially in areas where rice is grown (82, 93). Rice fields are areas where snails, domestic and wild ducks, cattle, and humans seasonally use the water, so the life cycle is maintained consistently (5, 84, 94–104). Species of *Trichobilharzia* are identified most often, though not exclusively (5). Rice fields are plowed by water buffalo and cattle in areas where *Indoplanorbis exustus* occurs, and hence, dermatitis may be caused by one of the species of *Schistosoma*, as noted above. Nonetheless, *Trichobilharzia* is still by far the most common etiological agent.

## MOLLUSCAN AND AVIAN HOST SPECIFICITY

Schistosomes have colonized many families of snails as first intermediate hosts (12, 62). Mammalian schistosomes use 3 families of snails, compared to 15 families used by avian schistosomes, as summarized in Table 1. The majority of schistosome species are transmitted by the pulmonate snail families Physidae, Lymnaeidae, and Planorbidae (10, 70, 105). Interestingly, two snail families contain species (e.g., *Biomphalaria* and *Indoplanorbis* in the Planorbidae family and *Stagnicola* in the Lymnaeidae family) that can host both avian and mammalian schistosomes that cause dermatitis (40, 51, 70, 104, 106).

Previous papers have reviewed the details of host specificity in mammalian schistosomes (Fig. 1, clades B and C) (107, 108). Because avian schistosomes are the major group of schistosomes causing dermatitis, our own discussion focuses on their avian and snail hosts. For two reasons, these avian schistosome species comprise most of the dermatitis reports: first, many avian hosts seasonally migrate, consequently disseminating avian schistosomes as they fly (domestic ducks can serve as definitive hosts particu-

larly for *Trichobilharzia*); and second, some snail hosts are habitat generalists (e.g., *Physa* [syn. *Physella*] *acuta* and *Lymnaea stagnalis*) that are now globally distributed. As a result, the opportunities for birds and snails to come into contact across time and space are vast. For example, *P. acuta* (host to *Gigantobilharzia huronensis*, *Trichobilharzia physellae*, and *Trichobilharzia querquedulae*) thrives in both natural and altered environments, with a wide tolerance for water temperature and chemistry, including the conditions found in ponds, drainage ditches, rivers, marshes, and ephemeral water (109–111). In Europe, the lymnaeid snail *L. stagnalis* (host of *Trichobilharzia szidati*) has also been linked to cases of dermatitis in people who acquired it while working with aquaria (112, 113), providing evidence of the snail's ability to persist, in addition to its local global presence.

From an evolutionary perspective, the vagility and habitat specificity of most bird hosts, in concert with the availability of snails in aquatic habitats, are likely mechanisms for widespread host switching in snails, and thus for diversification of avian schistosomes (12, 114). Our knowledge of the current schistosome-snail associations indicates that once a schistosome is hosted by a particular species of snail, it possesses little ability to utilize more than a few species within that genus (e.g., *Radix*, *Stagnicola*, and *Physa*) (115). Two exceptions are *Dendritobilharzia pulverulenta*, which uses *Anisus vortex* in Europe (116) and *Gyraulus parvus* in North America (117), both of which are small, related planorbid snails, and *Trichobilharzia regenti*, which employs lymnaeid snails of the genus *Radix* in Europe and *Austropeplea tomentosa* in New Zealand (118). There is little evidence of schistosome species crossing snail families, naturally or experimentally (for an exception, see references 119 and 120). *Trichobilharzia franki* was reported to be widespread across Europe, but detailed molecular studies are showing that it may represent several species related to snail host use (70, 121, 122).

Cercarial dermatitis is caused not only by species of schistosomes from indigenous snails but also by those from invasive or introduced snails. For example, *Haminoea japonica* (originally from Japan but now off the California coast) and *Ilyanassa obsoleta* (originally from the east coast but now on the west coast of North America) are responsible for annual dermatitis outbreaks at marine swimming beaches (25, 64). In freshwater, *L. stagnalis* is found commonly in the northern Eastern hemisphere, yet in the northern Western hemisphere it is only locally common. When *L. stagnalis* is found to be infected, the infecting species is related to a common European species, *Trichobilharzia szidati* (70, 123). Interestingly, *P. acuta* is one of the most invasive pulmonate snails and can host at least four species of avian schistosomes in North America (10, 70), yet there are no reports of this snail hosting schistosomes in their invasive range (outside North America). It is also noteworthy that most of the schistosome species transmitted by physid snails have thus far been found only in North America (at least based on genetic comparisons) (e.g., *G. huronensis*, *T. physellae*, and one undescribed lineage of schistosome [10]). The lymnaeid snail genera *Stagnicola* (found in North America) and *Radix* (found in the Eastern Hemisphere) are the main snail hosts for most species of *Trichobilharzia*; in fact, thus far, *Radix* maintains most of the reported species diversity of *Trichobilharzia* (122). *Trichobilharzia regenti* has been recognized to cause dermatitis in Lake Wanaka in New Zealand, and it may have been introduced from Europe in wild duck breeds (*Anas platyrhynchos*) used for hunting. It is now found commonly in the endemic nonmigratory scaup *Aythya novaeseelandiae* and the snail *Austropeplea tomentosa* (118). Schistosomes seem to be specific to particular snail hosts at the species or genus level, but not as much to their avian or mammalian hosts, though loose specificity of definitive host use exists at higher taxonomic levels (Table 1) (e.g., *Schistosoma haematobium* in humans, *Bivitellobilharzia* in elephants, and *Allobilharzia* in swans).

The diversity of avian schistosomes found around the world is in no small part due to the ability of thousands of migratory birds to carry their parasites across several latitudes and longitudes, exposing commonly encountered snails. This propensity to migrate large distances distinguishes avian schistosomes from mammalian schistosomes in terms of distribution, diversification, and host use, perhaps with the exception of *Schistosoma mansoni* (in terms of long-distance migration only) (124). Yet the schistosome species found in a wide range of avian host orders (e.g., *Bilharziella* and *Dendritobilharzia*) (Table 1) are not the ones recurrently responsible for outbreaks and are also species that are not genetically diverse compared to other species (69, 125, 126). Currently, *D. pulverulenta* might be the most widespread single species of avian schistosome, crossing both the Northern and Southern Hemispheres (117, 125, 127, 128).

The most derived clade, or most recently evolved clade, in clade D has three genera: *Allobilharzia*, *Anserobilharzia*, and *Trichobilharzia* (Fig. 1). *Allobilharzia*, from swans, and *Anserobilharzia*, from geese, both have a circumpolar distribution (69, 70, 105, 129, 130). *Anserobilharzia brantae*, which is common in North America (in the Canada goose [*Branta canadensis*] and the snow goose [*Chen caerulescens*]) but also found in Europe (in the greylag goose [*Anser anser*]), has been identified in at least one outbreak of dermatitis in the United States (10). The area was a eutrophic municipal lake/pond with a dense population of *Gyraulus parvus* snails and Canada geese. The third genus, *Trichobilharzia*, the most species-rich genus in the family, is found almost exclusively in ducks. It should be noted that there are several species of *Trichobilharzia* reported from other avian families (*Trichobilharzia corvi* from passeriforms, along with other species [131, 132]), but based on the morphology of adults and eggs and snail host use (when known), these probably represent new genera, or these avian hosts are not the primary (competent) hosts or might be aberrant cases (70, 78). Within the genus *Trichobilharzia*, several clades are specific to certain groups of ducks: for example, *Trichobilharzia stagnicolae* and *Trichobilharzia mergi* are found in mergansers (Merginae) (70, 133), *T. querquedulae* in the "blue-winged duck" clade (*Anas clypeata*, *Anas discors*, and *Anas cyanoptera*) (70, 134), *T. physellae* in an ecological group of diving ducks that includes ducks of the Aythinae and Merginae, and a common, undescribed species of *Trichobilharzia*, species A, in *Anas americana* (70). It is not yet clear which duck groups (phylogenetic or ecological) are more specific for *T. franki* or *T. szidati*. Interestingly, *T. regenti* has probably been reported from the most diverse duck species (135) and does not appear to have a preferred host within the Anatidae. In North America, *T. stagnicolae* and *T. physellae* are most often identified in dermatitis outbreaks (70).

## INTRAMOLLUSCAN DEVELOPMENT OF AVIAN SCHISTOSOMES

As noted above, *Trichobilharzia* is the most diverse schistosome genus and has most often been implicated in outbreaks of cercarial dermatitis. As a result, studies on the avian schistosome-snail in-

termediate host relationship have focused primarily on species of the genus *Trichobilharzia*. Additionally, long-term laboratory maintenance of *T. szidati* and *L. stagnalis* enabled the experiments that uncovered the intimate molecular interactions of avian schistosomes and their snail hosts. (The *Trichobilharzia ocellata* organism used as an experimental model in European laboratories for the last few decades is identical to *T. szidati*, and the latter name is preferred and is used here [123]. If *T. ocellata* is used in the text body, then it refers to the non-European isolates of the parasite.)

After hatching from eggs in an aquatic environment, schistosome miracidia search for and invade an appropriate snail host species. This behavior must be accomplished quickly, as miracidia have a temperature-dependent limited life span of around 20 h, as reported for, e.g., *T. stagnicolae* (136). Studies on the miracidial behavior of *T. szidati* have shown a progression of steps from host finding to penetration and migration. Miracidia respond to environmental stimuli, such as light or gravity, that direct them to the microhabitat occupied by the host snails (137). Snails release various chemical compounds that form an "active space" around them and serve as chemoattractants for the miracidia. Miracidia recognize macromolecular glycoconjugates, termed miracidium-attracting glycoproteins (MAGs) or miraxones, that consist of a protein core and carbohydrate chains linked O-glycosidically via *N*-acetyl-D-galactosamine and serine/threonine (138, 139). The attractant for miracidia is encoded in these carbohydrate moieties. Upon entering the "active space" of the snail, miracidia modify their movement by increased random turns within the increasing attractant gradient and by a turn-back form of swimming within the decreasing attractant gradient (140). This mode of orientation (chemokinesis), observed, e.g., in *T. szidati* or *T. franki* (115, 138), results in the first contact of the miracidia with the snail, which is then followed by repeated investigation/probing of the snail surface and, finally, miracidial attachment (140). Penetration of the snail surface follows, although the factors contributing to this process, such as the components of miracidial penetration glands, remain largely unknown.

After penetrating the surface epithelium of the snail, miracidia of avian schistosomes transform into mother sporocysts that give rise to daughter sporocysts, which migrate to the snail hepatopancreas, where production of the final larval stage, the cercariae, takes place (13). The prepatent period lasts about 3 to 10 weeks, depending on several factors, such as the miracidial dose or temperature (13, 141). Infection by avian schistosomes may lead to alterations of the snail internal defense system (IDS), metabolism, and endocrine functions. Such alterations have been studied widely in *L. stagnalis*, the intermediate host of *T. szidati* (142–145).

The snail IDS is based solely on innate immune mechanisms composed of humoral and cellular limbs. Lectins are essential humoral components, whereas hemocytes represent the main effector cells (146, 147). Both limbs of the *L. stagnalis* IDS appear to be activated and then suppressed during early and late stages, respectively, of a *T. szidati* infection (143). *In vitro*, hemocytes failed to encapsulate and destroy *T. szidati* sporocysts (148). It has been suggested that parasite excretory-secretory products participate in the modulation of hemocyte activities (149). Disruption of hemocyte signaling pathways, such as protein kinase C (PKC) and extracellular signal-regulated kinase (ERK) pathways, may also influence hemocyte activities (150–152). For example, carbohydrates known to be present on larval surfaces of *T. szidati* and *T. regenti*, such as D-galactose and L-fucose (153–156), affected hemocyte PKC and ERK signaling in *L. stagnalis*, which suggests an immunosuppressive role (157, 158). However, experiments investigating the direct effect of *Trichobilharzia* larvae on hemocyte signaling have not been performed. Alterations of humoral defense components also occur during infections by avian schistosomes, and at least two molecules, molluscan defense molecule (MDM) and granularin, have been investigated in this respect (159, 160). Both molecules, produced in *L. stagnalis* by granular cells of connective tissue, are related to phagocytic activity of hemocytes. MDM enhances phagocytosis of hemocytes, and the expression of a corresponding gene for MDM is downregulated in *L. stagnalis* infected with *T. szidati* (159, 161). In contrast, treatment of hemocytes with granularin decreases phagocytic activity, and the encoding gene is upregulated in parasitized snails (160, 161).

In the snail host, avian schistosomes also interfere with metabolism, such as causing abnormal body growth, and endocrine functions, such as causing a reduction of egg laying (161). In *L. stagnalis*, these processes are regulated by neuroendocrine cells in the central nervous system (CNS) (162, 163). *Trichobilharzia szidati* releases an undescribed substance that induces the snail host to produce schistosomin (a peptide of 8.7 kDa consisting of 79 amino acids) from its connective tissue and hemocytes (145). Schistosomin acts as a neuropeptide that interferes with some hormones, such as calfluxin, a neuropeptide that stimulates the influx of $Ca^{2+}$ into the mitochondria of albumen gland cells (164, 165). As a consequence, ovulation and egg laying are inhibited in the snail. Excitability of neuroendocrine cells (light green cells [LGCs]) responsible for growth (162) increases in response to schistosomin (166). As a result, the body size of infected snails may become considerably larger than that of uninfected snails (142, 161). Other neuropeptides (FMRFamide-related peptides) are also upregulated during infection of *L. stagnalis* by *T. szidati*, and these peptides, via inhibition of neuroendocrine cells, may be responsible for the suppression of snail metabolism and reproduction (167). All these changes in infected snails may provide energy resources and space that can be exploited by the schistosomes for development (145, 161).

The survival rate of infected snails releasing schistosome cercariae as the agent of cercarial dermatitis varies among species. A limited number of studies have focused on survival rates of avian schistosome-infected versus uninfected snails. As an example, 90% of *L. stagnalis* snails infected experimentally with a single miracidium of *T. ocellata* (North American isolate) were alive at 28 weeks of age (three infected snails were alive for 19 months), whereas all uninfected snails were dead (168). In contrast, *L. stagnalis* or *Planorbarius corneus* snails naturally infected with *T. szidati* or *Bilharziella polonica*, respectively, lived a shorter time, on average, than the corresponding uninfected individuals (169, 170).

## VERTEBRATE HOST FINDING AND PENETRATION

Cercariae emerging from the snail intermediate host are the infective stage to the definitive host and are also the stage responsible for causing cercarial dermatitis. A cercaria is a multicellular larva comprised of an oblong body and a slender tail that is bifurcated (furcocercous) at the posterior end (Fig. 2). Cercariae of schistosomes leave their snail hosts actively. For this purpose, they employ a pair of specialized unicellular escape glands that are obvious in mature cercariae within sporocysts. Once the cercariae have emerged, only their ducts lined by microtubules are visible, sug-

**FIG 2** Furcocercaria of *Trichobilharzia regenti* with protruded acetabulum (arrow), lateral view. Bar, 200 μm. (Courtesy of J. Bulantová, reproduced with permission.)

gesting a release of granular gland content likely containing histolytic enzymes during their migration through the snail tissue (171).

Once in the water, schistosome cercariae express a complex pattern of behaviors that is composed of movement cycles that are repeated in defined frequencies (172). In general, they express negative geotaxy and positive phototaxy, which result in the concentration of cercariae just beneath the water surface, where appropriate definitive hosts may occur (173). Surface tension of the water enables clinging of cercariae via their ventral sucker (acetabulum) (173). This resting phase is interrupted by phases of active swimming (173). The effect of light on the behavior of swimming cercariae was studied in detail for *Trichobilharzia szidati*. Cercariae are able to react to a moving shadow stimulus (produced by a potential host) by a burst of forward swimming (body first) away from the source of light, to deeper levels, where they can encounter the feet of duck definitive hosts. A shadow stimulus applied in the active phase inhibits swimming and prolongs the following passive phase (173). Two types of photoreceptors, located near the dorsal surface of the body, were described for this species: a pair of lens-covered pigment cup ocelli and a special type of unpigmented, rhabdomeric photoreceptors, composed of three cells arranged in a three-dimensional (3-D) configuration. The lens-covered pigment cup ocelli probably serve to detect the direction of incoming light and to control the direction of swimming in relation to a light source, while the unpigmented photoreceptors serve as monitors for light intensity (174). The pigmented ocelli are also present in other genera of avian (*Bilharziella*, *Dendritobilharzia*, *Gigantobilharzia*, and *Austrobilharzia*) and mammalian (*Schistosomatium* and *Heterobilharzia*) schistosomes but are absent in *Schistosoma*.

Moving shadows also trigger a readiness for cercarial attachment to substrates, further stimulated by thermal and chemical host cues (172). As for the latter signals, compounds in the host skin (ceramides and cholesterol) stimulate enduring contact of cercariae of *T. szidati* with the host skin (173, 175). There is variability among schistosome species in responses to light, shadow, physical, and chemical cues, such that for different species, some of the signals may not work or may include some additional ones, such as touch, water turbulence, and/or additional chemical compounds (172, 176). The variation among the different avian schistosome species reflects the diversity in biology and ecology of schistosomes and their adaptations to the spectra of avian hosts (172, 176).

Invasion of the bird or mammal skin is initiated by the cercariae receiving the proper signal. Surprisingly, there were few differences between the avian *T. szidati* and human *S. mansoni* organisms in their pattern of invasive behavior toward living human skin; most cercariae did not penetrate the skin immediately after attachment but performed a leech-like creeping which lasted 0 to 80 s for *T. szidati* and 15 s to 5.58 min for *S. mansoni* (177). Such behavior guided the cercariae to skin wrinkles or hair follicles, where most penetration sites were located (178) (Fig. 3). Penetration behavior and production of secretions stimulate neighboring cercariae to use the same entry site on the skin (177, 178). Invasion of the skin is facilitated by secretions of cercarial penetration glands released from openings at the apex of the muscular head organ by spasmodic contractions of cercarial body musculature (179). The head organ performs concurrent thrust movements against the skin surface while the cercaria is firmly attached by the ventral sucker (176, 178, 180). Signals for skin invasion seem to be universal for schistosomes—fatty acids, especially polyunsaturated fatty acids containing 18 carbons and two or three *cis* double bonds (linoleic and linolenic acids), which are bound in cell membranes and occur as free molecules on the surface of human and bird skin (176).

For *T. szidati*, the tail is shed within 0 to 105 s after the onset of penetration, sometimes during creeping. This shedding seems to be generated at least partially by contractions of a muscular collar at the body-tail junction, which plays a role in the closure of the cercarial hind body after tail shedding. Cercarial penetration occurs in a nearly surface-parallel direction, while the spined ventral sucker supports squeezing of the cercarial body into the opening in the skin caused by histolytic gland secretions. Full penetration of living human skin was achieved within a mean of 4 min (83 s to 13.3 min), which was significantly faster than the case for the human parasite *S. mansoni* (6.58 min, on average) (177). Faster penetration of avian schistosome cercariae might be a consequence of these parasites' adaptation to lower concentrations of fatty acids in duck skin; therefore, reaction to higher concentrations in human skin may induce faster invasion (177, 178). Another explanation may be that different histolytic enzymes are used for penetration (see below). Skin penetration success rates

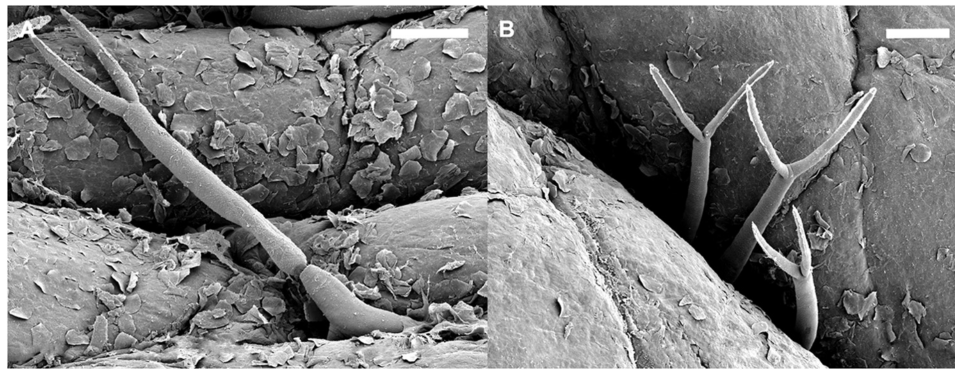


**FIG 3** Scanning electron micrographs of cercariae of *Trichobilharzia regenti* penetrating the skin of a duck leg. (A) An individual larva entering the skin; the tail is still preserved. (B) Tails of three cercariae penetrating the skin in a group. Bars, 100 μm. (Courtesy of J. Bulantová, reproduced with permission.)

may vary greatly. In experiments with *T. szidati* and human volunteers, the highest penetration success rate was 49% underneath the forearm (181).

The ultrastructure and chemical composition of avian schistosome cercarial penetration glands and their secretions have been poorly studied relative to the case for human schistosomes (*Schistosoma*). In fact, most of our knowledge is based on only two species of *Trichobilharzia*. There are five pairs of unicellular penetration glands: three pairs are located behind the ventral sucker (postacetabular glands), and two pairs are located around the ventral sucker (circumacetabular or preacetabular glands). These glands are filled with secretory vesicles that are released through the gland processes at the surface of the head organ. A 3-D model of *T. regenti* acetabular glands shows that they occupy more than one-fourth of the cercarial body volume (postacetabular glands, ca. 15%; and circumacetabular glands, ca. 12%). Differences were observed in the appearance of granular material/secretory vesicles contained in the glands, pH value, and the ability to bind various dyes and fluorescent markers (180, 182).

In *T. szidati*, proteolytic activity was detected in cercarial gland secretions induced by linoleic acid. This activity was linked with an orthologue of a chymotrypsin-like serine peptidase, named cercarial elastase and characterized from *S. mansoni* and *S. haematobium* cercariae. A protein on blots of *T. szidati* cercarial secretions as well as in histological sections of penetration glands immunologically cross-reacted with antibodies against elastases of *S. mansoni* and *S. haematobium* (183, 184). However, in another study, the reaction of antibodies raised against elastase of *S. mansoni* was observed neither with the penetration glands of *T. szidati* nor with the cercarial secretions on Western blots (180). In contrast, high activities of cysteine peptidases were noticed in induced cercarial secretions and homogenates for *T. szidati* and *T. regenti* (180, 185). In addition, the presence of cercarial elastase in the latter species was not confirmed by screening of a cDNA library (186). On the other hand, a papain-like cysteine peptidase, termed cathepsin B2, was found in the postacetabular penetration glands of *T. regenti*. This enzyme was shown to cleave proteins of the host skin, similar to the case with *S. japonicum* (187, 188). Its expression was even higher in intravertebrate stages (schistosomula and adults), suggesting that there are multiple roles of this enzyme during the life cycle (189). It seems that the use of particular peptidase families for skin penetration and tissue invasion is diverse among schistosomes (190) and may confer host specificity.

In the postacetabular penetration glands of *T. szidati* and *T. regenti* cercariae, a lectin(s) specific for β-1,3- and β-1,4-linked saccharide chains and their sulfated derivatives is present, though its biological function is still unknown (180, 191). It is interesting that there are high concentrations of calcium in the circumacetabular glands of both species (180). Several hypotheses suggest that the role(s) of calcium in the glands (including those of *S. mansoni*) may be to regulate gland peptidase activity, stimulate glycocalyx removal, interact with connective tissue proteoglycans, regulate host blood coagulation, or polymerize the adhesive substance from postacetabular glands. However, none of these hypotheses (except for a regulation of peptidase activity) has been confirmed adequately (180, 192, 193). Following contact with the host skin or a linoleic acid-coated surface (L. Mikeš, unpublished data), cercariae start to expel small amounts of gland content during the creeping movement, which is "printed" as the cercariae touch the surface—these "kissing marks" are made of a sticky substance. In *S. mansoni*, this substance is a product of the postacetabular glands and is composed of neutral and acidic mucosubstances (194). This product might serve adhesive or enzyme-directive functions. Similar material is produced by cercariae of *Trichobilharzia* spp. yet differs in chemical composition (180). Finally, the production of three types of eicosanoids by *T. szidati* cercariae is stimulated by linoleate (195). Eicosanoids may have a role in host invasion (vasodilatation), and their involvement in immune evasion was proven by the inhibition of superoxide production by human neutrophils (195).

During cercarial penetration, dramatic changes of surface structures and metabolism lead to the transformation of the cercaria to a schistosomulum. The thick glycocalyx that served as a protective layer for the free-living cercaria is shed, as its carbohydrate-rich composition is a target of the host complement cascade. In the schistosome species studied so far, the glycocalyx is markedly rich in fucose residues (154, 196–198). There is an obvious loss of saccharide moieties at the surfaces of transformed schistosomula of *T. szidati* and *T. regenti*, leading to reduced immunoreactivity and attractiveness for fucose-specific lectins (154, 155, 199, 200). Also, similar to the case for human schistosomes and members of other families of blood flukes, the trilaminar surface membrane of the outer cercarial tegument gradually changes to the doubled heptalaminar membrane of the schistosomulum, which has a protective function against the host immune system (199). In *T. regenti* stimulated by linoleate *in vitro*, shedding of the

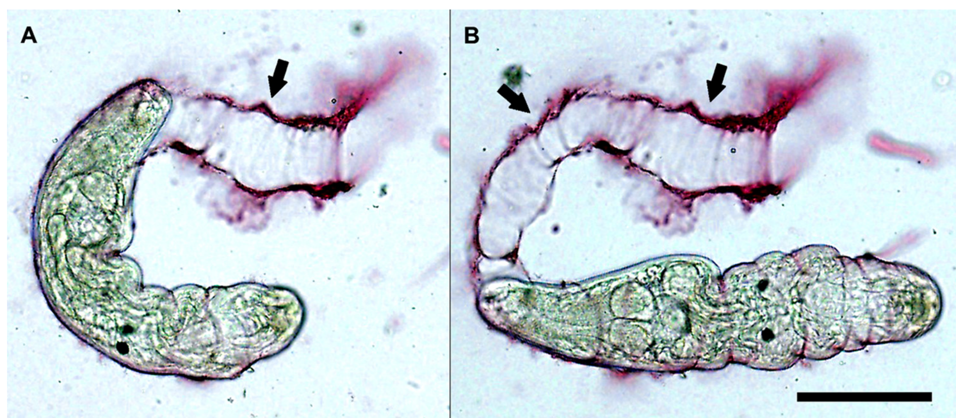


**FIG 4** Living *Trichobilharzia regenti* cercaria *in vitro*, shedding its glycocalyx upon stimulation by linoleate. The sticky products of the penetration glands, stained with lithium carmine, adhere to the surface of the body, and as the cercaria crawls forward by periodical constrictions (A) and extensions (B), the glycocalyx and bound secretions are removed from the surface in a sleeve-like manner. Arrows indicate detached sleeve-like remnants of glycocalyx and gland products. Bar, 100 μm. (Courtesy of J. Chaloupecká, reproduced with permission.)

glycocalyx starts at the anterior of the cercaria, surrounding the openings of penetration glands (J. Chaloupecká and L. Mikeš, unpublished data). Sticky products of the glands adhere to the surface of the body, and as the cercaria crawls forward, the glycocalyx, with the gland products, is shed in a sleeve-like manner, until it is detached at the end of the hind body (Fig. 4). Transformed cercariae/schistosomula then lose their osmotic resistance toward water and become dependent on isosmotic conditions of the host (Chaloupecká and Mikeš, unpublished data). Whether any compounds of gland secretions take part directly (e.g., enzymatically) in the process of glycocalyx shedding is still unclear.

It should be mentioned that cercariae of avian schistosomes readily penetrate other (soft) tissues, and peroral infections of birds (definitive hosts) and mice (accidental hosts) with cercariae of *T. regenti* and *T. szidati* have been confirmed (L. Kolářová, K. Blažová, V. Pech, and P. Horák, unpublished data). This phenomenon is also known for mammalian schistosomes of the genus *Schistosoma* (201–203). It is not clear, however, how much these peroral infections contribute to the transmission of schistosomes under natural conditions. Due to the features of the esophageal mucosa, the penetration of *Trichobilharzia* cercariae does not require the penetration glands to be emptied completely, and their tails may be preserved for some time. The stimuli triggering this penetration behavior remain unknown.

## PATHOGENICITY OF AND IMMUNE REACTIONS AGAINST AVIAN SCHISTOSOMES

### Survival and Migration in Avian Hosts

Once the cercariae have transformed into schistosomula and reached their final location, it is not clear how long avian schistosomes live in their definitive hosts. For example, in experimental infections of ducks, adults of *Trichobilharzia parocellata* were found at 86 days postinfection (p.i.) (204). Whereas experimental infections with *T. szidati* and *T. regenti* last for about 3 to 5 weeks (13, 205), the adult worms of a Canadian isolate of *T. ocellata* were found in the liver at 370 days p.i. (206). Data describing the schistosome life span and length of time for egg release in a bird host will be necessary in considering the epidemiology of dermatitis. Nonetheless, avian schistosomes have a preference for two major habitats within the avian host: the visceral venous system (mesenteric, renal, cloacal, and portal vessels) and the nasal passages (except for *Dendritobilharzia*, which is found in the arterial system) (13).

Migration and localization have been characterized for a few visceral schistosomes and only one (*T. regenti*) of the eight nasal schistosomes (205, 207). Visceral schistosomes in birds have a migration pattern similar to those of *Schistosoma* spp. in mammals. After skin penetration, schistosomula of *T. szidati* navigate toward deeper skin layers by following dark and higher concentrations of D-glucose and L-arginine (208, 209). Once a blood capillary is found, the worms penetrate it and migrate to the heart and lungs. In the lungs, the worms enter free air space and then reenter the blood system (206, 210). Finally, visceral blood vessels (usually portal and mesenteric veins) are the preferred habitat (13). However, there are at least two exceptions, as follows: (i) the adults of *T. szidati*/*T. ocellata* leave the blood system and enter the layers of the host intestinal wall and mucosa (206, 211) and (ii) *Dendritobilharzia pulverulenta* prefers the arterial system of its hosts, where it is found in the lower dorsal aorta and the femoral arteries (212). For the nasal schistosome *T. regenti*, migration is dramatically different. Schistosomula leave the skin and then seek and penetrate peripheral nerves (Fig. 5) to migrate to the spinal cord and brain of their host. From there, the adult worms appear intra- and extravascularly in the nasal mucosa (207).

### Pathology Caused by Visceral Species in Birds and Mammals

Most pathological studies of avian schistosomes in the avian host have been detailed for experimental birds, though there are a few reports from wild birds. As for accidental mammalian hosts, only experimental infections have been evaluated. The schistosomula migrate through the heart and lungs of birds and mammals, and only in the avian host do they reach their final destination. Migration through host lungs has been shown to cause damage (16, 210, 213). Infections of duck and mouse lungs by *T. szidati* are accompanied by hemorrhages in the periphery of the lungs (210). Migration of schistosomula in the lungs of ducks leads to formation of lymphocytic lesions and an influx of macrophages, heterophils,

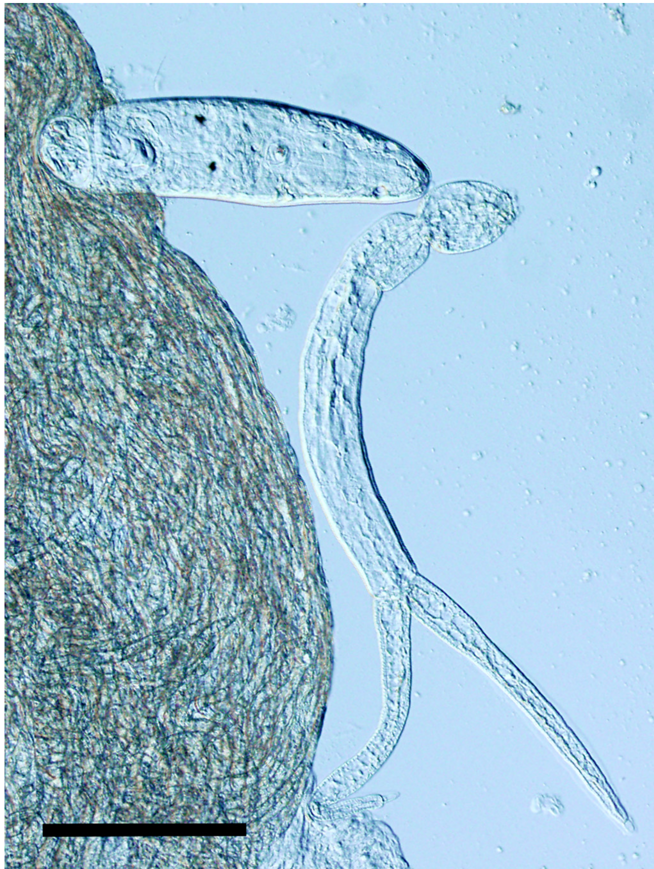

**FIG 5** Cercaria of *Trichobilharzia regenti in vitro*, penetrating a peripheral nerve isolated from a duck. The tail is already detached, and the head organ burrows into the nerve. Bar, 200 μm. (Courtesy of J. Bulantová, reproduced with permission.)

and eosinophils into the afflicted tissue (210). However, in mouse lungs, schistosomula do not evoke a specific inflammatory reaction; only alveolar congestion and edema are observed (210). Damage to the lung tissue in general, and formation of alveolar congestion in particular, might be caused by schistosomula that leave the blood system and localize extravascularly in the alveolar walls. Their inability to reenter the blood system might be linked to their relative size and loss of orientation in a noncompatible mouse host (210). Similarly, migrations to mammalian lungs and accompanied hemorrhages have been observed in hamsters, guinea pigs, rabbits, and rhesus monkeys experimentally exposed to three species of *Trichobilharzia* (213). Pulmonary infections of chickens and pigeons with the marine schistosome *Ornithobilharzia canaliculata* led to the development of lesions in the arterial and venous vascular systems (lymphocytic endarteritis, periarteritis, and segmental proliferation of the vascular endothelium), hyperplasia of smooth muscles in tertiary bronchi, and thickening of alveolar septa. Cellular infiltrates consisted mainly of histiocytes, heterophils, and lymphocytes (16). In contrast to the case with visceral schistosomes, infections of duck and mouse lungs with the nasal species *T. regenti* probably represent an ectopic localization of schistosomula (207, 214, 215).

As for patent infections (exclusively in birds), major pathology is caused by granulomas around eggs and only partly by adult worms. Obliterative endophlebitis caused by adult schistosomes (probably *Trichobilharzia filiformis*) in the intestinal veins of mute swans (*Cygnus olor*) has been recorded (216). The intestinal surface showed various stages of villous atrophy with intestinal mucosal lesions, associated with infiltration of the lamina propria of the jejunum and ileum by lymphocytes and plasma cells and by a smaller number of heterophils and eosinophils. Eggs were observed multifocally in the lamina propria of the small and large intestines, and their presence triggered mild to severe granulomatous reactions (216). Eggs laid by the adult worms of *Austrobilharzia variglandis* in experimentally infected chickens caused edema, cellular infiltration, and hyperplasia of smooth muscle of the muscularis externa of the intestine. Around the eggs, mononuclear cells formed early granulomas with a few eosinophils and heterophils, followed by accumulation of giant and epithelioid cells (217).

Similarly, examination of Atlantic brant geese (*Branta bernicla hrota*) infected with *Trichobilharzia* sp. (probably *Anserobilharzia brantae*, based on current taxonomy) revealed the development of granulomas around eggs located in the colon (218). Granulomas were also observed in the duodenum and small intestine of pigeons infected with *Ornithobilharzia canaliculata* (16). Three species of ducks infected with *T. physellae* showed no associated tissue reaction in the vicinity of adults located in mesenteric veins, but a granulomatous reaction around the eggs was detected occasionally in the mucosa and submucosa of the intestine (219). On the other hand, the most serious lesions and fibroplasia of the portal triads and adjacent parenchyma were observed in the livers of those ducks and were attributed to mature *T. physellae* (219). In the case of pigeons infected with *O. canaliculata*, granulomatous lesions surrounding collapsed eggs were observed in the liver parenchyma (16). Intestinal pathology may be accompanied by poor nutritional conditions, as noticed in pigeons infected with *O. canaliculata* (16). Exceptionally, a more serious manifestation of the infection in naturally infected wild ducks was reported where the adults and eggs of *T. physellae* caused partial to complete paralysis of the cervical, wing, and leg muscles, foul-smelling diarrhea, and half-closed pasted eyelids (219).

### Pathology Caused by Nasal Species in Birds and Mice

Histological examination of the nasal tissue of birds showed the extra- and intravascular locations of adult worms of *T. regenti* (220), with their first appearance on day 13 p.i. (205). Immature eggs appeared from day 15 p.i., and at day 19 p.i., eggs were fully developed and observed extravascularly in the nasal mucosa, with the maximum number of eggs seen on day 22 p.i. (221). The area surrounding the eggs was infiltrated by numerous eosinophils, heterophils, histiocytes, and multinucleated giant cells and a few plasma cells and mononucleated cells (220). The formation of granulomas around the eggs was noted from day 22 p.i. (221). Miracidia that hatched directly in the host tissue were surrounded by lymphocytes, eosinophils, and heterophils, without granuloma formation (221). While an infected bird is drinking/feeding, only the miracidia leave the tissue to enter the water, which represents an exceptional mode of transmission among schistosomes (205). The presence of adults only did not initiate an influx of immune cells to their vicinity, but the presence of large worms and eggs caused the development of focal hemorrhages throughout the nasal mucosa (207, 221). Probably the more devastating aspect of the pathology of this species is the effect on the CNS of hosts (birds

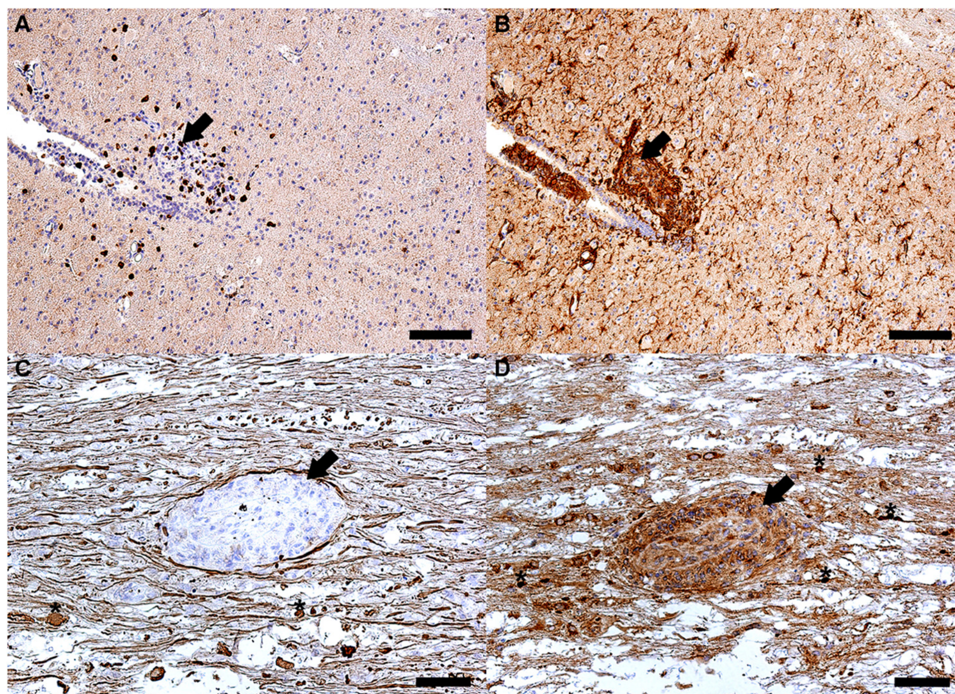


**FIG 6** Destruction of a schistosomulum (arrows) in the thoracic part of the spinal cord of a BALB/c mouse at 21 days p.i. (longitudinal sections). (A and B) Inflammatory lesion consisting of $CD3^+$ lymphocytes (dark spots) (A) and microglia cells (brown-stained ramified cells) (B) that were detected by use of anti-mouse $CD3^+$ and anti-mouse Iba-1 antibodies, respectively. Nuclei of other cells were stained blue by hematoxylin. (C and D) Tissue around the schistosomulum contains damaged axons. Axonal damage was accompanied by formation of spheroids (asterisks) in the site of axonal disruption and was visualized immunohistochemically by use of anti-mouse nonphosphorylated and phosphorylated neurofilament antibodies (SMI-311 and SMI-312, respectively) (C) and anti-mouse β-amyloid precursor protein antibodies (D). Bars, 100 μm (A and B) and 50 μm (C and D). (The figure was created by L. Lichtenbergová.)

and experimental mammals). As stated above, cercariae penetrate the skin and migrate to the nasal passages via the CNS rather than the circulatory system.

CNS infections of ducks and mice by *T. regenti* may lead to the development of various transient or permanent neuromotor symptoms, such as weak to severe leg paralysis and balance/orientation disorders (207, 214, 215). In avian hosts, schistosomula in the CNS initiated an accumulation of inflammatory cells, such as eosinophils and heterophils, that represented the most abundant cell infiltrates, yet minimal damage to nervous system cells was detected (220). In a few cases, however, damage to the CNS was observed in birds with visceral schistosomes. For example, granulomatous encephalitis in mute swans, caused by *Dendritobilharzia* sp., has been described (222, 223). Schistosome eggs found in the cerebrums and cerebellums of naturally infected swans were surrounded by giant cells, macrophages, lymphocytes, and, to a lesser extent, heterophils and fibroblasts (223). The presence of *Dendritobilharzia* eggs in the CNS represents an ectopic localization.

Because of the pathology caused by *T. regenti*, and thus the implications for human health, most experimental work has been done in mice rather than birds. For up to 3 days p.i., migration of schistosomula through the murine nervous tissue did not evoke inflammation or tissue damage, and all detected parasites were intact (215, 224). A host reaction to the infection was visible on days 6 and 7 p.i. The presence of parasites led to the accumulation of immune cells, predominantly microglia, macrophages, and neutrophils and, to a lesser extent, $CD3^+$ lymphocytes (215, 224). Proliferating astrocytes formed "glial scars" at the sites of previously migrating schistosomula (215). Ongoing infection was associated with a more intense inflammatory reaction in white and gray matter of the spinal cord. Microglia, macrophages, neutrophils, eosinophils, and $CD3^+$ lymphocytes participated in the formation of inflammatory lesions surrounding the disintegrating schistosomula, and damage to the axons was detected (215) (Fig. 6). The localization of schistosomula outside the solid tissue, in the subarachnoidal space of the spinal cord and the brain and in the cavity of the 4th ventricle of the brain, led neither to damage nor to inflammation of the adjacent nervous tissue (215). It seems that schistosomula located in the cavities of CNS were able to delay destruction by the immune cells. Nevertheless, most of the worms were eliminated by 21 days p.i. (215). Challenge infections triggered a strong immune response, which efficiently and rapidly eliminated the schistosomes (215, 224).

In immunodeficient SCID mice, primary infections as well as reinfections did not evoke a significant skin reaction, and the schistosomula often escaped from the skin to the CNS (224), where migrating schistosomula caused axonal damage and an influx of immune cells (215). In comparison to the case with immunocompetent mice, the schistosomula survived longer in the CNS, probably due to the absence of T and B lymphocytes (215, 224), cells that may represent important effectors in destruction of schistosomula. Larger numbers of schistosomula in the CNS and their extended time of migration via nervous tissue resulted in a higher rate of occurrence of paralysis of immunodeficient SCID mice (215).

The above-mentioned damage to the nervous tissues of birds and mice demonstrates that not only the eggs but also the other stages of schistosomes are responsible for major pathology. In this particular case, just the schistosomula (migrating juveniles) of *T. regenti* can be regarded as the most pathogenic stage of the parasite.

### Skin Immune Response and Cercarial Dermatitis

Skin immune reactions of birds to the penetration of avian schistosome cercariae are insufficiently described. In any case, birds do respond to penetrating cercariae, as shown in a histological observation of chicken skin infected by *Ornithobilharzia canaliculata*. Severe infiltrations of the dermis by histiocytes and heterophils and aggregation of lymphocytes around dilated capillaries in the dermis were recorded (16). Dead and destructed schistosomula surrounded by heterophils and histiocytes were found in the epidermises of chickens at 12 h p.i. At 24 h p.i., lymphocytes, histiocytes, and heterophils still persisted in the dermis, but the number of immune cells decreased (16).

Immunohistopathology of cercarial dermatitis in humans recognized three phases of cellular responses (leucocytic, lymphocytic, and histiocytic) against *Trichobilharzia* larvae (225). However, because studies on humans are rarely performed (112, 225, 226), a mouse model was established to provide more-detailed studies on immunohistopathology. Primary infection of mice with *T. regenti* causes an acute inflammatory reaction with edema and vasodilatation (227). Parasites located in the dermis are surrounded by large inflammatory cellular foci that are formed by neutrophils, macrophages, mast cells, major histocompatibility complex class II (MHC II) antigen-presenting cells, and a small number of $CD4^{+}$ lymphocytes (227). Repeated infections cause perivasculitis, folliculitis, and substantially more influx of the same cell types that are noted after primary infection (227). Extensive skin inflammation leads to the formation of large abscesses and subsequently to dermal and epidermal necrosis. Sites of previous cercarial penetration are characterized by intraepidermal pustulae and parakeratosis (227).

*In vitro* culture of skin biopsy specimens from primary mouse infections by *T. regenti* revealed a release of the acute-phase cytokines interleukin-1β (IL-1β) and IL-6 and an increased production of IL-12 (227). Larger amounts of IL-12 correlated with elevated production of gamma interferon (IFN-γ) in the cell culture supernatant (antigen-stimulated lymphocytes) from the skin draining lymph nodes (227). IFN-γ and IL-12 are associated with Th1 cell differentiation, and IL-1β and IL-6 are important in Th17 polarization (228). Although the role of Th17 in host tissue immunopathology has been described for some infections by helminths (e.g., human schistosomes) (228), participation of Th17 cells in the processes associated with skin penetration by avian schistosomes needs to be clarified.

Reinfections of mice with *T. regenti* were accompanied by edema that developed as a consequence of local vascular permeability caused by histamine released from activated mast cells (227). Histamine has a regulatory function in the Th1 and Th2 polarization of the immune response (229). Mast cells also rapidly released a large amount of IL-4, which has been detected in the supernatants of skin biopsy specimens from *T. regenti*-reinfected mice (227). Like mast cells, basophils degranulate and release IL-4 as a response to the presence of *T. regenti* antigens (230). Elevation of total serum IgE levels implies that histamine and IL-4 production by mast cells and basophils occurs in an IgE-dependent manner (227, 230). Dominance of the Th2 response was also supported by an elevation of antigen-specific IgG1 antibodies and a decrease of IgG2b antibodies (Th1 associated) in the sera of mice reinfected with *T. regenti* (230). Cercarial dermatitis in reinfected mice is therefore Th2 polarized, with a response comprised of an early type I hypersensitivity reaction and late-phase skin inflammation (227).

It has been shown in several cases that mammals (including humans) are unsuitable hosts for avian schistosomes, such that the worms cannot mature and reproduce (except for *Austrobilharzia variglandis*, which is able to reach sexual maturity in the lungs of *Meriones unguiculatus* gerbils [231]). Nevertheless, cercarial dermatitis is probably not the only interaction of avian schistosomes and mammals. Dermatitis develops as an immune (allergic) reaction of the already sensitized person; it represents a powerful protection of the body against worms in the skin. However, in a naive (nonsensitized) or immunodeficient experimental host, at least some worms survive, leave the skin, and migrate throughout the body (213, 232). Mild to severe consequences of such migration may appear (see above); most importantly, *T. regenti* is neurotropic and can cause damage to the central nervous system (155, 207, 214, 215, 220, 224). To date, no information about migration of avian schistosomes in human bodies is available. It therefore seems that laboratory animals (mice, rats, etc.) are indispensable for assessing all risks associated with infections of mammals (humans) by avian schistosomes.

## DETECTION AND IDENTIFICATION OF AVIAN SCHISTOSOMES

Prior to or during the dermatitis season, especially following a dermatitis outbreak, a standard protocol for the detection of schistosome cercariae involves the collection of and screening for cercarial emergence in snails. Usually, in a laboratory, individual snails are placed in a beaker/small wells with clean water and exposed to a lamp to stimulate shedding of cercariae. Subsequently, cercariae are collected and identified under a light microscope. If no cercariae emerge from the snails, there are three options: the snails can be dissected to find the schistosomes, the snail tissue can be pooled and molecular techniques applied to detect a schistosome infection, and/or additional snails can be collected from affected and surrounding areas (233, 234). Detection of microorganisms directly from water samples is developing rapidly, but among schistosomes, these techniques have been optimized for human schistosomes (235, 236). Methods thus far to detect avian schistosomes, *Trichobilharzia* in particular (237, 238), involve concentrating the cercariae from a water sample and using a PCR assay to detect a single cercaria in plankton (0.5 g) and snail tissues (0.25 g) (239). Using definitive host-seeking behavior, cercariae can be lured to a trap that contains linoleic acid, a known stimulus for cercarial penetration (240). Irrespective of a dermatitis outbreak, identifying which adult worms can be found in the habitat can be completed by examination of feces for eggs by using sedimentation/flotation (241) or Kato-Katz fecal smear methods (242) and/or postmortem examination of the arterial/venous system, nasals, liver, or kidney of the host. For nasal schistosome species, lavage of the nasal cavity of birds represents a method of choice. After rinsing out the nasal cavity with saline or water, freely moving miracidia (and partly the liberated eggs) can be detected in the wash fluid (13). The above-described examination of snails

and birds for avian schistosomes is thoroughly summarized in reference 243.

Examination of water or the snail or bird host is important for species delineation. Not only is there a highly diverse population of schistosomes, especially avian schistosomes, that are responsible for dermatitis, but these species differ markedly in their morphology and pathogenicity in birds and experimental animals (12, 13). Identification of schistosomes, particularly avian schistosomes, is challenging. Traditional methods using morphology (adults mostly, but also eggs and cercariae) are usually not sufficient to separate species, particularly within a genus, as these characteristics can be missing or minute, and their recognition often depends on the experience of the observer. However, some characteristics have been found to be informative for rough species identification, such as the position of internal organs and tegument spination of adults (13), morphology of eggs (244), or distribution of sensory papillae on cercariae (68, 198).

In addition to the morphological features, there are some nonmorphological criteria that can be used to differentiate species, for example, the behavior of cercariae (245), compatibility with the snail hosts, and organ/tissue affinity of adults in birds (135). If eggs and adults from a host are examined, it is not always clear which adults/eggs are conspecific or which match cercariae from snails unless experimental trials are conducted. Experimental infections with cercariae from snails to obtain adult worms for morphological assessment are ideal, but these are time-consuming and often yield low prevalences of infection. Because of the reported species, morphological, and pathogenic diversity of avian schistosomes (12, 13), from an epidemiological perspective on dermatitis, there is a need for species delineation and host use, and molecular techniques have proven a necessary and excellent tool for more rapid identifications (63, 70, 135). Such techniques have allowed us to genetically connect larval stages from snails to adult stages from birds, greatly advancing the epizootology and epidemiology of avian schistosomes in particular (10, 40, 123).

Molecular identification of an avian schistosome provides clues to the host source and biology and possible targets for control. There have been steady efforts and testing of markers that have revealed some consistency and validity for species identification of schistosomes (123, 246, 247). Molecular phylogenetics has had a major impact on the taxonomy and discovery of new lineages of avian schistosomes, particularly the major etiological agents of cercarial dermatitis, i.e., species of *Trichobilharzia* (10, 40, 63, 67, 69, 70, 121–123, 125, 129, 135, 248–251). Most of the effort has been conducted using three gene regions. To date, the nuclear ribosomal DNA D1-D2 regions of 28S, the internal transcribed spacer (ITS) region, and the mitochondrial *cox1* gene have been tested as molecular markers for systematics (40, 63, 67, 69, 70, 105, 123, 248, 249, 251), epidemiology (252), and diagnostics (121, 253). Sequencing of gene regions such as the nuclear ITS region and the mitochondrial *cox1* gene not only has linked life cycle stages but also has suggested that our recognition of the diversity of avian schistosomes continues to grow (10, 12, 40, 65–67, 69, 70, 105, 129, 133, 135).

While the molecular identification of avian schistosomes is still in the early stages, these markers have thus far proved successful in attributing most samples to a known species. At the level of (infra)populations, genetic diversity of *T. szidati* cercariae from 7 snails collected at 3 localities in Russia was recently shown by use of randomly amplified polymorphic DNA (RAPD) (254). At this time, neither microsatellite markers nor next-generation sequencing protocols have been developed that would allow detection of specific populations or host strains. The development of genome-wide sequencing protocols for population genetic analyses would greatly aid in identifying the epidemiological determinants of cercarial dermatitis outbreaks. Although genetic identifications have provided a framework for circumscribing species and for rapid detection, caution must be exercised, since genetic identification alone is not sufficient in the absence of data on disease dynamics and morphology (255, 256). A species designation should ideally reflect all data available, i.e., host species, location, and morphological characteristics of adult worms and eggs. In addition, comparative molecular analyses must be performed to obtain reliable and convincing results of species identification.

Most importantly, specimens and other data, e.g., genetic data, should be archived in a permanent museum collection or an archivable Web-based database for data resulting from a specimen, such as the sequence archive GenBank. Voucher samples of any life cycle stage of these schistosomes (or any parasite and host) should be preserved and deposited in a permanent museum collection (257–259). This is imperative for several reasons: the most important is for the question that has not yet been asked. In the event that the parasite sample does not match known species or has odd features, further work may be necessary. Access to images and measurements, plus an additional sample(s), will be used in further sequencing. Moreover, documentation of any species of schistosome coming through a clinic is an important record that can contribute to epidemiological studies, especially if it is associated with people affected by dermatitis.

## CLINICAL FEATURES, DIAGNOSIS, TREATMENT, AND PROPHYLAXIS OF HUMAN INFECTIONS

As for the clinical symptoms and signs, penetration of avian schistosome cercariae into mammalian (human) skin may initiate an immediate prickling sensation that persists for approximately 1 h (4, 260). The development and intensity of the subsequent allergic reaction depend on the number and duration of previous cercarial contacts, as well as individual susceptibility (4, 7, 260). The primary contact with cercariae may lead to either an imperceptible (7, 261) or mild skin reaction, with the development of small and transient macules, maculopapules, or inconspicuous papules of about 1 to 2 mm after 0.5 to 2 days p.i. A delayed reaction in the form of small papules can be observed in some persons as late as 8 days p.i. (7, 238).

Repeated infections cause a more pronounced cutaneous reaction followed by diffuse edema and development of erythematous papules or papulovesicles (4, 261). More specifically, the first transitory macules (up to 10 mm) and primary itching can appear as soon as 4 to 20 min after exposure. Thereafter (1 to 15 h p.i.), macules are replaced by papules (about 3 to 8 mm), and an intense itching (secondary itching) is experienced. In addition, erythema and edema may occur in the afflicted area for a few days. Vesicles of about 1 to 8 mm may form on papules at 2 to 3 days p.i. and may rupture as a result of scratching. As a consequence, bacterial superinfection may result in formation of pustules. Papules usually regress and disappear at 4 to 10 days p.i., leaving a pigmented spot (about 1 to 4 mm) on the skin for weeks (7, 225, 238); however, in some cases, the symptoms may persist for about 20 days p.i. (7, 238, 262, 263). Every macula/papula is a reaction against the penetrating cercaria(e) and thus represents the part of the body in

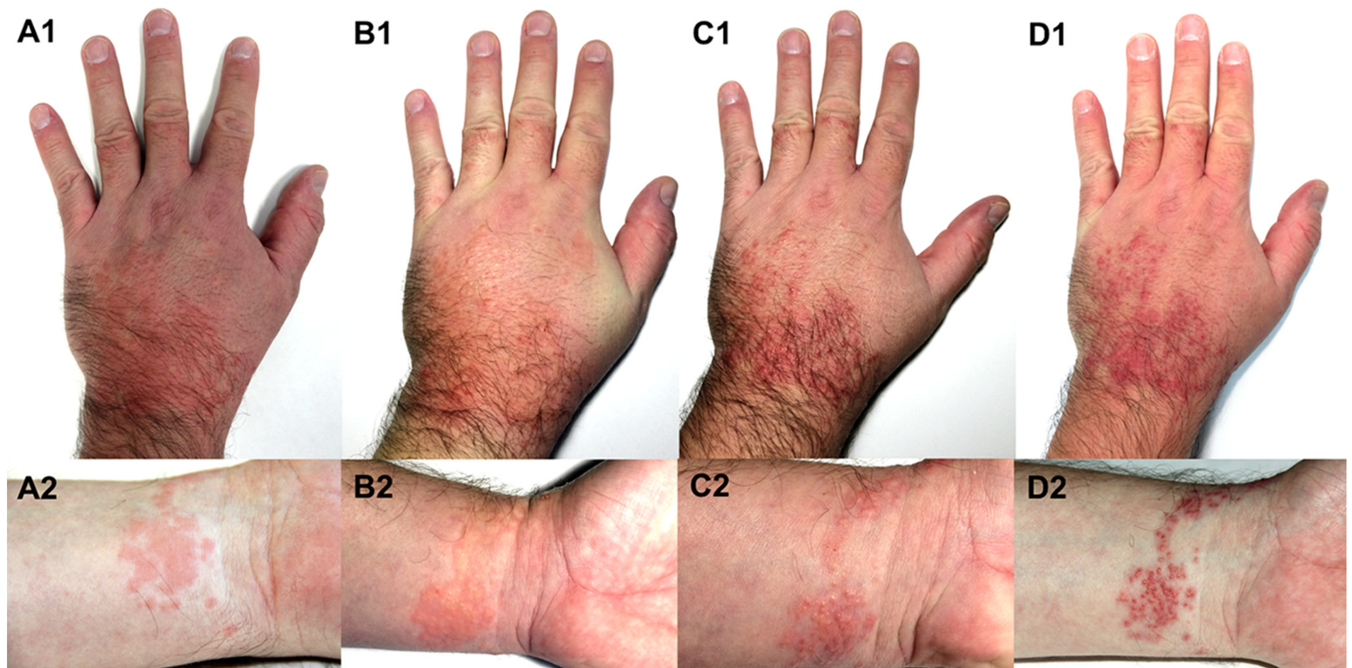


**FIG 7** Development of cercarial dermatitis on the dorsal (1) and ventral (2) parts of the left hand of a sensitized volunteer infected experimentally by *Trichobilharzia szidati* (the whole hand was immersed into a beaker containing water with cercariae). Images were taken at 1 (A), 2 (B), 3 (C), and 4 (D) days postexposure. Formation of macules (A) and papules with vesicles (B to D) can be seen. No penetration/reaction was recorded for the palm. Noticeable swelling of the hand is shown mainly in panel B1. (The figure was created by H. Kulíková and P. Horák.)

direct contact with cercariae (Fig. 7). An attack by many cercariae may be accompanied by generalized reactions, such as limb and lymph node swelling, nausea, diarrhea, and fever (7, 238).

Diagnosis is rather problematic. Skin reactions to cercariae from freshwater or marine environments may resemble insect bites, bacterial dermatitis, contact dermatitis, or skin reactions against nematocysts of larval cnidarians (sea anemones, thimble jellyfish, etc.) (7). Anamnestic data (suggesting recent contact with water reservoirs) and maculopapular skin eruption on the body parts that were in contact with water are important indicators. Direct proof of schistosome infection may be shown by skin biopsy of the papulae no later than 48 h p.i. Individual papulae should be excised/shaved off under local anesthesia, put in Bouin's fixative, cut into 10-μm sections, and stained with hematoxylin-eosin (225, 226). As for basic laboratory tests, increased eosinophil counts and elevated levels of total IgE may indicate an attack by avian schistosomes (7, 230, 238). Specific immunological/serological assays (skin test, "Cercarienhüllenreaktion" [a precipitation reaction surrounding the cercarial body in the presence of a specific antibody], indirect fluorescent-antibody test [IFAT], enzyme-linked immunosorbent assay [ELISA], and complement fixation test) to detect penetration by avian schistosomes are not sufficiently specific or sensitive (7, 238). Exceptionally, some reaction has been obtained with sera of dermatitis patients and a heterologous antigen—*Schistosoma mansoni* cercariae—used in Cercarienhüllenreaktion and IFAT (263, 264). Selection of a reliable (recombinant) antigen or primer for serological or DNA-based tests, respectively, is in progress (our unpublished data).

Therapy for afflicted areas includes only symptomatic (not causal) treatment of the condition in the form of soothing agents. For example, water chestnut planters in India use mustard oil to relieve the itching and rash in mild cases of dermatitis (265). In serious cases, application of systemic antihistamines (tablets or gels, e.g., hydroxyzine) or mild corticosteroids (e.g., 0.1% triamcinolon cream or 1% hydrocortisone ointment) may be considered (7, 226, 262, 266).

There are several recommendations to protect individuals. Wearing rubber waders or gloves or neoprene diving suits is 100% reliable, although not always appropriate or realistic. Upon contact with cercariae in water, the number of penetrating larvae can be reduced if action is taken within seconds to a few minutes (see the part on vertebrate host penetration above). For example, thorough toweling and exposure of skin to the sun are recommended for bathers immediately after leaving water. In the case of an accidental exposure in the laboratory, skin can be washed with 70% ethanol or warm water (as much as one can tolerate) and soap (our personal experience). Several chemicals have been tested as barriers to cercarial penetration. For example, LipoDEET, a long-acting liposome formulation of DEET (*N*,*N*-diethyl-*m*-toluamide), a common, safe, and available insect repellent, has been used successfully to prevent penetration of *S. mansoni* (267). However, a cream formulation of DEET was poorly effective against *T. szidati* penetrating living human skin (181). Two other formulations were effective: (i) SafeSea lotion against jellyfish stings, where the effective compound may be H1-antihistamine diphenhydramine; and (ii) niclosamide in a dosage as low as 0.1% in water-resistant sunscreens. We hypothesize that sunscreens based on plant oils (nonmedicated) will trigger cercarial penetration due to a high content of unsaturated fatty acids. In this regard, a negative effect in terms of human protection was observed for dimethicones (polydimethylsiloxanes and silicone oils), which are common ingredients in many skin care products (181).

## ECOLOGICAL FACTORS INFLUENCING THE OCCURRENCE OF AVIAN SCHISTOSOMES AND CERCARIAL DERMATITIS

Due to climate changes and land alterations, the seasonal window for parasite transmission may become longer, in addition to changes in the behavioral and physiological patterns of parasite hosts. For example, the accelerated growth of both snail and trematode larval populations (71) or changes in phenology of aquatic migratory birds to sedentary (268–270) can increase chances for outbreaks of cercarial dermatitis in both space and time.

### Global Warming and Eutrophication

At least in temperate climates, schistosome cercariae occur seasonally once the ambient temperature is high enough for snail activity and thus cercarial emergence (271). Also, snails infected during late summer can survive through the winter season and serve as a source of cercariae in spring (13, 272, 273). Global warming is predicted to have a positive effect on trematode intramolluscan stages, because their development is strongly temperature dependent, leading to increased cercarial emission rates. For example, a shorter prepatent period in relation to higher temperatures was demonstrated in experimental infections with *T. szidati* (141, 274). A slight rise in temperature will increase developmental rates and transmission success (number of cercariae available to find the definitive host). For example, a 5-fold increase in cercarial emergence with a 10°C increase in temperature was recorded for *Trichobilharzia* sp. from *Radix peregra* (275). In addition, a link has been suggested between the rise of 0.8°C within the last century and the increased frequency of cercarial dermatitis and *Trichobilharzia* prevalence at higher latitudes in Europe (9, 276). What does not change is the life span of cercariae, which is limited to the restricted source of reserve glycogen (cercariae do not take up any food). In this regard, temperature strongly affects cercarial survival. For example, the half-life of *T. szidati* was 16 h at 16°C but only 7.8 h at 25°C (169), and the life span of *Trichobilharzia arcuata* was 72 h at 4°C but only 48 h at 26°C (277). Infected snails change their thermal microhabitat selection, e.g., *Lymnaea stagnalis* infected with *T. szidati* prefers a colder water temperature than that preferred by uninfected snails (19.97°C versus 25°C). This may be an adaptation for either the snail or the schistosome, because at these temperatures larval development is slower and the rate of cercarial emission is lower, leading to less tissue damage in the snail (169, 170).

Eutrophication promotes excessive plant growth and decay, causing alterations in the dynamics of freshwater communities and thus leading to an increased risk of acquiring cercarial dermatitis (11, 278–280). Greater biomass of primary producers is associated with faster development and growth of snails due to the increase in food availability (280–282). Dense snail populations may increase the probability that a miracidium will find a snail and lead to a higher prevalence of infection. In addition, aquatic birds are attracted to such nutrient-rich environments, also contributing to an increase in parasite transmission (283). Using data from a mark-recapture study of *L. stagnalis* in eutrophic fishponds, rapid trematode recruitment into the snail populations was demonstrated (284). Maximum annual rates of colonization/recruitment for *T. szidati* were shown to reach up to 300%, such that the odds of trematode establishment in an individual snail were 3 times per year versus once in 10 years in other, mainly marine trematode-snail systems (284, 285).

Large snail populations with a high prevalence of avian schistosomes have been reported for several localities in Europe (71, 286–288), North America (289, 290), and Australia (291). Usually, natural preserves support diverse and abundant populations of potential hosts of schistosomes, so these areas may serve as hot spots for outbreaks of cercarial dermatitis. Although eutrophication contributes to infection risk, cases of cercarial dermatitis or findings of avian schistosomes have also been reported from oligotrophic or mesotrophic systems (8). In addition, other abiotic and biotic factors and human-induced habitat alterations may influence the occurrence of schistosomes and cercarial dermatitis, such as altered hydrology conditions with water-level fluctuation, ice cover, acidification, or dam constructions (268, 292, 293), anthropogenic pollutants (268, 294–297), biodiversity change in terms of introducing nonindigenous species that may affect endemic parasites (298), host susceptibility or resistance (9), predation upon trematode free-swimming larval stages by fish and other aquatic animals (295, 299, 300), or interspecific competition of parasites within the same snail host (301–303).

### Recreational Activities and Cercarial Dermatitis

The data in the literature suggest that lakes represent high-risk areas for cercarial dermatitis, as they are attractive for a large number of people, usually for recreational purposes (8). Cercarial dermatitis develops as a result of sensitization of the human immune system (232, 260), and repeated exposures to cercariae influence the occurrence and intensity of subsequent infections. For example, longer exposure in water increases cercarial penetration via frequent water visits and more time spent in shallow water (4, 304–306). While gender does not influence the risk of infection (4, 304, 305, 307), some studies found a higher risk among children of less than 15 years of age, since they tend to spend more time in shallow water (280, 308, 309). There are rare cases of nonsensitive individuals that may be due to host desensitization (310) or individual nonsusceptibility/nonattractivity to cercariae (4, 311).

Locally, the highest risk of infection usually occurs in shallow, warm, and vegetation-rich shore areas, where the snails accumulate and release cercariae (308, 309). Cercariae of avian schistosomes are concentrated just beneath the water surface (see the information on host finding), so swimming in deeper water may reduce the infection probability (280). However, snail preferences for habitat differ: *Lymnaea stagnalis* is found mostly in patches of aquatic vegetation, feeding on periphyton; *Radix* spp. accumulate in vegetation-free areas, on stones or muddy sediments; and physid snails prefer detritus as a source of food (312, 313). In addition, cercariae of avian schistosomes can be transported by wind and water currents for several kilometers (2, 304, 305). Therefore, taking the local situation into account is essential for risk assessment, because conditions unsuitable for one snail species may provide an ideal habitat for another.

Season and time of day were shown to have considerable effects. Most cases of cercarial dermatitis correlate with high air and water temperatures during the summer months, when schistosome development in snails is amplified and emission rates of cercariae are stimulated by sunlight (4, 11, 279, 280, 304, 305, 309, 314). Production can reach several thousands of cercariae/snail/day (169, 300, 315), and these emission rates may even counterbalance the loss of cercarial capability to survive and infect hosts at high temperatures (see above). Irrespective of the season, outbreaks of cercarial dermatitis have been reported in geothermally heated lakes and ponds in Iceland (71). Time of day proved to be another risk factor. Production of schistosome cercariae is usually discontinuous—particular species

may differ in circadian rhythms of cercarial release. This is usually attributed to the peak activity of the preferred definitive hosts. Thus, *Trichobilharzia* and some other genera infecting aquatic birds leave the snail host during the light period of the day, mainly in the morning hours (2, 4, 280, 305). Increased illumination, temperature, and snail locomotory activity trigger cercarial emergence (169, 211, 316, 317). Patterns of cercarial emergence can be changed almost immediately by reversing the dark-light regimen, suggesting that light has a decisive influence on the direction of the process (317, 318). The number of cercariae released from snails may vary greatly depending on the parasite/host species and the phase (age) of infection; large species of snails represent a higher risk, as more cercariae are produced per snail and released into the environment (10, 275, 315). This may also explain why some species of avian schistosomes that use smaller species of snails are not often detected during an outbreak.

### Control Measures Related to the Ecology of Avian Schistosomes

In order to reduce the risk of cercarial dermatitis, either water use during peak cercarial emergence should be avoided or the life cycle of avian schistosomes should be interrupted. Interruption of the schistosome life cycle has been tried on a number of occasions. One option is to reduce the prevalence of adult avian schistosomes in birds by eliminating ducks from high-risk areas. However, public acceptance of regular hunting or repelling of birds (ducks) at recreational lakes during summer months is uncertain. Another option is to treat birds with the antihelminthic drug praziquantel. Birds are captured in the field by use of modified dive traps and subsequently treated with praziquantel and then released. Recapture results showed a significant reduction of infections in mallards (319–321). Furthermore, in the subsequent year, there was a marked decline in prevalence of avian schistosomes in snails (320). However, treatment of the entire duck population is time-consuming and labor-intensive.

Reduction or elimination of snail populations is another strategy of cercarial dermatitis control by interrupting the life cycle. In the past, application of commercial molluscicides, such as niclosamide or copper sulfate, was common (319). However, repeated and unlimited treatments had a deleterious effect on animal communities. In addition, after repeated exposures, some snails may become resistant to copper sulfate or avoid the treatment by burrowing into the mud (322, 323). Manual collection of snails (309, 324) or destruction of their habitats by local removal of littoral vegetation (2) represents a less harmful approach to snail control. Large-scale destruction of snail populations by use of heavy machines along the shores of Annecy Lake (France) and Cultus Lake (Canada) led to substantial reductions of cercarial dermatitis (2). As for the biological control of snail populations, use of molluscivorous fish or prawns as natural snail predators is promising for long-term control (325–328), although it represents a risk for native flora and fauna if nonindigenous organisms are introduced.

Free-swimming miracidia and cercariae may represent a promising target for life cycle interruption. Snail finding and recognition by miracidia are based markedly on recognition of host molecules called miraxones (see the information on intramolluscan development), which led to a proposal to use miracidial traps containing miraxones (329). Unfortunately, due to the high level of diversity of avian schistosomes and presumed variability of snail recognition (139), this proposal proved unrealistic. Similarly, cercarial traps based on species-specific host-finding behavior (see the information on vertebrate host finding) might provide local protection (330). For example, fatty acid-stimulated transformation to the schistosomulum stage could be initiated in these traps, leading to the loss of resistance against a hypo-osmotic water environment (178, 331, 332). As far as trophic interactions of organisms are concerned, predation by small aquatic animals may represent a promising means of biological control. Schistosome miracidia and cercariae may serve as prey for larval aquatic insects, crustaceans, oligochaetes, shrimps, and fish (271, 333–338). In particular, the annelid *Chaetogaster limnaei sensu lato*, living commensally or parasitically on the shell surface or in the mantle and pulmonary cavities of freshwater snails, may act as an efficient predator and prevent penetration of miracidia (339, 340) or feed on cercariae as they are released from the snail (341, 342).

Finally, competitive interactions among trematode larval communities within an individual snail can substantially influence establishment and survival of schistosome intramolluscan stages. In terms of interspecific interactions, particular species of trematodes can be dominant, usually by producing competitively aggressive rediae, or subordinate because they have less aggressive sporocysts and no redial stage. Schistosomes belong to the latter group, and their sporocysts are eliminated via the predatory interactions of redia-producing trematodes, e.g., echinostomes (301, 343). Yet schistosomes may exert a dominant effect in some cases. The avian schistosome *Trichobilharzia brevis* persists in coinfections with two dominant echinostomes, *Echinostoma audyi* and *Hypoderaeum dingeri* (343), and can cause developmental suppression of the latter species. The nonpredatory exclusion of *H. dingeri* by *T. brevis* has also been shown experimentally (344). Intertrematode competition was recently identified as an important determinant in transmission of avian schistosomes. For example, competitive exclusion was estimated to result in an 18.0% reduction of *Trichobilharzia szidati* in *Lymnaea stagnalis* (345). Interestingly, *T. szidati* may represent a subordinate species, yet it frequently cooccurs in snails with other trematodes. Therefore, *T. szidati* may be either an obligate secondary invader of snails with a compromised immune system or a schistosome that can coexist in double infections, as was demonstrated for other species of *Trichobilharzia* and *Austrobilharzia* (302, 343, 346).

## PERSPECTIVES

The more we know about avian schistosome diversity and distribution, the better we are positioned to understand their evolutionary history and potential for the future dynamics of dermatitis outbreaks. These days, we have practical means for their identification and can better gauge the likelihood that some of these species may emerge in new contexts to cause unexpected problems. An effort to describe the avian schistosome species diversity in their hosts in the circumpolar regions, where most migratory birds spend the summer (and young birds become infected), seems to be a critical component to understanding the epidemiology of cercarial dermatitis. In addition, the condition itself and the host-parasite interaction require further characterization at the molecular level. At least three examples of future applications can be mentioned. (i) A more detailed knowledge of cercarial penetration mechanisms may help in the prevention of dermatitis by introducing new formulations containing inhibitory molecules. (ii) Characterization of biologically active secretions produced by avian schistosomes may help us to understand the molecular basis of tissue pathology in avian and mammalian hosts.

(iii) Newly designed primers or carefully selected antigens may be developed for reliable antibody- or DNA-based diagnostic tools. Such tools may be used to screen people or animals with health problems following recent water contact to assess the risk associated with exposure to cercariae of avian schistosomes.

## ACKNOWLEDGMENTS

The University of New Mexico partially supported this study through a National Institutes of Health grant to Eric S. Loker (R01 AI101438) and a National Science Foundation grant to S.V.B. (DEB 1021427). M.S. is grateful for financial support from the Institute of Parasitology (RVO; grant 60077344), Biology Centre of the Academy of Sciences of the Czech Republic. The scientific activities of P.H., L.M., L.L., and V.S. were financially supported by Charles University in Prague (research program P41/PrFUK, UNCE grant 204017, GAUK grant 435911, and grant SVV 260074/2014) and the Czech Science Foundation (grant 13-29577S).

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**Petr Horák,** M.Sc., Ph.D., is a Professor of Parasitology and Head of the Department of Parasitology at the Faculty of Science, Charles University in Prague, Czech Republic. He obtained his parasitology/biology degree from the Charles University in Prague. The President of the Czech Republic appointed Petr Horák as a Professor of Parasitology in 2005. Professor Horák has presented and published on many topics, dealing with parasitic helminths in general and cercarial dermatitis/avian schistosomes in particular. His teaching activities lie in general parasitology and the biology of helminths. His research is focused on functional morphology of helminths, trematodes, and avian schistosomes (systematics, life cycles, and host-parasite interactions). Details of his personal data can be found at http://www.petrhorak.eu.

**Libor Mikeš,** M.Sc., Ph.D., is an Assistant Professor of Parasitology at the Department of Parasitology, Faculty of Science, Charles University in Prague, from which he received his master's and doctoral degrees. His international experience includes, e.g., stays at the Keele University, United Kingdom, and the Institute for the Biotechnology of Infectious Diseases, UTS, Sydney, Australia. Dr. Mikeš is currently the President of the Czech Society for Parasitology. His basic academic interests lie in the general biology of helminths (with a special focus on the trematode superfamilies Schistosomatoidea and Diplostomoidea). He is also involved in teaching molecular and cellular interactions between parasites and hosts and biochemical parasitology, and he leads several field courses in parasitology. His research and publication activities are focused mainly on the biochemistry of helminths (proteomics, peptidases of trematodes and monogeneans, and glycobiology of larval trematodes) and immune reactions of hosts against avian schistosomes.

**Lucie Lichtenbergová,** M.Sc., Ph.D., is a Postdoctoral Fellow at the Department of Parasitology, Faculty of Science, Charles University in Prague. She finished her master's study at the Masaryk University in Brno, Czech Republic, and obtained her Ph.D. from the Charles University in Prague, Czech Republic. Since her doctoral study, she has focused on the research of interactions of avian schistosomes and their natural avian and accidental mammalian hosts. Immunology and histopathology are her centers of interest.

**Vladimír Skála,** M.Sc., is a Ph.D. student at the Department of Parasitology, Faculty of Science, Charles University in Prague, Czech Republic. His master's degree was obtained at the same institution, and his master's thesis focused on the morphology and development of the monostome fluke *Notocotylus attenuatus* (Digenea: Notocotylidae). At present, he is investigating the defensive activity of hemocytes of two lymnaeid snails transmitting avian schistosomes and the immunomodulation caused by trematodes in their snail hosts. His research is carried out in collaboration with Anthony J. Walker from Kingston University, London, United Kingdom. Vladimír has presented the results of his research at several national and international scientific meetings.

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**Miroslava Soldánová,** M.Sc., Ph.D., received a Ph.D. in parasitology at the Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic, in 2011. Simultaneously, she started as a Research Assistant at the Institute of Parasitology, Biology Centre, Academy of Sciences of the Czech Republic, where she currently continues as a Postdoctoral Fellow in the research team focused on trematode ecology and systematics. Her 1-year postdoctoral fellowship (2012–2013) at the University of Duisburg-Essen (Faculty of Biology, Aquatic Ecology, Germany) focused on the impact of eutrophication on the composition of trematode communities in large lake ecosystems in the Ruhr River, screening of freshwater snails in relation to trematode infection with avian schistosomes, and assessment of potential risks of cercarial dermatitis for humans. Her fields of interest comprise parasite ecology (trematode community structure and organization in freshwater molluscs), environmental parasitology (use of parasite communities in molluscs as bioindicators), and trematode taxonomy.

**Sara Vanessa Brant,** Ph.D., is an Assistant Research Professor and Senior Collections Manager of the Museum of Southwestern Biology, Division of Parasites, at the University of New Mexico. She obtained her parasitology/biology M.S. and Ph.D. from the University of Nebraska-Lincoln. Her main research focus is the evolutionary and ecological diversity of trematodes, in particular those in the blood fluke families (Schistosomatidae, Spirorchiidae, Sanguinicolidae). Her recent efforts have been funded to address global avian schistosome diversity and their host interactions. She is also involved in efforts to motivate and promote the importance of placing both parasite and host vouchers in permanent museum collections for future studies.

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# Influence of *Trichobilharzia regenti* (Digenea: Schistosomatidae) on the Defence Activity of *Radix lagotis* (Lymnaeidae) Haemocytes

**Vladimír Skála[1]*, Alena Černíková[2], Zuzana Jindrová[1,3], Martin Kašný[1,4], Martin Vostrý[1,5], Anthony J. Walker[6], Petr Horák[1]**

**1** Charles University in Prague, Faculty of Science, Department of Parasitology, Prague, Czech Republic, **2** Charles University in Prague, Faculty of Science, Institute of Applied Mathematics and Information Technologies, Prague, Czech Republic, **3** Charles University in Prague, 1st Faculty of Medicine, Institute of Immunology and Microbiology, Prague, Czech Republic, **4** Masaryk University, Faculty of Science, Department of Botany and Zoology, Brno, Czech Republic, **5** Institute of Haematology and Blood Transfusion, Prague, Czech Republic, **6** Molecular Parasitology Laboratory, School of Life Sciences, Kingston University, Kingston upon Thames, Surrey, United Kingdom

## Abstract

*Radix lagotis* is an intermediate snail host of the nasal bird schistosome *Trichobilharzia regenti*. Changes in defence responses in infected snails that might be related to host-parasite compatibility are not known. This study therefore aimed to characterize *R. lagotis* haemocyte defence mechanisms and determine the extent to which they are modulated by *T. regenti*. Histological observations of *R. lagotis* infected with *T. regenti* revealed that early phases of infection were accompanied by haemocyte accumulation around the developing larvae 2–36 h post exposure (p.e.) to the parasite. At later time points, 44–92 h p.e., no haemocytes were observed around *T. regenti*. Additionally, microtubular aggregates likely corresponding to phagocytosed ciliary plates of *T. regenti* miracidia were observed within haemocytes by use of transmission electron microscopy. When the infection was in the patent phase, haemocyte phagocytic activity and hydrogen peroxide production were significantly reduced in infected *R. lagotis* when compared to uninfected counterparts, whereas haemocyte abundance increased in infected snails. At a molecular level, protein kinase C (PKC) and extracellular-signal regulated kinase (ERK) were found to play an important role in regulating these defence reactions in *R. lagotis*. Moreover, haemocytes from snails with patent infection displayed lower PKC and ERK activity in cell adhesion assays when compared to those from uninfected snails, which may therefore be related to the reduced defence activities of these cells. These data provide the first integrated insight into the immunobiology of *R. lagotis* and demonstrate modulation of haemocyte-mediated responses in patent *T. regenti* infected snails. Given that immunomodulation occurs during patency, interference of snail-host defence by *T. regenti* might be important for the sustained production and/or release of infective cercariae.

**Citation:** Skála V, Černíková A, Jindrová Z, Kašný M, Vostrý M, et al. (2014) Influence of *Trichobilharzia regenti* (Digenea: Schistosomatidae) on the Defence Activity of *Radix lagotis* (Lymnaeidae) Haemocytes. PLoS ONE 9(11): e111696. doi:10.1371/journal.pone.0111696

**Editor:** Daniel Doucet, Natural Resources Canada, Canada

**Received** July 4, 2014; **Accepted** October 7, 2014; **Published** November 5, 2014

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**Data Availability:** The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

**Funding:** The study was financially supported by the Charles University in Prague (Grant GAUK no. 435911, research programmes PRVOUK - P41/PrF, UNCE - 204017 and SVV 260074/2014). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* Email: mich007@email.cz

## Introduction

Aquatic snails serve as intermediate hosts of many trematodes, including those important in veterinary and human medicine. Compatibility between such parasites and the host snail is partially governed by innate immunological processes that comprise cellular and humoral components. Mobile phagocytic cells called haemocytes play the major role in mediating the cellular defence response whereas lectins are considered as the most essential recognition molecules of humoral response [1], [2]. Haemocyte-mediated defence responses that are important for eliminating foreign invaders such as parasites include phagocytosis, encapsulation, and production of reactive oxygen species (ROS) [1], [3], [4].

Phagocytosis is used to eliminate small non-self particles, primarily bacteria; however, pieces of trematode tegument are also known to be actively engulfed by haemocytes after encapsulation [3]. The phagocytic response also triggers generation of ROS [5], [6]. Among the ROS, hydrogen peroxide ($H_2O_2$) is an important metabolite known for killing sporocysts of the human parasite *Schistosoma mansoni* [4]. At the molecular level, snail haemocyte defence responses are regulated by complex networks of intracellular signalling pathways, including the evolutionarily conserved protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) pathways [7–10]. Activation of PKC, p38 MAPK and/or extracellular signal-regulated kinase (ERK) is required for efficient phagocytosis and $H_2O_2$ production by snail haemocytes; other kinases such as phosphatidylinositol 3-kinase also play a crucial role in these processes [7], [9–12].

During infection, compatible trematodes alter snail host defence responses presumably to help ensure survival and replication of the parasite. Phagocytic activity of haemocytes is decreased e.g. in the gastropods *Biomphalaria glabrata* and *Lymnaea stagnalis* infected with *Echinostoma paraensei* [13] and *Trichobilharzia szidati* [14], respectively. In the prosobranch snail, *Littorina littorea*, infection with *Himasthla elongata* reduces haemocyte ROS production, which correlates with increased haemocyte number in the snail circulation [15]. Such alterations of host defence mechanisms might be caused by trematode-derived components interfering with signalling pathways of snail haemocytes [16]. This hypothesis is supported by results showing that *S. mansoni* excretory-secretory products (ESPs) generated during development of miracidia to mother sporocysts impair $H_2O_2$ production in *B. glabrata* haemocytes [10] and disrupt ERK signalling in these cells [17].

*Radix lagotis* is an important intermediate host of the nasal bird schistosome *Trichobilharzia regenti* [18], [19], a causative agent of cercarial dermatitis in humans [20]. Following penetration into the snail, *T. regenti* miracidia develop to mother sporocysts, which in turn produce daughter sporocysts [21]. This latter stage gives rise to cercariae that are released into the water during the patent phase of infection. As far as immunological aspects of infection are concerned, snail defence responses related to the initiation of *T. regenti* infection, and changes in *R. lagotis* haemocyte activities in the patent phase of infection are unknown.

The present paper combines histological observations of juvenile *R. lagotis* snails infected with *T. regenti* miracidia, with comparisons of haemocyte abundance and haemocyte phagocytic activity and $H_2O_2$ production between uninfected and infected snails in the patent phase of *T. regenti* infection. At the molecular level, basal PKC and ERK phosphorylation in haemocytes from both snail groups was compared and their possible roles in regulation of haemocyte phagocytic activity and $H_2O_2$ production explored. Such complementary approaches provide the first and integrated insight into the immunobiology of *R. lagotis* snails demonstrating modulation of defence responses during infection of snails with the compatible trematode parasite.

## Methods

### Uninfected and *T. regenti*-infected *R. lagotis*

Uninfected *R. lagotis* were maintained in the laboratory at ambient room temperature (19–22°C; RT) in aquaria filled with aerated tap water and were fed fresh lettuce *ad libitum*. Juvenile and adult snails (together with the eggs laid) were reared together.

Juvenile snails with shell heights 5–8 mm were infected with *T. regenti* miracidia obtained as described by Horák *et al*. (1998) [18]. The snails were placed individually into wells of a 24-well culture plate (Nunc) containing tap water and each exposed to 3–8 miracidia for 5 h, with 15 miracidia used to infect each snail for histological analysis. After exposure, the snails were placed in a separate aquarium for 5 weeks, and they were then checked under a direct light source for shedding of *T. regenti* cercariae. Snails releasing cercariae (infected snails) were then maintained in a further separate aquarium.

### Light microscopy

Two juvenile *R. lagotis* were dissected for each infection time point studied, 1, 2, 3, 5, 12, 16, 20, 36, 44, 60 and 92 h post–exposure (p.e.) of snails to *T. regenti* miracidia. The soft body of each snail was carefully removed from its shell and fixed in Bouin-Hollande fixative at RT for 24 h. The specimens were then embedded in JB-4 resin (Polysciences), sections cut to 2 µm with a microtome (Finesse ME, Shandon Scientific) and stained with Wright-Giemsa (Polysciences). Finally, sections were individually embedded in DPX medium (Sigma), examined under an Olympus BX 51 light microscope and digital images captured using a DP70 digital camera system.

### Transmission electron microscopy

For transmission electron microscopy (TEM), juvenile *R. lagotis* were dissected 5 and 15 h p.e. and fixed in 2.5% glutaraldehyde (Sigma) in complete sterile snail saline (SSS+: 3 mM Hepes, 3.7 mM NaOH, 36 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, pH 7.8, 100 mOsm; [5]) at 4°C for 24 h. The specimens were then post–fixed in 1% $OsO_4$ (Polysciences) in SSS+ for 2 h, washed three times in SSS+, dehydrated in ethanol (50%, 80%, 96%, twice each for 15 min, and 100% three times each for 5 min) and acetone (100%, three times each for 5 min). Subsequently, the tissue was incubated in 100% acetone:Spurr mixture at increasing Spurr concentrations: 2:1 for 2 h, 1:1 for 5 h, 1:2 for 12 h, followed by pure Spurr resin three times each for 12 h. Then, the material in fresh Spurr resin was transferred to plastic capsules and incubated at 60°C for 48 h. The embedded samples were first sectioned at 2 µm thick sections with a Finesse ME microtome, stained with 1% toluidine blue (Polysciences) and observed under a light microscope (Olympus BX 51). When larvae of *T. regenti* were detected, 60–70 nm thick sections were prepared using ultramicrotome Ultracut E (Reichert-Jung). These sections were stained with uranyl acetate and lead citrate [22] and evaluated under TEM JEOL 1011 microscope. Digital images were captured using associated software.

### Haemolymph extraction and enumeration of haemocytes in uninfected and infected *R. lagotis*

Uninfected and infected *R. lagotis* with shell heights 1.0–1.6 cm were selected for haemolymph extraction with infected snails extracted no later than 2 months post-patency. The snails were washed with distilled water, dried, and haemolymph was extracted by head-foot retraction [23].

Haemocyte numbers were quantified for individual uninfected and infected snails. Haemolymph from each snail was pooled on parafilm (Sigma) and diluted 1:1, 2:1, or 3:1 (one part = 10 µl) in incomplete sterile snail saline (SSS-) where 2 mM $MgCl_2$ and 4 mM $CaCl_2$ were omitted, and 2% ethylenediaminetetra-acetic acid (EDTA; Sigma) added (SSS-/EDTA) to reduce haemocyte aggregation/adhesion; SSS-/EDTA buffer was exclusively used for counting haemocytes. Enumeration was carried out with Bürker haemocytometers and haemocyte numbers were expressed as haemocytes/ml of haemolymph. The data were analysed for normality (Shapiro-Wilk normality test) using R 2.13.0 statistical software (www.r-project.org). Spearman's correlation test was used to assess the relationship between shell heights and haemocyte numbers of individual snails. Haemocyte numbers between the snail groups were compared using Wilcoxon signed-rank test (non-parametric two-sample test; Wilcoxon test).

### Preparation of haemocyte monolayers

Haemolymph from uninfected and infected snails (shell heights 1.3–1.6 cm) was extracted in alternating order to ensure similar conditions for both haemolymph types while the monolayers were prepared. Aliquots of haemolymph drawn from the snails were pipetted directly into the wells of a 96-well culture plate (Nunc) containing 50 µl SSS+ to achieve a final volume of 250 µl/well (final ratio: 4 parts haemolymph: 1 part SSS+). Ten to forty snails were required to obtain sufficient haemolymph for each mono-

layer. Haemocytes were left to settle and adhere to the bottom of the wells for 30 min at RT. Monolayers were then washed with SSS+ (see below) and their quality checked under a microscope (Olympus IX 71). Any wells containing haemocyte clumps or discontinuous monolayers were not used. When haemocyte numbers per well were enumerated, aliquots of haemolymph were also collected on parafilm and diluted with equal amount of SSS-/EDTA; haemocytes were then enumerated as described above.

## Phagocytosis assays

Haemocyte monolayers were washed three times with 250 µl SSS+ and equilibrated in 190 µl SSS+ for 30 min at RT. 10 µl of *Escherichia coli* bioparticles (pHrodo red; Molecular Probes) prepared following manufacturer's instructions were then added to each well and plates incubated at RT in the dark for 2 h. These bioparticles are non-fluorescent outside cells, but become fluorescent in phagosomes. Therefore, no washing was necessary after incubation and intracellular fluorescence was immediately quantified using Tecan Infinite M200 microplate reader at 545 nm excitation and 600 nm emission. The signal of *E. coli* bioparticles alone in wells was also measured in each assay and the value subtracted from all values obtained from wells containing haemocytes and *E. coli* bioparticles.

Phagocytic activity of haemocytes from uninfected and infected snails was then expressed per volume of haemolymph (200 µl) and per 50,000 haemocytes, in case infection altered haemocyte number. Uninfected snails were also used to study the effects of inhibition of PKC and ERK signalling on phagocytic activity. Haemocyte monolayers were pre-incubated for 30 min at RT with 1 µM or 10 µM inhibitor of PKC (GF109203X; Sigma), MEK (U0126; Cell Signalling Technology - CST), which is the immediate upstream activator of ERK, or in DMSO vehicle alone (0.05%; Sigma) prior to adding bioparticles. Effects of inhibition assays were evaluated in terms of haemolymph volume (200 µl).

Using R 2.13.0 statistical software, raw fluorescence intensity data for each measurement were analysed for normality (Shapiro-Wilk normality test). Wilcoxon test was then used to compare the phagocytic activity between uninfected and infected snails, whereas paired t-test was applied to data when assessing the effect of GF109203X and U0126 on phagocytosis by *R. lagotis* haemocytes. For graphic representation, the data for uninfected snails were assigned a value of 100%.

## Hydrogen peroxide assays

Haemocyte monolayers were prepared and haemocyte numbers/well enumerated as described above except that 50 µl haemolymph and 12.5 µl SSS+ were used per well. After washing monolayers twice with 250 µl SSS+, haemocytes were left to equilibrate for 30 min at RT in 100 µl SSS+. $H_2O_2$ output by haemocytes was monitored using the Amplex red hydrogen peroxide/peroxidase assay kit (Molecular Probes) in which Amplex red reacts with $H_2O_2$ to produce the red-fluorescent product, resorufin. Working solutions of the assay mixture that were prepared in SSS+ contained: 0.1 U $ml^{-1}$ horseradish peroxidase (HRP), 50 µM Amplex red reagent, and either 0.1% DMSO or 10 µM PMA (phorbol 12-myristate 13-acetate; Sigma) in DMSO. PMA was used because in other molluscs this phorbol ester increases ROS production by haemocytes [10], [24], [25]. 100 µl of the respective working solution was added to each individual haemocyte monolayer and the plate was incubated in the dark for 30 min at RT. For inhibition assays using uninfected snails, haemocytes were exposed to 5 µM GF109203X, U0126 or DMSO (vehicle) alone (0.025%) for 30 min at RT prior to adding the working solution containing PMA. The final concentration of DMSO after adding the working solutions was 0.1% in all cases.

Fluorescence was monitored at 520 nm and 615 nm excitation and emission, respectively, in a microplate reader (Tecan Infinite M200) for 60 min. $H_2O_2$ output by uninfected and infected snail haemocytes was evaluated per volume of haemolymph (50 µl) and haemocyte number with adjustment to 50,000 cells. Inhibition assays were evaluated per volume of haemolymph (200 µl).

The data sets were tested for normality (Shapiro-Wilk normality test) and for equality of variances (Two-variances F-test). Two-sample t-test or Wilcoxon test was used to compare basal and PMA-modulated $H_2O_2$ production between uninfected and infected snails. Experiments investigating the effects of PKC and ERK inhibition on $H_2O_2$ production were analysed using either parametric or nonparametric paired tests. Since the tests at different time points are dependent, a Fisher's combination test using inverse normal method [26] was used for further processing of p-values. The resulting test statistic was compared to Pocock's critical value 2.49. If the test statistic was higher than this critical value, a significant difference between data sets was confirmed.

## SDS-PAGE and western blot analysis

Haemocyte monolayers prepared as detailed above were washed three times with 250 µl SSS+, and left to equilibrate in 250 µl SSS+ at RT for 30 min. The SSS+ was then removed and haemocytes lysed by adding 25 µl of hot (95°C) SDS-PAGE sample buffer. Proteins were separated by gel electrophoresis (10% Mini-Protean TGX precast gel; Bio-Rad) and transffered to Immun-Blot PVDF membrane (Bio-Rad) using Trans-Blot turbo blotting system (Bio-Rad). Membranes were blocked with 5% non-fat dried milk (Bio-Rad) in 0.1% Tween/Tris-buffered saline (TTBS) at RT for 45 min, and incubated overnight at 4°C in either anti-phospho-PKC (pan) (βII Ser660) rabbit polyclonal antibodies or anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2) rabbit monoclonal antibodies (CST) (1:1000 in TTBS). These antibodies were previously validated for detection of exclusively phosphorylated (activated) forms of PKC and ERK in *L. stagnalis* haemocytes [7], [27], and were also used in other studies of molluscs [17]. Following further incubation at RT for 2 h, membranes were washed 3×5 min in TTBS and incubated for 2 h at RT in anti-rabbit IgG HRP-conjugated secondary antibodies (1:4000 in TTBS) (CST). Immunoreactive bands were then visualised using SuperSignal West Dura extended duration substrate (Thermo Scientific) and a LAS 4000 Luminescent image analyser. Blots were stripped in Restore Western blot stripping buffer (Thermo Scientific) for 2 h at RT, and re-probed overnight in p44/p42 MAPK (Erk1/2) antibody (CST) (1:1000 in TTBS), which recognizes ERK regardless of its phosphorylation state. Finally, the blots were stripped and re-probed with anti-actin antibodies (Sigma) (1:4000 in TTBS) for 1 h at RT to confirm equal loading of proteins between lanes.

The intensities of immunoreactive bands were analysed using Multi Gauge 3.2. software. The values for PKC and ERK phosphorylation and for total ERK in haemocytes of uninfected snails were standardised as 100% and differences in PKC and ERK phosphorylation and in total ERK from infected snails calculated. The data were evaluated for normality (Shapiro-Wilk normality test) and for equality of variances (Two-variances F-test). Two-sample t-test was then applied using R 2.13.0 statistical software.

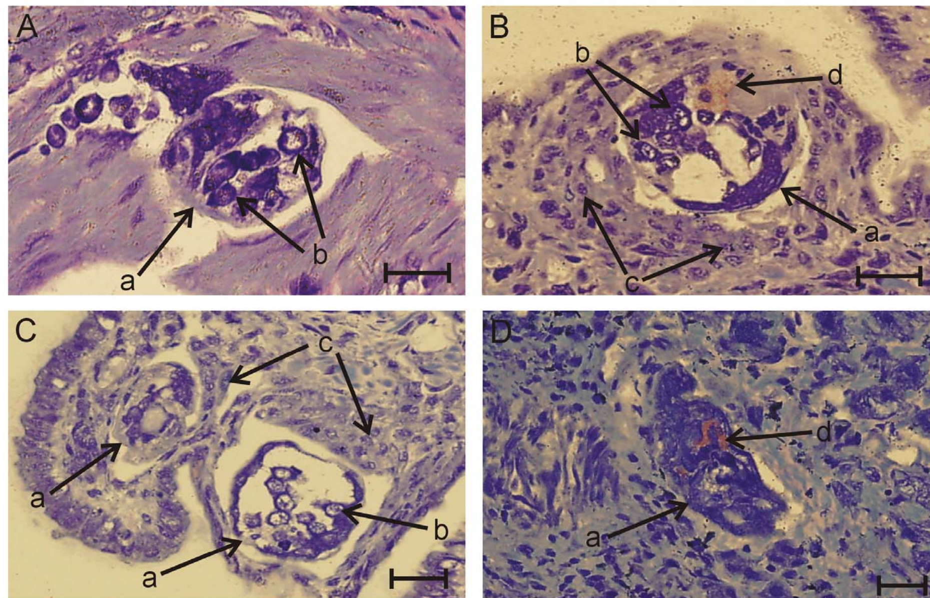


**Figure 1. *Trichobilharzia regenti* larvae within the tissue of *Radix lagotis* revealed by light microscopy between 1–92 h p.e.; Wright-Giemsa stained sections.** (A) Miracidium of *T. regenti* (a) containing germ cells (b) occurs within the snail tissue without haemocyte infiltration 1 h p.e. (B) and (C) Haemocytes (c) are present in the vicinity of developing *T. regenti* mother sporocyst (a) 2 and 16 h p.e., respectively; germ cells (b) and gland structure (d) of the parasite are visible. (D) The area around *T. regenti* mother sporocyst (a) contains no haemocytes 92 h p.e. Gland structure (d) is located in the body of the parasite. Scale bar = 20 μm. The images shown are representative of the situation seen in all sections observed during these experiments.
doi:10.1371/journal.pone.0111696.g001

## Results

### Histological observations of *R. lagotis* experimentally infected with *T. regenti*

Histological observations of *R. lagotis* experimentally infected with *T. regenti* provided insights into the encapsulation responses within the snail tissue between 1 and 92 h p.e. Haemocytes were not evident in close proximity to the parasite at 1 h p.e. (Figure 1A). However, considerable accumulation of haemocytes was observed close to the developing *T. regenti* between 2 and 16 h p.e. (Figure 1B–C). Haemocytes appeared to surround the developing mother sporocysts irregularly in several layers; however, it was not clear whether the cells were directly attached to the parasite surface. Thereafter, at 20 and 36 h p.e. the haemocytic response against the parasite appeared to decline (data not shown) and while the haemocytes occurred individually in the vicinity of mother sporocysts, they did not accumulate in layers. At the latter time points, 44, 60 and 92 h p.e. no haemocytes were observed close to *T. regenti* (Figure 1D).

Transmission electron microscopy of *T. regenti* mother sporocysts within the snail tissue at 5 and 15 h p.e. (Figure 2; 15 h p.e. shown) showed that the larvae remained apparently undamaged despite numerous haemocytes being adjacent to the parasite (Figure 2A). Furthermore, some haemocytes were in a tight contact with sporocyst surface microvilli, and microtubular aggregates were observed within their phagosomes (Figure 2A–B).

### Haemocyte number in uninfected and *T. regenti*-infected *R. lagotis*

Evaluation of haemocyte number/ml haemolymph in 23 individuals of uninfected and infected *R. lagotis* demonstrated that the concentration of circulating haemocytes did not correlate with the shell height of the snails (Figure 3). Considerable variation in haemocyte number was observed within the extracted haemolymph for snails of similar size. In uninfected snails, the lowest haemocyte concentration was $4.2 \times 10^4$ cells/ml (shell height 1.40 cm) whereas the highest was $74.9 \times 10^4$ cells/ml (shell height 1.57 cm) (Figure 3). In infected snails, the lowest haemocyte concentration was $4.7 \times 10^4$ cells/ml (shell height 1.04 cm) whereas the highest was $180.4 \times 10^4$ cells/ml (shell height 1.26 cm) (Figure 3). Statistical analysis revealed that mean haemocyte number/ml haemolymph of infected snails was 79% greater than that of uninfected snails ($45.9 \times 10^4$ cells/ml vs. $25.6 \times 10^4$ cells/ml; $p < 0.05$).

### Defence responses of haemocytes from uninfected and *T. regenti*-infected *R. lagotis*

To explore the effects of *T. regenti* infection on haemocyte defence, we measured phagocytic activity and $H_2O_2$ production by haemocytes derived from uninfected and *T. regenti*-infected *R. lagotis*. Haemocyte phagocytic activity was determined by the ability of these cells to internalise *E. coli* bioparticles (Figure 4A). Comparisons made in a physiological context, which consider activity per volume of haemolymph (200 μl), revealed that phagocytosis by haemocytes from infected snails was not significantly different from that of uninfected snails (Figure 4B). However, when the phagocytic activity was compared taking into account the different numbers of haemocytes in the extracted haemolymph, with more haemocytes present as a result of parasite infection, phagocytosis by infected snail haemocytes was reduced significantly to approximately 50% of that of uninfected snails ($p < 0.05$; Figure 4B).

For $H_2O_2$ production we studied basal and PMA-stimulated output by haemocytes from uninfected and infected snails (Figures 5–6). Evaluation per volume of haemolymph (50 μl) revealed that the basal output of $H_2O_2$ by haemocytes from infected snails was similar to that of uninfected snails, despite the infected snails possessing greater numbers of haemocytes/ml

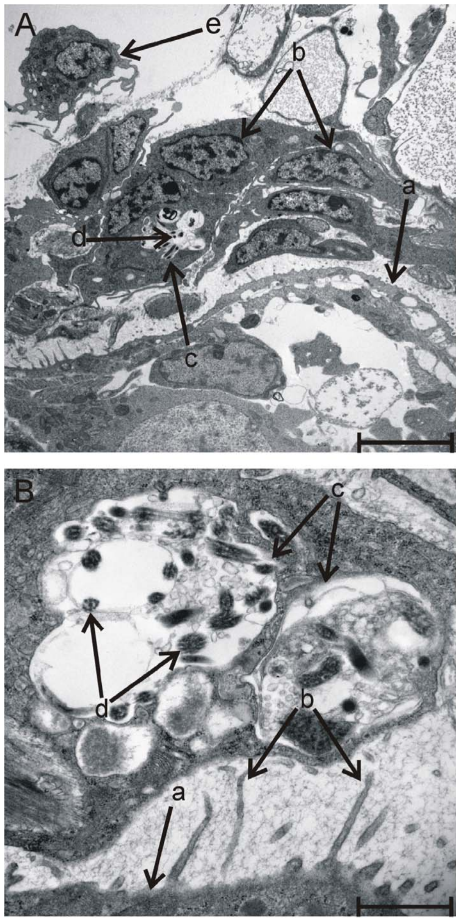


**Figure 2. *Trichobilharzia regenti* mother sporocysts within the tissue of *Radix lagotis* 15 h p.e.; TEM images.** (A) Mother sporocyst of *T. regenti* (a) is surrounded by haemocytes with remarkable nuclei (b). Phagosome (c) of one haemocyte with internalised microtubular aggregates (d) is visible (B in detail). Another haemocyte (e) is located near the parasite. Scale bar = 5 μm. (B) Microvilli (b) are present on the surface of *T. regenti* mother sporocyst (a). Haemocyte adjacent to the sporocyst contains phagosomes (c) with microtubular aggregates (d). Scale bar = 1 μm.
doi:10.1371/journal.pone.0111696.g002

(Figure 5). In contrast, when the data were adjusted for haemocyte number (50,000), the cells from uninfected snails produced significantly more $H_2O_2$ than those from infected snails at each time point after 20 min ($p<0.05$; Figure 5).

In the presence of 5 μM PMA (an activator of PKC) haemocyte $H_2O_2$ production increased 270% and 240% when considering haemolymph volume (50 μl) in uninfected and infected snails after 60 min, respectively (Figure 6); the difference between snail groups was not statistically significant. In contrast, when considering haemocyte number (50,000) $H_2O_2$ production by haemocytes from uninfected snails in the presence of PMA was approximately 2-fold that of haemocytes from infected snails at all time points studied after 20 min ($p<0.01$; Figure 6).

## PKC and ERK activation in haemocytes from uninfected and *T. regenti*-infected *R. lagotis*

Because signalling pathways are known to regulate haemocyte defence responses such as phagocytosis and $H_2O_2$ output [7], [9–12], and because these defence responses were supressed in *R. lagotis* haemocytes as a result of *T. regenti* infection, we aimed to determine PKC and ERK activation in haemocyte monolayers derived from uninfected and infected *R. lagotis*. Western blotting of haemocyte proteins with anti-phosphospecific PKC and ERK antibodies, which detect only the active forms of these kinases in snails [7], [8], [27], followed by densitometric analysis of immunoreactive bands from several independent blots revealed that PKC and ERK phosphorylation were reduced by 57% and 55%, respectively, in haemocytes from infected snails when compared to those from uninfected snails ($p<0.01$; Figure 7A–B). We reasoned, therefore that ERK expression might also be suppressed. However, western blots performed to determine the quantity of ERK in haemocytes using antibodies that detect ERK irrespective of its phosphorylation state (Figure 7C) demonstrated that mean levels of ERK were 24% higher in infected snails when compared to uninfected ones, although this difference was not statistically significant. Unfortunately, lack of a suitable anti-PKC antibody for snails prevented evaluation of total PKC protein levels.

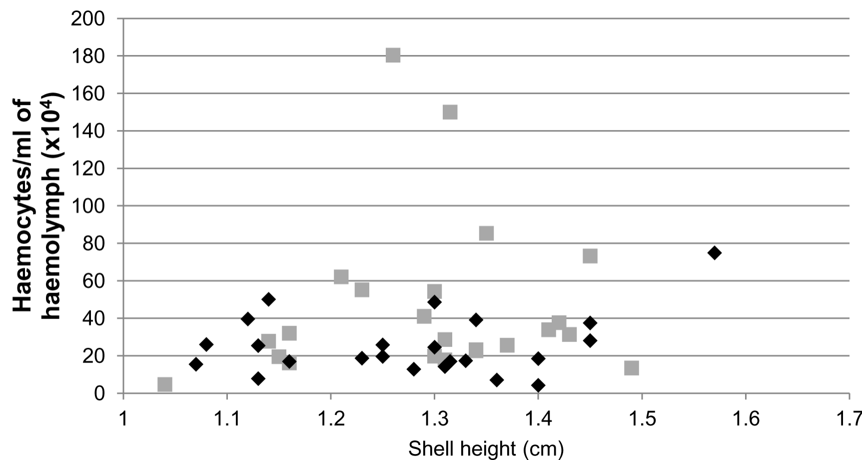


**Figure 3. Number of haemocytes/ml of haemolymph of individual uninfected (black diamond) and *Trichobilharzia regenti* infected (grey box) *Radix lagotis*.** The numbers of haemocytes/ml from individual snails with different shell heights were enumerated using a Bürker haemocytometer.
doi:10.1371/journal.pone.0111696.g003

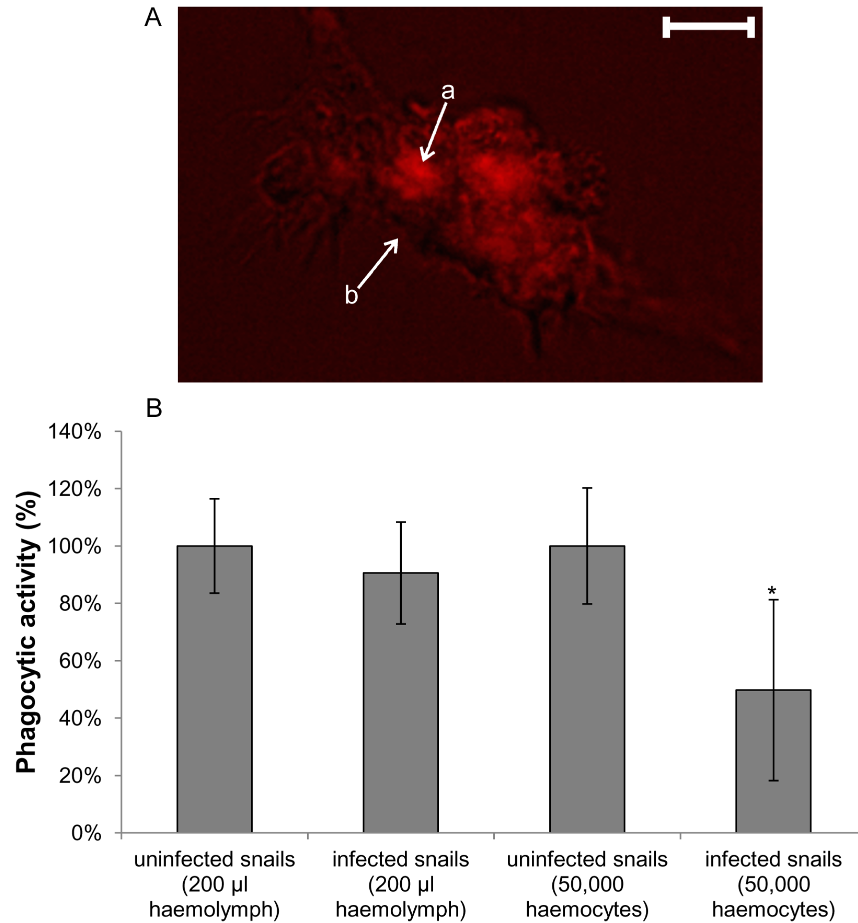


**Figure 4. Phagocytosis of *E. coli* bioparticles by haemocytes from uninfected and *Trichobilharzia regenti* infected *Radix lagotis*.** Phagocytic activities were assessed by incubating *E. coli* bioparticles with haemocyte monolayers and assessing the relative fluorescence of internalised particles after 2 h using a microplate reader. (A) The combined (phase-contrast and fluorescence) image of *E. coli* bioparticles (a) within a haemocyte (b); scale bar = 10 µm. (B) Data were evaluated per volume of haemolymph (200 µl) and per number of haemocytes (50,000) (shown as mean values ± SEM; n = 7) with uninfected snails considered as having 100% activity. *p<0.05 when compared to uninfected snails (50,000 haemocytes); Wilcoxon test.

doi:10.1371/journal.pone.0111696.g004

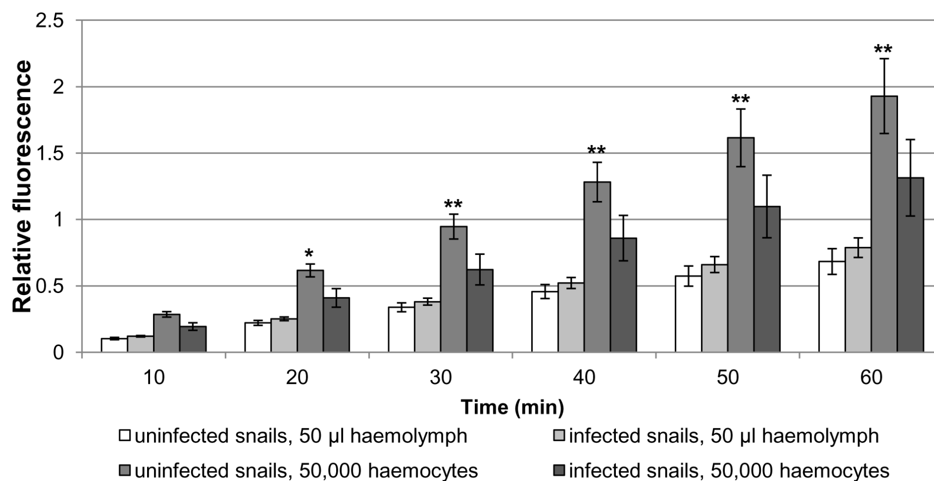


**Figure 5. Basal $H_2O_2$ production in haemocytes from uninfected and *Trichobilharzia regenti* infected *Radix lagotis*.** $H_2O_2$ output by haemocyte monolayers was detected by Amplex red and the intensity of fluorescence measured by microplate reader over 60 min. The mean relative fluorescence values are shown (± SEM; n = 7) and represent the increase in $H_2O_2$ production over time. Data were evaluated per volume of haemolymph (50 µl) and per number of haemocytes (50,000). *p<0.05, **p<0.01, when compared to infected snails (50,000 haemocytes); two-sample t-test or Wilcoxon test combined with Fishers's combination test.

doi:10.1371/journal.pone.0111696.g005

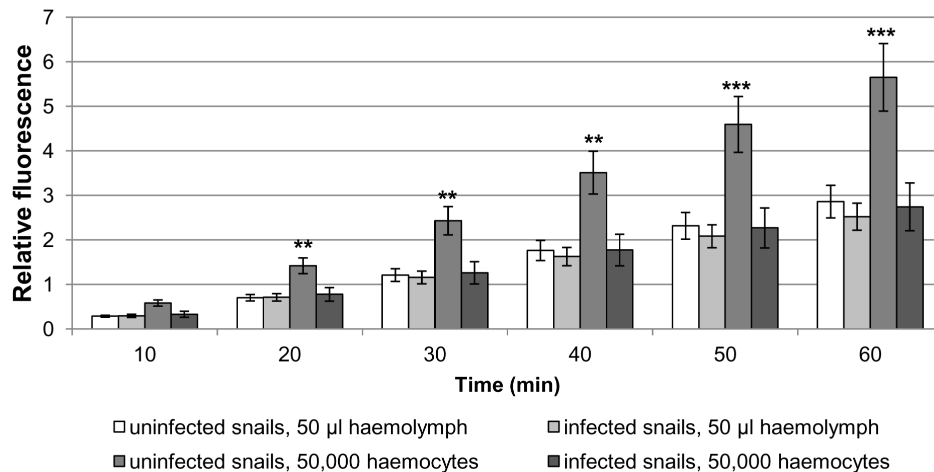


**Figure 6. PMA-stimulated $H_2O_2$ production in haemocytes from uninfected and *Trichobilharzia regenti* infected *Radix lagotis*.** $H_2O_2$ output by haemocyte monolayers treated with 5 µM PMA was detected by Amplex red and the intensity of fluorescence was measured by microplate reader over 60 min. The mean relative fluorescence values are shown (± SEM; n = 7) and represent the increase in $H_2O_2$ production over time. Data were evaluated per volume of haemolymph (50 µl) and per number of haemocytes (50,000). **p<0.01, ***p<0.001, when compared to infected snails (50,000 haemocytes); two-sample t-test or Wilcoxon test combined with Fishers's combination test.
doi:10.1371/journal.pone.0111696.g006

### Effect of PKC and MEK inhibitors on phagocytosis and $H_2O_2$ production

To investigate the possible role of PKC and ERK in the regulation of phagocytosis by *R. lagotis* haemocytes, cells from uninfected snails were incubated with the PKC or MEK inhibitors GF109203X or U0126, respectively, compounds that have been shown to decrease PKC or ERK phosphorylation (activation) in *L. stagnalis* haemocytes [7], [27]. Phagocytosis was blocked in a dose-dependent manner, with 1 µM and 10 µM GF109203X significantly suppressing uptake of bioparticles by approximately 35% and 70%, respectively ($p<0.01$, $p<0.001$; Figure 8A). U0126 at 1 µM and 10 µM concentration significantly reduced phagocytic activity of haemocytes by approximately 33% and 67%, respectively ($p<0.01$, $p<0.001$; Figure 8B).

Treatment of haemocytes from uninfected *R. lagotis* with 5 µM PMA resulted in a 212% increase in $H_2O_2$ production after 60 min in contrast to an 80% increase in the absence of PMA; thus at this time point PMA stimulated $H_2O_2$ output approximately 2.6-fold when compared to controls ($p<0.001$; Figure 9). Next, the ability of PKC (GF109203X; 5 µM) and MEK (U0126; 5 µM) inhibitors to affect haemocyte $H_2O_2$ production was tested. GF109203X substantially attenuated $H_2O_2$ release by PMA-stimulated haemocytes when compared to haemocytes treated with DMSO (vehicle) and PMA at all time points ($p<0.01$, $p<0.001$; Figure 9), reducing $H_2O_2$ output to levels similar to those seen under basal conditions. In addition, DMSO did not significantly affect PMA-stimulated $H_2O_2$ production when compared to that of cells treated with PMA only. U0126 also significantly reduced PMA-stimulated $H_2O_2$ production by *R. lagotis* haemocytes (Figure 10). After 60 min, the increase in $H_2O_2$ production as a result of PMA exposure was reduced by 37% ($p<0.001$; Figure 10).

## Discussion

### Histological observation of *T. regenti* in *R. lagotis*

We evaluated by histology haemocyte migratory/encapsulation responses triggered in *R. lagotis* by the bird schistosome, *T. regenti*. The timing of this haemocyte response should be interpreted with a tolerance of +5 h, because the snails were exposed to *T. regenti* miracidia for 5 h, after which the specimens were fixed at different time points between 1 and 92 h p.e. Taking this into account, between 1 and 6 h p.e. the response varied where some larvae were encapsulated by haemocytes while others appeared without haemocytic infiltration. In the case of *Biomphalaria alexandrina* infected with *S. mansoni*, haemocytes were also not observed around some miracidia while others underwent encapsulation 6 h p.e. [28]. Haemocytes then surrounded developing *T. regenti* sporocysts in our study between 7 and 21 h p.e., and their occurrence started to fluctuate at the latter time points. This encapsulation, however, did not lead to killing of the parasites that appeared to be morphologically intact. Similarly, compatible *S. mansoni* larvae have also been seen encapsulated but not destroyed by *B. glabrata* haemocytes in in vitro experiments or by *Biomphalaria tenagophila* fibrous cells observed in vivo [29–31]. Haemocytes of *R. lagotis* might be attracted towards *T. regenti* by ciliary plates shed during miracidium-mother sporocyst transformation [21]. A role of *T. szidati* ciliary plates in activating *L. stagnalis* haemocytes has been previously suggested [32], [33]. Furthermore, within haemocytes we observed microtubular aggregates that likely corresponded to the remnants of phagocytosed ciliary plates. Ciliary plates of *S. mansoni* miracidia are also phagocytosed by *B. glabrata* haemocytes [34].

Then, up until 41 h p.e., the haemocytic response against the developing *T. regenti* appeared to decline and no haemocytes were observed in the proximity of larvae between 44 and 97 h p.e. Haemocyte motility might be affected by parasite-derived components such as ESPs, which in the case of *E. paraensei* repel *B. glabrata* haemocytes [35]. Based on our observations, we suggest that the developing sporocysts of *T. regenti* escaped the cellular defence response of *R. lagotis* enabling successful parasite development. However, it is also possible that not all larvae that penetrated the snails were observed and some of these might have been destroyed after encapsulation. Both normally developing and encapsulated sporocysts of *S. mansoni* within *B. glabrata* have previously been observed [36].

Interestingly, in our laboratory-reared *T. regenti*, approximately 90% of *R. lagotis* snails become infected with the parasite while

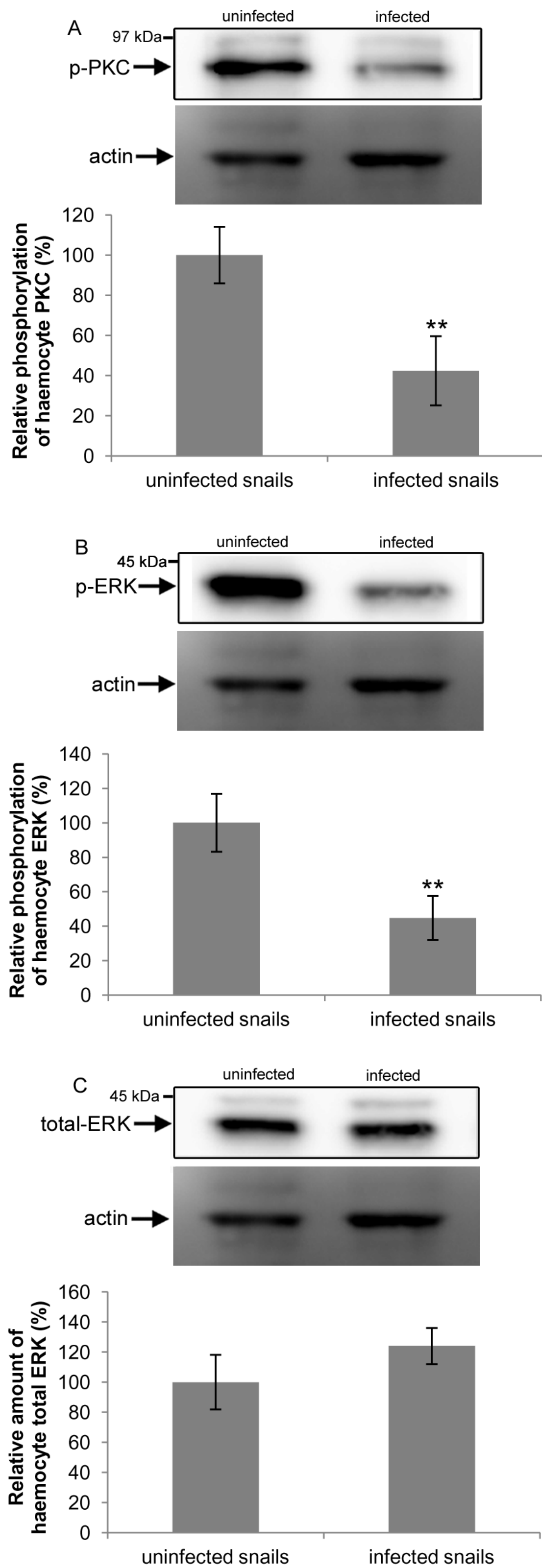


**Figure 7. PKC and ERK phosphorylation and total ERK levels in haemocytes from uninfected and *Trichobilharzia regenti* infected *Radix lagotis*.** Representative blots showing (A) PKC and (B) ERK phosphorylation in adherent haemocytes from uninfected and infected snails. (C) Levels of total ERK in uninfected and infected snails. Band intensities were measured and the mean (± SEM) haemocyte PKC and ERK phosphorylation (n = 10) and total ERK levels (n = 5) calculated (shown in the graphs) with uninfected values considered as 100%. **$p < 0.01$ when compared to haemocyte PKC and ERK phosphorylation levels in uninfected snails; two-sample t-test.
doi:10.1371/journal.pone.0111696.g007

the remainder appear resistant (data not shown). This phenomenon, reduced compatibility, may be a consequence of long-term passage of the parasite in laboratory conditions [37]. However, reduced compatibility may already arise earlier as shown for the first generation of offspring of *B. alexandrina* snails susceptible to *S. mansoni* [38].

## Quantities of circulating haemocytes from uninfected and *T. regenti*-infected *R. lagotis*

Haemocyte numbers/ml haemolymph, phagocytic activity and $H_2O_2$ production were compared between uninfected and infected *R. lagotis* snails, with infected snails studied during the patent period of infection by *T. regenti*. Correlation analysis revealed that the levels of circulating haemocytes were not influenced by age (shell height) of individual *R. lagotis* from both groups. In contrast, older specimens of *Lymnaea acuminata* f. *rufescens*, *Indoplanorbis exustus* and *Ruditapes decussatus* have been shown to possess significantly higher haemocyte counts per volume of haemolymph than the younger individuals [39], [40].

Despite variation in haemocyte concentration in uninfected and infected *R. lagotis*, the infected snails had significantly more (1.8-

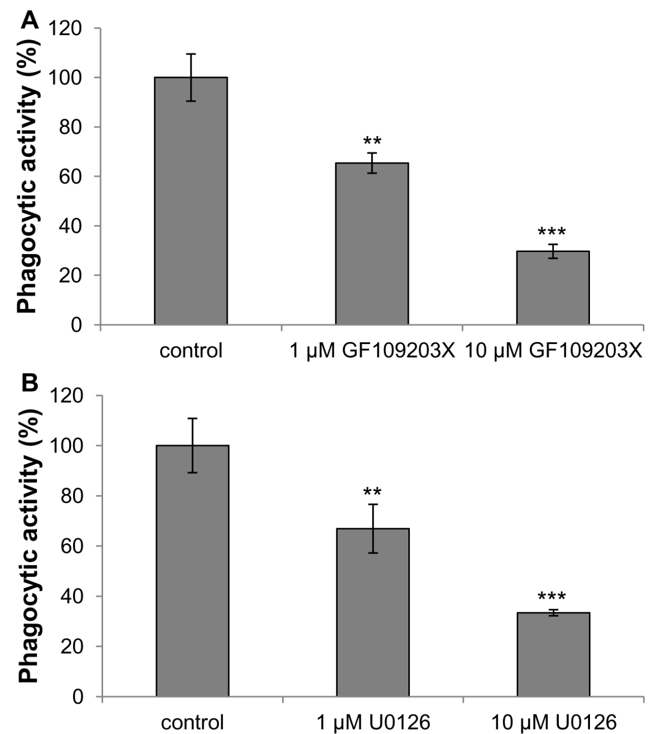


**Figure 8. Effect of PKC (GF109203X) and MEK (U0126) inhibitors on phagocytosis by haemocytes from uninfected *Radix lagotis*.** Haemocyte monolayers were pre-incubated with (A) GF109203X, (B) U0126, or vehicle (DMSO; shown as controls) prior to challenge with *E. coli* bioparticles. The intracellular fluorescence resulting from phagocytosis was measured using a microplate reader and mean (± SEM; n = 7) levels of phagocytosis expressed in relation to control (100%) values. **$p < 0.01$, ***$p < 0.001$, when compared to control values; paired t-test.
doi:10.1371/journal.pone.0111696.g008

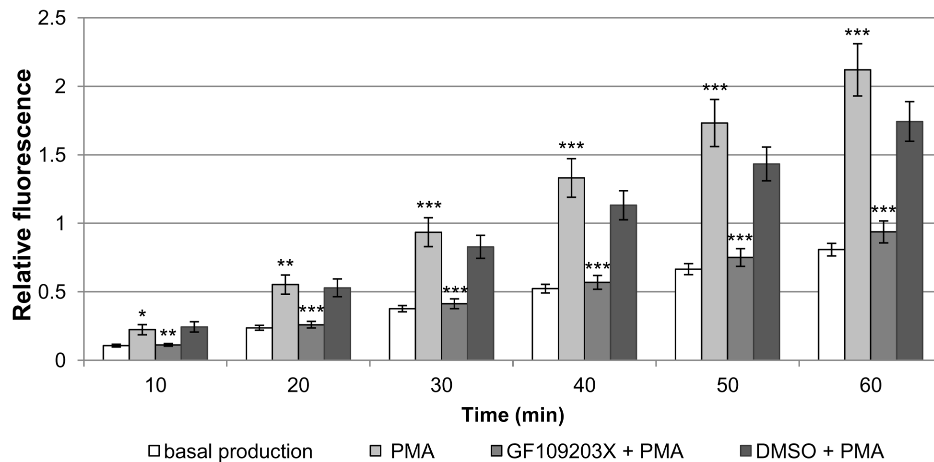


**Figure 9. PMA-stimulated $H_2O_2$ production in haemocytes from uninfected *Radix lagotis*, and the effect of PKC inhibition on $H_2O_2$ production.** $H_2O_2$ output by haemocyte monolayers in the presence of PMA (5 μM), GF109203X (5 μM) and PMA, DMSO (vehicle) and PMA, or SSS+ alone was detected by Amplex red and the intensity of fluorescence was measured by microplate reader over 60 min. The mean (± SEM; n = 7) relative fluorescence values shown represent the increase in $H_2O_2$ production over time in the various treatments. *$p<0.05$, **$p<0.01$, ***$p<0.001$, for PMA values compared to basal production, and **$p<0.01$, ***$p<0.001$ for GF109203X+PMA compared to DMSO+PMA; paired t-test or paired-samples Wilcoxon test combined with Fishers's combination test.
doi:10.1371/journal.pone.0111696.g009

fold) circulating haemocytes/ml haemolymph, when compared to their uninfected counterparts. Similar differences in haemocyte number were previously found between uninfected and *H. elongata*-infected *L. littorea* [15]. Infection of *B. glabrata* with *E. liei* or *E. paraensei* also results in increased numbers of haemocytes in the circulation [41], [42]. On the other hand, haemocyte concentrations appeared constant in *L. stagnalis* snails infected with *Diplostomum spathaceum* [43], suggesting that increased haemocyte number is not a general response of snails to trematode infection.

## Comparison of defence activities of haemocytes from uninfected and *T. regenti*-infected *R. lagotis* and the influence of PKC and ERK activities

In-vitro experiments with haemocytes from either uninfected or *T. regenti*-infected *R. lagotis* were particularly challenging because preparation of cell monolayers from haemolymph pools maintained on ice as done for *L. stagnalis* and *B. glabrata* [7], [17] was intractable for *R. lagotis*. The majority of haemocytes clumped during such manipulation and, therefore, aliquots of haemolymph expelled during head-foot retraction were transferred directly from the snails to the wells. Furthermore, variation in the numbers of

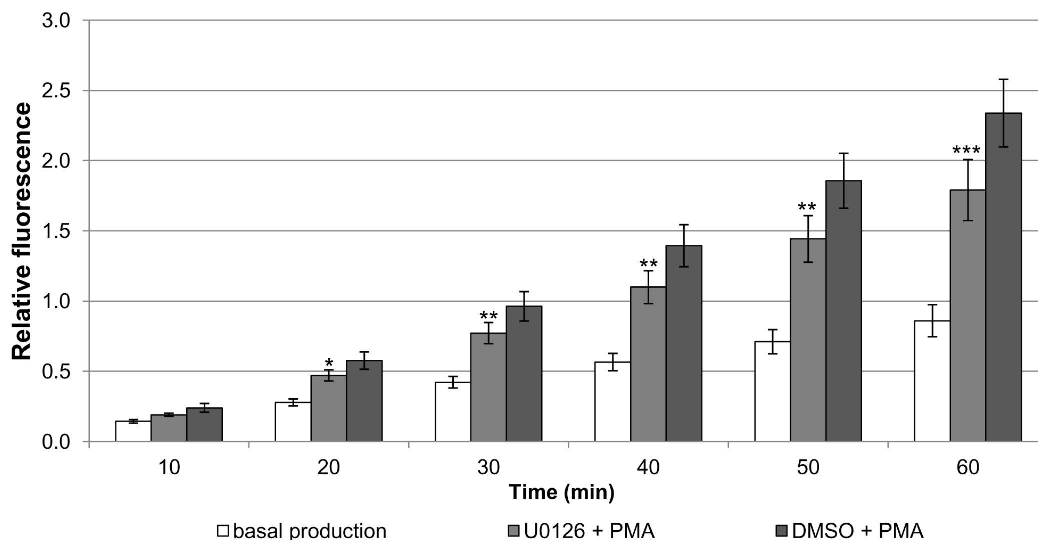


**Figure 10. The effect of MEK inhibition on PMA-stimulated $H_2O_2$ production in haemocytes from uninfected *Radix lagotis*.** $H_2O_2$ output by haemocyte monolayers in SSS+ alone, U0126 (5 μM) and PMA, or DMSO (vehicle) and PMA was detected by Amplex red and the intensity of fluorescence was measured by microplate reader over 60 min. The mean (± SEM; n = 3 for SSS+ otherwise n = 7) relative fluorescence values shown represent the increase in $H_2O_2$ production over time in the various treatments. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ for U0126+PMA compared to DMSO+PMA; paired t-test or paired-samples Wilcoxon test combined with Fishers's combination test.
doi:10.1371/journal.pone.0111696.g010

circulating haemocytes in *R. lagotis* necessitated haemocyte counting in each experiment. Wells with haemolymph from infected snails usually contained almost twice the number of cells in haemolymph from uninfected snails.

Phagocytosis of *E. coli* bioparticles, evaluated using equal numbers of haemocytes (50,000), was approximately 50% lower in infected snails when compared to that of uninfected snails, although when considering haemolymph volume (200 µl) phagocytic activities were similar. Because bioparticles were used in excess and were found free in the incubation medium after exposure to haemocytes, we conclude that the phagocytic activity of haemocytes was not limited by *E. coli* bioparticles availability, but was supressed as a result of *T. regenti* infection. However, it remains to be determined whether individual haemocytes exhibited lower phagocytic activity generally or whether some populations were more affected than others. The increased concentration of haemocytes in infected snails which was 1.8-fold higher in comparison to uninfected snails likely compensated for the overall decreased phagocytic capacity.

Haemocytes obtained from *B. glabrata* or *L. stagnalis* infected with *E. paraensei* or *T. szidati*, respectively, also possess reduced phagocytic activity [13], [14], [44]. Such suppression was observed several days or weeks after exposure to parasites. Furthermore, phagocytic activity of haemocytes was reduced in haemocytes exposed to parasite-derived ESPs [45], [46]. Although specific bioactive molecules of *T. regenti* were not investigated in our study, the phagocytic capacity of *R. lagotis* haemocytes might be affected by products of daughter sporocysts or cercariae as these stages persist in snails in the patent phase of infection.

The PKC and ERK pathways have been found to be essential for efficient phagocytosis by haemocytes of *L. stagnalis*, *B. glabrata* or *Mytilus galloprovincialis* [7], [47], [48]. We therefore explored the possible regulatory role of PKC and ERK in phagocytosis by *R. lagotis* haemocytes. Inhibitors of PKC (GF109203X) and MEK (U0126) significantly blocked haemocyte phagocytic activity in a dose-dependent manner. At 1 µM and 10 µM, GF109203X decreased phagocytosis by 35% and 70% whereas U0126 by 33% and 67%, respectively. This supports the involvement of PKC and ERK in phagocytosis of *E. coli* bioparticles by *R. lagotis* haemocytes. Furthermore, levels of PKC and ERK phosphorylation (activation) were 57% and 55% lower, respectively, in haemocytes from infected snails compared to uninfected snails following adhesion. Thus, the reduced phagocytic activity of haemocytes from infected snails might be caused (at least partly) by supressed PKC and ERK activation in these cells. Because the level of total (phosphorylated and non-phosphorylated) ERK was not reduced in these cells it is possible that the expression of upstream signalling elements might be suppressed; these could include integrin which is known to activate ERK and to be important in cell adhesion [49], [50]. The expression of PKC protein was not studied in the current work since available antibodies are generally ineffective at recognizing PKCs in snail haemocytes (unpublished results).

Infected and uninfected *R. lagotis* haemocytes were further compared in their capacity to generate $H_2O_2$. Amplex red utilized in our study was previously used for monitoring $H_2O_2$ production by snail haemocytes [9], [24]. Basal and PMA-stimulated $H_2O_2$ production did not differ significantly between uninfected and infected snails when considering only the volume of haemolymph (50 µl). On the other hand, basal $H_2O_2$ production calculated per number of haemocytes (50,000) was significantly different, with haemocytes from uninfected snails producing more $H_2O_2$ as early as 20 min. Similarly, PMA-stimulated $H_2O_2$ production by haemocytes from uninfected snails increased significantly with time from 20 min, being approximately 2-fold higher after 60 min when compared to that of haemocytes from infected snails. The reduced capacity of haemocytes from infected snails to generate $H_2O_2$ might be important for *T. regenti* survival, as $H_2O_2$ was previously shown to be an important ROS involved in in-vitro killing of *S. mansoni* sporocysts [4]. In *L. littorea*, haemocytes from snails infected with *H. elongata* produce 2-fold less superoxide [15], a precursor of $H_2O_2$ [4], [51]. As with phagocytosis, it is possible that *R. lagotis* compensate for decreased $H_2O_2$ generation by haemocytes by increasing their number in the circulation. Nevertheless, whether all haemocytes or their proportion were inhibited remains unknown as well as components of *T. regenti* responsible for such alteration. In *B. glabrata*, PMA-stimulated production of $H_2O_2$ was significantly reduced when haemocytes were simultaneously exposed to PMA and ESPs of *S. mansoni* [10].

As PMA is an activator of PKC, a role of this kinase in the regulation of $H_2O_2$ production by haemocytes from uninfected *R. lagotis* snails was further investigated; participation of ERK signalling in this process using the MEK inhibitor (U0126) was also explored. Haemocytes exposed to GF109203X displayed substantially reduced PMA-stimulated $H_2O_2$ production that was similar to levels comparable with basal (unstimulated) $H_2O_2$ output. U0126 also significantly affected PMA-stimulated $H_2O_2$ output by snail haemocytes, although at less extent than GF109203X. Thus, PKC and ERK appear to play a role in regulating $H_2O_2$ production by *R. lagotis* haemocytes. PKC and ERK signalling were previously found to be crucial in regulation of $H_2O_2$ production by haemocytes of *B. glabrata* [10], [24] and *L. stagnalis* [9]. As already mentioned for haemocytes of infected snails, basal levels of PKC and ERK phosphorylation (activation) were significantly lower than in haemocytes of uninfected snails; lower $H_2O_2$ production by haemocytes from infected snails could therefore be the result of lower PKC and ERK activities in response to the parasite. Our study and a previous report suggesting that ESPs may attenuate PKC and ERK phosphorylation in snail haemocytes [17] support the notion that parasites modulate haemocyte defence pathways at the level of cell signalling [16] and possibly at multiple phases during development.

The present paper provides the first insights into the immunobiology of the snail *R. lagotis*, an important intermediate host of the nasal bird schistosome *T. regenti*. Histological study of the *R. lagotis* response against *T. regenti* showed that haemocytes are able to accumulate near the invading larvae, but they do not destroy the parasite. This enables further development of trematode larvae, leading to patent phase of *T. regenti* infection in snails. The phagocytic activity and capacity for $H_2O_2$ generation were suppressed in haemocytes of infected snails. Importantly, PKC and ERK that appear to regulate such responses in *R. lagotis* were also shown to be less active in haemocytes from infected snails. It is hypothesized that attenuation of both responses in haemocytes is partially compensated by increased concentration of haemocytes in the circulation of infected snails, enabling the snail to fend off other pathogens such as bacteria. Further research is needed to understand how this impacts survival and continued cercarial production of *T. regenti* in *R. lagotis*, and to determine the parasite-derived molecules responsible for alterations in *R. lagotis* haemocyte responses.

## Acknowledgments

We would like to thank Veronika Siegelova, M.Sc., (Charles University in Prague) for laboratory maintenance of snails and parasites.

## Author Contributions

Conceived and designed the experiments: VS ZJ MK MV AJW PH. Performed the experiments: VS ZJ MV. Analyzed the data: VS AC ZJ MV. Contributed reagents/materials/analysis tools: VS AJW PH. Wrote the paper: VS AC ZJ MK MV AJW PH.

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Developmental and Comparative Immunology 79 (2018) 137–141

Contents lists available at ScienceDirect

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Short communication

# Extracellular trap-like fiber release may not be a prominent defence response in snails: evidence from three species of freshwater gastropod molluscs

Vladimír Skála [a, b, *], Anthony J. Walker [c], Petr Horák [a]

[a] Charles University, Faculty of Science, Department of Parasitology, Prague, Czech Republic
[b] Charles University, First Faculty of Medicine, Institute of Immunology and Microbiology, Prague, Czech Republic
[c] Kingston University, School of Life Sciences, Pharmacy and Chemistry, Molecular Parasitology Laboratory, Kingston Upon Thames, Surrey, United Kingdom

A R T I C L E I N F O

*Article history:*
Received 14 July 2017
Received in revised form
18 October 2017
Accepted 19 October 2017
Available online 22 October 2017

*Keywords:*
Extracellular trap-like fiber (ET-like fiber)
*Lymnaea stagnalis*
*Radix lagotis*
*Planorbarius corneus*
Snail immunity
Haemocytes

A B S T R A C T

The discovery that mammalian neutrophils generate extracellular chromatin fibers that entrap/kill bacteria supported a new paradigm for innate immunity in animals. Similar findings in other models across diverse taxa have led to the hypothesis that the phenomenon is ancient and evolutionary conserved. Here, using a variety of synthetic (e.g. peptidoglycan) and biological (e.g. trematode larvae) components to investigate extracellular trap-like (ET-like) fiber production *in vitro* by haemocytes of *Lymnaea stagnalis*, *Radix lagotis* and *Planorbarius corneus* snails, ET-like fibers were rarely observed. We suggest, therefore, that ET-like fibers play a marginal role in defence of these snail species and thus the fiber production may not be a critical process underpinning immunity in all invertebrate species.

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## 1. Introduction

Reticulated DNA fibers produced by neutrophils (neutrophil extracellular traps; NETs), eosinophils (extracellular traps; ETs) and other cells of the vertebrate innate immune system are considered important structures that facilitate the elimination of bacteria and eukaryotic unicellular/multicellular parasites extracellularly (von Köckritz-Blickwede and Nizet, 2009; Zawrotniak and Rapala-Kozik, 2013; Hermosilla et al., 2014). In invertebrates, immunity typically relies on haemocytes that cooperate with humoral recognition factors such as lectins and fibrinogen-related proteins to deliver the defence response. While extracellular nucleic acids can bolster immunity as shown in the greater wax moth *Galleria mellonella* (Altincicek et al., 2008), ET-like fibers resembling NETs of vertebrates have recently also been found to mediate defence of *Litopenaeus vannamei* (Ng et al., 2013) and *Carcinus maenas* (Robb et al., 2014) haemocytes. Interestingly, mesogleal cells of the sea anemone *Actinia equina* (Robb et al., 2014), and sentinel cells of the social amoeba *Dictyostelium discoideum* (Zhang et al., 2016) have also been shown to release DNA fibers extracellularly. In molluscs, ET-like fibers have been reported in bivalves (*Mytilus edulis*, *Crassostrea gigas*) (Robb et al., 2014; Poirier et al., 2014), and gastropods (*Arion lusitanicus*, *Limax maximus* and *Achatina fulica*) in which the fibers entrapped metastrongyloid larvae (Lange et al., 2017). In the latter case, different types of ET-like fibers (i.e. aggregated, spread and diffuse) were observed, with histones and myeloperoxidase as fiber constituents (Lange et al., 2017).

In the current study, we employed haemocytes of *Lymnaea stagnalis* and two other species of freshwater gastropod snails, *Radix lagotis* and *Planorbarius corneus* to elucidate ET-like fiber production in snails that serve as intermediate hosts of trematode larvae. For comparative purposes, we used *Mytilus edulis* haemocytes that are known to release ET-like fibers.

## 2. Materials and methods

### 2.1. ET-like fiber release by Mytilus edulis haemocytes

Haemocytes of *M. edulis* were utilized for initial experiments.

* Corresponding author. Charles University, Faculty of Science, Department of Parasitology, Prague, Czech Republic.
*E-mail address:* mich007@email.cz (V. Skála).

https://doi.org/10.1016/j.dci.2017.10.011
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Haemolymph was extracted and haemocyte monolayers were prepared as previously described (Robb et al., 2014) in 96-well tissue culture plates (Nunc) employing 250 μl haemolymph/well diluted (1:1) with 0.05 M Tris-HCl buffer, pH 7.6, supplemented with 2% glucose, 2% NaCl, 0.5% EDTA. Haemocytes were incubated with 20 μM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) at 10 °C for 48 h, stained with 1 μM Sytox green (Thermo Fisher Scientific) that effectively binds DNA of dead cells (Thakur et al., 2015) and examined for ET-like fiber release under a fluorescence microscope (Olympus IX71).

### 2.2. Snails and haemocytes

Laboratory-reared *L. stagnalis* and *R. lagotis* were maintained at 19–22 °C in aerated aquaria, and fed fresh lettuce *ad libitum*. *Planorbarius corneus* snails were obtained from a local pond (Prague) and examined for cercarial shedding; infected snails were excluded from experiments. Haemolymph from snails was extracted according to Sminia (1972). Samples from L. *stagnalis* and *P. corneus* were pooled on ice, diluted 2:1 with sterile snail saline (SSS; Adema et al., 1991) and 250 μl transferred into individual wells of a 96-well plate. Experiments with *P. corneus* were also conducted in Chernin's balanced salt solution (CBSS; Chernin, 1963). Haemolymph from *R. lagotis* was handled as described previously (Skála et al., 2014). The haemocyte number per well was approx. $2.8 \times 10^5$ for *L. stagnalis*, $6 \times 10^4$ for *R. lagotis* and $1.2 \times 10^5$ for *P. corneus*, enumerated using a Bürker haemocytometer.

### 2.3. Preparation of parasite material

Miracidia of *Trichobilharzia regenti* were obtained *via* the laboratory life cycle according to Horák et al. (1998), fixed in 2% (v/v) paraformaldehyde for 30 min and free aldehyde groups blocked in 1% glycine at 4 °C overnight (Zahoor et al., 2008). The larvae were then washed twice with SSS and stored at −20 °C. Homogenised miracidia were prepared by sonicating miracidia for three cycles (7W, 20 s each, Vibracell-72405 100-W ultrasonicator, Bioblock Scientific, France) in SSS followed by determination of protein concentration using Quant-iT Protein Assay Kit (Invitrogen).

### 2.4. Haemocyte exposure

*Lymnaea stagnalis* haemocyte monolayers were treated with SSS containing peptidoglycan (PGN; 0.1, 1.0 and 10.0 μg/ml), *E. coli* lipopolysaccharide serotype 0111:B4 (LPS; 0.1, 1.0 and 10.0 μg/ml), PMA (0.1, 1.0, 10.0 μM), D-galactose or L-fucose (200, 400, 800 nM, 1 and 10 μM), D-galactose-L-fucose in combination (800 nM of each in SSS) (all purchased from Sigma-Aldrich), live/heat-killed *Staphylococcus saprophyticus* at ~10, ~100 and ~1000 bacteria/haemocyte, or miracidial homogenate (1 or 10 μg/ml). All incubations were performed at room temperature (e.g. Plows et al., 2005) for 3 h and 24 h. Three independent experiments were performed with one replicate for each condition/duration. The haemocytes were then stained with 1 μM Sytox Green in SSS for 20 min and the entire cell populations examined visually under the fluorescence microscope; haemocytes producing ET-like fibers were enumerated.

For intact parasite exposure, 200 miracidia in 100 μl SSS were transferred to individual wells of a chamber slide (Lab-Tek); 200 μl complete *L. stagnalis* haemolymph were added and after 1 h incubation, 100 μl supernatant were replaced by 100 μl fresh haemolymph. This step was done to enhance the continuous migration of haemocytes towards the parasite. Incubation times/Sytox green staining were as above; the experiments were performed twice independently. Finally, specimens were embedded in Vectashield (Vector Laboratories), examined using a Zeiss LSM880 laser scanning confocal microscope, and images analysed using FIJI Image J (Schindelin et al., 2012).

Haemocyte monolayers obtained from *R. lagotis* and *P. corneus* were incubated in SSS containing PMA (0.1, 1, 5, 10 μM), LPS (0.1, 1.0, 10.0 μg/ml), or heat-killed *S. saprophyticus* at ~100 bacteria/haemocyte.

## 3. Results and discussion

Initial experiments were performed with haemocytes of *M. edulis*, previously shown to produce ET-like fibers (Robb et al., 2014), to demonstrate fiber release in our laboratory. Similar to Robb et al. (2014), PMA clearly induced ET-like fiber release (Fig. 1A and B in Supplementary Materials) that was ETotic.

Next, snail haemocytes were exposed to PMA or LPS, compounds that were shown previously to stimulate effective NETs/ET-like fiber formation (von Köckritz-Blickwede and Nizet, 2009; Robb et al., 2014; Ng et al., 2013). Other components (e.g. L-fucose/D-galactose) were employed because they are linked to snail-trematode interactions (Plows et al., 2005).

The screening assays revealed that *L. stagnalis*, *R. lagotis* and *P. corneus* haemocytes produced only low numbers of extracellular DNA fibers (Table 1) and, therefore, other components associated with ET-like fibers such as histones (Ng et al., 2013; Robb et al., 2014) were impossible to investigate. However, given that occasional DNA fibers were observed in all species studied (Fig. 1) we define the fibers as 'ET-like' as in other invertebrates (Ng et al., 2013; Robb et al., 2014; Poirier et al., 2014; Lange et al., 2017).

That compounds such as PGN or PMA failed to elicit robust ET-like fiber production in *L. stagnalis* was surprising (Table 1). Similarly, PMA did not stimulate ET-like fiber formation by *C. gigas* haemocytes (Poirier et al., 2014). Exposure of haemocytes to 20 μM PMA in SSS or in modified SSS (SSS supplemented with D-trehalose (1 g/L), D-glucose (1 g/L) (Sigma-Aldrich) and antibiotics (penicillin/streptomycin; Lonza)), enabling longer-term *L. stagnalis* haemocyte survival for 48 h also did not evoke haemocyte ETotic responses (data not shown). On the other hand, *M. edulis* haemocytes produced fibers when exposed to 50 μM PMA for 48 h (Robb et al., 2014). In *R. lagotis*, haemocytes exposed to PMA produced only few ET-like fibers (Table 1, Fig. 1H). This finding was unexpected because PMA induces the respiratory burst in *R. lagotis* haemocytes (Skála et al., 2014), a reaction considered essential for ET-like fiber formation (Robb et al., 2014; Poirier et al., 2014).

Although LPS significantly induced NETs/ET-like fiber formation in mammalian neutrophils or shrimp haemocytes (von Köckritz-Blickwede and Nizet, 2009; Ng et al., 2013), only two ET-like fibers were produced by *L. stagnalis* haemocytes (Table 1, Fig. 1A). Additionally, no ET-like fibers were observed when these haemocytes were treated with 25 μg/ml LPS in modified SSS for 24 h, and the protocol of Brinkmann et al. (2010) was used to visualise the fibers (data not shown). With *P. corneus*, one ET-like fiber was observed when haemocytes were exposed to 10 μg/ml LPS in CBSS for 24 h (Fig. 1F) whereas nine fibers were observed in SSS (Table 1). Thus, these different culture media did not seem to largely influence the outcome with respect to ET-like fiber formation.

PMA and LPS activate protein kinase C (PKC) in *L. stagnalis* haemocytes (Walker and Plows, 2003; Wright et al., 2006), which stimulates NO production (Wright et al., 2006). Such responses might, at least in part, explain the inability of PMA and LPS to effectively promote ET-like fiber production. However, D-galactose and L-fucose attenuate PKC and extracellular-signal regulated kinase (ERK) activation in *L. stagnalis* haemocytes, with subsequent suppression of phagocytosis (Plows et al., 2005). These sugars are present on the surface of the helminth *T. regenti* (Blažová and Horák, 2005; Chanová et al., 2009), an incompatible parasite that

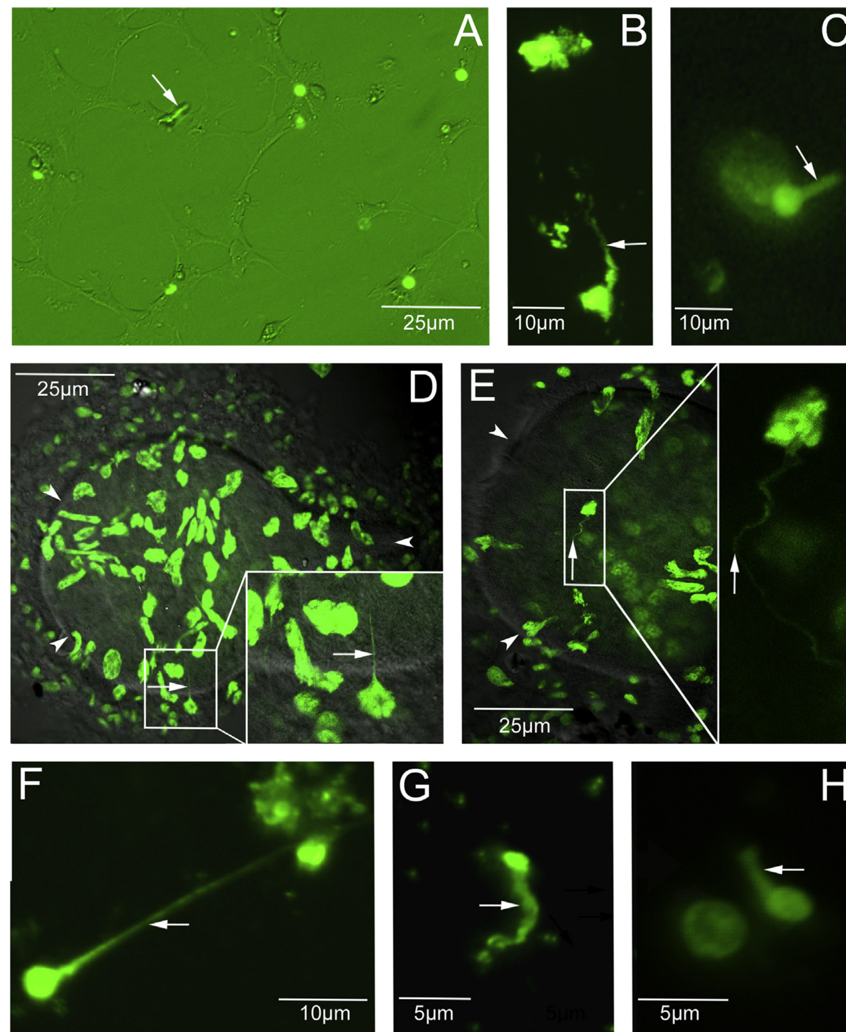


**Fig. 1.** Extracellular trap-like (ET-like) fiber production by haemocytes of *Lymnaea stagnalis* **(A–E)**, *Planorbarius corneus* **(F–G)** and *Radix lagotis* **(H)**. Green fluorescence represents DNA positive material - cell nuclei and ET-like fibers (arrows). **(A)** Low magnification of haemocyte monolayer shows that one cell produces ET-like fiber when treated with LPS (1 µg/ml) for 3 h. **(B, C)** ET-like fibers produced after the treatment of cells with homogenised *Trichobilharzia regenti* miracidia (10 µg/ml) for 3 h **(B)** and 24 h **(C)**. **(D, E)** Encapsulation of *T. regenti* miracidia (arrowheads) by snail haemocytes, and expulsion of ET-like fibers (arrows) against the parasite during 3 h confrontation; detailed view in the insets. **(F)** ET-like fiber produced after the treatment of haemocytes with LPS (10 µg/ml) in CBSS for 24 h. **(G)** ET-like fiber formed in the presence of *Staphylococcus saprophyticus* for 3 h. **(H)** ET-like fiber produced after the treatment of cells with PMA (5 µM) for 3 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

penetrates but does not survive in *L. stagnalis*. However, exposure to these sugars did not affect ET-like fiber production (Table 1).

As the soluble compounds did not substantially stimulate ET-like fiber formation by the snail haemocytes, we tested pathogen-haemocyte combinations. Heat-killed *S. saprophyticus* bacteria were phagocytosed by the snail haemocytes (Fig. 1C in Supplementary Materials) whereas several ET-like fibers were produced with unclear function (Table 1); similar results were also obtained with live *S. saprophyticus* (data not shown). In contrast, ET-like fibers produced by *C. gigas* haemocytes were shown to entrap *Listonella anguillarum* (Poirier et al., 2014), whereas fibers produced by *L. vannamei* trapped and killed *E. coli* (Ng et al., 2013).

Experiments using fixed *T. regenti* miracidia and whole snail haemolymph showed that haemocytes encapsulate the parasite (Fig. 1D-E). Moreover, confocal microscopy revealed that several haemocytes expelled ET-like fibers against *T. regenti* during 3 h exposure (Fig. 1D–E). In gastropods, haemocyte derived ET-like fibers were demonstrated previously in *A. fulica*, which trapped viable *Angiostrongylus vasorum* larvae *in vitro* (Lange et al., 2017). Release of ET-like structures was also observed *in vivo* in the mucous extrapallial space of *L. maximus* in response to invading *A. vasorum* (Lange et al., 2017). However, in our study, only a few *L. stagnalis* haemocytes produced ET-like fibers against *T. regenti* (Fig. 1D–E) and thus the fibers are unlikely the main defence tool for parasite elimination. Although attempted, evaluation of *T. regenti* and *L. stagnalis* interactions in snail histological sections was technically demanding (results not shown) and, therefore, the extent of ET-like fiber production *in vivo* remains unknown. Finally, homogenised *T. regenti* miracidia did not stimulate significant ET-like fiber production (Table 1, Fig. 1B–C).

To conclude, we examined the ability of several compounds and pathogens to elicit ET-like fiber production in the freshwater snails *L. stagnalis*, *R. lagotis* and *P. corneus in vitro*. ET-like fiber production has previously been reported in several invertebrates including molluscs. Together with reports on vertebrates, it is postulated that NETs/ET-like fiber release is a widely shared and effective defence mechanism among animals. The findings presented here highlight variation in ET-like fiber-based innate immune mechanisms in

**Table 1**
An overview of compounds/pathogens and conditions used to stimulate extracellular trap-like fiber production by haemocytes of the freshwater snail species *Lymnaea stagnalis*, *Radix lagotis* and *Planorbarius corneus*.

| species | compound/pathogen in SSS buffer | condition | duration (h) | no. of ET-like fibers observed |
|---|---|---|---|---|
| *Lymnaea stagnalis* | phorbol 12-myristate 13-acetate | 0, 0.1, 1, 10 (μM) | 3 | 0, 0, 0, 0 |
| | | | 24 | 0, 0, 0, 0 |
| | lipopolysaccharide | 0, 0.1, 1, 10 (μg/ml) | 3 | 0, 1, 2, 2 |
| | | | 24 | 0, 1, 0, 0 |
| | peptidoglycan | 0, 0.1, 1, 10 (μg/ml) | 3 | 0, 6, 7, 4 |
| | | | 24 | 1, 1, 6, 3 |
| | D-galactose | 0, 200, 400, 800 (nM); 1, 10 (μM) | 3 | 2, 3, 4, 7; 0, 0 |
| | | | 24 | 2, 1, 8, 5; 0, 0 |
| | L-fucose | | 3 | 2, 5, 0, 1; 0, 0 |
| | | | 24 | 2, 1, 1, 2; 0, 0 |
| | D-galactose/L-fucose | 0, 800/800 (nM) | 3 | 2, 6 |
| | | | 24 | 2, 0 |
| | *S. saprophyticus* | 0, 10, 100, 1000 bacteria/haemocyte | 3 | 0, 0, 0, 0 |
| | | | 24 | 0, 4, 0, 0 |
| | homogenised *T. regenti* miracidia | 0, 1, 10 (μg/ml) | 3 | 1, 6, 7 |
| | | | 24 | 0, 3, 6 |
| *Radix lagotis* | phorbol 12-myristate 13-acetate | 0, 0.1, 1, 5, 10 (μM) | 3 | 2, 1, 2, 3, 3 |
| | | | 24 | 2, 0, 0, 0, 0 |
| | *S. saprophyticus* | 0, 100 bacteria/haemocyte | 3 | 2, 0 |
| | | | 24 | 0, 0 |
| *Planorbarius corneus* | lipopolysaccharide | 0, 0.1, 1, 10 (μg/ml) | 3 | 0, 9, 0, 0 |
| | | | 24 | 0, 0, 0, 0 |
| | *S. saprophyticus* | 0, 100 bacteria/haemocyte | 3 | 0, 1 |
| | | | 24 | 1, 0 |

invertebrates, since no significant ET-like fiber production was achieved with the investigated haemocytes following exposure to a wide range of stimulants used in other studies. For further confirmation, *in vivo* studies are required.

## Funding

The study was financially supported by the Charles University research programmes UNCE-204017, SVV 244-260432/2017, PROGRES Q43 and by a research grant of the Malacological Society of London to VS.

## Acknowledgments

We would like to thank Veronika Siegelová (Charles University) for laboratory maintenance of snails and parasites, and Veronika Harsová (Charles University) for excellent technical support. We also would like to thank Dr. Petr Petráš (The National Institute of Public Health) for kind provision of *Staphylococcus saprophyticus*.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.dci.2017.10.011.

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## Conclusions

The review of the literature revealed that the immune interactions between *T. regenti* and compatible/incompatible snail hosts have not previously been studied. Therefore, the results presented in the thesis provide the first insight into the immune reactions of snails against *T. regenti* and provide an important contribution to knowledge regarding compatibility/incompatibility in snail-schistosome interactions.

**The most significant findings:**

• Most studies on bird schistosome-snail intermediate host relationships have focused on *Trichobilharzia szidati*-*Lymnaea stagnalis*. In *L. stagnalis*, *T. szidati* interferes with the immune system or metabolism in order to ensure its survival and reproduction.

• Penetration of *T. regenti* into compatible *R. lagotis* snails activates haemocytes that migrate and aggregate near the parasite. Failure of haemocytes to eliminate *T. regenti* enables parasite development to the cercarial stage in the patent phase of infection.

• Modulation of *R. lagotis* haemocyte activities, as demonstrated for phagocytosis and hydrogen peroxide production, might be important for the continuous production and/or release of infective cercariae.

• Activation of cellular signalling cascades is important for defence activities of *R. lagotis* haemocytes. Attenuation of cell signalling in snails during the patent phase of infection by *T. regenti* might be responsible for immunomodulation.

• Haemocytes of incompatible *L. stagnalis* snails encapsulate *T. regenti* miracidia and produce a few extracellular trap-like (ET-like) fibers; formation of ET-like fibers represents a novel type of immune response.

•ET-like fibers are probably not crucial for *T. regenti* elimination by *L. stagnalis* haemocytes.

• Comparatively, *R. lagotis* and *P. corneus* haemocytes produce a low number of ET-like fibers and thus these structures are likely not always pivotal in the defence of invertebrates.

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