Biology and ecology of trematodes parasitizing aquatic snails in the Ruhr reservoir system in Germany

Dissertation
zur
Erlangung des Doktorgrades
Dr. rer. nat.

der Fakultät für
Biologie
an der

Universität Duisburg-Essen

vorgelegt von
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November 2015

Angaben zur Prüfung

Die der vorliegenden Arbeit zugrunde liegenden Experimente wurden in der Abteilung für Aquatische Ökologie der Universität Duisburg-Essen durchgeführt.

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Tag der mündlichen Prüfung: 21.12.2015

Acknowledgements

Although only my name appears on the cover of this dissertation, such a work is never possible without the support, contribution and help of many other people, whom I owe my gratitude to.

First and foremost, I want to thank my supervisor Bernd Sures for the great support throughout the years, and it has been quite some years, for the stimulating ideas and discussions and for the guidance to (sometimes spontaneously) develop the thesis in new directions. I am also very grateful for the many chances I had to work on projects abroad and to be able to present my results at conferences around the world. It has been a pleasure!

Massive thanks go to Ana Pérez-Del-Olmo, Aneta Kostadinova and Mirka Soldánová, the Spanish-Bulgarian-Czech connection for introducing me to this wormy world of parasites and generously teaching and supporting me throughout my studies. Without you this thesis would not be. Thanks for all your input and support, and for keeping me alive on cookies the first day I joined for lab and fieldwork. Ana and Mirka, many thanks for patiently teaching me during your postdocs in Essen and beyond. Aneta, thanks for your immense support and great mentoring.

I thank everybody from the fantastic Aquatic Ecology group in Essen for the enjoyable time. In particular, I would like to thank my home away from home, aka the crazy office: Michelle Keppel, Kerstin Dangel and Michael Hohenadler for many thoughtful conversations on everything but work. Jessica Schwelm, Verena Altmann and Jana Köchling for their tireless efforts during the many sampling trips and the long and often late hours in the lab. Without you this would not have been possible either. Many thanks also go to Daniel Grabner for patiently explaining everything molecular, to Birgit and Kira for keeping everything going, and to Mark Schumann, Manoo Bahmani and Jörg Kaminski for technical support during the experiments and field work. Cheers to the vinyl and sports clubs Mark, Christian (#CKF) and Oli, and the Wasserturm WG.

Many thanks go to everybody in Tomáš Scholz's Laboratory of Helminthology in České Budějovice, in particular to Aneta Kostadinova, Mirka Soldánová, Simona Georgieva and Anna Faltýnková for sharing their vast knowledge on trematode taxonomy with me; and of course to all the other wonderful people from all over the world I was able to meet during my stays at the institute and could have beers with at Singer's Pub or during the Helminthological Days meetings. Furthermore, I thank Martin Kalbe and Franz Jirsa for their encouraging feedback at the meetings in Boiensdorf and Neusiedl from the very beginning of my thesis to the discussion of final results.

I am very grateful for a scholarship by the Deutsche Bundesstiftung Umwelt (DBU) that made this work possible. Special thanks go to Volker Wachendörfer. Furthermore, I acknowledge funding by the Faculty of Biology at the University of Duisburg-Essen as well as travel grants by the German Academic Exchange Service (DAAD) and the Leopoldina German National Academy of Sciences.

Last but certainly not least, I thank my friends for always being there for me and, most importantly, my family for their love and support.

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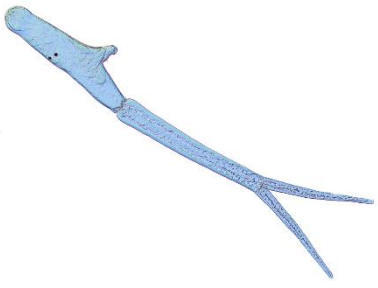

1. Summaries

1.1 Summary

Parasites are integral and important elements of ecosystems that occur in virtually every habitat on this planet (Poulin 1999, Hudson et al. 2006). However, except for the obviously important role of some species as disease agents, parasites have long been neglected and considered unimportant in the context of most ecological studies. Only in recent years, studies have begun to investigate the ecological importance of parasites, e.g. their structuring forces in trophic transmissions and food webs (Lafferty et al. 2008) or their contribution to an ecosystem's biomass (Kuris et al. 2008, Thieltges et al. 2008). It has been argued, that it is even impossible to fully understand ecosystems without considering the parasites therein (Lafferty et al. 2006a). Furthermore, based on their often complex life cycles and strong interaction in ecosystems, parasites may serve as useful bioindicators to assess environmental conditions and changes (Lafferty 1997, Vidal-Martínez et al. 2010, Nachev & Sures, 2015).

Environmental changes, such as climate change and global warming are expected to have major impacts on parasites and on entire ecosystems, often with yet unpredictable consequences (Marcogliese 2001, 2008). It is, therefore, essential that we understand host-parasite systems at the ecosystem level. Although there are some exceptionally well-studied aquatic ecosystems that focus on the roles of parasites in these habitats and allow predictions of the impact and possible effects of environmental changes (e.g. Kuris et al. 2008, Preston et al. 2013, Thieltges et al. 2013, Lagrue et al. 2015), our knowledge of host-parasite interaction in man-made waterbodies, such as impounded lakes and reservoirs, is still very limited and large-scale studies focussing on the ecological role of parasites in such systems are lacking. This is especially critical, since such waterbodies fulfil vital roles in densely populated areas, where they serve as drinking water storages, recreational areas and constitute important biodiversity hot spots. The Ruhr area in Germany is one of the largest and most densely populated urban areas in Europe and the eponymous extensive reservoir system of the Ruhr and its tributaries plays a vital role for the whole region. Our knowledge of the ecological role of parasites in these aquatic habitats is still fairly limited, however.

In order to bridge this gap, this thesis aims at assessing the biology and ecology of trematodes parasitizing aquatic snails in the Ruhr reservoir system in Germany. Trematodes in snails have been identified as a particularly promising group of parasites that lend themselves to investigating and assessing environmental conditions and ecosystem changes in aquatic systems (Huspeni & Lafferty 2004), due to their complex multiple-host life cycles that enable us to study the complex roles of parasites in ecosystems at many levels and in great detail. This thesis, therefore, aims at addressing the importance of trematodes in the Ruhr reservoir systems by identifying larval trematode infections

in snails in the reservoirs of the Ruhr river system. The individual studies of this work provide (i) detailed integrative approaches to taxonomically questionable trematode groups to assess the full diversity in the ecosystem, (ii) an analysis of the productivity and emergence of trematode cercariae to assess the parasites' functional role in the ecosystem, (iii) an evaluation of the risk factors of swimmer's itch via a detailed case study of one of the Ruhr lakes, and finally (iv) an assessment of the overall diversity, distribution and community structure of larval trematodes and the identification of potential trematode transmission pathways between their host groups. Altogether, this holistic approach, encompassing trematode taxonomy, their functional part in ecosystems, the role of medically relevant species and their contribution to an ecosystem's diversity will provide a detailed and comprehensive insight into the complex role of trematodes in an important reservoir system in Europe.

In order to assess the diversity and distribution patterns of trematodes in the Ruhr area, snails were collected at several sampling sites in five reservoirs of the Ruhr river catchment area in Germany, Baldeneysee, Hengsteysee, Hennetalsperre, Sorpetalsperre and Versetalsperre, during the summer months of the years 2012 and 2013. Sampling and analyses were mostly focused on gastropod snails belonging to two families, Lymaeidae and Planorbidae, since they proved to harbour the most diverse trematode fauna in Europe (Faltýnková & Haas 2006, Soldánová et al. 2011, Brown et al. 2011). A total of 3,171 lymnaeid snails belonging to four species, *Radix auricularia*, *Stagnicola palustris*, *Radix peregra* and *Lymnaea stagnalis*, and 2,176 planorbid snails belonging to two species, *Gyraulus albus* and *Segmentina nitida*, were collected and screened for trematode infections. Trematode stages were identified according to morphological features and, where necessary, with the aid of molecular methods.

Of the 5,347 sampled snails, 1,049 showed patent or prepatent infections with trematodes, resulting in a total prevalence of 19.6%. Detailed integrative approaches of molecular and morphological methods revealed the presence of cryptic species within the trematode genera *Echinostoma*, *Petasiger* and *Diplostomum*. The analysis of *Echinostoma* spp. cercariae allowed the delineation of two cryptic species of the '*revolutum'* group from larval stages found in *R. auricularia*, *R. peregra* and *S. palustris*. Likewise, analyses of infections from *G. albus* revealed the distinctive status of three species of *Petasiger* from this host that occurred in sympatry at one locality, highlighting the high diversity within this trematode genus, even at small spatial scales. Most distinctively, seven different species of *Diplostomum* were found in the lymnaeid snail populations from the Ruhr reservoirs, three named species, *D. spathaceum*, *D. pseudospathaceum* and *D. parviventosum*, and four species-level lineages, '*Diplostomum* sp. Clade Q' and '*D. mergi'* Lineages 2-4. Furthermore, the detailed approach provided evidence that the cercariae of '*D. mergi*' Lineage 1 of Georgieva et al. (2013a) are actually *D. parviventosum*. Remarkably, the snail species *L. stagnalis* and *S. palustris*

harboured only one species, *D. pseudospathaceum*, while *R. auricularia* populations revealed a highly diverse picture with six different lineages of *Diplostomum*, and it remains to be investigated why the diversity of *Diplostomum* in these hosts presents such contrary situation. Altogether, these integrative approaches of molecular and morphological data advance our knowledge of the taxonomic situation of these trematode taxa and reveal the remarkably high cryptic diversity of these parasites in the Ruhr reservoir system.

However, the knowledge of the sheer diversity of trematodes in snails does not provide information on their functional role within an ecosystem. In order to assess the contribution of trematode cercariae to the biomass in European freshwaters, cercarial emergence of the bird schistosome *Trichobilharzia szidati* from naturally infected *L. stagnalis* was studied. The study revealed an average daily emergence rate of 2,621 cercariae per snail, with emissions peaks of up to 29,560 cercariae. Calculated for an individual snail's lifetime this summed up to a cumulative cercarial biomass of 4.8 g, a mass equivalent of or even exceeding the snail's own body weight, illustrating the ecological importance a single trematode species that contributes a considerable amount of cercarial mass to an aquatic ecosystem. Since *T. szidati* is only one out of 37 species that produce large amounts of cercariae, the overall cercarial biomass emitted into the Ruhr reservoirs is comparable to the impressive numbers recently calculated for marine (Thieltges et al. 2008), estuary (Kuris et al. 2008) and North American freshwater ecosystems (Preston et al. 2013). The results of this study demonstrate how trematodes, despite their small individual size, significantly contribute to the biotic productivity in the Ruhr freshwater system.

Since bird schistosomes are the causative agents of swimmer's itch, a re-emerging disease in Europe (Soldánová et al. 2013), the disease risk factors in the Ruhr area based on the occurrence, distribution and biology of these parasites was assessed for one reservoir (Baldeneysee). Two bird schistosome species, *Trichobilharzia franki* and *T. szidati,* could be detected at several sampling sites in Baldeneysee, where abundant lymnaeid snail populations were present. Although these species showed only low prevalence, human infections are well possible, due to the high numbers of cercariae that can be released from individual infected snail hosts in short periods of time, as shown in the laboratory emission studies.

With a total of 37 species, the trematode species richness in the Ruhr reservoir system was considerably higher than the trematode species richness in snail intermediate hosts described from most other well-studied ecosystems (e.g. Faltýnková 2005, Faltynkova & Haas 2006, Thieltges et al. 2006, Żbikowska 2007, Kuris et al. 2008, Lagrue & Poulin 2015). Altogether, *Radix auricularia* harboured by far the most prevalent, species rich and diverse trematode fauna of all studied hosts, supporting the assumption that *R. auricularia* plays the most important role in the life cycle of trematodes in large reservoirs and lakes, comparable to the dominant role of *L. stagnalis* described for

small pond systems. Similarly, *G. albus* harboured more diverse and prevalent trematode communities compared to *S. nitida*, which contrasts with the situation observed for these two species in small ponds (Faltýnková et al. 2008a). The analysis of the data from the Ruhr, therefore, suggests a characteristic host-parasite dynamics in large reservoir systems.

The majority of trematode species in the Ruhr requires fish-eating or anatid birds as final hosts and almost all trematode species have life cycles involving trophic transmission of the parasites to their respective final host, which provides information on trophic interactions and energy flow in the ecosystem. Since trematodes with trophic transmission strategies often directly or indirectly alter their host's behaviour in order to facilitate transmission success to the next host (see e.g. Lafferty & Morris 1996), they actively shape the structure of food webs through which they are transmitted. Therefore, the rich and abundant trematode fauna in the Ruhr freshwaters plays a highly complex role in the food web connectivity in the Ruhr reservoirs.

Overall, the selected aspects of trematodes in the Ruhr river system studied in the context of this thesis provide a broad and comprehensive overview of these parasites in a freshwater ecosystem that is typical for freshwater reservoirs in Europe for the first time. Trematodes are deeply embedded in and active elements of the ecological processes that shape and structure ecological communities, energy flow and the biodiversity of complex ecosystems and the rich trematode fauna in the Ruhr contributes to key aspects that make this ecosystem more diverse, productive and stable, and thus healthy.

1.2 Zusammenfassung

Hintergrund

Parasiten stellen zentrale und wichtige Akteure in Ökosystemen dar und kommen weltweit in nahezu jedem Habitat vor (Poulin 1999, Hudson et al. 2006). Abgesehen von der offensichtlich bedeutenden Rolle mancher Parasitenarten als Krankheitserreger, werden Parasiten in ökologischen Studien häufig vernachlässigt oder für unbedeutend befunden. Erst in den letzten Jahren wurde der ökologischen Bedeutung von Parasiten vermehrt Beachtung geschenkt und Studien haben beispielsweise ihre strukturgebende Rolle in Nahrungsnetzen (Lafferty et al. 2008) oder ihren Beitrag zur Biomasseproduktion in Ökosystemen im Detail beleuchten können (Kuris et al. 2008, Thieltges et al. 2008). Es ist somit nahezu unmöglich Ökosysteme überhaupt vollständig zu verstehen, ohne die darin vorkommenden Parasiten zu beachten (Lafferty et al. 2006a). Darüber hinaus können Parasiten durch ihre häufig komplexen Lebenszyklen und die starke Vernetzung mit ihren Wirten als Bioindikatoren genutzt werden, die Auskunft über den Zustand eines Ökosystems oder mögliche Umweltveränderungen liefern können (Lafferty 1997, Vidal-Martínez et al. 2010, Nachev & Sures, 2015).

Umweltveränderungen wie der Klimawandel und die globale Erwärmung werden massive Auswirkungen auf Ökosysteme und deren Biozönosen inklusive der Parasiten haben, deren Konsequenzen gegenwärtig nur schwer vorherzusagen sind (Marcogliese 2001, 2008). Es ist daher von grundlegender Bedeutung, dass wir die ökosystemaren Zusammenhänge und Funktionen von Wirt-Parasiten Systeme verstehen. Einige aquatische Ökosysteme (marine Systeme, Ästuare, Süßwasserseen) wurden hinsichtlich der Rolle von Parasiten bereits eingehend untersucht und liefern weitreichende Informationen, die zum besseren Verständnis der komplexen Interaktionen und zukünftige Umweltveränderungen beitragen (z.B. Kuris et al. 2008, Preston et al. 2013, Thieltges et al. 2013, Lagrue et al. 2015). Unser Wissen über die Rolle von Parasiten in anthropogenen Gewässern, wie Talsperren und Stauseen, ist jedoch stark begrenzt und weitreichende Studien über die ökologische Funktion von Parasiten in diesen Systemen in Europa fehlen gänzlich. Dies ist besonders bedenklich, da solche Gewässer in dicht besiedelten Gebieten zentrale und grundlegende Funktionen erfüllen und der Trinkwasserversorgung, Naherholung sowie der Schaffung lokaler Biodiversitäts-Hotspots dienen. Das Ruhrgebiet in Deutschland stellt eine der größten und dicht besiedelten urbanen Regionen Europas dar, und die Ruhr und ihre Nebenflüsse verfügen über ein umfassendes Netzwerk aus Talsperren und Stauseen, die der Wasserversorgung der Region dienen. Über die ökologische Bedeutung von Parasiten der Ruhr liegen bislang keine umfassenden Studien vor.

Die vorliegende Dissertation soll diese Lücke schließen und die ökologische Rolle von Trematoden in aquatischen Schnecken in den Ruhrstauseen erfassen. Digene Trematoden eignen sich besonders, um die Strukturen in Ökosystemen zu untersuchen, da sie durch ihre komplexen Lebenszyklen mit verschiedenen Wirten Einblicke in die Rolle von Parasiten auf verschiedenen Ebenen ermöglichen.
Die einzelnen Studien dieser Arbeit liefern (i) detaillierte integrative Untersuchungen taxonomisch fragwürdiger Trematodengruppen, um die potentielle kryptische Diversität der Parasiten erfassen zu können, (ii) eine Analyse der Produktivität von Zerkarien, um die funktionelle Rolle von Trematoden in den Gewässern beurteilen zu können, (iii) eine Bewertung der Risikofaktoren der Badedermatitis anhand einer Fallstudie aus einem der Ruhrgewässer und (iv) eine Studie der Diversität, Verteilung und Struktur der Trematodengemeinschaft in Schnecken in der Ruhr. Dieser übergreifende Ansatz, der taxonomische, funktionelle, humanrelevante sowie ökologische Aspekte einschließt, soll einen detaillierten und umfassenden Einblick in die komplexe Rolle von Trematoden in Stauseen und Talsperren in Europa ermöglichen.
Um die Diversität und Verteilung von Trematoden in der Ruhr zu erfassen, wurden aquatische Schnecken in fünf Gewässern der Ruhr und ihrer Nebenflüsse, Baldeneysee, Hengsteysee, Hennetalsperre, Sorpetalsperre und Versetalsperre in den Sommermonaten der Jahre 2012 und 2013 gesammelt und auf Infektionen mit Larvalstadien der Trematoden untersucht. Das Hauptaugenmerkt wurde dabei auf Gastropoden der Familien Lymnaeidae und Planorbidae gelegt, die als wichtigste erste Zwischenwirte für Trematoden in Europa dienen (s. Faltýnková & Haas 2006, Soldánová et al. 2011, Brown et al. 2011). Insgesamt wurden 3171 lymnaeide Schnecken der vier Arten *Radix auricularia*, *Stagnicola palustris*, *Radix peregra*, und *Lymnaea stagnalis* sowie 2176 planorbide Schnecken der Arten *Gyraulus albus* und *Segmentina nitida* gesammelt und untersucht. Trematodeninfektionen wurden anhand morphologischer Merkmale und, sofern notwendig, mittels molekularbiologischer Methoden identifiziert.
Insgesamt zeigten 1049 der 5347 untersuchten Schnecken Infektionen mit Larvalstadien von Trematoden, was einer Gesamtprävalenz von 19,6% entspricht. Detaillierte morphologische und molekularbiologische Untersuchung offenbarten kryptische Trematodenarten innerhalb der Gattungen *Echinostoma*, *Petasiger* und *Diplostomum*. So erlaubten die Untersuchungen der Zerkarien von *Echinostoma* spp. die Beschreibung zweier kryptischer Arten der '*revolutum*' Gruppe aus *R. auricularia*, *R. peregra* und *S. palustris*. Die genaue Betrachtung der larvalen Trematodenstadien aus *G. albus* bestätigte den distinkten Status von drei Arten der Gattung *Petasiger*, die sympatrisch an einer Probestelle vorkamen und somit die hohe Trematodendiversität auf kleinstem geographischem Raum verdeutlichen. Insgesamt sieben verschiedene Arten der Gattung *Diplostomum* konnten in den Schlammschneckenpopulationen der Ruhrstauseen nachgewiesen werden, darunter drei beschriebene Arten, *D. spathaceum*, *D. pseudospathaceum* und *D. parviventosum* sowie vier weitere

Abstammungslinien, '*Diplostomum* sp. Clade Q' und '*D. mergi'* Lineages 2-4. Die detaillierten morphologischen und molekularen Analysen erlaubten es dabei, '*D. mergi'* Lineages 1 von Georgieva et al. (2013a) als *D. parviventosum* zu identifizieren. Bemerkenswerterweise waren die Schneckenarten *L. stagnalis* und *S. palustris* mit nur einer einzigen Art, *D. pseudospathaceum*, infiziert, während *R. auricularia* eine ungleich diversere Fauna mit sechs verschiedenen *Diplostomum* Arten beherbergte. Diese ungleiche Verteilung der Trematodenarten bedarf weiterer Untersuchungen und bietet ein aufschlussreiches Modellsystem zur Untersuchung von Wirt-Parasit Spezifität und Co-Evolution nahe verwandter Arten. Insgesamt tragen die taxonomischen Studien dieser Arbeit zum besseren Verständnis der untersuchten Trematodengattungen bei und zeigen die hohe kryptische Diversität dieser Parasiten in den Ruhrstauseen.

Das Wissen um die bloße Diversität von Trematoden in ihren Zwischenwirten liefert jedoch keine Anhaltspunkte über die funktionelle Rolle der Parasiten in den Ökosystemen. Um den Beitrag der Trematodenlarven aus Schnecken in europäischen Frischwassersystemen besser beurteilen zu können, wurde die Zerkarienproduktion der Vogelschistosomen *Trichobilharzia szidati* aus *L. stagnalis* untersucht. Die Studie zeigte eine mittlere tägliche Produktionsrate von 2621 Zerkarien pro Schnecke, jedoch konnten Produktionsspitzen mit bis zu 29 560 individuelle Larven pro Schnecke und Tag nachgewiesen werden. Berechnet für die Lebensdauer einer einzelnen Schnecke ergibt die mittlere Produktion eine kumulative Zerkarienbiomasse von 4,8 Gramm, was dem Eigengewicht der Wirtsschnecke entspricht oder dieses sogar übersteigt. Da *T. szidati* nur eine der insgesamt 37 in der Ruhr gefunden Arten ausmacht, von denen alle große Zerkarienzahlen produzieren, ist die Gesamtproduktivität der Trematoden in diesen Stauseen durchaus vergleichbar mit der hohen Parasitenbiomasse, die für marine (Thieltges et al. 2008), ästuare (Kuris et al. 2008) und nordamerikanische Süßwasserökosysteme (Preston et al. 2013) beschrieben wurde. Die Studie zeigt somit die ökologische Bedeutung von Trematoden, die trotz ihrer geringen individuellen Größe einen substantiellen Beitrag zur Biomasse in den aquatischen Ökosystemen der Ruhr leisten.

Da Vogelschistosomen als Erreger der Badedermatitis in Europa eine bedeutende Rolle spielen (Soldánová et al. 2013), wurden die Infektionsrisiken für Menschen in der Ruhr evaluiert. Hierzu wurden am Beispiel des Baldeneysees das Vorkommen und die Verteilung von Vogelschistosomen erfasst und Risikofaktoren aufgrund der Biologie der Parasiten ermittelt. Zwei relevante Arten, *Trichobilharzia franki* und *T. szidati*, konnten an verschiedenen Stellen im Baldeneysee, an denen jeweils abundante Schneckenpopulationen vorkamen, nachgewiesen werden. Obwohl beide Arten charakteristisch niedrige Prävalenz zeigten, ist ein Infektionsrisiko in diesem Gewässer gegeben, besonders da einzelne infizierte Schnecken in der Lage sind, große Mengen infektiöser Zerkarien abzugeben, wie die Experimente zur Zerkarienproduktion zeigen konnten.

Mit insgesamt 37 Arten ist die Trematodenfauna in Schnecken in der Ruhr deutlich artenreicher als die Gemeinschaften in Schnecken der meisten anderen bisher untersuchten Ökosysteme (z.B. Faltýnková 2005, Faltýnková & Haas 2006, Thieltges et al. 2006, Żbikowska 2007, Kuris et al. 2008, Lagrue & Poulin 2015). Insgesamt verfügte *Radix auricularia* über die mit Abstand artenreichste und diverseste Trematodengemeinschaft und stellt somit den wichtigsten ersten Zwischenwirt in den Ruhrstauseen dar. Dies stützt die Annahme, dass *R. auricularia* in großen Seen und Stauseen eine ähnlich bedeutende Rolle im Lebenszyklus von Trematoden spielt, wie *Lymnaea stagnalis* in kleinen Gewässern und Tümpeln. Gleichermaßen zeigte sich bei planorbiden Wirten ein ähnliches Bild und *Gyraulus albus* wies deutlich artenreichere und prävalentere Trematodengemeinschaften auf als *Segementina nitida*. Verglichen mit der dominanteren Rolle von *S. nitida* in kleinen Gewässern und Teichen (Faltýnková et al. 2008a), zeigten diese Arten somit in Stauseen eine gegensätzliche Rolle. Die Analyse der Daten aus den Ruhrstauseen legt somit nahe, dass diese Gewässertypen über eine charakteristische Wirt-Parasit Dynamik verfügen.

Die Mehrheit der Trematodenarten aus der Ruhr benötigen zur Vervollständigung ihrer Lebenszyklen fischfressende Vögel oder Entenvögel als Endwirte und fast alle Trematoden verfügen über trophische Übertragungswege zu ihren Endwirten. Diese Informationen lassen wertvolle Rückschlüsse über die trophischen Beziehungen und Energieflüsse innerhalb des Ökosystems zu. Da Trematoden mit tropischen Übertragungsstrategien häufig das Verhalten ihrer Wirte manipulieren oder beeinflussen, um die Übertragungswahrscheinlichkeit in den nächsten Wirt zu begünstigen (Lafferty & Morris 1996), tragen sie aktiv zur Strukturierung der Nahrungsnetze bei, durch die sie übertragen werden. Der artenreichen und abundanten Trematodenfauna in Schnecken fällt somit eine zentrale und komplexe Rolle in der Konnektivität und Strukturierung von Nahrungsnetzen und Energieflüssen in diesem Ökosystem zu.

In ihrer Gesamtheit ermöglichen die im Rahmen dieser Dissertation erarbeiteten Aspekte von Trematodeninfektionen in Schnecken der Ruhrstauseen zum ersten Mal einen übergreifenden und detaillierten Überblick über die wichtige und vielschichtige Rolle dieser Parasiten in Stauseen in Europa. Trematoden stellen aktive Elemente in Ökosystemen dar, in denen sie maßgeblich zur Biodiversität, Biomasse und Struktur der Lebensgemeinschaften und somit zur Stabilität und Funktionalität von Ökosystemen beitragen.

2. Introduction

2. Introduction

Ecology can be defined as the study of intra- and interspecific interactions between organisms and the interaction between organisms and their non-living environment (Poulin 2007), and ecologists are dealing with a wide range of topics from the diversity and distribution of species to nutrient cycling and energy flow in ecosystems, or the role humans play in these interactions. Consequently, ecologists have traditionally been interested in all forms of life from prokaryotic bacteria to macro-eukaryotes, e.g. large mammals. Parasitic organisms live in intimate contact with another organism, the host, on which they are energetically dependent and on which they exert some sort of harm (Thieltges et al. 2013). Parasites are, therefore, consumers that use their hosts' resources and thus are integral elements of food webs (Lafferty et al. 2006b, Kuris et al. 2008). Hence the study of parasites falls well within the wide field of ecology. However, parasites and their relationship to and interactions with hosts have traditionally been studied by a comparably small group of scientists, parasitologists, and the exchange of ideas between ecologists and parasitologists has long been rather limited (Poulin 2007).

Exceptions to this are parasite species that affect humans, livestock or crops as pathogens. These have often been extensively studied from veterinary, phytopathological or medical perspectives. Indeed, parasitic pathogens are responsible for some of the world's most important diseases of humans, plaguing millions of people and constituting major global health problems. Malaria alone has accounted for 200 million cases and an estimated 584,000 deaths, mostly among African children, in 2013 (WHO 2014), and helminth infections in humans are estimated to affect up to 4.3 billion people worldwide, i.e. more than half of the planet's population (Crompton 1999). Highlighting the medical relevance of these parasites, the 2015 Nobel Prize in Physiology or Medicine was jointly awarded to researchers working on therapies against Malaria and helminth infections.

Except for this obviously important role of some species as disease agents, parasites have long been neglected and considered unimportant in the context of most ecological studies, and vice versa new ecological ideas and theories were often ignored by parasitologists (Poulin 2007). However, the last 25 to 30 years have seen a slow but steady advancement of our understanding of parasites as important and integral elements of ecosystems (Poulin 1999, Hudson et al. 2006), leading to a new area of research at the junction of ecology and parasitology (Thomas et al. 2009), called Ecological Parasitology.

Parasites are highly diverse and occur in literally every ecosystem, and parasitism is regarded as the most popular lifestyle on Earth (Hechinger & Lafferty 2005 and references therein). Since we know little of the total diversity on our planet (with about 90% of the estimated 8.7 million species still

awaiting description, Mora et al. 2011), we cannot precisely know the total number of parasitic species but current estimations are ranging from one third to over half the diversity on the planet (reviewed in Poulin 2014) and practically all free-living metazoan species harbour one or more parasite species (Poulin & Morand 2004). At the ecosystem level parasites have been shown to be important structuring forces in trophic transmissions and food webs (Lafferty et al. 2006b). They make up a large proportion of the cumulative biomass and thus considerably contribute to the energy flow within ecosystems (Kuris et al. 2008, Thieltges et al. 2008). Furthermore, parasites provide vital 'ecosystem services', such as regulation of host abundance or even concentration of pollutants (Sures 2003, Dobson et al. 2008). It has been argued, therefore, that it is even impossible to fully understand ecosystems without considering parasites (Lafferty et al. 2006a). Moreover, based on their often complex life cycles and strong interaction in ecosystems, parasites may serve as useful bioindicators to assess environmental conditions and changes (Lafferty 1997, Vidal-Martínez et al. 2010, Nachev & Sures, 2015).

Our environment is rapidly and drastically changing due to anthropogenic pressures, such as population growth, increased pollution, growing needs of resources and, maybe most challenging, global warming. Especially climate change and global warming are regarded to have major impacts on parasites with many, often unforeseeable, consequences on parasite transmission patterns, life-history traits, virulence, and on entire ecosystems (Marcogliese 2001, 2008). Moreover, we do not know how these complex changes in ecosystems will affect us in the future. Examples have shown how environmental alterations, such as eutrophication of water bodies due to agriculture, industrialization or urbanization, can affect parasite communities and trigger complex and often drastic changes that can restructure whole ecosystems. A well-documented example are local extinctions of amphibian populations in North American freshwaters that were caused by increased prevalence of the trematode *Riberioa ondatrae*. These parasites utilise amphibians as a second intermediate host in which they cause malformations that make infected animals easy prey for predatory birds, the parasite's final host. As a result of anthropogenic nutrient enrichment in these ecosystems, suitable conditions for abundant snail intermediate host populations were created, offering an ideal habitat for the asexual reproduction of *Riberioa ondatrae* cercariae that then infect amphibian tadpoles and cause malformations (Johnson & Chase 2004, Johnson et al. 2007). Such examples show the essentiality of understanding host-parasite systems at the ecosystem level.

While there are some exceptionally well-studied aquatic ecosystems that focus on the roles of parasites in these systems and allow predictions of the impact and possible effects of environmental changes (e.g. Kuris et al. 2008, Preston et al. 2013, Thieltges et al. 2013, Lagrue et al. 2015), these systems are geographically far apart and highly different (marine, intertidal with brackish water, or freshwater). On the other hand, our knowledge of host-parasite interaction in other ecosystems, such as man-made waterbodies (e.g. impounded lakes, reservoirs, channels etc.), is still very limited and

large-scale studies focussing on the role of parasites in such systems are scarce. However, especially impounded lakes and reservoir systems play important roles in densely populated areas, where they serve as water storage for drinking water supply and are widely used for recreational activities, e.g. swimming or water sports. Furthermore, such man-made freshwater systems constitute important biodiversity hot spots, especially in urbanised areas. With more than 5 million people and an area of approximately 4,400 km^2 the metropolitan Ruhr area is one of the most densely populated and largest urban agglomerations in Europe. The eponymous extensive river system of the Ruhr and its tributaries consists of a network of interconnected reservoirs and impounded lakes (hereinafter referred to as reservoirs) that play a vital role for the whole region. However, our knowledge of the ecological role of parasites in these aquatic habitats is still fairly limited. It is therefore crucial that we understand the integral role of parasites to fully comprehend the ecology of such systems. In order to study complex interactions in ecosystems, a group of parasites is required that can adequately reflect this complexity and enable comparisons between different habitats.

Digenean trematodes are a particularly promising group of parasites that lend themselves to investigating and assessing environmental conditions and ecosystem changes in aquatic systems (Huspeni & Lafferty 2004). They are ubiquitous and the most common metazoan parasites in aquatic ecosystems (Koehler et al. 2012), which makes them valuable bionindicators to compare different ecosystems. With an estimated number of 24,000 known species (Dobson et al. 2008), digenean trematodes are amongst the most species-rich parasite groups. Most importantly, trematodes typically have complex multiple-host life cycles, with a wide variety of vertebrate species serving as definitive hosts, molluscs (mainly gastropods, rarely bivalves) as first intermediate host, and a large group (depending on trematode species, usually fishes or invertebrates) as potential second intermediate host (Figure 1). Such multiple-host life cycle enables us to study the complex roles of parasites in ecosystems at many levels and in great detail. Moreover, due to these complex life cycles with snails serving as common first intermediate hosts, trematode communities in snails reflect the richness and abundance of free-living assemblages and are suitable bionindicators of the diversity of free-living species (Hechinger et al. 2007).

Adult digeneans live in the vertebrate definitive host, in which they reproduce sexually, typically via cross-fertilisation. Most trematode life cycles are partially aquatic and eggs produced by the adult parasites are excreted into the aquatic environment, usually with the final host's faeces, and contain a single short-lived larval stage each, the miracidium. For aquatic trematodes the ciliated miracidium hatches in the water and actively seeks out and infects a suitable first intermediate mollusc host, in which the miracidium transforms into a mother sporocyst and asexual reproduction occurs, either via daughter sporocysts or rediae. In the course of this process, large parts of the snail tissue are exploited by the parasites that replace the digestive gland or the gonads, usually resulting in castration

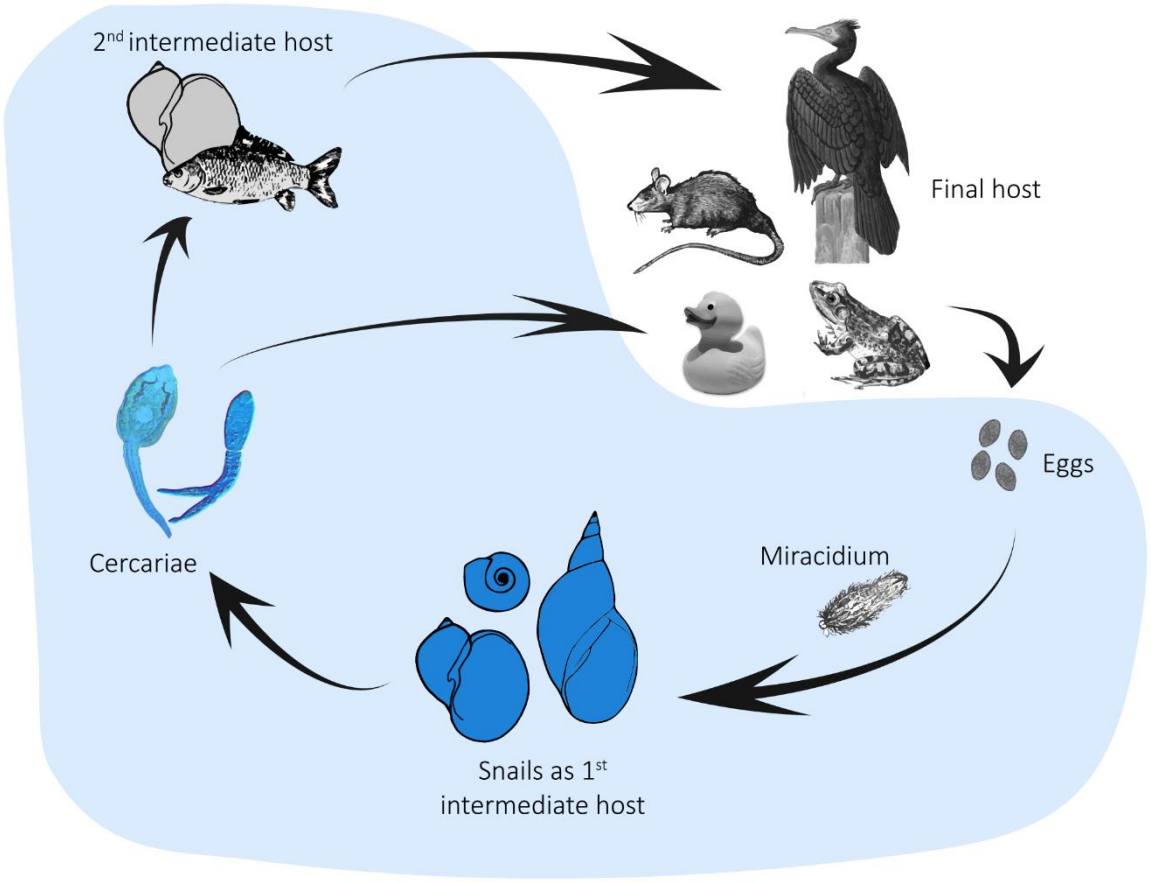

Figure 1 Schematic drawing of a typical aquatic life cycle of digenean trematodes showing the free-living parasite stages and the different host groups. 1st intermediate snail hosts and free-swimming trematode larval stages, the cercariae that are subject of this study are highlighted in blue. Area highlighted in light blue indicates stages that usually occur in water.

of the host snail. Each sporocyst or redia produces numerous infective dispersive stages, the cercariae, which can be released from an infected snail host in large numbers and over long periods of time. Since cercariae have limited energy reserves and are short-lived, it is crucial that cercarial emergence time is triggered by environmental conditions (e.g. sunshine or temperature) that will enhance the likeliness of encountering the next target host and the individual cercarial behaviour is adapted to enhancing the transmission success (Combes et al. 1994). Depending on the transmission strategy, cercarial morphology is very diverse and can often be used to distinguish trematode species. After release from the snail host, cercariae disperse in the aquatic environment to seek out a suitable second intermediate host and upon contact shed their tail and encyst in or on the host as metacercariae. The second intermediate host is usually part of the definitive host's diet and the parasites are trophically transmitted to the next host. In order to facilitate transmission to the next host, some trematode species can alter the behaviour of the infected intermediate host, making parasitized prey more susceptible to predation by final host predators (Lafferty & Morris 1996). Alternatively cercariae of some species, such as trematodes of the family Schistosomatidae, directly seek out and penetrate their

definitive host or encyst on aquatic plants waiting to be directly eaten by a suitable definitive host (e.g. *Notocotylus attenuatus*). Once in the definitive hosts, the trematodes migrate to their preferred site of infection where they reach sexual maturity, reproduce and produce eggs, thereby completing the life cycle. This generalized life cycle applies to the majority of trematode species but variations and exceptions can occur depending on the life strategies of certain species, e.g. the land-based life cycle of *Dicrocoelium dendirticum* relies on fully embryonated eggs being directly eaten by a terrestrial snail.

Despite their importance in aquatic habitats, the ecology of trematodes has never been extensively studied in complex interconnected reservoir systems in densely populated areas that are subject to intense anthropogenic pressure and use. A preliminary study of trematodes from the Ruhr area in Germany revealed a diverse trematode fauna with species-rich and abundant trematode communities, including the presence of bird schistosomes that can cause swimmer's itch in humans (Soldánová et al. 2010), indicating that the relatively young man-made reservoirs of the Ruhr river system may offer ideal conditions to study the role of trematodes in reservoirs. Such a single study can however only provide a first glimpse at the complex role of trematodes in an ecosystem, leaving many questions unanswered and many issues unresolved that require further attention.

Traditionally morphological characteristics of adult trematodes, cercariae and metacercariae have been used to identify trematode species and study the parasites' diversity. However, morphological species identification can be problematic, especially in groups with morphologically similar cryptic species. The incorporation of molecular data has facilitated unambiguous species identification, diagnoses of problematic taxa and the discovery of cryptic species (Georgieva et al. 2013a). Furthermore, molecular tools allow the elucidation of complex trematode life cycles by matching sequences from different developmental stages of the parasite (e.g. metacercariae, cercariae and adults) that were sampled at different times and/or locations (Criscione et al. 2005). Integrative approaches that combine both morphological and molecular approaches are most promising, since they allow accurate identifications and descriptions as well as thorough comparisons with already described specimens. The first study of trematodes from the Ruhr area identified trematode diversity solely based on morphological species identification. Since taxonomy of many trematode groups has been shown to be much more complex than previously estimated and the diversity much higher than morphological assessment alone can reveal, it is likely that these samplings do not reflect the whole diversity of trematodes in the studied system. The high cryptic diversity recently shown in North American and European *Diplostomum* spp. suggests this (Locke et al. 2010, Georgieva et al. 2013a, Blasco-Costa et al. 2014, Pérez-del-Olmo et al. 2014), since these parasites were prevalent in the above-mentioned study from Germany. It remains a crucial question whether taxonomical in-depth studies based on integrative morphological and molecular approaches can provide additional insights and reveal hidden trematode diversity.

However, the knowledge of the full diversity of trematode species in a system does not give us information on their functional role in the respective ecosystem. Trematodes have been shown to contribute to a large extend to the biomass in aquatic ecosystems and play important parts in structuring food webs and energy flow (Thieltges et al. 2008, Preston et al. 2013, Lambden & Johnson 2013), yet no studies on this exist from German freshwaters. It remains to be studied what functional role trematodes play in reservoir systems and what the biomass contribution of individual trematode species amounts to in such habitats.

Furthermore, some trematode species are medically important disease agents, such as human schistosomes that cause Bilharziasis or Schistosomiasis with an estimated 230 million people infected and a further 500 million at risk of infection worldwide (Grimes et al. 2015). In Europe, bird schistosomes are the causative agents of swimmer's itch or cercarial dermatitis, an allergic skin inflammation caused by the free-swimming cercariae that is considered a re-emerging disease in Europe (reviewed in Soldánová et al. 2013, Appendix II). These aspects highlight the importance to study snail-trematode interactions in aquatic ecosystems also from a human health perspective. The first study on trematode communities from the Ruhr revealed the presence of bird schistosomes, which exhibited a high prevalence at some of the sampling sites (Soldánová et al. 2010). Since these systems are man-made waterbodies that play important roles in densely populated areas where some of the reservoirs are used for recreational activities, such as swimming, surfing or canoeing, the relevance of trematodes for humans needs to be assessed in the context of given local factors in order to provide a conclusive risk assessment of human infections.

Finally, since it is impossible to fully understand ecosystems without considering its parasites (Lafferty et al. 2006a), it is crucial to assess the organization and dynamics of trematodes and the overall diversity of trematode assemblages in snails in the Ruhr reservoir system. The extensive river system of the Ruhr and its tributaries consists of a network of interconnected reservoirs, and it remains to be tested whether the diversity of trematode assemblages in snails is equally distributed among the connected waterbodies and different snail hosts or whether there are structural patterns in the trematode assemblage and community structure. Altogether, such an approach would help to advance our understanding of the complex host-parasite interactions in reservoir systems and would reveal valuable information on final host occurrence and trophic interactions in the ecosystem.

This thesis therefore aims at addressing the importance of trematodes in the Ruhr reservoir systems by identifying larval trematode infections of four lymnaeid (*Radix auricularia*, *Radix peregra*, *Stagnicola palustris* and *Lymnaea stagnalis*) and two planorbid snail species (*Gyraulus albus* and *Segmentina nitida*). Thereby, I will, quite literally, open a 'can of worms'. However, more than creating a situation that causes a lot of problems and confusion, this shall reveal the complex role trematodes play in reservoir ecosystems. The following individual studies of this work provide (i) detailed

integrative approaches to taxonomically questionable trematode groups to assess the full diversity in the ecosystem, (ii) an analysis of the productivity and emergence of trematode cercariae to assess the parasites' functional role in the ecosystem, (iii) an evaluation of the risk factors of swimmer's itch via a detailed case study of one of the Ruhr lakes and finally (iv) an assessment of the overall diversity, distribution and community structure of larval trematodes and the identification of potential trematode transmission pathways between their host groups. These aspects will be individually addressed and discussed in detail in the following chapters and summarized and debated in an overall discussion. Altogether, this holistic approach, encompassing trematode taxonomy, their functional part in ecosystems, the role of medically relevant species and their contribution to an ecosystem's diversity will provide a detailed and comprehensive insight into the complex role of trematodes in an important reservoir system in Europe.

3. Aims and objectives

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Aims

The aims of this study are to address essential aspects of trematode communities in snail hosts in the Ruhr reservoir system in order to give a comprehensive overview of their role in this ecosystem. To be able to do so, taxonomic, functional, public health and ecological aspects of trematode infections in six snail host species were studied in detail. Together, these aspects, addressed in individual studies, will provide a broad but detailed insight into the role of trematodes in interconnected man-made waterbodies in Europe and expand our knowledge on this important group of parasites. In order to do so, larval trematode infections of four lymnaeid (*Radix auricularia*, *Radix peregra*, *Stagnicola palustris* and *Lymnaea stagnalis*) and two planorbid snail species (*Gyraulus albus* and *Segmentina nitida*) from five waterbodies of the Ruhr reservoir system were identified and studied.

Objectives

The individual objectives of this work are as follows:

I. Taxonomic aspects

Application of thorough integrative approaches combining morphological and molecular methods to taxonomically problematic trematode groups or specimens that showed conspicuous morphological features to reveal potential cryptic diversity and provide detailed descriptions of cercariae

II. Functional aspects

Assessment of the productivity and emergence of cercariae, using the example of *Trichobilharzia szidati* cercariae from *Lymnaea stagnalis*, by determining (i) temporal cercarial emission patterns and (ii) the average daily output rate of cercariae per snail that will allow accurate estimations of the biomass of cercariae released into the ecosystem

III. Public health aspects

Identification of the occurrence of the main causative agents of simmer's itch, *Trichobilharzia* spp., in Lake Baldeney (Baldeneysee) and estimation of risk factors based on parasite and host biology and the given local situations

IV. Ecological aspects

Assessment of the overall diversity and distribution of larval trematode assemblages in the six studied snail species and analysis of trematode component community composition and structure in the Ruhr river system, as well as the identification of trematode transmission pathways between their hosts

4. Materials and methods

4. Materials and methods

This chapter presents a general overview of the sampling approach and the individual methods applied in the laboratory in order to identify trematode infections in the individual snail populations and to assess their role in the Ruhr river system. More detailed accounts of materials and methods of the individual studies are presented in the respective chapters.

Study area and sampling

In order to assess the taxonomic diversity and distribution patterns of trematode in the Ruhr area, we collected snails at several sampling sites in five reservoirs of the Ruhr river catchment area in Germany: Baldeneysee (51°24' 20.08"N, 7°2'22.47"E); Hengsteysee (51°24'52.17"N, 7° 27'42.55"E); Hennetalsperre (51°19'50.97"N, 8°15'46.82"E); Sorpetalsperre (51°20'15.01"N, 7°56'46.18"E); and Versetalsperre (51°10'55.71"N, 7°40'57.12"E) (Figure 2). In order to be able to accurately estimate the potential risk of swimmer's itch in Baldeneysee, some additional sampling sites were selected at this lake to assess the occurrence of bird schistosomes of the genus *Trichobilharzia* (see Chapter III).

All waterbodies were constructed during the first half of the 20th century along the Ruhr river and its tributaries as drinking water reservoirs, natural river water treatment plants and to regulate the water flow of the whole river system. Today, such reservoir systems are typical for man-made water bodies in Central Europe and fulfil viable functions in densely populated areas, such as drinking water supply, the generation of hydropower, or are used for recreational activities. All reservoirs and impounded lakes in this area are centrally operated by the Ruhr River Association (Ruhrverband), making it the largest multi-reservoir system in Germany (Ruhrverband 2015). Table 1 provides information on the individual reservoirs.

Table 1 General characteristics of the study reservoirs and water quality data.

		Baldeney-see	Hengstey-see	Sorpetal-sperre	Hennetal-sperre	Versetal-sperre
Reservoir data[a]	**Construction (year)**	1931-1933	1917-1929	1926-1935	1901-1905 (1950-1955)	1929-1951
	Surface area (km^2)	2.64	1.36	3.30	2.10	1.8
	Depth (m)	3.14 (mean)	1.94 (mean)	up to 57	up to 51	up to 52
	Volume (Mio. m^3)	7.6	3.3	70	38.4	32.8
Water quality data	**Eutrophication status**	eutrophic	eutrophic	mesotrophic–oligotrophic[b]	mesotrophic[b]	oligotrophic[b]

[a] Ruhrverband (2015)
[b] AWWR & Ruhrverband (2013)

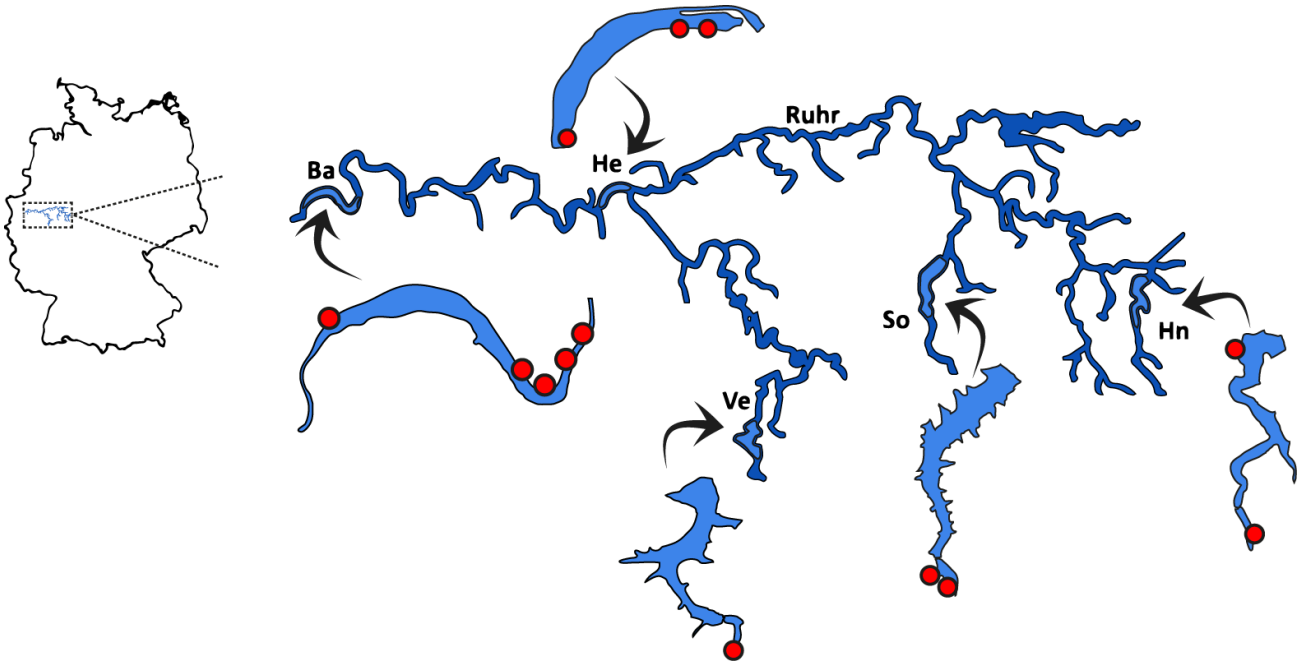

Figure 2 Map of the Ruhr area and the reservoir system studied. Individual sampling sites are indicated by red dots. *Abbreviations*: Ba, Baldeneysee; He, Hengsteysee; Ve, Versetalsperre; So, Sorpetalsperre; Hn, Hennetalsperre.

Each sampling site was visited repeatedly (11 times) during the summer months (May – September) in two consecutive years, 2012 and 2013. At each site, snails were collected by hand or with the aid of hand-nets from stones, sediment and aquatic vegetation. Although sampling was not quantitative, the sample sizes reflected the abundance of the individual hosts at the sampling sites, since the sampling effort (i.e. time spent at each locality) was comparable at each site.

A total of 6,569 snails belonging to 19 species from eight families were collected during that time (Table 2). The most abundant species belonging to the families Planorbidae and Lymnaeidae that are known to serve as important intermediate hosts for digenean trematodes were selected for thorough examination for trematode infections (highlighted in bold in Table 2). In total 5,347 snails [1,909 *Radix auricularia* (L.), 349 *R. peregra* (Müller), 668 *Stagnicola palustris* (Müller), 245 *Lymnaea stagnalis* (L.), 1,981 *Gyraulus albus* (Müller) and 195 *Segmentina nitida* (Müller)] were used in the following studies.

Table 2 Snail species collected in the Ruhr reservoirs during the sampling campaigns in 2012 and 2013. Snail species analysed for trematode infections are highlighted in bold.

Family	Snail spp.	Number sampled
Planorbidae	*Ancylus fluviatilis*	159
	Anisus vortex	175
	Bathyomphalus contortus	176
	Gyraulus albus	1,981
	Planorbarius corneus	121
	Planorbis planorbis	4
	Segmentina nitida	195
Lymnaeidae	*Galba truncatula*	61
	Lymnaea stagnalis	245
	Radix auricularia	1,909
	Radix peregra	349
	Stagnicola palustris	668
Sphaeriidae	*Sphaerium* sp.	3
Viviparidae	*Viviparus contectus*	6
Hydrobiidae	*Potamopyrgus antipodarum*	2
Physidae	*Physa fontinalis*	263
	Physella acuta	1
Bithyniidae	*Bithynia tentaculata*	170
Acroloxidae	*Acroloxus lacustris*	81

Trematode identification

After each sampling trip all snails were placed in individual cups with filtered lake water in the laboratory at a temperature of 20°C and exposed to a light source (LED lamps) for two to five days to induce cercarial shedding (see Figure 3). Snails that did not emit cercariae during that time were dissected and carefully checked for prepatent infections. Trematode stages were first identified alive under an Olympus BX51 microscope with the help of appropriate identification keys or other relevant primary sources (e.g. Faltýnková et al. 2007, 2008a, Niewiadomska 1986, Niewiadomska & Kiseliene 1994) and documented with an Olympus UC30 digital camera. For further investigation of specimens that could not be accurately identified and for species belonging to taxonomically problematic groups,

trematode material was fixed in molecular grade ethanol and hot and cold 4% formaldehyde solution for molecular and morphological studies, respectively. Trematode genera that are known to be taxonomically problematic or groups that showed conspicuous morphological features were subjected to thorough morphological and molecular studies to reveal potential cryptic diversity (see Chapter I). Following Bush et al. (1997) parasite prevalence (P) was calculated in each study as the proportion of infected host individuals in relation to total number of host individuals in a population ($P=n_{inf}/N*100$, with n_{inf} = infected snails and N = all snails in a population).

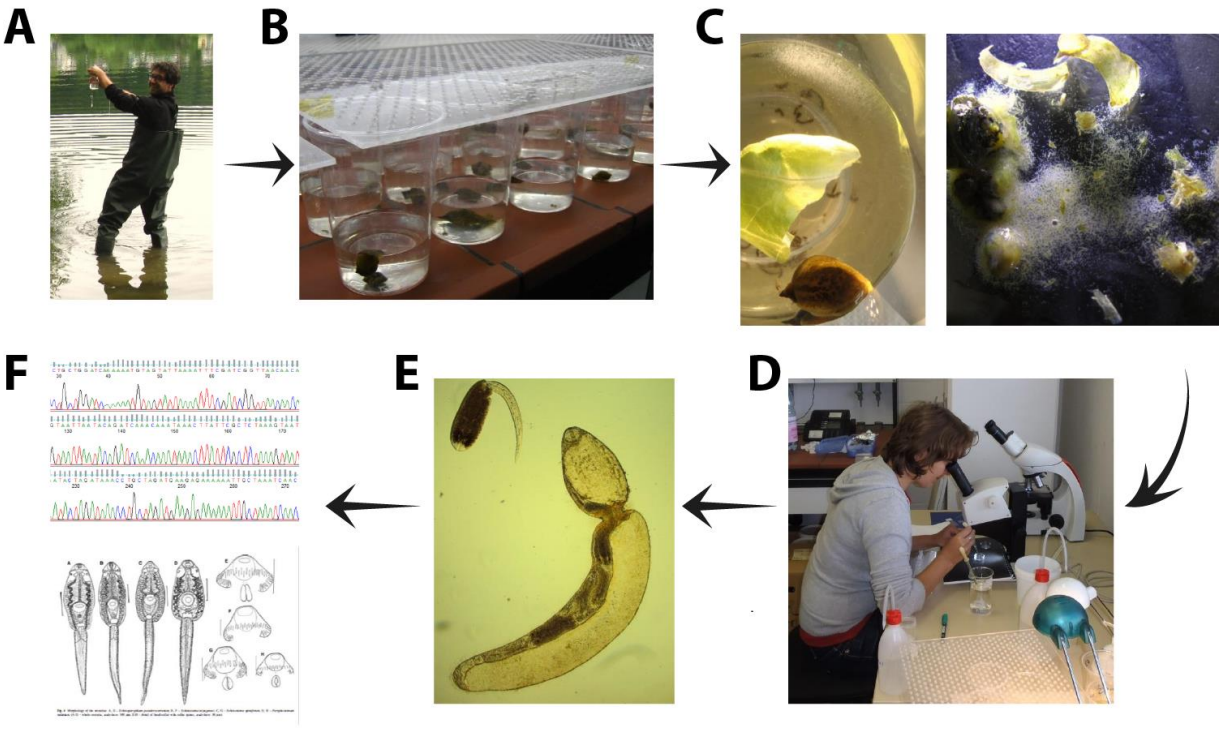

Figure 3 From lake to laboratory: Sampling and identification of trematode infections in snails. A, collecting snails at the lake; B, separating snails in individual cups for cercarial emission; C, released cercariae in cup (left) and intramolluscan trematode stages in dissected snail (right); screening snails for trematode infections; E: trematode redia and cercaria under light microscopy; F: identification of trematode species based on morphology and/or molecular tools.

For the analysis of the cercarial surface morphology and body spination by scanning electron microscopy (SEM), formalin-fixed cercariae were post-fixed in 2% osmium tetroxide for two hours, washed in 0.1 M phosphate buffer, dehydrated through an acetone series, point-dried and sputter-coated with gold. SEM photographs were taken with a JEOL JSM-7401F field emission scanning electron microscope. All SEM photographs were taken at the Laboratory of Electron Microscopy at the Biology Centre of the Czech Academy of Sciences in České Budějovice. In order to obtain metrical data of live and formalin-fixed isolates, measurements from light and scanning electron microscope photographs were taken with the program ImageJ v.1.47 (Abramoff et al. 2004).

For molecular analyses, total genomic DNA was isolated from ethanol-fixed cercariae or rediae obtained from single snail individuals. Polymerase chain reaction (PCR) amplifications of different target gene regions (mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunit 1 [*nad*1], partial fragments of the barcode region of the mitochondrial cytochrome oxidase subunit 1 gene [*cox*1], and the internal transcribed spacer 1 and 2 including the 5.8S subunit of the ribosomal RNA-gene [ITS1- 5.8S-ITS2]) were performed using Ready-To-Go-PCR Beads (GE Healthcare, UK) and the appropriate primers (see Chapter I for full details, e.g. on specific primers and thermocycling profiles used for individual trematode groups). PCR amplicons were purified using Qiagen QIAquick™ PCR Purification Kits (Qiagen Ltd, UK) and sequenced directly for both strands using the respective PCR primers. Sequencing was performed on an ABI Prism 3130xl automated sequencer using ABI Big Dye chemistry (ABI Perkin Elmer, UK) according to the manufacturer's protocol. Contiguous sequences were assembled with MEGA v6 (Tamura et al., 2013) and submitted to GenBank. Distance-based neighbour-joining (NJ), maximum likelihood (ML) and Bayesian inference (BI) analyses were used for tree reconstruction based on newly-generated and published sequences for the individual trematode groups studied (see Chapter I).

More detailed information on the methodology of the individual studies are presented in the respective chapters, i.e. on the cercarial emission experiment (Chapter II), the assessment of risk factors of swimmer's itch (Chapter III), and the calculation of diversity indices of parasite assemblages and component communities in the different snail host species (Chapter IV).

5. Chapter I

Taxonomy of selected trematode groups – a closer look

5. Chapter I

Taxonomy of selected trematode groups – a closer look

This chapter contains the three following studies that investigate the diversity of taxonomically questionable groups by applying an integrative approach, combining thorough morphological and molecular methods.

5.1 Georgieva, S.*, Selbach, C.*, Faltýnková, A., Soldánová, M., Sures, B., Skírnisson, K., & Kostadinova, A. (2013). New cryptic species of the '*revolutum*' group of *Echinostoma* (Digenea: Echinostomatidae) revealed by molecular and morphological data. *Parasites & Vectors, 6*(1), 64. *Authors contributed equally

5.2 Selbach, C., Soldánová, M., Georgieva, S., Kostadinova, A., Kalbe, M., & Sures, B. (2014). Morphological and molecular data for larval stages of four species of *Petasiger* Dietz, 1909 (Digenea: Echinostomatidae) with an updated key to the known cercariae from the Palaearctic. *Systematic Parasitology, 89*(2), 153–66.

5.3 Selbach, C., Soldánová, M., Georgieva, S., Kostadinova, A., & Sures, B. (2015). Integrative taxonomic approach to the cryptic diversity of *Diplostomum* spp. in lymnaeid snails from Europe with a focus on the '*Diplostomum mergi*' species complex. *Parasites & Vectors, 8*, 300.

Parasites & Vectors

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New cryptic species of the '*revolutum*' group of *Echinostoma* (Digenea: Echinostomatidae) revealed by molecular and morphological data

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Abstract

Background: The digenean species of *Echinostoma* (Echinostomatidae) with 37 collar spines that comprise the so-called '*revolutum*' species complex, qualify as cryptic due to the interspecific homogeneity of characters used to differentiate species. Only five species were considered valid in the most recent revision of the group but recent molecular studies have demonstrated a higher diversity within the group. In a study of the digeneans parasitising molluscs in central and northern Europe we found that *Radix auricularia*, *R. peregra* and *Stagnicola palustris* were infected with larval stages of two cryptic species of the '*revolutum*' complex, one resembling *E. revolutum* and one undescribed species, *Echinostoma* sp. IG. This paper provides morphological and molecular evidence for their delimitation.

Methods: Totals of 2,030 *R. auricularia,* 357 *R. peregra* and 577 *S. palustris* were collected in seven reservoirs of the River Ruhr catchment area in Germany and a total of 573 *R. peregra* was collected in five lakes in Iceland. Cercariae were examined and identified live and fixed in molecular grade ethanol for DNA isolation and in hot/cold 4% formaldehyde solution for obtaining measurements from fixed materials. Partial fragments of the mitochondrial gene nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) were amplified for 14 isolates.

Results: Detailed examination of cercarial morphology allowed us to differentiate the cercariae of the two *Echinostoma* spp. of the '*revolutum*' species complex. A total of 14 partial *nad*1 sequences was generated and aligned with selected published sequences for eight species of the '*revolutum*' species complex. Both NJ and BI analyses resulted in consensus trees with similar topologies in which the isolates from Europe formed strongly supported reciprocally monophyletic lineages. The analyses also provided evidence that North American isolates identified as *E. revolutum* represent another cryptic species of the '*revolutum*' species complex.

Conclusion: Our findings highlight the need for further analyses of patterns of interspecific variation based on molecular and morphological evidence to enhance the re-evaluation of the species and advance our understanding of the relationships within the '*revolutum*' group of *Echinostoma*.

Keywords: *Radix auricularia*, *Radix peregra*, *Stagnicola palustris*, *Echinostoma*, Cryptic species, Europe

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Background

The digenean species of *Echinostoma* Rudolphi, 1809 (Echinostomatidae) with 37 collar spines that comprise the so-called *Echinostoma* '*revolutum*' complex, qualify as cryptic (*sensu* Bickford *et al.* [1]; see also Pérez-Ponce de León and Nadler [2] for a recent review) due to the interspecific homogeneity of characters used to differentiate species. Only five species, the Eurasian *Echinostoma revolutum* (Frölich, 1802), *E. echinatum* (Zeder, 1803) and *E. jurini* (Skvortsov, 1924), the North American *E. trivolvis* (Cort, 1914) and the African *E. caproni* Richard, 1964, were considered valid in the most recent revision of the group using for species delimitation a single morphological feature of the larval stages (the number of pores of the para-oesophageal gland-cells in the cercaria), the specificity towards the first intermediate host (at the familial level), the ability to infect avian or mammalian hosts (or both) and geographical range on a global scale (continents) [3-5] (but see Kostadinova and Gibson [6] for a critical review). It is worth noting that *E. echinatum* has not been formally described and justified in a taxonomic publication and is not recognised as valid [see 6 for details]. However, recent molecular studies have demonstrated a higher diversity within the '*revolutum*' species complex. Thus one African species, *Echinostoma deserticum* Kechemir et al., 2002, and a yet unidentified species from New Zealand were distinguished based on molecular data [7] (see also [8]), and *E. trivolvis* was found to represent a species complex [9]. Additional data on the geographical distribution of the *Echinostoma* spp. have also been obtained. *E. revolutum* was recorded in Australia [7] and North America [10,11], *Echinostoma paraensei* Lie & Basch, 1967 in Australia and South America [7], and *E.* cf. *robustum* in North and South America [11].

The pioneer molecular studies, predominantly based on laboratory strains, have revealed that the mitochondrial *nad*1 gene provides a better resolution for investiga-

Table 1 List of species/isolates of the '*revolutum*' species complex used in this study, their hosts, localities and GenBank accession numbers

Species	Host	Locality	Accession no.	Reference
***Echinostoma* sp. IG**	*Radix peregra* (isolate RPI1)	Nordic House (Iceland)	KC618448	Present study
***Echinostoma* sp. IG**	*Radix auricularia* (isolate RAG1)	Hengsteysee (Germany)	KC618449	Present study
***Echinostoma* sp. IG**	*Radix auricularia* (isolate RAG2)	Hengsteysee (Germany)	KC618450	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPI2)	Lake Myvatn (Iceland)	KC618451	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPI3)	Lake Myvatn (Iceland)	KC618452	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPI4)	Lake Myvatn (Iceland)	KC618453	Present study
Echinostoma revolutum	*Stagnicola palustris* (isolate SPG1)	Hengsteysee (Germany)	KC618454	Present study
Echinostoma revolutum	*Radix auricularia* (isolate RAG3)	Hennetalsperre (Germany)	KC618455	Present study
Echinostoma revolutum	*Radix auricularia* (isolate RAG4)	Hennetalsperre (Germany)	KC618461	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPG1)	Hennetalsperre (Germany)	KC618456	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPG2)	Hennetalsperre (Germany)	KC618457	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPG3)	Hennetalsperre (Germany)	KC618458	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPG4)	Hennetalsperre (Germany)	KC618460	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPG5)	Hennetalsperre (Germany)	KC618459	Present study
Echinostoma caproni	na	Cameroon	AF025838	Morgan & Blair [7,13]
Echinostoma caproni	na	Madagascar, Egypt	AF025837	Morgan & Blair [7,13]
Echinostoma caproni	*Rattus norvegicus*	Cairo (Egypt)	AJ564378	Marcilla et al. (unpublished)
E. deserticum*	na	Niger	AF025836	Morgan & Blair [7,13]
Echinostoma* cf. *friedi	*Planorbis* sp.	Wales (UK)	AY168937	Kostadinova *et al.* [14]
Echinostoma friedi	*Mesocricetus auratus* (exp.)	Pons, Valencia (Spain)	AJ564379	Marcilla *et al.* (unpublished)
Echinostoma paraensei	na	Brazil	AF025834	Morgan & Blair [7,13]
Echinostoma revolutum	*Radix peregra/Columba livia* (exp.)	Bulgaria	AY168933	Kostadinova *et al.* [14]
Echinostoma revolutum	*Lymnaea elodes/Gallus gallus* (exp.)	Shock Lake, Indiana (USA)	GQ463082	Detwiler *et al.* [11]
Echinostoma revolutum	*Lymnaea elodes*	Pond A, Indiana (USA)	GQ463088	Detwiler *et al.* [11]
Echinostoma revolutum	*Lymnaea elodes*	Pond A, Indiana (USA)	GQ463090	Detwiler *et al.* [11]
Echinostoma revolutum	*Lymnaea elodes*	Pond A, Indiana (USA)	GQ463086	Detwiler *et al.* [11]

Table 1 List of species/isolates of the '*revolutum*' species complex used in this study, their hosts, localities and GenBank accession numbers *(Continued)*

Echinostoma revolutum	*Lymnaea elodes*	Shock Lake, Indiana (USA)	GQ463084	Detwiler *et al.* [11]
Echinostoma revolutum	*Ondatra zibethicus*	Virginia (USA)	JQ670862	Detwiler *et al.* [11]
Echinostoma revolutum	na	"Germany, Europe"	AF025832	Morgan & Blair [7,13]
Echinostoma robustum **	*Lymnaea elodes*	Minnesota (USA)	GQ463054	Detwiler *et al.* [11]
Echinostoma robustum **	*Biomphalaria glabrata/G. gallus* (exp.)	Brazil	GQ463055	Detwiler *et al.* [11]
Echinostoma robustum **	*Lymnaea elodes*	Pond A, Indiana (USA)	GQ463053	Detwiler *et al.* [11]
Echinostoma trivolvis	*Ondatra zibethicus*	Virginia (USA)	JQ670860	Detwiler *et al.* [9]
Echinostoma trivolvis	*Ondatra zibethicus*	Virginia (USA)	JQ670852	Detwiler *et al.* [9]
Echinostoma trivolvis	*Ondatra zibethicus*	Virginia (USA)	JQ670854	Detwiler *et al.* [9]
Echinostoma trivolvis	*Ondatra zibethicus*	Virginia (USA)	JQ670858	Detwiler *et al.* [9]
Echinostoma trivolvis	*Ondatra zibethicus*	Virginia (USA)	JQ670856	Detwiler *et al.* [9]
Echinoparyphium recurvatum	*Radix peregra*	Wales (UK)	AY168944	Kostadinova *et al.* [14]
Echinoparyphium aconiatum	*Lymnaea stagnalis*	Finland	AY168945	Kostadinova *et al.* [14]

* Syn. *Echinostoma* sp. I Africa of Morgan and Blair [17,13]; ** *sensu* Detwiler *et al.* [11].

ting relationships within the problematic *Echinostoma* '*revolutum*' species complex in comparison with the nuclear rRNA spacers and the mitochondrial *cox*1 gene [12,13]. The subsequent DNA-based studies [7,9-11,14] have provided a framework for investigating genetic variation in natural *Echinostoma* spp. populations and revealed novel data on the cryptic variation, identification and geographical distribution of the species of the '*revolutum*' complex.

However, in contrast with the wealth of sequences gathered recently from North America, which have revealed high diversity (six cryptic lineages) within the '*revolutum*' complex of *Echinostoma* [9,11], data from European natural populations are virtually lacking. Thus, of the eight species described and/or recorded from Europe, *i.e. E. revolutum*, *E. paraulum* Dietz, 1909, *E. jurini* (Skvortsov, 1924), *E. miyagawai* Ishii, 1932, *E. robustum* Yamaguti, 1935, *E. bolschewense* (Kotova, 1939), *E. nordiana* (Baschkirova, 1941), *E. friedi* Toledo et al., 2000 [3,5,15-22], sequence data are available only for *E. revolutum* [7,12-14] and *E. friedi* (GenBank AJ564379).

In a study of the digeneans parasitising molluscs in central and northern Europe we found that *Radix auricularia* (Linnaeus, 1758), *Radix peregra* (Müller, 1774) and *Stagnicola palustris* (Müller, 1774) were infected with larval stages of two species of the *Echinostoma* '*revolutum*' complex of cryptic species, one resembling *E. revolutum* sensu stricto (s.s.) and one undescribed species (see also [23]). Here we describe the cercariae of these two species and provide morphological and molecular evidence for their delimitation. Further, we extend the approaches of Morgan and Blair [7,13], Kostadinova *et al.* [14] and Detwiler *et al.* [11] to the relationships within the '*revolutum*' species complex inferred from the *nad*1 gene with the newly-generated sequence data from natural infections in snails in Europe. Phylogenetic analyses revealed the presence of

Table 2 Prevalence of *Echinostoma* spp. from natural infections in *Radix* spp. and *Stagnicola palustris* in Germany and Iceland

Species	Host	Locality	Prevalence (%)
Echinostoma revolutum	*Radix peregra*	Lake Myvatn (Iceland)	2.31
	Radix auricularia	Hennetalsperre (Germany)	1.92 - 10.00
	Radix peregra	Hennetalsperre (Germany)	37.50[a]
	Stagnicola palustris	Hengsteysee (Germany)	0.74
***Echinostoma* sp. IG**	*Radix peregra*	Nordic House (Iceland)	0.94
	Radix auricularia	Baldeneysee (Germany)	1.32 (2009)[b]
	Radix auricularia	Hengsteysee (Germany)	2.00 - 2.90 (2009)[b]
	Radix auricularia	Hengsteysee (Germany)	1.56 (2011)[b]

[a] Sample size small (n = 16); [b] Year indicated for different surveys of the same snail host.
Values are calculated for homogenous distinct samples only.

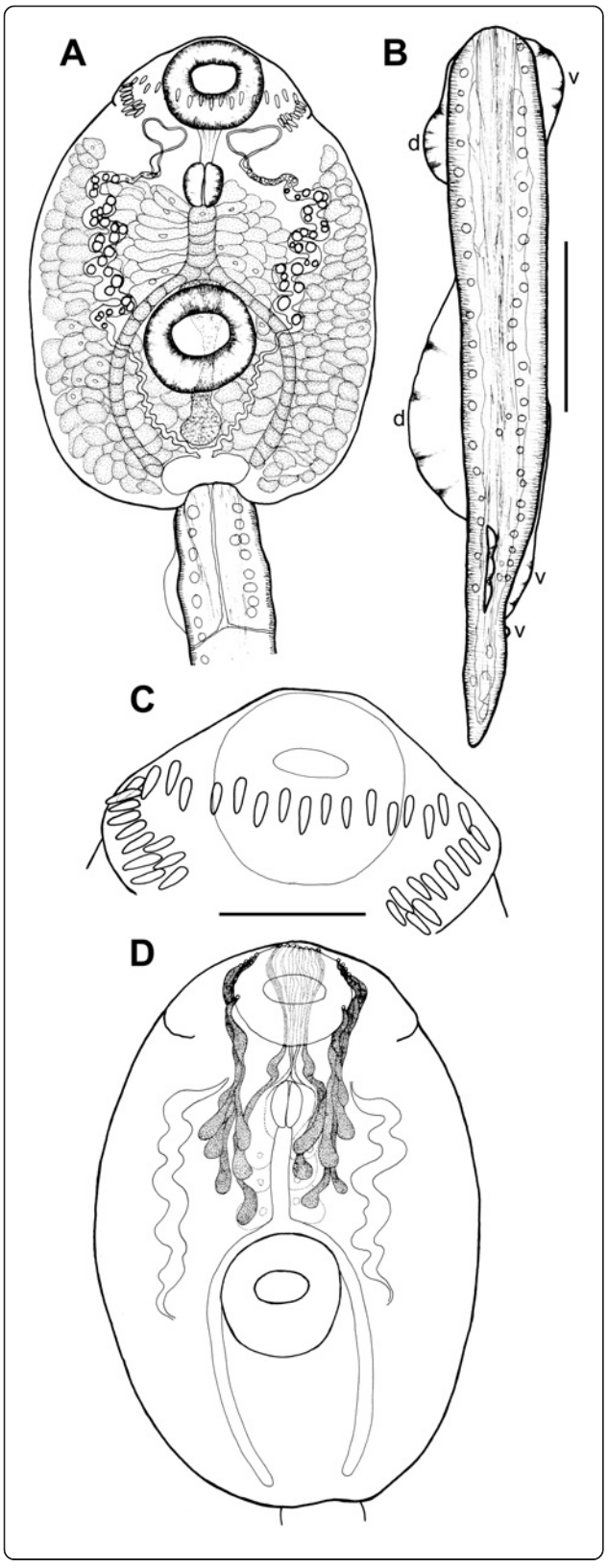

Figure 1 ***Echinostoma*** **sp. IG, drawings of live cercaria. A**. Body, ventral view. **B**. Tail, lateral view (note that only one of the two ventro-lateral fin-folds is illustrated). **C**. Head collar. **D**. Schematic illustration of the para-oesophageal gland-cells. *Abbreviations*: d, dorsal fin-fold; v, ventral fin-fold. *Scale-bars*: **A**, **B**, 100 μm; **C**, 50 μm.

additional cryptic lineages of the *Echinostoma* '*revolutum*' species complex.

Methods

Sample collection

Totals of 2,030 *R. auricularia,* 357 *R. peregra* and 577 *S. palustris* were collected during 2009–2012 in seven reservoirs of the River Ruhr catchment area (North Rhine-Westphalia, Germany): Baldeneysee (51°24′20.08"N, 7°2′22.47"E); Harkortsee (51°23′40.56"N, 7°24′8.27"E); Hengsteysee (51°24′52.17"N, 7°27′42.55"E); Hennetalsperre (51°19′50.97"N, 8°15′46.82"E); Kemnader See (51°25′19.05"N, 7°15′43.07"E); Sorpetalperre (51°20′ 15.01"N, 7°56′46.18"E); and Versetalsperre (51°10′55.71"N, 7°40′57.12"E). Seven distinct samples of *R. peregra* (a total of 573 snails) were collected in five localities in Iceland: Lakes Family Park (64°08′15"N, 21°52′03"W) and Nordic House (64°08′19"N, 21°56′45"W) in Reykjavik; Opnur (63°58′43"N, 21°10′37"W); Raudavatn (64°05′35"N, 21°47′14"W); and Helgavogur, Lake Myvatn (65°38′04"N, 16°55′28"W) in May and August 2012. Snails were collected randomly with a strainer or picked by hand from stones and floating vegetation along the shore at several sampling sites at each reservoir. In the laboratory, snails were labelled and placed individually into beakers with a small amount of lake water, and kept under a light source for up to 5 days to stimulate emergence of cercariae. Thereafter, snails were measured, dissected and examined for prepatent infections.

Morphological data

Cercariae were examined and identified live using the data from the keys of Faltýnková *et al.* [24,25] and other relevant primary sources [3,18-22]. Digital photographs of live cercariae (and rediae) were taken with a digital camera of an Olympus BX51 microscope. Vital stains (Neutral Red and Nile Blue sulphate) were used for visualisation of the para-oesophageal gland-cells of the cercariae. Measurements (in micrometres) were taken from the digital images with the aid of QuickPHOTO CAMERA 2.3 image analysis software or the program ImageJ [26]. Upon preliminary identification, two samples of cercariae (rediae) per isolate were fixed: (i) in molecular grade ethanol for DNA isolation and sequencing; and (ii) in hot/cold 4% formaldehyde solution for obtaining measurements from fixed materials. Snails

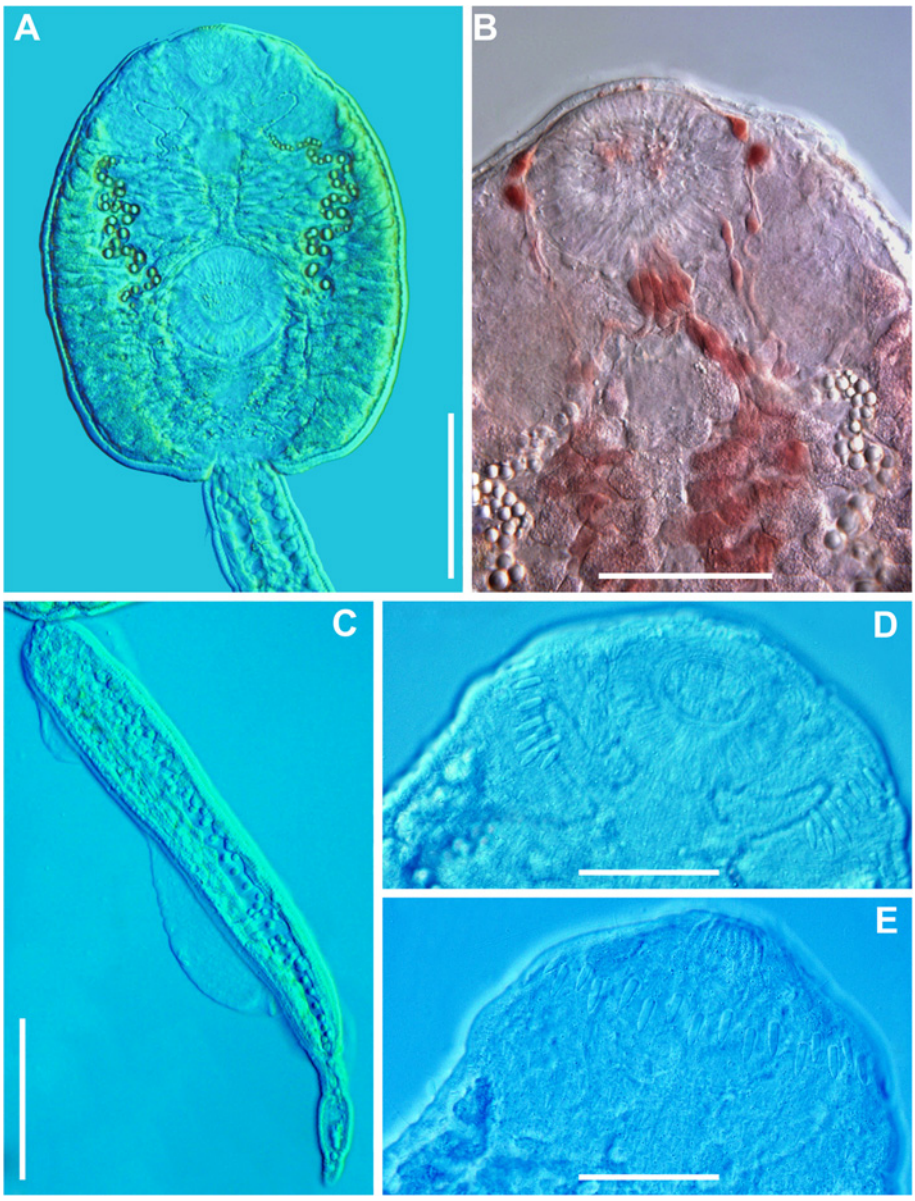

Figure 2 ***Echinostoma*** **sp. IG, microphotographs of live cercaria. A**. Body, ventral view. **B**. Dorsal view showing para-oesophageal gland-cells and outlets (staining with Neutral Red) **C**. Tail, lateral view. **D**. Head collar, ventral view showing angle and lateral spines. **E**. Head collar, dorsal view, showing dorsal collar spines. *Scale-bars*: **A**, **C**, 100 μm; **B**, **D**, **E**, 50 μm.

were identified using Glöer [27]. Although *R. peregra* and *R. ovata* (Draparnaud, 1805) have recently been treated as junior synonyms of *R. balthica* (Linnaeus, 1758) we used the name *R. peregra* following the molecular studies of Bargues *et al.* [28] and Huňová *et al.* [29] which provide sequences for snails sampled in both central Europe and Iceland.

Molecular data

Total genomic DNA was isolated from ethanol-fixed single rediae and/or 10–50 pooled cercariae obtained from a single snail individual by placing the samples in 200 μL of a 5% suspension of deionised water and Chelex® containing 0.1 mg/mL proteinase K, followed by incubation at 56°C for 3 h, boiling at 90°C for 8 min, and centrifugation at 14,000 g for 10 min. Polymerase chain reaction (PCR) amplifications of partial fragments of the mitochondrial gene nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) were performed in 25 μl reactions using Ready-To-Go-PCR Beads (GE Healthcare, UK) containing ~2.5 units of puReTaq DNA polymerase, 10 mM Tris–HCl (pH 9.0), 50 mM KCl,

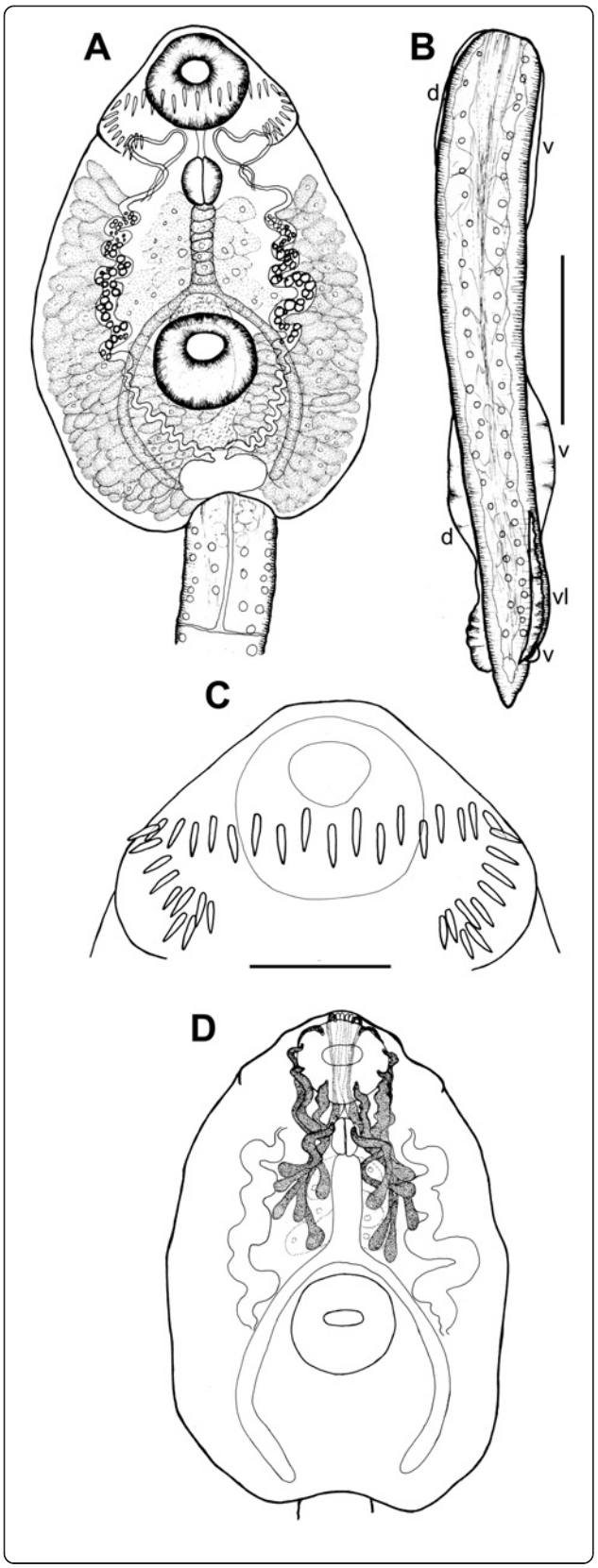

Figure 3 ***Echinostoma revolutum*****, drawings of live cercaria. A**. Body, ventral view. **B**. Tail, lateral view (note that only one of the two ventro-lateral fin-folds is illustrated). **C**. Head collar. **D**. Schematic illustration of the para-oesophageal gland-cells. *Abbreviations*: d, dorsal fin-fold; v, ventral fin-fold; vl, ventro-lateral fin-fold. *Scale-bars*: **A**, **B**, 100 μm; **C**, 50 μm.

1.5 mM $MgCl_2$, 200 mM of each dNTP and stabilisers including BSA, 10 mM of each PCR primer, and 50 ng of template DNA. The following PCR primers were used: forward NDJ11 (equivalent to JB11 in [13]) 5′-AGA TTC GTA AGG GGC CTA ATA-3′ and reverse NDJ2a: 5′-CTT CAG CCT CAG CAT AAT-3′ [14]. The thermocycling profile comprised initial denaturation at 95°C for 5 min, followed by 35 cycles with 30 s denaturation at 94°C, 20 s primer annealing at 48°C, and 45 s at 72°C for primer extension, with a final extension step of 4 min at 72°C.

PCR amplicons were purified using Qiagen QIAquick™ PCR Purification Kit (Qiagen Ltd, UK) and sequenced directly for both strands using the PCR primers. Sequencing was performed on an ABI Prism 3130xl automated sequencer using ABI Big Dye chemistry (ABI Perkin-Elmer, UK) according to the manufacturer's protocol. Contiguous sequences were assembled and edited using MEGA v5 [30] and submitted to GenBank (accession numbers shown in Table 1).

Newly-generated and published *nad*1 sequences for *Echinostoma* spp. (see Table 1 for details) were aligned using Clustal W implemented in MEGA v5 with reference to the amino acid translation, using the echinoderm and flatworm mitochondrial code [31]. Species boundaries were assessed via neighbour-joining (NJ) analyses of Kimura-2-parameter distances using MEGA v5 (nodal support estimated using 1,000 bootstrap resamplings) and Bayesian inference (BI) analysis using MrBayes 3.2 [32,33]. The best-fitting model of nucleotide substitution estimated prior to BI analysis using jModelTest 2.1 [34] was the Hasegawa-Kishino-Yano model including estimates of invariant sites and among-site rate heterogeneity (HKY + I + G).

BI log-likelihoods were estimated with default prior probabilities and likelihood model settings (nst = 2; rates = invgamma; ngammacat = 4) over 10^6 generations via 4 simultaneous Markov Chain Monte Carlo chains (nchains = 4) with a sampling frequency of 100. The first 25% of the samples were discarded (sump burnin = 2500) as determined by the stationarity of lnL assessed with Tracer v. 1.4 [35]; the remaining trees were used to construct the 50% majority-rule consensus tree and to estimate the nodal support as posterior probability values [36]. Genetic distances (uncorrected p-distance) were calculated with MEGA v5.

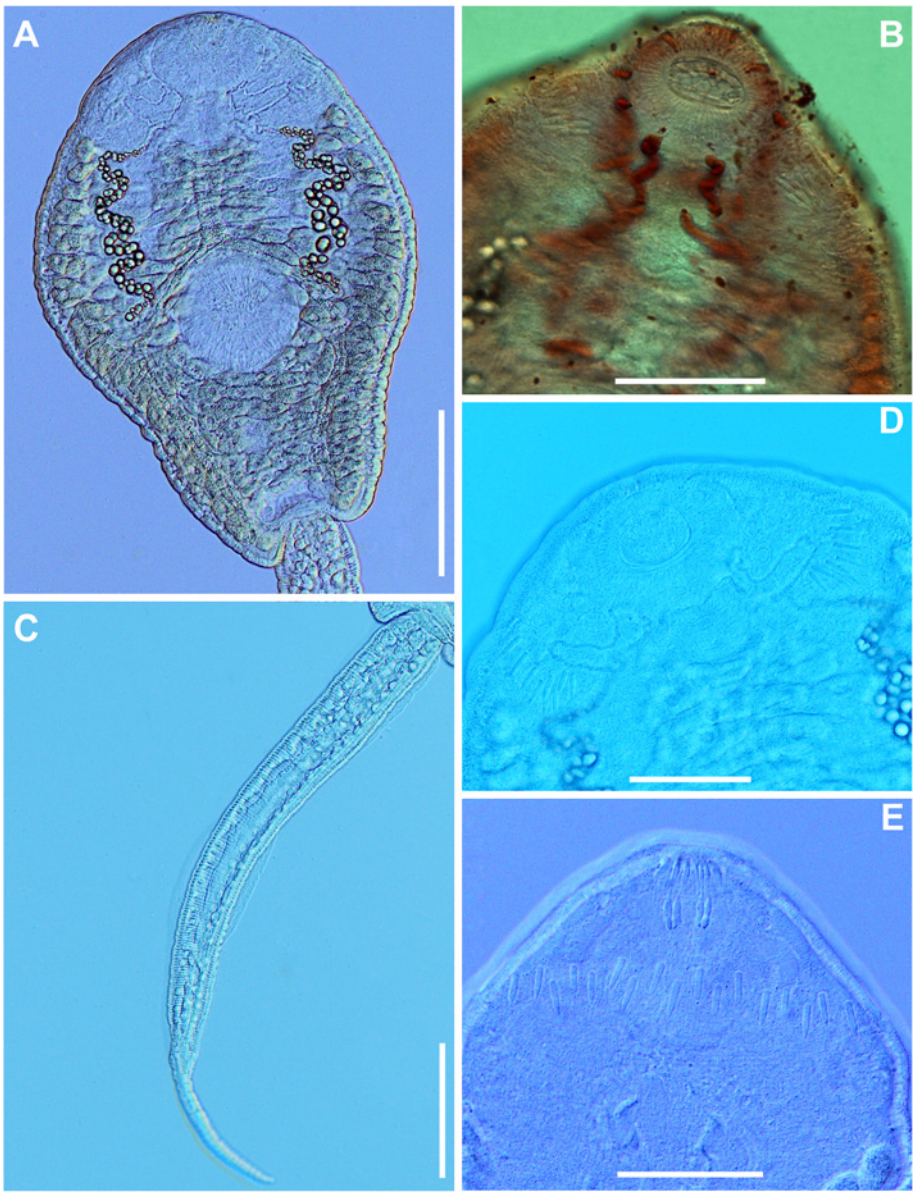

Figure 4 *Echinostoma revolutum*, microphotographs of live cercaria. A. Body, ventral view. **B**. Ventral view showing outlets of para-oesophageal gland-cells (staining with Neutral Red). **C**. Tail, lateral view. **D**. Head collar, ventral view showing angle and lateral spines. **E**. Head collar, dorsal view showing dorsal collar spines. *Scale-bars*: **A**, **C**, 100 μm; **B**, **D**, **E**, 50 μm.

Results

Morphological identification of infections in natural snail populations

We found larval stages of *Echinostoma* spp. in the snail populations sampled in three of the seven reservoirs in the River Ruhr drainage in Germany and in two of the five lakes in Iceland (see Table 2 for details on hosts and localities). Three lymnaeid snail species acted as first intermediate hosts of *Echinostoma* spp. of the '*revolutum*' species complex in the areas studied: *R. peregra* in the lakes in Iceland and *R. auricularia*, *R. peregra* and *S. palustris* in the reservoirs in Germany. Prevalences were usually low (typically 1-3%) but occasionally higher values were registered (Table 2).

Detailed examination of cercarial morphology allowed us to identify two types of echinostomatid cercariae among the isolates sampled in Iceland and Germany (Figures 1, 2, 3, 4, 5). Both types belong to the '*revolutum*' species complex of *Echinostoma* which is characterised by the following features of the cercariae: (i) 37 collar spines with an arrangement 5-6-15-6-5 (5 angle and 6 lateral spines on each side and 15 dorsal spines in a double row; Figures 1C, 2D,E, 3C, 4D,E); (ii) tail with a tip forming a highly contractile attenuated process and seven prominent

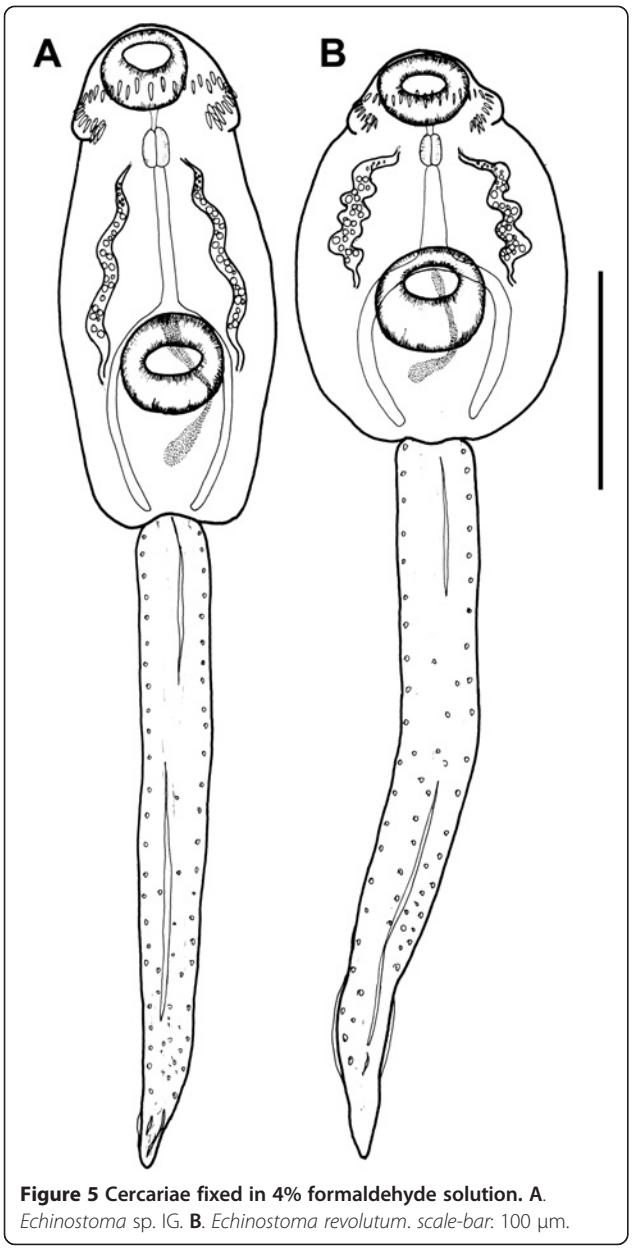

Figure 5 Cercariae fixed in 4% formaldehyde solution. A. *Echinostoma* sp. IG. **B**. *Echinostoma revolutum*. *scale-bar*: 100 μm.

tegumental fin-folds (2 dorsal, 3 ventral and 2 ventro-lateral; Figures 1B, 2C, 3B, 4C); and (iii) a flame-cell formula 2[(3 + 3 + 3) + (3 + 3 + 3)] = 36.

Eleven isolates (three ex *R. peregra* from Iceland, plus two ex *R. auricularia*, five ex *R. peregra* and one ex *S. palustris* from Germany) were identified as *E. revolutum* based on cercarial morphology and especially the presence of 12 small para-oesophageal gland-cells with long ducts, located between pharynx and ventral sucker [24] (Figures 2B, 4B). However, seven isolates of cercariae, one ex *R. peregra* from Iceland and six ex *R. auricularia* from Germany, further referred to as *Echinostoma* sp. IG (indicating the origin of the isolates *i.e.* Iceland and Germany) exhibited slight differences from the isolates identified as *E. revolutum* as follows: (i) collar spines with blunt (Figures 1C, 2D,E) *vs* sharp (Figures 3C, 4D,E) tips; (ii) para-oesophageal gland-cell outlets opening at the margin of the oral sucker only (one dorsal pair, four dorsolateral pairs, and one ventro-lateral pair; see Figures 1D, 2B) *vs* openings present on the ventral surface of the body (one pair at the level of pharynx; the remaining *i.e.* one dorsal pair, one dorsolateral pair, and three ventro-lateral pairs opening at the margin of the oral sucker, see Figures 3D, 4B); and (iii) distal dorsal tail fin-fold large *vs* less prominent (length 40-60% of tail length *vs* 20-38%; width c.70% of tail width *vs* 20-30%; compare Figures 1B, 2C and 3B, 4C; Table 3). Comparison of the metrical data obtained for live cercariae revealed that *Echinostoma* sp. IG had a shorter tail, with distinctly larger distal dorsal fin-fold and shorter distal ventral fin-fold (Table 3). Furthermore, although it was difficult to observe the fin-folds in fixed material thus rendering differentiation difficult, the cercariae of *Echinostoma* sp. IG were characterised by a distinctly more elongate, narrower body and a shorter tail (Figure 5; Table 3); this represents another distinguishing feature for the two European species studied by us.

Molecular analysis

A total of 14 partial *nad*1 sequences was generated (11 for *E. revolutum* and 3 for *Echinostoma* sp. IG; Table 1). These sequences were aligned with selected published sequences representing the data available for eight species of the '*revolutum*' species complex of *Echinostoma* generated from both laboratory strains [13] and natural isolates [9,11,14]; two otherwise unpublished sequences were also retrieved from GenBank (see Table 1 for details). The aligned dataset included 39 sequences and was comprised of 472 nt positions after trimming the ends to match the shortest aligned sequences. Sequences for *Echinoparyphium* spp. of Kostadinova *et al.* [14] were used as outgroups (Table 1).

Both NJ and BI analyses resulted in consensus trees with similar topologies (see Figure 6 for a phylogeny inferred from genetic distances and BI). The newly-generated sequences for *E. revolutum* formed a strongly supported clade which included a sequence for *E. revolutum* (s.s.) of Kostadinova *et al.* [14] (see also [6]). On the other hand, the sequences for the isolates identified as *Echinostoma* sp. IG formed a strongly supported reciprocally monophyletic lineage, basal to *Echinostoma* spp., which also incorporated the sequence for an isolate from Wales (UK) provisionally identified as *Echinostoma* cf. *friedi* by Kostadinova *et al.* [14]. The isolates comprising this lineage also exhibited the highest levels of divergence from the isolates of *Echinostoma* spp. analysed (p-distance range 17.2-21.6%; divergence from *E. friedi* (AJ564379) (p-distance range 18.9-19.1%).

Table 3 Comparative metrical data (in μm) for live and fixed cercariae of *Echinostoma* sp. IG and *E. revolutum* from natural infections in *Radix* spp. and *Stagnicola palustris* in Germany and Iceland

Species	*Echinostoma* sp. IG			*E. revolutum*		
	Live material	Fixed material		Live material	Fixed material	
	Range	Range	Mean	Range	Range	Mean
Body length	260 – 362	228 – 292	254	303 – 427	159 – 234	188
Body width (max.)	184 – 249	90 – 97	94	193 – 251	107 – 125	112
Oral sucker length	45 – 63	36 – 46	42	56 – 71	38 – 52	45
Oral sucker width	50 – 66	37 – 45	42	53 – 68	37 – 49	42
Ventral sucker length	54 – 72	43 – 54	48	63 – 83	47 – 66	55
Ventral sucker width	57 – 81	44 – 47	46	58 – 83	48 – 60	54
Pharynx length	25 – 29	16 – 25	20	27 – 36	20 – 24	21
Pharynx width	22 – 26	12 – 19	15	25 – 29	13 – 14	13
Oesophagus length	56 – 89	61 – 96	78	54 – 103	30 – 55	40
Tail length	334 – 353	296 – 378	344	364 – 417	316 – 405	367
Tail width (at base)	44 – 49	30 – 34	32	39 – 52	20 – 36	27
Tail-tip length	67 – 83	–	–	35 – 93	–	–
Proximal dorsal fin-fold length	49 – 63	50	–	41 – 153	–	–
Proximal dorsal fin-fold width	14 – 15	–	–	5 – 13	8 – 11	9
Distal dorsal fin-fold length	147 – 212	106 – 154	120	72 – 159	–	–
Distal dorsal fin-fold width	30 – 35	14 – 21	16	7 – 16	–	–
Proximal ventral fin-fold length	47 – 90	73	–	51 – 116	85	–
Proximal ventral fin-fold width	12 – 15	–	–	4 – 6	5	–
Distal ventral fin-fold length	44 – 64	41	–	74 – 202	99 – 157	125
Distal ventral fin-fold length	6 – 18	8	–	7 – 14	–	–

Unexpectedly, the European isolates of *E. revolutum* and those obtained from natural infections in *Lymnaea elodes* and *Ondatra zibethicus* (L.) in North America by Detwiler *et al.* [11] formed two strongly supported sister lineages. This solution (both NJ and BI analyses) was consistent with the distinctly higher inter-lineage divergence (p-distance; 4.9–6.8%) compared with intra-lineage divergence (p-distance range, European isolates: 0–2.1%, North American isolates: 0.4–1.1%). These data indicate that the North American isolates represent another cryptic species of the '*revolutum*' species complex.

Another unexpected result was that the sequence for *Echinostoma revolutum* of Morgan and Blair [7,13] (AF025832; isolate from Europe) exhibited a strong association with the sequence for *Echinostoma friedi* of Marcilla *et al*. (unpublished, GenBank AJ564379) based on an isolate of this species recently described by these authors [22] from Spain (p-distance 0.8%; divergence from nearest neighbours, *i.e. Echinostoma robustum* sensu Detwiler *et al.* [11], of 4.9-9.1%. The clade comprising the former two European isolates and those of *E. robustum* from North America exhibited a complex structure suggesting the existence of at least three species (subclade support indicated in Figure 6).

Discussion

The combined morphological and DNA-based approaches in this first intensive screening of *Radix* spp. for infections with *Echinostoma* spp. allowed us to delineate two cryptic species of the '*revolutum*' complex in central and northern Europe. Furthermore, comparative sequence analyses depicted three additional cryptic lineages in North America.

Both distance- and model-based phylogenies provided high support for reciprocal monophyly of *Echinostoma* sp. IG. The isolates of this lineage, that evidently represents a new species, awaiting further formal description after a discovery of the adult parasite stage, were found to be clearly distinguishable among the European isolates by using both morphological and molecular evidence. Although the identification of the European isolates of *Echinostoma* spp. followed the standard taxonomic practice, the detection of the new cryptic species required substantial taxonomic expertise. This involved detailed knowledge on the variation of the features used for species delimitation based on thorough morphological examination of a large number of cercariae from each isolate. The corroboration of our hypothesis for the distinct species status of the two species of *Echinostoma*

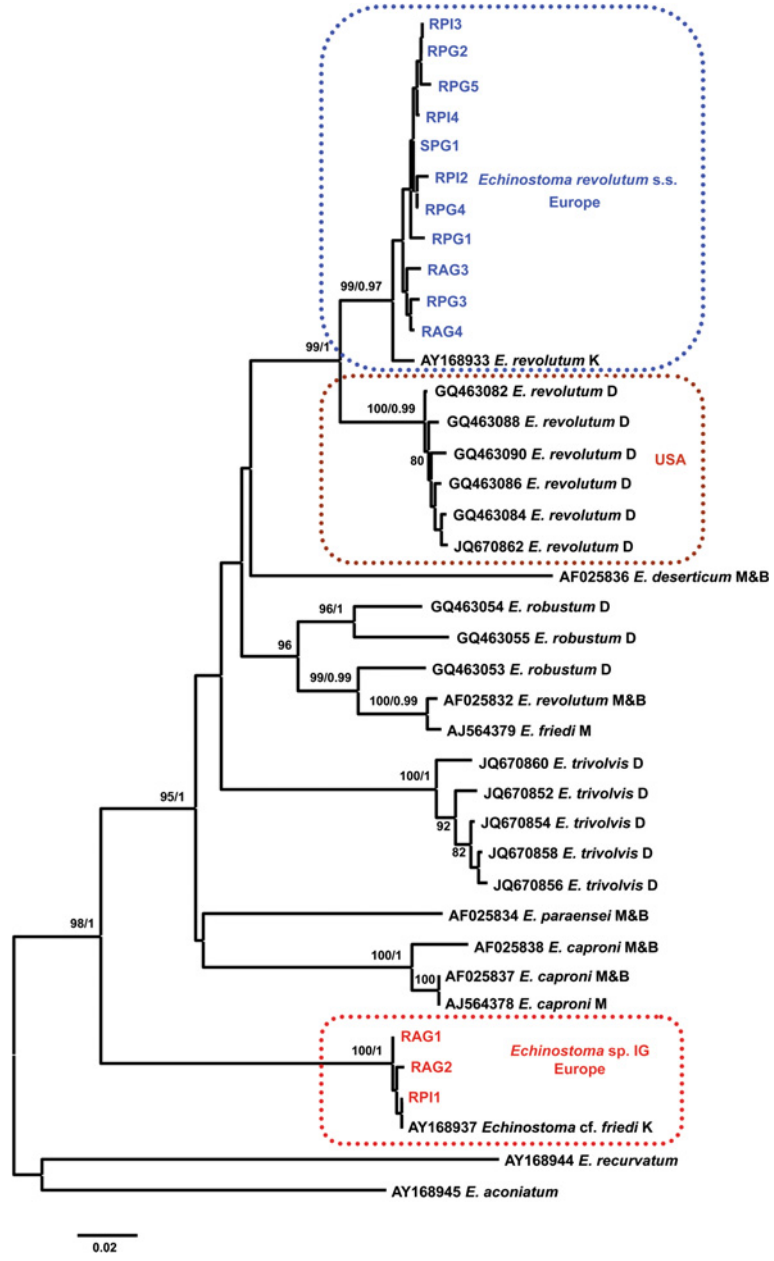

Figure 6 Neighbour-joining (NJ) phylogram reconstructed using the newly-generated and retrieved from GenBank *nad1* sequences (472 nt positions) for *Echinostoma* spp. of the '*revolutum*' species complex. Outgroup: *Echinoparyphium* spp. Nodal support (bootstrap values > 70% shown only) inferred from 1,000 replicates; these are followed by posterior probabilities from BI analysis. The scale-bar indicates expected number of substitutions per site. Sequence identification as in GenBank followed by a letter: D, Detwiler et al. [9,11]; K, Kostadinova *et al.* [14]; M, Marcilla *et al.* (GenBank); M & B, Morgan & Blair [7,13].

parasitising snail populations in Germany and Iceland on the basis of molecular data thus may appear secondary.

However, the distinguishing features are difficult to detect and/or subject to variation (reviewed in Kostadinova and Gibson [6]). For example, Kanev [3] described 16 ducts and pores of para-oesophageal gland-cells in the cercariae of *E. revolutum* ex *Lymnaea stagnalis*; of these, 12 were located on the oral sucker and four on the ventral surface. On the other hand, we detected only 12 small para-oesophageal gland-cells in the cercariae of *E. revolutum* ex *Radix* spp.; Faltýnková *et al.* [24] also provided this number for *E. revolutum* ex *L. stagnalis*. It is worth noting that recent field studies indicate that *E. revolutum* most commonly occurs in *L. stagnalis* in Europe [23,24], infections with this species have occasionally been reported in the past from *R. auricularia*, *R. peregra* and *R.*

ovata (Draparnaud, 1805) [22,37-45]. Further molecular study would reveal whether *Echinostoma* spp. of the '*revolutum*' species complex parasitising *L. stagnalis* and *Radix* spp. are conspecific or represent as yet undiscovered cryptic species. We believe that 'reciprocal illumination' *sensu* Hennig [46] of morphological characters upon a molecular-based species delimitation has a strong potential for delineating species boundaries within the '*revolutum*' complex of cryptic species.

Echinostoma sp. IG was found to be conspecific with an isolate from Wales (UK) provisionally identified as *Echinostoma* cf. *friedi* by Kostadinova *et al.* [14]. The lineage comprising this and the newly-sequenced isolates occupied a basal position (as in Kostadinova *et al.* [14]) and this is in sharp contrast with the phylogenetic solution based on *nad*1 gene of Detwiler *et al.* [11]. These authors wrote that "A comparison of samples identified as *E. robustum* (U58102) and *E. friedi* (AY168937) reveals that they are found within the same monophyletic clade and thus do not qualify as distinct species according to a phylogenetic definition. Additionally, they are genetically similar (0.009 genetic divergence, ND1 ..." and concluded that "the sample tentatively identified as *E. friedi* in Kostadinova *et al.* (2003) is genetically very similar to *E. robustum*". Our results clearly indicate that the sequence for *E. friedi* from its type-locality in Spain (AJ564379; Marcilla *et al.* unpublished sequence in GenBank) and for the European isolate labelled as *E. revolutum* (AF025832) of Morgan and Blair [7,12,13] represent conspecific isolates; the genetic divergence between these two isolates was 0.8%, *i.e.* substantially lower than that (*i.e.* 18.9-19.1%) between the lineage containing *E.* cf. *friedi* (AY168937) of Kostadinova *et al.* [14] and the European isolate labelled as *E. revolutum* (*nad*1 sequence AF025832; ITS sequence U58102) by Morgan and Blair [7,12,13]. We believe, therefore, that Detwiler *et al.* [11] have in fact used the otherwise unpublished sequence for *E. friedi* of Marcilla *et al.* (AJ564379) but have mislabelled it (as AY168937).

Kostadinova *et al.* [14] indicated a tentative affiliation to *E. robustum* of the isolates of the 'Australian-German' clade of *Echinostoma* spp. of Morgan and Blair [7], but suggested that this specific identification is pending a redescription of both larval and adult stages. The present results indicate that suggesting synonymy for the European isolate studied by Morgan and Blair [7,12,13] and *E. friedi* should await examination of a larger number of molecularly characterised natural isolates of the European species of the '*revolutum*' complex since our knowledge on cryptic diversity in this group is still limited. This suggestion is supported by the discovery of two genetically distinct, geographically separated lineages of *E. revolutum*: *E. revolutum* s.s. from Europe and *E. revolutum* of Detwiter *et al.* [11] from North America, thus demonstrating that the suggestion for the cosmopolitan distribution of this species [11] appears to be a result of cryptic variation. Indeed, these authors noted that their results of network analyses indicate gene flow and population expansion within North America but not on a global scale. The taxonomy of the North American species can be further scrutinised using the morphological data available for cercariae and/or experimentally developed adults [11,47].

Conclusion

The results of our study suggest that further analyses of patterns of interspecific variation based on a combination of molecular and well-documented morphological data would enhance the re-evaluation of the species and advance our understanding of the relationships within the '*revolutum*' group of *Echinostoma*.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CS, MS and KS obtained the samples. CS, AF, MS and SG undertook the morphological study. SG carried out the sequencing and phylogenetic analysis. CS, SG, AF and MS prepared the first draft of the MS. KS, BS and AK conceived and coordinated the study and helped to draft the MS. All authors read and approved the final manuscript.

Acknowledgements

We thank Blanka Škoríková for her kind help with the figures and Jana Köchling, Verena Altmann, Jessica Schwelm and Dr Ana Pérez-del-Olmo, for their assistance in sampling. This study was supported by the Czech Science Foundation (AF, AK, MS, SG, grant P505/10/1562); the 'Sichere Ruhr' project as part of the Bundesministerium für Bildung und Forschung (BMBF) program 'Sustainable Water Management' (BS, grant 02WRS1283); and the Research Fund of the University of Iceland (KS). CS benefited from a Deutsche Bundesstiftung Umwelt (DBU) PhD fellowship.

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Received: 31 January 2013 Accepted: 5 March 2013
Published: 13 March 2013

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doi:10.1186/1756-3305-6-64
Cite this article as: Georgieva *et al.*: New cryptic species of the '*revolutum*' group of *Echinostoma* (Digenea: Echinostomatidae) revealed by molecular and morphological data. *Parasites & Vectors* 2013 **6**:64.

Syst Parasitol (2014) 89:153–166
DOI 10.1007/s11230-014-9513-4

Morphological and molecular data for larval stages of four species of *Petasiger* Dietz, 1909 (Digenea: Echinostomatidae) with an updated key to the known cercariae from the Palaearctic

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Received: 26 June 2014 / Accepted: 17 July 2014

Abstract Large-tailed echinostomatid cercariae of the genus *Petasiger* Dietz, 1909 (Digenea: Echinostomatidae) from the planorbid snails *Gyraulus albus* (Müller) and *Planorbis planorbis* (L.) collected in Germany and the Czech Republic and metacercariae from *Gasterosteus aculeatus* L. (Gasterosteiformes: Gasterosteidae) collected in Canada are characterised morphologically and molecularly. The rediae, cercariae and metacercariae are described in detail and compared with the existing data on the larval stages of *Petasiger* spp. Comparative molecular analyses using 28S rDNA and *nad*1 mitochondrial sequences supported the distinct status of four species of *Petasiger*. Molecular and morphological evidence for their distinction and an updated key to the known large-tailed cercariae of *Petasiger* from the Palaearctic are provided.

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Introduction

Echinostomatids of the genus *Petasiger* Dietz, 1909 constitute a relatively large group of digenean trematodes (33 nominal species, of these 23 species described from the Palaearctic; see Faltýnková et al., 2008). The most recent revision of the genus recognised a total of 18 valid species (Faltýnková et al., 2008). Of these, seven have been described or recorded in Europe: two species possessing 27 collar spines [*P. exaeretus* Dietz, 1909 and *P. phalacrocoracis* (Yamaguti, 1939)] and five species with 19 collar spines [*P. grandivesicularis* Ishii, 1935, *P. islandicus* Kostadinova & Skírnisson, 2007, *P. megacanthus* (Kotlán, 1922), *P. neocomense* Fuhrmann, 1927 and *P. pungens* (Linstow, 1893)] (Faltýnková et al., 2008).

Species of *Petasiger* utilise snails of the family Planorbidae Rafinesque as first intermediate hosts, fish as second intermediate hosts and fish-eating birds as definitive hosts. However, in spite of the numerous records of *Petasiger* spp. in the bird hosts, most noticeably grebes (Faltýnková et al., 2008), data on the

occurrence of parasite life-history stages in their intermediate hosts are limited. To date, the life-cycles of only three species have been elucidated in the Palaearctic. The life-cycles of *P. neocomense* and *P. grandivesicularis* were completed experimentally in the laboratory (identification of the adult stage not confirmed for *P. neocomense*, see Karmanova, 1971; Kostadinova & Chipev, 1992). Recently Georgieva et al. (2012) elucidated the life-cycle of *P. islandicus* by matching sequences for the mitochondrial gene nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) and the 28S rRNA gene from all parasite life-cycle stages, i.e. cercariae and rediae ex *Gyraulus* cf. *laevis (*Alder), metacercariae ex *Gasterosteus aculeatus* L. and adults ex *Podiceps auritus* (L.) from Iceland. These authors also provided comparative sequences for *P. neocomense* and *P. phalacrocoracis*. Six otherwise unidentified large-tailed echinostomatid cercariae have been described in Europe: *Cercaria thamesensis* Khan, 1960 and *Cercaria hamptonensis* Khan, 1960 ex *Planorbis planorbis* (L.) from the River Thames, UK (Khan, 1960), *Cercaria rashidi* Nasir, 1962 and *Cercaria titfordensis* Nasir, 1962 ex *Planorbis carinatus* Müller from lakes in Birmingham, UK (Nasir, 1962), *Petasiger* sp. of Ginetsinskaya & Dobrovolskij (1964) ex *P. planorbis* in the Volga Delta, Russia (Ginetsinskaya & Dobrovolskij, 1964) and *Petasiger* sp. of Kostadinova (1997) ex *P. planorbis* in Lake Durankulak, Bulgaria.

In the course of a study of parasites in planorbid snails in Germany and the Czech Republic, we found large-tailed echinostomatid cercariae in two snail species, *Gyraulus albus* (Müller) and *P. planorbis*; additionally, metacercariae of *Petasiger* were collected from *G. aculeatus* from Vancouver Island, Canada. Comparative analyses of the sequences obtained from the cercarial and metacercarial isolates revealed the presence of four species of *Petasiger*. This paper provides molecular and morphological evidence for their distinction and an updated key to the known large-tailed echinostomatid cercariae from the Palaearctic.

Materials and methods

Sample collection

Sampling of planorbid snails in Germany was carried out at five reservoirs of the River Ruhr: Baldeneysee (51°24′20.08″N, 7°2′22.47″E), Hengsteysee (51°24′52.17″N, 7°27′42.55″E), Hennetalsperre (51°19′50.97″N, 8°15′46.82″E), Sorpetalsperre (51°20′ 15.01″N, 7°56′46.18″E) and Versetalsperre (51°10′55.71″N, 7°40′57.12″E) between May and September 2012 and 2013. A total of 2,749 planorbid snails belonging to seven species was examined: *Gyraulus albus* (Müller) (1,919 snails), *Segmentina nitida* (Müller) (195), *Bathyomphalus contortus* (L.) (176), *Anisus vortex* (L.) (175), *Ancylus fluviatilis* Müller (159), *Planorbarius corneus* (L.) (121) and *Planorbis planorbis* (L.) (4). Additionally, ten large *P. planorbis* were examined from Kleiner Plöner See (54°09′42.17″N, 10°22′45.09″E) near Plön, Germany in 2012, and further 72 *P. planorbis* and 57 *P. corneus* were collected at Pond Černiš (49°00′10.3″N 14°25′57.9″E) in the Nature Reserve Vrbenské Ponds (Czech Republic) in July 2013. Snails were identified according to Glöer (2002).

Of all snail hosts examined, only two species were infected with *Petasiger* spp.: *G. albus* (seven in Hennetalsperre and one in Hengsteysee) and *P. planorbis* (two in Kleiner Plöner See and one in Pond Černiš). Additionally, metacercariae of *Petasiger* sp. were recovered from the oesophageal wall of *G. aculeatus* from Gosling Lake (50°03′30.3″N, 125°30′12.1″W), Vancouver Island (Canada) by MK; these were used for comparative purposes.

Morphological data

Samples of all isolates were fixed in molecular grade ethanol for DNA isolation and sequencing, and in 4% formaldehyde solution for measurements from fixed materials. Formalin-fixed cercariae were stained with iron-acetocarmine, dehydrated through an alcohol series, cleared in dimethyl phthalate and mounted in Canada balsam. Light microscopy photographs of live and fixed isolates (rediae, cercariae and metacercariae) were taken with a digital camera of an Olympus BX51 microscope. Measurements were taken from pictures of both live and fixed materials with the software ImageJ (Abramoff et al., 2004). All measurements are in micrometres and are given as the range followed by the mean in parentheses. In addition to measurements, ratios of (i) tail length in relation to body length (TL/BL) and (ii) tail length in relation to maximum tail width (TL/TW) were calculated for both live and fixed cercariae.

Molecular data

Total genomic DNA was isolated from 10–15 pooled ethanol-fixed cercariae as described by Georgieva et al. (2013). Polymerase chain reaction (PCR) amplifications were performed in 25 µl reactions using illustra puReTaq Ready-To-Go PCR beads (GE Healthcare, UK). Partial fragments of the mitochondrial gene nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) were amplified (*c*.500 nt) using primers NDJ11 (forward; 5′-AGA TTC GTA AGG GGC CTA ATA-3') and NDJ2a (reverse; 5′-CTT CAG CCT CAG CAT AAT-3′) (Kostadinova et al., 2003) and the following thermocycling profile: initial denaturation at 95°C for 5 min followed by 35 cycles with denaturation at 94°C for 30 s, primer annealing at 48°C for 20 s, primer extension at 72°C for 45 s and a final extension step at 72°C for 4 min. Partial 28S rDNA sequences (domains D1-D3; *c*.1200 nt) were amplified for a subset of isolates from *nad*1-derived lineages using primers ZX-1 (forward; 5′-ACC CGC TGA ATT TAA GCA TAT-3′) (Bray et al., 2009) and 1500R (reverse; 5′-GCT ATC CTG AGG GAA ACT TCG-3′) (Tkach et al., 2003) and the following thermocycling conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles with denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, primer extension at 72°C for 2 min and a final extension step at 72°C for 7 min. PCR amplicons were purified directly with a QIAquick PCR purification kit (Qiagen Ltd, UK) following the manufacturer's instructions. PCR fragments were sequenced directly for both strands using the PCR primers with ABI BigDye chemistry (ABI Perkin-Elmer), alcohol-precipitated, and run on an ABI Prism 3130xl automated sequencer. Contiguous sequences were assembled and aligned in MEGA v.6 (Tamura et al., 2013), and submitted to GenBank under accession numbers KM191799 - KM191817.

Two alignments were analysed. The first comprised *nad*1 sequences (474 nt, 15 sequences) for ten isolates plus five published sequences (Kostadinova et al., 2003; Georgieva et al., 2012) for *P. islandicus* and *Echinostoma revolutum* (Frölich, 1802) (used as the outgroup) from Europe. The second alignment (1,261 nt, 12 sequences) comprised 28S rDNA sequences for nine isolates and three published sequences (Kostadinova et al., 2003; Georgieva et al., 2012) for European isolates of *P. islandicus*, *P. phalacrocoracis* and *E. revolutum* (used as the outgroup). Neighbour-joining (NJ), maximum likelihood (ML) and Bayesian inference (BI) analyses were conducted separately for the two datasets. Prior to analyses, evolutionary substitution models were analysed with jModelTest 2.1.4 (Darriba et al., 2012) using Akaike Information Criteria (AIC) for selection of best-fitting models; these were HKY+Γ (*nad*1 dataset) and GTR+I+Γ (28S dataset). Maximum likelihood analyses were carried out in PhyML 3.0 (Guindon et al., 2010). BI analyses were performed with MrBayes version 3.2 (Ronquist et al., 2012). Log likelihoods were estimated over 10,000,000 generations using Markov chain Monte Carlo (MCMC) searches on two simultaneous runs of four chains, sampling trees every 1,000 generations. The first 2,500 trees were discarded as "burn-in" and a consensus topology and nodal support estimated as posterior probability values (Huelsenbeck et al., 2001) were calculated from the remaining trees.

Molecular data

A total of 12 isolates of *Petasiger* spp. was sequenced (eight ex *G. albus*, three ex *P. planorbis* and one ex *G. aculeatus*) resulting in successful generation of ten *nad*1 and nine 28S rDNA sequences (see Table 1 for details). The trees inferred from the NJ, ML and BI analyses of the partial *nad*1 gene provided strong support for four reciprocally monophyletic lineages of *Petasiger* spp. (Fig. 1A) with the metacercarial isolate as earlier divergent. Two clades (*Petasiger* sp. 1 and 2) comprised cercarial isolates ex *G. albus* from Hennetalsperre whereas the third (*Petasiger* sp. 3) comprised cercarial isolates from both *G. albus* and *P. planorbis* (see Table 1). The levels of intraspecific divergence were substantially lower (p-distance; overall range 0–1.5%) compared with those of interspecific divergence (14.0–28.5%). No *nad*1 sequences were obtained for one isolate ex *G. albus* and for the Czech isolate ex *P. planorbis* but the 28S sequences for these isolates were identical with that for *Petasiger* sp. 1 (isolate GANH1).

Partial sequences of the 28S rRNA gene were obtained from representative isolates of the *nad*1-derived clades and aligned with the available sequences on GenBank (Olson et al., 2003; Georgieva et al., 2012) for *P. islandicus*, *P. phalacrocoracis* and

Table 1 Summary data for the isolates of *Petasiger* spp. from planorbid snails in central Europe and fish in Canada used for generation of the new *nad*1 and 28S rDNA sequences

Species	Isolate	Stage[a]	Host species	Locality	GenBank accession number	
					*nad*1	28S
Petasiger sp. 1	GAHN1	C	*Gyraulus albus*	Lake Hennetalsperre[b]	KM191808	KM191801
Petasiger sp. 1	GAHN2	C	*Gyraulus albus*	Lake Hennetalsperre[b]	KM191809	
Petasiger sp. 1	GAHN3	C	*Gyraulus albus*	Lake Hennetalsperre[b]		KM191799
Petasiger sp. 1	PPC	C	*Planorbis planorbis*	Pond Černiš[c]		KM191800
Petasiger sp. 2	GAHN4	C	*Gyraulus albus*	Lake Hennetalsperre[b]	KM191810	KM191802
Petasiger sp. 2	GAHN5	C	*Gyraulus albus*	Lake Hennetalsperre[b]	KM191811	
Petasiger sp. 3	GAHN6	C	*Gyraulus albus*	Lake Hennetalsperre[b]	KM191812	KM191803
Petasiger sp. 3	PPKPS1	C	*Planorbis planorbis*	Lake Kleiner Plöner See[b]	KM191813	KM191804
Petasiger sp. 3	PPKPS2	C	*Planorbis planorbis*	Lake Kleiner Plöner See[b]	KM191814	
Petasiger sp. 3	GAHN7	C	*Gyraulus albus*	Lake Hennetalsperre[b]	KM191815	KM191805
Petasiger sp. 3	GAHE	C	*Gyraulus albus*	Lake Hengsteysee[b]	KM191816	KM191806
Petasiger sp. 4	GAG	M	*Gasterosteus aculeatus*	Lake Gosling[d]	KM191817	KM191807

[a] Life-cycle stage (C, cercaria; M, metacercaria); [b]Germany; [c]Czech Republic; [d]Canada

E. revolutum (used as the outgroup). The phylogenetic analyses provided support for the complex of species possessing 19 collar spines and for two clades (*Petasiger* sp. 1 and 3) depicted in the analyses of the *nad*1 data (Fig. 1B) with the isolate of *P. phalacrocoracis* (possessing 27 collar spines) as earliest divergent (not supported by ML). However, the relationships within the group with 19 collar spines were not all resolved. No intra-lineage divergence was detected; the interspecific divergence within the clade of *Petasiger* spp. with 19 collar spines was between 0.7 and 2.2% (9–27 nt); the divergence between the latter clade and *P. phacrocoracis* ranged between 3.8 and 5.3% (38–53 nt). Detailed descriptions of the isolates sequenced are provided below.

Family Echinostomatidae Looss, 1899
Subfamily Echinostomatinae Looss, 1899
Genus *Petasiger* Dietz, 1909

Petasiger sp. 1

First intermediate host: *Gyraulus albus* (Müller), *Planorbis planorbis* (L.) (Planorbidae).

Localities: Hennetalsperre, Germany; Pond Černiš, Czech Republic.

Voucher material: IPCAS D-707 (hologenophore).

Representative DNA sequences: KM191808 - KM191809 (*nad*1); KM191799 - KM191801 (28S rDNA).

Description (Figs. 2A, 3A, 4A, 5A–B)

Redia

Body of daughter redia slightly brownish, elongate-saccular, 776–3,541 × 182–343 (1,801 × 254). Collar 96–153 (128) wide, divided into 4 lobes (2 lateral, 1 dorsal and 1 ventral). Pharynx 54–99 × 51–95 (74 × 71). Intestine saccate, reaching to locomotory processes; the latter 2, situated in last quarter of body.

Cercaria

[Measurements from 19 live specimens.] Body colourless, elongate-oval, 111–257 × 77–163 (175 × 117). Entire body surface covered with minute spines. Collar 58–97 (80) wide, with 19 collar spines (formula 4–11–4; 4 angle spines on each ventral lappet, 13–16 × 2; 11 marginal spines in single row, 12–15 × 2–3).

Tail massive, light brownish, elongate, with almost parallel sides, 1,194–2,280 (1,804) long, much longer

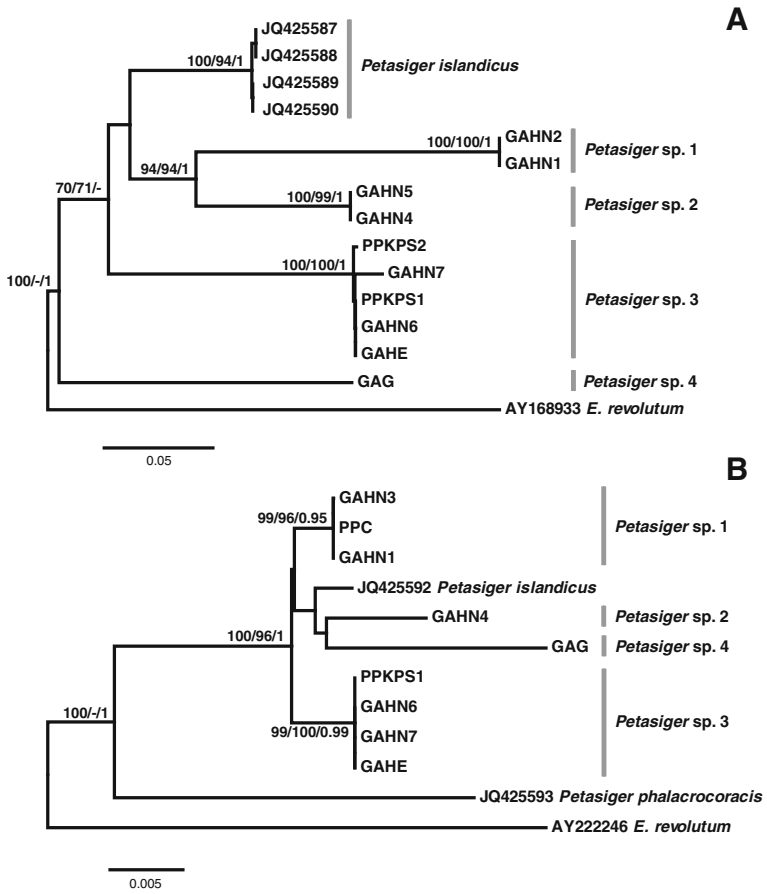

Fig. 1 Neighbour-joining (NJ) phylogram reconstructed using the newly-generated and retrieved from GenBank sequences for *Petasiger* spp. A, *nad*1 dataset (474 nt positions); B, 28S rDNA dataset (1,261 nt). Nodal support [bootstrap values > 70% (NJ, ML) and posterior probablility values > 0.95 (BI) shown only] is provided as NJ/ML/BI. The scale-bar indicates the expected number of substitutions per site. Isolates are coded as in Table 1; *Echinostoma revolutum* was used as an outgroup

than body [TL/BL = 10–17 (14)], with maximum width 146–285 (220), much longer than wide [TL/TW = 6–12 (9)]. Two fine tegumental membranes present along three quarters of tail (1 dorsal and 1 ventral), interrupted in some specimens (Fig. 5A, B). Tail musculature consists of a group of fine longitudinal muscle bands throughout length of tail and numerous circular and oblique muscle fibres. Clearly separated large brown cells present along median axis of tail in immature cercariae.

Oral sucker ventro-subterminal, 41–51 × 40–57 (45 × 48). Ventral sucker muscular, post-equatorial, 40–55 × 38–55 (45 × 46). Prepharynx distinct; pharynx elongate-oval, 16–24 × 11–18 (19 × 14); oesophagus long, bifurcates anterior to ventral sucker; caeca narrow, reach anterior margin of excretory vesicle. Cystogenous gland-cells numerous, with rhabditiform contents, occupy most of body posterior to pharynx. Penetration gland-cells few (5–6), small, indistinct, on either side of oesophagus. Genital primordia in 2 cellular masses anterior and posterior to ventral sucker, connected by chain of cells.

Excretory vesicle bipartite; main (ascending) collecting ducts wide, filled with 37–70 refractive granules 4–15 in diameter (most formed by fusion of 2–3 smaller ones) between posterior margin of pharynx and mid-level of ventral sucker, narrowing posteriorly, connecting to anterior borders of excretory vesicle; accessory excretory vesicle present in narrow anterior region of tail. Flame-cell formula not determined.

Measurements from 6 fixed cercariae: Body 148–206 × 54–87 (177 × 71); collar 47–62 (53) wide; oral sucker 30–39 × 36–41 (35 × 38); ventral sucker 35–42 × 28–41 (38 × 36); tail 1,130–1,471

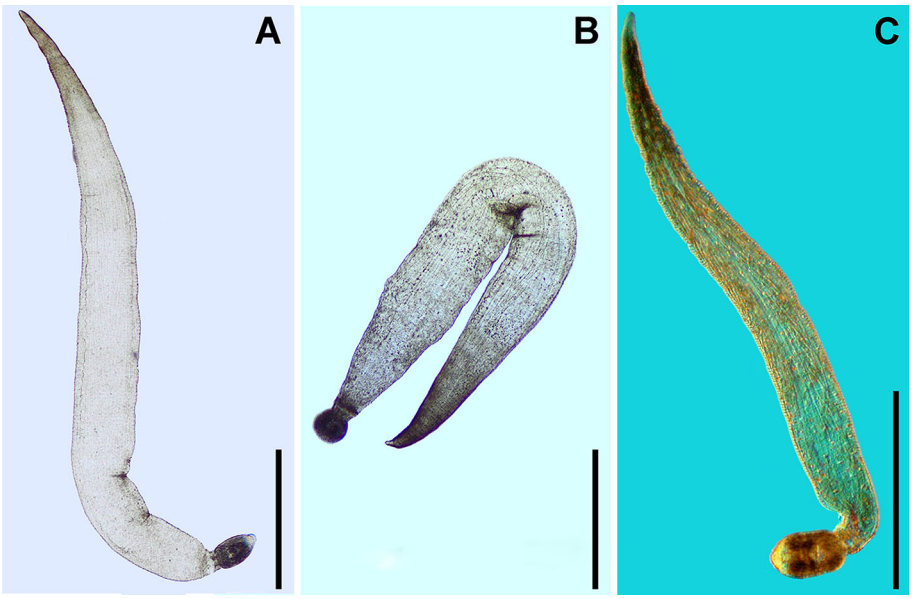

Fig. 2 Live cercaria, total view. A, *Petasiger* sp. 1; B, *Petasiger* sp. 2; C, *Petasiger* sp. 3. *Scale-bars*: 500 μm

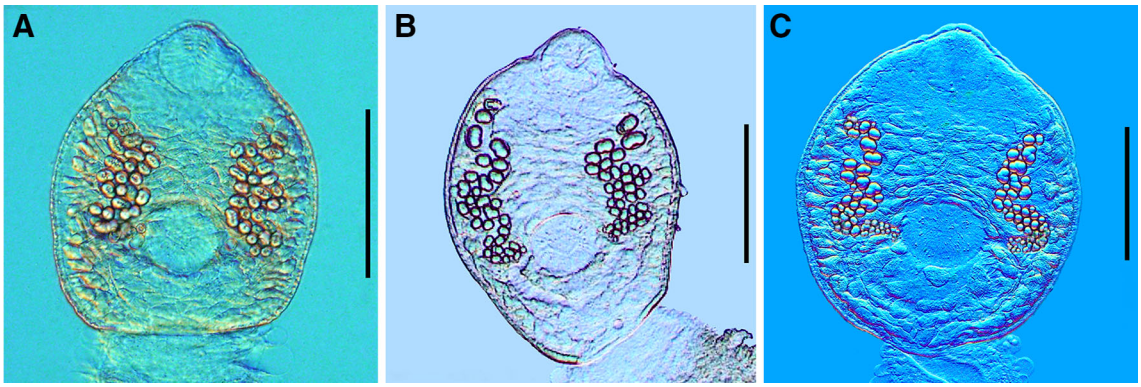

Fig. 3 Live cercaria, body. A, *Petasiger* sp. 1; B, *Petasiger* sp. 2; C, *Petasiger* sp. 3. *Scale-bars*: 100 μm

(1,345) long, with maximum width 169–197 (183); TL/BL = 5–10 (8); TL/TW = 6–9 (7).

Remarks

The cercariae of *Petasiger* sp. 1 and *Petasiger* sp. 2 (described below) both possess fine membranes on the tails (a feature not previously described in other large-tailed echinostomatid cercariae), collar spines of similar shape and size (Fig. 4A, B) and are morphologically very similar with only a few non-overlapping distinguishing features. The body of the cercaria of *Petasiger* sp. 1 is longer [live material, 111–257 *vs* 101–110 (mean 175 *vs* 105 μm)] and the ratio TL/BL differs [10–17 *vs* 16–20 (mean 14 *vs* 18)]. Comparisons with *Petasiger* sp. 3 (described below) revealed a number of distinctive differences in cercarial morphology that allow distinguishing the species. The tail of the cercaria of *Petasiger* sp. 1 is longer [1,194–2,280 *vs* 1,176–1,858 (mean 1,804 *vs* 1,486 μm)] and wider [146–285 *vs* 103–200 (mean 220 *vs* 145 μm)] than in *Petasiger* sp. 3; the ratio TL/BL is also higher in the former [10–17 *vs* 7–12 (mean 14 *vs* 10)] (Fig. 2A, C). Fine tegumental dorsal and ventral

membranes are present on the tail of the cercaria of *Petasiger* sp. 1 (*vs* absent in *Petasiger* sp. 3). The body of the cercaria of *Petasiger* sp. 1 is colourless whereas the cercaria of *Petasiger* sp. 3 contains small yellow pigmented granules on either side of the prepharynx and oesophagus and the collar spines of *Petasiger* sp. 1 are slightly thinner (2–3 µm) with a sharp tip, compared with the somewhat wider (3–4 µm) blunt spines of *Petasiger* sp. 3 (Fig. 4A, C).

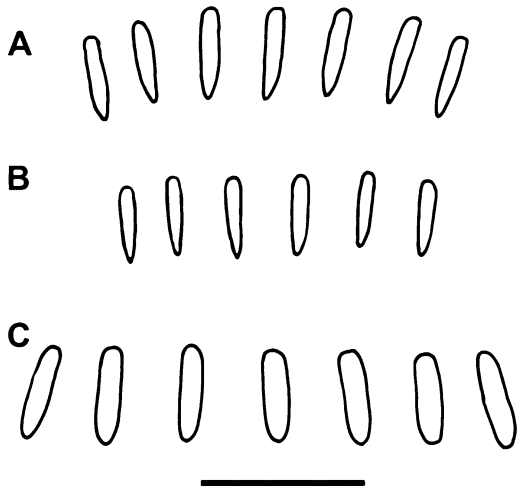

Fig. 4 Dorsal collar spines of cercaria. A, *Petasiger* sp. 1; B, *Petasiger* sp. 2; C, *Petasiger* sp. 3. *Scale-bar*: 25 µm

Within the group of large-tailed cercariae with 19 collar spines described from the Palaearctic, the cercariae of *P. islandicus* and *Cercaria titfordensis* (syn. *Petasiger neocomense* of Karmanova, 1971 *sensu* Kostadinova & Chipev, 1992) with their wide, leaf-like tails differ markedly from those of *Petasiger* sp. 1 possessing almost parallel sides. In addition to the different shape, the cercarial tail in *P. islandicus* and *C. titfordensis* is much shorter than in *Petasiger* sp. 1 (fixed material, 740–972 and 544–736 *vs* 1,130–1,471 µm, respectively). Cercariae of *Petasiger grandivesicularis* and *Petasiger* sp. of Kostadinova (1997) show different, non-overlapping ratios TL/BL (6–7 and 4–6, respectively, *vs* 10–17 in *Petasiger* sp. 1) and can thus be clearly distinguished from the latter. Furthermore, the body of the cercaria of *P. grandivesicularis* is larger [length 203–394 *vs* 111–257 (mean 274 *vs* 175 µm)]. The cercaria of *Petasiger* sp. of Kostadinova (1997) has a shorter [753–1,380 *vs* 1,194–2,280 (mean 945 *vs* 1,804 µm)] and narrower tail [128–142 *vs* 146–285

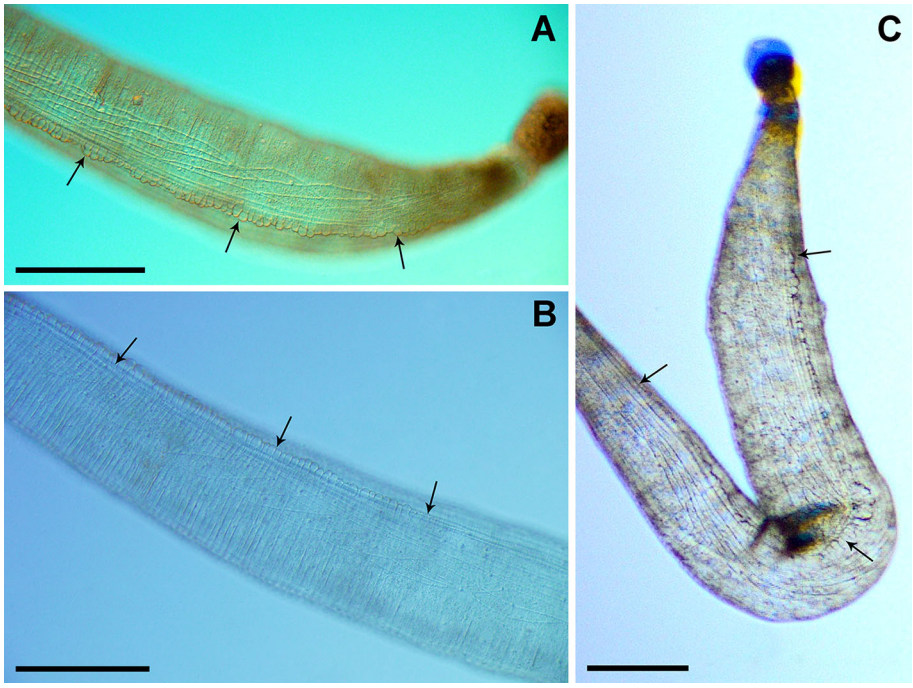

Fig. 5 Details of cercarial tail with fine ventral and dorsal tegumental membranes (*arrows*). A, B, *Petasiger* sp. 1; C, *Petasiger* sp. 2. *Scale-bars*: 200 µm

(mean 133 *vs* 220 μm)] than the cercaria of *Petasiger* sp. 1 and contains yellow pigment in the body at the level of the pharynx that is not present in *Petasiger* sp. 1. The cercaria of *Petasiger* sp. described by Ginetsinskaya & Dobrovolskij (1964) has 19 spines and a long tail with parallel sides similar to *Petasiger* sp. 1 (1,300–1,500 *vs* 1,194–2,280 μm) but exhibits a lower ratio TL/BL (10 *vs* 10–17). Since the description by Ginetsinskaya & Dobrovolskij (1964) does not contain information allowing detailed comparisons, this form is not included in the key below.

Although the cercariae described by Khan (1960), *C. hamptonensis* and *C. thamesensis*, were reported as having 20 collar spines, there is a possibility that this is due to miscounts and the cercariae belong to the group with 19 collar spines instead. In that case, *C. thamesensis* could be clearly distinguished from the cercaria of *Petasiger* sp. 1 by the much shorter [925–1,125 *vs* 1,194–2,280 (mean 1,065 *vs* 1,804 μm)], leaf-like (*vs* with almost parallel sides), colourless (*vs* brownish) tail. *Cercaria hamptonensis* is described with a tail with almost parallel sides comparable to *Petasiger* sp. 1 but its body is much longer [233–380 *vs* 111–257 (mean 290 *vs* 175 μm)] resulting in a much lower ratio TL/BL [4–9 *vs* 10–17 (mean 14)]. Furthermore, yellow pigment is described to be diffused throughout body and tail of *C. hamptonensis* but is absent in *Petasiger* sp. 1.

Petasiger sp. 2

First intermediate host: *Gyraulus albus* (Müller) (Planorbidae).
Locality: Hennetalsperre, Germany.
Voucher material: IPCAS D-708 (hologenophore).
Representative DNA sequences: KM191810 - KM191 811 (*nad*1); KM191802 (28S r DNA).

Description (Figs. 2B, 3B, 4B, 5C)

Redia
Body of daughter redia slightly brownish, elongate-saccular, 1,551–2,372 × 294–311 (1,962 × 302). Collar 116–153 (135) wide, divided into 4 lobes (2 lateral, 1 dorsal and 1 ventral). Pharynx 69 × 63–67 (69 × 65). Intestine saccate, reaching to locomotory processes; the latter 2, located in last quarter of body.

Cercaria
[Measurements from 2 live specimens.] Body colourless, elongate-oval, 101–110 × 122–125 (105 × 123). Entire body surface covered with minute spines. Collar 54–79 (67) wide, with 19 collar spines (formula 4–11–4: 4 angle spines on each ventral lappet, 14 × 3; 11 marginal spines in a single row, 12–13 × 2).

Tail massive, light brownish, elongate, with almost parallel sides, 1,665–2,239 (1,952) long, much longer than body [TL/BL = 16–20 (18)], with maximum width 253–279 (266), much longer than wide [TL/TW = 7–8 (7)]. Two fine tegumental membranes present along three quarters of tail (1 dorsal and 1 ventral), interrupted in some specimens (Fig. 5C). Tail musculature consists of a group of fine longitudinal muscle bands throughout length of tail and numerous circular and oblique muscle fibres.

Oral sucker ventro-subterminal, 45–48 × 43–50 (46 × 47). Ventral sucker muscular, post-equatorial, 50 × 50. Prepharynx distinct; pharynx elongate-oval, 18 × 13; oesophagus long, bifurcates anterior to ventral sucker; caeca narrow, reach anterior margin of excretory vesicle. Cystogenous gland-cells numerous, with rhabditiform contents, occupy most of body posterior to pharynx. Penetration gland-cells few, small, indistinct, on either side of oesophagus. Genital primordia in 2 cellular masses anterior and posterior to ventral sucker, connected by chain of cells.

Excretory vesicle bipartite; main (ascending) collecting ducts wide, filled with 80–83 refractive granules 3–17 in diameter (most formed by fusion of 2–3 smaller ones) between posterior margin of pharynx and mid-level of ventral sucker, narrowing posteriorly, connecting to anterior borders of excretory vesicle; accessory excretory vesicle present in narrow anterior region of tail. Flame-cell formula not determined.

Measurements from 6 fixed cercariae: Body 123–145 × 60–82 (137 × 67); collar 41–52 (46) wide; oral sucker 28–38 × 28–34 (31 × 31); ventral sucker 30–43 × 29–44 (35 × 33); tail 1,025–1,551

(1,159) long, with maximum width 110–138 (128); TL/BL = 7–13 (9); TL/TW = 8–10 (9).

Remarks

The cercariae of *Petasiger* sp. 2 and *Petasiger* sp. 3 show a number of distinctive differences that allow to distinguish the two forms. The tail of the cercaria of *Petasiger* sp. 2 is longer [live material, 1,665–2,239 *vs* 1,176–1,858 (mean 1,952 *vs* 1,486 μm)] and wider [253–279 *vs* 103–200 (mean 266 *vs* 145 μm)] than in *Petasiger* sp. 3; the ratio TL/BL is also considerably higher in the former [16–20 *vs* 7–12 (mean 18 *vs* 10)] (see Fig. 2B, C for comparison). Fine tegumental dorsal and ventral membranes are present on the tail of the cercaria of *Petasiger* sp. 2 (*vs* absent in *Petasiger* sp. 3). The body of the cercaria of *Petasiger* sp. 2 is colourless whereas the cercariae of *Petasiger* sp. 3 contain small yellow pigmented granules on both sides of the prepharynx and oesophagus and the collar spines are slightly thinner (2–3 μm), with a sharp tip [*vs* somewhat wider (3–4 μm) blunt spines in *Petasiger* sp. 3] (Fig. 4B, C).

Within the group of large-tailed cercariae with 19 collar spines described from the Palaearctic, the cercariae of *P. islandicus* and *C. titfordensis* with their short, wide, leaf-like tails differ markedly from *Petasiger* sp. 2, possessing an elongate tail with almost parallel sides (fixed material, 740–972 and 544–736 *vs* 1,025–1,551 μm in *Petasiger* sp. 2, respectively). The cercariae of *Petasiger grandivesicularis* and *Petasiger* sp. of Kostadinova (1997) show different, non-overlapping ratios TL/BL (6–7 and 4–6, respectively, *vs* 16–20 in *Petasiger* sp. 2) and can thus be clearly distinguished. The cercarial body of *P. grandivesicularis* is colourless, two to four times longer [203–394 *vs* 101–110 (mean 274 *vs* 105 μm)] and also slightly wider [120–219 *vs* 122–125 (mean 159 *vs* 123 μm)] than in *Petasiger* sp. 2 and the tail is much shorter [753–1,380 *vs* 1,665–2,239 (mean 945 *vs* 1,952 μm)] and narrower [128–142 *vs* 253–279 (mean 133 *vs* 266 μm)]. The cercaria of *Petasiger* sp. of Ginetsinskaya & Dobrovolskij (1964) has a shorter tail (1,300–1,500 *vs* 1,665–2,239 μm in *Petasiger* sp. 2), distinctly larger body (179–190 *vs* 101–110 μm) and a considerably lower ratio TL/BL (10 *vs* 16–20).

The cercaria of *Petasiger* sp. 2 can be differentiated from *C. hamptonensis* and *C. thamesensis* based on other features than the questionable number of spines. The short, leaf-like, colourless tail of *C. thamesensis* is clearly distinguishable from the much longer, almost parallel-sided tail in *Petasiger* sp. 2 [925–1,125 *vs* 1,665–2,239 (mean 1,065 *vs* 1,952 μm)]. *Cercaria hamptonensis* differs from *Petasiger* sp. 2 in having a body more than twice as long [233–380 *vs* 101–110 (mean 290 *vs* 105 μm)] and a substantially lower ratio TL/BL [4–9 *vs* 16–20 (mean 18)].

Petasiger sp. 3

First intermediate host: *Gyraulus albus* (Müller), *Planorbis planorbis* (L.) (Planorbidae).
Localities: Hennetalsperre, Hengsteysee, Kleiner Plöner See, Germany.
Voucher material: IPCAS D-709 (hologenophore).
Representative DNA sequences: KM191812 - KM191816 (*nad*1); KM191803 - KM191806 (28S rDNA).

Description (Figs. 2C, 3C, 4C)

Redia
Body of daughter redia brownish, elongate, 434–1,994 × 115–335 (1,097 × 221). Collar 113–164 (138) wide, divided into 4 lobes (2 lateral, 1 dorsal and 1 ventral). Pharynx 48–90 × 42–88 (66 × 62). Intestine saccate, reaching posterior to locomotory processes; the latter 2, situated in last quarter of body.

Cercaria
[Measurements from 15 live specimens.] Body elongate–oval, with yellow pigment in anterior part on both sides of prepharynx and oesophagus, 135–225 × 96–184 (172 × 130). Entire body surface covered with minute spines. Collar 80–115 (103) wide, with 19 collar spines (formula 4–11–4; 4 angle spines on each ventral lappet, 12–16 × 3–4; 11 marginal spines in single row, 13–16 × 3–4).

Tail massive, light brownish, elongate, with almost parallel sides, 1,176–1,858 (1,486) long, much longer than body [TL/BL = 7–12 (10)], with maximum width 103–200 (145), much longer than wide [TL/TW = 9–13 (11)]. Membranes on tail absent. Tail musculature consists of a group of fine longitudinal muscle bands throughout length of tail and numerous circular and oblique muscle fibres. Clearly

separated large transparent cells present along median axis of tail in immature cercariae.

Oral sucker ventro-subterminal, 34–50 × 44–59 (45 × 52). Ventral sucker muscular, post-equatorial, 44–59 × 48–62 (53 × 56). Prepharynx short; pharynx elongate-oval, 19–26 × 10–20 (22 × 16); oesophagus long, bifurcates anterior to ventral sucker; caeca narrow reach anterior margin of excretory vesicle. Cystogenous gland-cells numerous, with rhabditiform contents, occupy most of body posterior to pharynx, accessory excretory vesicle in narrow anterior region of tail. Penetration gland-cells few (5–6), small, indistinct, on either side of oesophagus. Genital primordia in 2 cellular masses anterior and posterior to ventral sucker, connected by chain of cells.

Excretory vesicle bipartite; main (ascending) collecting ducts wide, filled with 55–92 refractive granules 3–15 in diameter (most formed by fusion of 2–5 smaller ones) between posterior margin of pharynx and mid-level of ventral sucker, narrowing posteriorly, connecting to anterior borders of excretory vesicle. Flame-cell formula not determined.

Measurements from 11 fixed cercariae: Body 119–170 × 68–98 (151 × 80); collar 50–56 (54) wide; oral sucker 32–39 × 32–36 (36 × 34); ventral sucker 27–40 × 27–40 (34 × 35); tail 862–1,296 (1,116) long, with maximum width 81–121 (100); TL/BL = 6–10 (7); TL/TW = 7–14 (11).

Remarks

The cercaria of *Petasiger* sp. 3 keys down to *C. rashidi* in the key to the large-tailed cercariae of Kostadinova & Chipev (1992). Both forms show matching morphological features. Thus, the ratios TL/BL and TL/TW vary within overlapping ranges [TL/BL 7–12 in *Petasiger* sp. 3 *vs* 6–13 in *C. rashidi*; TL/TW 9–13 *vs* 9 (published drawing)]. The body length of fixed cercariae of *Petasiger* sp. 3 and *C. rashidi* is similar (119–170 *vs* 112–168 μm) but *Petasiger* sp. 3 appears slightly narrower than *C. rashidi* (68–98 *vs* 88–136 μm). The tail of formalin-fixed *Petasiger* sp. 3 is shorter than reported for *C. rashidi* (862–1,296 *vs* 1,448–1,640 μm). However, we believe this difference is due to the fixation of *Petasiger* sp. 3 in cold formalin in which the body, and especially the tail, can shrink. The method of Nasir (1962) of relaxing the cercariae with Neutral Red before fixing in hot formalin seems to provide results much more consistent with our measurements of the tail from live cercariae (1,176–1,858 μm). The number of refractive granules in the main collecting ducts of the excretory vesicle differs slightly in both descriptions (55–92 formed by fusion of 2–5 smaller ones in *Petasiger* sp. 3 *vs* 42–64 formed by fusion of 2–3 smaller ones in *C. rashidi*); however, both shape and number of granules can be very variable between cercariae of the same species. The collar spines are of similar sizes in *Petasiger* sp. 3 and *C. rashidi* (dorsal spines 13–16 *vs* 12–14 μm; angle spines 12–16 *vs* 16 μm) and the width of both oral sucker (32–39 *vs* 29–33 in fixed cercariae) and ventral sucker (27–40 *vs* 31–35 in fixed cercariae) is overlapping. Most noticeably, both cercariae possess characteristic small yellow granules on either side of the prepharynx that are absent in most *Petasiger* spp. Based on the key of Kostadinova & Chipev (1992) and the descriptions and drawings of Nasir (1962), we consider *C. rashidi* conspecific with *Petasiger* sp. 3.

The cercaria of *Petasiger* sp. 3 differs distinctly from the cercariae of *P. islandicus* and *C. titfordensis* possessing wide, leaf-like tails, (TL/TW 9–13 *vs* 4–8 and 3–4, respectively). The characteristic yellow pigment in the body of *Petasiger* sp. 3 is not present in either *P. islandicus* or *C. titfordensis*. The cercaria of *P. grandivesicularis* shows a different ratio TL/BL (6–7 *vs* 7–12 in *Petasiger* sp. 3). Furthermore, the body of *P. grandivesicularis* is larger [length 203–394 *vs* 135–225 (mean 274 *vs* 172 μm) in *Petasiger* sp. 3] and also lacks yellow pigment. *Petasiger* sp. of Kostadinova (1997) is described with similar yellow pigment at the level of the pharynx but can be distinguished from *Petasiger* sp. 3 based on the non-overlapping ratio TL/BL (4–6 *vs* 7–12 in *Petasiger* sp. 3). Although within a slightly overlapping range, the cercarial tail of *Petasiger* sp. of Kostadinova (1997) is shorter than in *Petasiger* sp. 3 [753–1,380 *vs* 1,176–1,858 (mean 945 *vs* 1,486 μm)]. The cercaria of *Petasiger* sp. of Ginetsinskaya & Dobrovolskij (1964) has a tail of similar length (1,300–1,500 *vs* 1,176–1,858 μm in *Petasiger* sp. 3) and a similar ratio TL/BL (10 *vs* 7–12). However, the cercarial tail in *Petasiger* sp. 3 is distinctly more elongate [ratio TL/TW 9–13 (mean 11) *vs* 6 in *Petasiger* sp. (estimated from the published figure)] and the cercarial body is colourless (Ginetsinskaya & Dobrovolskij, 1964).

The cercaria of *Petasiger* sp. 3 can be distinguished from *C. hamptonensis* and *C. thamesensis* based on features other than the number of collar spines as

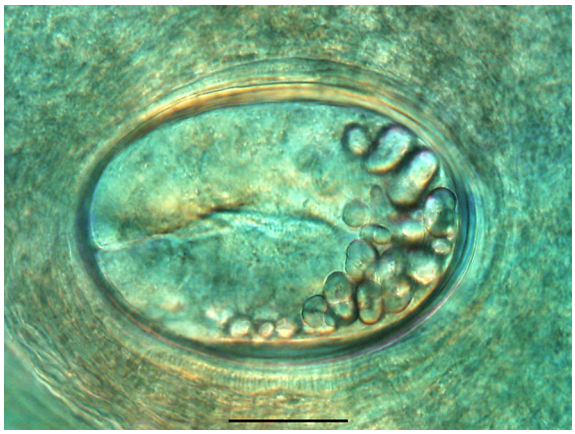

Fig. 6 Metacercaria of *Petasiger* sp. 4. *Scale-bar*: 25 μm

follows. The short, leaf-like, colourless tail of *C. thamesensis* is clearly distinguishable from the longer, light brownish tail with almost parallel sides of the cercaria of *Petasiger* sp. 3 [925–1,125 *vs* 1,176–1,858 (mean 1,065 *vs* 1,486 μm)]. Furthermore, the body of *C. hamptonensis* is much longer [233–380 *vs* 135–225 (mean 290 *vs* 172 μm)] and the ratio TL/BL is lower than in *Petasiger* sp. 3 [4–9 *vs* 7–12 (mean 10)].

Petasiger sp. 4

Second intermediate host: *Gasterosteus aculeatus* L. (Gasterosteiformes: Gasterosteidae).
Locality: Gosling Lake, Vancouver Island, Canada.
Representative DNA sequences: KM191817 (*nad*1); KM191807 (28S rDNA).

Description (Fig. 6)

Metacercaria
[Measurements from 3 fixed specimens encysted in oesophageal wall of the host.] Cyst elongate-oval, 76–90 × 50–57 (81 × 52), covered with thin layer (6–7) of parasite origin, surrounded by thick layer (19–20) of connective tissue of host origin. Body spined; collar bears 19 spines. Main collecting excretory ducts filled with large, composite excretory granules.

Remarks

The life-cycles of two North American species of *Petasiger* possessing 19 collar spines have been elucidated experimentally: *Petasiger nitidus* Linton, 1928 and *Petasiger chandleri* Abdel-Malek, 1952 (see Beaver, 1939; Abdel-Malek, 1953). Of these, the metacercaria of *P. nitidus* encysting in the oesophagus and lower pharynx of experimentally infected fish [*Ameiurus nebulousus* (Lesueur), *Notropis hudsonius* (Clinton), *Umbra limi* (Kirtland), *Lepomis pallidus* (Mitchell), *Ambloplites rupestris* (Rafinesque), *Perca flavescents* Mitchell, *Lebistes reticulatus* (Peters) and 'minnows'] appears similar to the metacercaria ex *G. aculeatus* in both shape and size (invariably ovoid, 85 × 68 μm), in possessing a thick outer layer of host tissue surrounding the cyst (up to 40 μm) and in the presence of large excretory granules in the main excretory ducts (Beaver, 1939).

The metacercaria of *P. chandleri* (considered a *species inquirenda* by Faltýnková et al., 2008) is slightly larger (100 × 73 μm) than that of *Petasiger* sp. 4. Furthermore, *P. chandleri* is described with 19–21 spines but this variation may be a result of lumping together the observations on the cercaria (Abdel-Malek, 1952) and adult (Abdel-Malek, 1953) (see Faltýnková et al., 2008). The species status of *P. chandleri* remains questionable until further material from the final host is described (Faltýnková et al., 2008). Based on the morphological data of the metacercariae, it is possible that *Petasiger* sp. 4 is conspecific with *P. nitidus*. However, species identification based on morphological features of the metacercariae is rather difficult and further findings of cercariae and adults of *Petasiger* spp. from North America that provide comparative molecular and morphological data are necessary to elucidate the life-cycle of *Petasiger* sp. 4 and confirm whether it is actually conspecific with *P. nitidus*. We provide the first molecular data for such a comparison.

Key to the large-tailed echinostomatid cercariae from the Palaearctic

1a Head collar with 20 spines; two groups of four angle spines each plus 12 marginal spines in single row (formula 4-12-4) 2
1b Head collar with 19 spines; two groups of four angle spines each plus 11 marginal spines in single row (formula 4-11-4) 3
2a Tail elongate (ratio TL/BL = 7–8), with almost parallel sides (ratio TL/TW = 10–14).

Yellow pigment in the body and tail. In *Planorbis planorbis* .... *Cercaria hamptonensis* Khan, 1960

2b Tail leaf-like (ratio TL/BL = 3–4; ratio TL/TW = 2–4). Body and tail colourless. In *Planorbis planorbis* .... *Cercaria thamesensis* Khan, 1960

3a Tail leaf-like 4

3b Tail elongate with almost parallel sides 5

4a Tail short (540–740 μm in fixed cercarie). Ratio TL/TW = 3–4. Body and tail colourless. In *Planorbis carinatus* .... *Cercaria titfordensis* Nasir, 1962 [syn. Cercaria of *Petasiger neocomense* of Karmanova (1971)]

4b Tail long (740–970 μm in fixed cercarie). Ratio TL/TW = 4–8. Body whitish, tail brownish. In *Gyraulus* cf. *laevis* ..... *Petasiger islandicus* Kostadinova & Skírnisson, 2007

5a Ratio TL/BL < 7 6

5b Ratio TL/BL > 7 7

6a Body with 2 ventral pits. Ratio TL/BL = 4–6; ratio TL/TW = 6–10. Cercariae negatively phototactic. In *Planorbis planorbis* .... *Petasiger* sp. of Kostadinova (1997)

6b Body lacking ventral pits. Ratio TL/BL = 6–7; ratio TL/TW = 10–15. Cercariae positively phototactic. In *Planorbis planorbis* .... *Petasiger grandivesicularis* Ishii, 1935

7a Tail with fine ventral and dorsal tegumental membranes. Body colourless 8

7b Tail lacking tegumental membranes. Yellow pigment on both sides of prepharynx. In *Gyraulus albus* and *Planorbis planorbis* .... *Petasiger* sp. 3 [syn. *Cercaria rashidi* Nasir, 1962]

8a Ratio TL/BL = 10–17. Body length >110 μm. In *Gyraulus albus* and *Planorbis planorbis* .... *Petasiger* sp. 1

8b Ratio TL/BL = 16–20. Body length <110 μm. In *Gyraulus albus* ..... *Petasiger* sp. 2

Discussion

The present material possesses features that are fully consistent with the morphology of *Petasiger* spp. whose life-cycles have been elucidated either experimentally or with the aid of sequence data (Kostadinova & Chipev, 1992; Georgieva et al., 2012). The main characteristics of the larval stages of *Petasiger* spp. of the group with 19 collar spines include: (i) daughter redia with a collar divided into four lobes, long saccate intestine, and two distinct locomotory processes; (ii) large-tailed cercaria with 19 spines (formula 4–11–4; four angle spines on each ventral lappet), cystogenous gland-cells with rhabditiform contents occupying most of the body and main ascending collecting ducts of the excretory system filled with large refractive granules, great mass of which are formed by fusion of smaller ones; and (iii) metacercaria encysting on the inner surface of pharyngeal region, anterior oesophagus or maxillar musculature of fish; cysts invariably ovoid, with a thin layer of parasite origin enveloped (partially or entirely) with thick connective tissue of host origin; main excretory collecting ducts of metacercaria filled with large, composite excretory granules.

Both morphological and molecular data supported the distinct status of the larval stages of the three *Petasiger* spp. from *G. albus* and *P. planorbis* in central Europe and of the metacercaria ex *G. aculeatus* in Canada. All three European species occurred in sympatry in one locality (Lake Hennetalsperre); this fact highlights the high diversity of *Petasiger* spp. even at small spatial scales. Further, two of the species developed in both hosts studied, *G. albus* and *P. planorbis*, thus indicating that a relaxed host specificity towards the snail intermediate host may be common in *Petasiger* spp. The concordance of the results from the two approaches applied in the present study stresses the importance of the combination of molecular and well-documented morphological data for the advancement of our understanding of the diversity and relationships of *Petasiger* spp.

However, we found it impossible to reach decisions regarding the identity of the four species studied due to the scarcity of comparative data. Of the five species with 19 collar spines parasitising grebes in Europe, i.e. *P. grandivesicularis*, *P. islandicus*, *P. megacanthus*, *P. neocomense* and *P. pungens* (Linstow, 1893), three were recorded in Germany: *P. neocomense* ex *Podiceps cristatus* (L.) (type-host) (see Odhner, 1910, as *Echinostomum pungens*); Odening, 1963); *P. pungens* ex *Tachybaptus ruficollis* (Pallas) (see Odening, 1962, 1965); and *P. grandivesicularis* ex *T. ruficollis* (see

Kostadinova, 1999). The life-cycle of the last species has been completed experimentally and our comparisons of the three cercariae described here with the cercaria described by Kostadinova & Chipev (1992) revealed substantial morphological differences that rule out a possibility for conspecificity.

The life-cycles of *P. neocomense* and *P. pungens* are not yet known. Karmanova (1971) described a large-tailed cercaria with 19 collar spines from the planorbid *Gyraulus acronicus* (Férrusac) in the Volga Delta (Astrakhan Reserve), which encysted in *Rutilus rutilus* (L.), *Scardinius erythrophthalmus* (L.) and *Alburnus alburnus* (L.). She identified the adults obtained in experimental infections of young grebes as *P. neocomense* but did not describe the adult. Kostadinova & Chipev (1992) suggested that *C. titfordensis* and the cercaria described by Karmanova (1971) belong to one and the same species but expressed concerns about the identification of the adults obtained experimentally by Karmanova due to the lack of morphological data and the contradictions in the Russian literature concerning the descriptions of *P. neocomense*, an opinion with which we agree (see key above). Nevertheless, both *C. titfordensis* and the cercaria described by Karmanova (1971) can be readily differentiated from the three cercariae described by us by the distinct shape of the tail and other morphological features.

Kostadinova (1997) described and compared the morphology of an unidentified cercaria of *Petasiger* ex *P. planorbis* occurring in sympatry with *P. grandivesicularis* in a lake on the Bulgarian Black Sea coast and suggested that it is probably a larval stage of *P. pungens*. However, although experimental infections of fish were successful, no adult worms were obtained. Therefore, based on the geographical distribution of the adults, we do not rule out the possibility that two of the cercariae described by us may represent *P. neocomense* and *P. pungens*. Nevertheless, our findings and the updated key to the large-tailed cercariae indicate a much higher diversity of larval rather than adult stages of *Petasiger* spp. in Europe (cercariae of nine *vs* adults of five species). Further studies focused on obtaining molecular data for the adult parasites of grebes would be influential for the identification of the larval stages in snails and fish and the elucidation of the life-cycles of *Petasiger* spp. (e.g. Georgieva et al., 2012).

Acknowledgements We thank two anonymous reviewers for their valuable comments and suggestions that improved our manuscript. We are grateful to Jana Köchling, Verena Altmann and Jessica Schwelm (University of Duisburg-Essen) and the students of the Parasitology Course 2012 (International Max Planck Research School for Evolutionary Biology, Plön, Germany) for their assistance during sampling and in the laboratory, and to Blanka Škoríková (Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic) for her kind help with the figures. Furthermore, we thank Dan I. Bolnick (University of Texas at Austin, Section of Integrated Biology, Austin, TX, USA) for providing sticklebacks from Vancouver Island. This study was supported by the Czech Science Foundation (grant P505/12/G112); the 'Sichere Ruhr' project as part of the Bundesministerium für Bildung und Forschung (BMBF) program 'Sustainable Water Management' (grant 02WRS1283). CS benefited from a Deutsche Bundesstiftung Umwelt (DBU) PhD scholarship.

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Selbach *et al. Parasites & Vectors* (2015) 8:300
DOI 10.1186/s13071-015-0904-4

Parasites & Vectors

RESEARCH Open Access

Integrative taxonomic approach to the cryptic diversity of *Diplostomum* spp. in lymnaeid snails from Europe with a focus on the '*Diplostomum mergi*' species complex

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Abstract

Background: Recent molecular studies have discovered substantial unrecognised diversity within the genus *Diplostomum* in fish populations in Europe and North America including three species complexes. However, data from the first intermediate host populations are virtually lacking. This study addresses the application of an integrative taxonomic approach to the cryptic species diversity of *Diplostomum* spp. in natural lymnaeid snail populations in Europe with a focus on the '*D. mergi*' species complex.

Methods: Totals of 1,909 *Radix auricularia*, 349 *Radix peregra*, 668 *Stagnicola palustris* and 245 *Lymnaea stagnalis* were sampled at five reservoirs of the Ruhr river system in Germany and screened for infections with *Diplostomum* spp. Cercariae were examined and identified alive, fixed and under scanning electron microscopy. Sequences from the barcode region of the cytochrome *c* oxidase subunit 1 (*cox*1) mitochondrial gene and from the internal transcribed spacer cluster (ITS1-5.8S-ITS2) of the rRNA gene were amplified for 51 and 13 isolates, respectively.

Results: Detailed morphological and molecular analyses provided evidence for three named species (*Diplostomum spathaceum*, *D. pseudospathaceum* and *D. parviventosum*), and a further four species-level lineages ('*D. mergi* Lineages 2–4' and '*Diplostomum* sp. Clade Q' in the lymnaeid snail populations from the Ruhr river basin. The paper provides the first descriptions of molecularly identified cercariae of *D. spathaceum* and of the cercariae of *D. parviventosum*, three lineages of the '*D. mergi*' species complex and of '*Diplostomum* sp. Clade Q'.

Conclusion: The integration of molecular and morphological evidence for *Diplostomum* spp. achieved in this study will serve as a baseline for species identification of these important parasites of snail and fish populations and thus advance further studies on the distribution of *Diplostomum* spp. in Europe.

Keywords: '*Diplostomum mergi*' species complex, *Diplostomum parviventosum*, *Diplostomum pseudospathaceum*, *Diplostomum spathaceum*, *Radix auricularia*, *Lymnaea stagnalis*, *Stagnicola palustris*, Cercariae, *cox*1, ITS, Europe

Background

The incorporation of molecular data has brought a major advancement in species taxonomy, due to the possibility to distinguish cryptic species and re-evaluate existing morphological identification criteria. Especially for trematode species with complex life-cycles, where sampling often provides only one stage of a parasite's life-cycle at a time (*e.g.* cercariae or metacercariae only), molecular analyses provide an effective means of species identification and inference of complete life-cycles by matching data from the different life-cycle stages [1, 2]. However, although larval trematodes in snails are potentially useful indicators of environmental conditions [3], they are difficult to identify and the taxonomic expertise is limited to few individuals [4]. This highlights the importance of providing accurate and accessible information on their identification.

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Diplostomum von Nordmann, 1832 is a major and important taxonomic group of widely distributed freshwater trematode parasites that utilise lymnaeid snails, fish and fish-eating birds to complete their life-cycles. Recent molecular studies have discovered higher than previously recognised diversity of *Diplostomum* spp. at small geographical scales. A total of 12 species was found in fishes in northern Canada based on molecular evidence: three named species, *Diplostomum indistinctum* (Guberlet, 1923), *Diplostomum huronense* (La Rue, 1927) and *Diplostomum baeri* Dubois, 1937, and a further nine unidentified species-level lineages of *Diplostomum* [5]. The first studies addressing integration of morphological assessment and molecular prospecting for species diversity of the genus in Europe resulted in molecular elucidation of the life-cycles of *Diplostomum spathaceum* (Rudolphi, 1819) (type-species) and *D. pseudospathaceum* Niewiadomska, 1984 and provided evidence for a substantial unrecognised genetic and morphological diversity, *i.e.* 15 species-level lineages including three complexes of genetically distinct lineages [6–9].

Six of these originate from the snail and fish populations studied in the River Ruhr drainage in Germany. Of particular importance is the finding of a number of isolates comprising three slightly divergent lineages within the '*D. mergi*' species complex [6]. However, most of these isolates, *i.e.* '*D. mergi* Lineage 3', originate from fish and due to the low sampling effort only few isolates from their lymnaeid snail hosts are available: a single isolate for '*D. mergi* Lineage 1' and three isolates for '*D. mergi* Lineage 2', all from *Radix auricularia* (L.).

The application of barcoding approach to species diversity of *Diplostomum* in Europe depends on the availability of sequence databases based on precisely identified isolates, a process that is currently impeded by the lack of taxonomic expertise (see Georgieva *et al.* [6] for detailed discussion). This highlights the need for combining molecular data with thorough morphological descriptions that will allow species delimitation and recognition in future studies. This study addresses the application of an integrative taxonomic approach to the cryptic species diversity of *Diplostomum* spp. in natural lymnaeid snail populations in Europe with a focus on the '*D. mergi*' species complex. Detailed morphological and molecular data gathered in an extensive sampling of four lymnaeid species in five reservoirs of the Ruhr and its tributaries provided evidence for three named species and four distinct lineages of *Diplostomum* spp. The thorough morphological descriptions of the cercariae of *D. parviventosum* Dubois, 1932, *D. pseudospathaceum*, *D. spathaceum* and of the three novel lineages of the '*D. mergi*' species complex and '*Diplostomum* sp. Clade Q', in association with the molecular delineation provided here, will serve as a baseline for species identification of these important parasites of snail and fish populations and thus advance further studies on the distribution of *Diplostomum* spp. in Europe.

Methods

Sample collection

A total of 3,171 lymnaeid snails of four species [1,909 *Radix auricularia* (L.), 349 *R. peregra* (Müller), 668 *Stagnicola palustris* (Müller), 245 *Lymnaea stagnalis* (L.)] was collected and examined for trematode infections during the summer months (May to September) in 2012 and 2013. Snails were collected at several sampling sites in five reservoirs of the River Ruhr catchment area in North Rhine-Westphalia, Germany: Baldeneysee (51°24′20.08″N, 7°2′22.47″E); Hengsteysee (51°24′52.17″N, 7°27′42.55″E); Hennetalsperre (51°19′50.97″N, 8°15′46.82″E); Sorpetalsperre (51°20′15.01″N, 7°56′46.18″E); and Versetalsperre (51°10′55.71″N, 7°40′57.12″E) (see Fig. 1 and Table 1 for details). At each sampling site snails were collected randomly with hand-nets or picked by hand from sediment, stones and floating aquatic vegetation along the shore. In the laboratory all snails were measured, labelled and placed in separate beakers with lake water under a light source to stimulate cercarial emission. Snails that did not emit cercariae for several days were dissected and examined for the presence of prepatent infections. Prevalence was calculated for distinct samples (*i.e.* collected from one site within a locality on a given date) comprising more than 15 snails.

Morphological data

Cercariae were identified alive under light microscope and cercariae of *Diplostomum* spp. were identified based on the morphological descriptions and the key of Niewiadomska & Kiselienė [10]. Detailed light microscopy photographs of cercariae of *Diplostomum* spp. were taken with an Olympus UC30 digital camera on an Olympus BX51 microscope and all visible (under light microscopy) features described by Niewiadomska & Kiselienė [10] were recorded. Cercariae and/or sporocysts of all isolates were fixed in molecular grade ethanol for DNA isolation and sequencing and in cold and hot 4 % formaldehyde solution for detailed analysis of the surface morphology and body spination by scanning electron microscopy (SEM) and for obtaining measurements from fixed materials. Formalin-fixed cercariae were post-fixed in 2 % osmium tetroxide for two hours, washed in 0.1 M phosphate buffer, dehydrated through an acetone series, point-dried and sputter-coated with gold for SEM. SEM photographs were taken with a JEOL JSM-7401 F field emission scanning electron microscope. Descriptions are based on examination of live and formalin-fixed material and digital photomicrographs from both light microscopy and SEM. Measurements were taken with the program

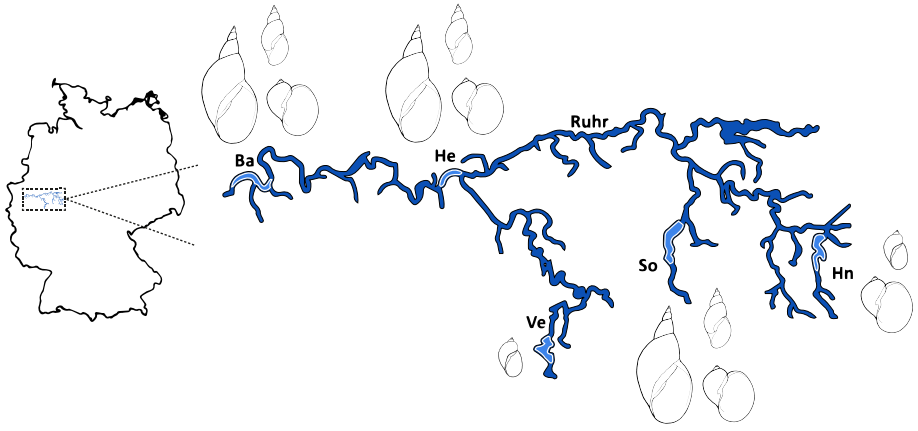

Fig. 1 Map of the Ruhr river system in Germany with sampling sites. *Abbreviations*: Ba, Baldeneyesee; He, Hengsteysee; Hn, Hennetalsperre; So, Sorpetalsperre; Ve, Versetalsperre. *Lymnaea stagnalis*; *Radix auricularia*; *Radix peregra*; *Stagnicola palustris*

ImageJ 1.47v [11]; measurements in the descriptions are based on live specimens; measurements from fixed material are provided in the tables. All measurements in the text and tables are in micrometres and are presented as the range followed by the mean in parentheses. The following abbreviations for the metrical features were used: BL, body length; BW, maximum body width; AOL, anterior organ length; AOW, anterior organ width; VSL, ventral sucker length; VSW, ventral sucker width; TSL, tail stem length; TSW, tail stem width (at base); FL, furca length (see Additional file 1: Figure S1 for a schematic illustration of a cercaria showing the metrical features). The following relative proportions (ratios) were calculated for both live and fixed cercariae and used in addition to these measurements: VSW/AOW, ventral sucker width to anterior organ width ratio; BL/TSL, body length to tail stem length ratio; TSL/FL, tail stem length to furca length ratio.

Molecular data

Total genomic DNA was isolated from 100–200 ethanol-fixed cercariae obtained from single snail individuals using the Chelex method (see [12] for details). Partial fragments of the barcode region of the *cox*1 mitochondrial gene were amplified via polymerase chain reaction (PCR) using Ready-To-Go PCR beads (GE Healthcare, UK) and the PCR primers Plat-diploCOX1F (5′-CGT TTR AAT TAT ACG GAT CC-3′) and Plat-diploCOX1R (5′-AGC ATA GTA ATM GCA GCA GC-3′) [13] as described in Georgieva *et al.* [6]. PCR amplifications of the ITS1-5.8S-ITS2 gene cluster were performed as above using the primers D1 (forward: 5′-AGG AAT TCC TGG TAA GTG CAA G-3′) and D2 (reverse: 5′-CGT TAC TGA GGG AAT CCT GGT-3′) [14].

PCR amplicons were purified using a QIAquick PCR purification kit (Qiagen Ltd, UK) and sequenced directly from both strands using the PCR primers (*cox*1) and the primers BD1 (forward: 5′-GTC GTA ACA AGG TTT CCG TA-3′) and BD2 (reverse: 5′-TAT GCT TAA ATT CAG CGG GT-3′) (ITS1-5.8S-ITS2; [14]) with ABI BigDye chemistry (ABI Perkin-Elmer, UK), alcohol-precipitated, and run on an ABI Prism 3130x1 automated sequencer. Contiguous sequences were assembled with MEGA v6 [15] and submitted to GenBank.

Sequences were aligned with Muscle implemented in MEGA v6. The 51 newly-generated *cox*1 sequences were aligned with reference to the amino acid translation, using the echinoderm and flatworm mitochondrial code [16] together with 28 sequences retrieved from GenBank, the latter comprising 1 – 4 representative sequences per species/lineage identified in previous studies in Europe [6, 17]; (see Additional file 2: Table S1 for details). The ITS1-5.8S-ITS2 sequences generated from selected isolates (n = 13) from the *cox*1-derived clades were aligned with 32 published sequences, representative for the species/

Table 1 Summary data for the lymnaeid snails examined/infected with *Diplostomum* spp. in five reservoirs of the River Ruhr catchment area in Germany

	Ba	He	So	Hn	Ve	Total
Total number of lymnaeid snails examined	437	1,772	357	292	313	3,171
Number of snails infected with *Diplostomum* spp.	8	66	4	–	–	78
Number of distinct samples with *Diplostomum* spp. infections	7 (0)*	29 (20)*	2 (2)*	–	–	38 (22)*

Abbreviations: Ba, Baldeneysee; He, Hengsteysee; Hn, Hennetalsperre; So, Sorpetalsperre; Ve, Versetalsperre
*Distinct samples with n ≥ 15 in which *Diplostomum* spp. infections were detected (number in parentheses)

lineages sequenced in Europe [6, 17, 18] and Canada [5, 14]. Sequences for *Tylodelphys* spp. were used as outgroups.

Distance-based neighbour-joining (NJ) and model-based Bayesian inference (BI) algorithms were used for tree reconstruction. Prior to analyses the best-fit nucleotide substitution models were selected in jModelTest 2.1.1 [19, 20]. These were the Hasegawa-Kishino-Yano model including estimates of invariant sites and among-site rate heterogeneity (HKY + I + G) for the *cox*1 dataset and the Hasegawa-Kishino-Yano model including estimates of invariant sites (HKY + I) for the ITS dataset. BI analyses were carried out with MrBayes 3.2 [21] using Markov Chain Monte Carlo (MCMC) searches on two simultaneous runs of four chains during 10^7 generations, sampling trees every 10^3 generations. The first 25 % of the sampled trees were discarded as with burn-in for each data set and the consensus tree topology and the nodal support were estimated from the remaining samples as posterior probability values [22]. Distance matrices (p-distance model, *i.e.* the percentage of pairwise character differences with pairwise deletion of gaps) were calculated with MEGA v6. The numbering scheme of Georgieva *et al.* [6] for the lineages of *Diplostomum* spp. was applied for consistency.

Results

Overview of infections with *Diplostomum* spp.

Examination of 3,171 lymnaeid snails belonging to four species revealed a total of 78 infections with *Diplostomum* spp. (overall prevalence of 2.5 %): 35 in *Radix auricularia*, 27 in *Lymnaea stagnalis* and 16 in *Stagnicola palustris*; no infections were found in *Radix peregra*. The majority of snails infected with *Diplostomum* spp. was collected in Hengsteysee, supporting the most abundant snail populations, whereas only few infected snails were found in Baldeneysee and Sorpetalsperre due to the lower snail density resulting in smaller sample size and none were recorded in either Hennetalsperre or Versetalsperre (Table 1). Prevalence of infections with *Diplostomum* spp. in distinct samples comprising more than 15 snails ranged from 1.0 to 16.7 % (Table 2). The data in Table 2 also reveal the high diversity of *Diplostomum* spp. in Hengsteysee.

Using the key and descriptions in Niewiadomska & Kiselienė [10] morphological identification of cercariae to the species level was achieved for two species, *D. parviventosum* ex *R. auricularia* and *D. pseudospathaceum* ex *L. stagnalis*. Subsamples of the remaining isolates were subjected to molecular identification based on sequence data [6, 17].

Molecular analyses

Partial *cox*1 sequences were obtained for most of the isolates (51; 65 %) collected in Hengsteysee and Sorpetalsperre (see Table 3 for details). Both NJ and BI analyses of the *cox*1 dataset (410 nt) recovered eight reciprocally monophyletic lineages (Figs. 2, 3). The predominant part of the isolates ex *R. auricularia* (n = 25; 76 %) clustered with the isolates comprising '*D. mergi*' species complex *sensu* Georgieva *et al.* [6] thus expanding substantially the content of the three lineages identified by these authors (Fig. 2). Nine isolates identified here as *D. parviventosum* based on morphology formed a strongly supported lineage ('*D. mergi* Lineage 1') together with the single isolate ex *R. auricularia* (JX986873) of Georgieva *et al.* [6], 11 further isolates clustered together with the three isolates ex *R. auricularia* (JX986874–JX986876) ('*D. mergi* Lineage 2' *sensu* Georgieva *et al.* [6]) and four isolates clustered with five metacercarial isolates ex *Salmo trutta fario* L. and *Gobio gobio* L. of the '*D. mergi* Lineage 3' *sensu* Georgieva *et al.* [6]; this lineage was joined by a single isolate (RaHe20; further referred to as '*D. mergi* Lineage 4'). The remaining isolates ex *R. auricularia* joined two additional strongly supported lineages: *D. spathaceum* (7 isolates) and 'Clade Q' *sensu* Georgieva *et al.* [6] (one isolate) represented by one cercarial isolate ex *R. auricularia* (RA97) and two metacercarial isolates ex *R. rutilus* (RR43 and RR45) from Lake Constance, all reported as *D. spathaceum* (see Behrmann-Godel [17]) but annotated as *D. mergi* in the GenBank. All isolates ex *L. stagnalis* and *S. palustris* were identified as and clustered with isolates of *D. pseudospathaceum* (Fig. 3).

The mean intraspecific divergence within the *cox*1 dataset examined ranged between 0.30 and 0.95 %, *i.e.* below the range for mean divergence in the interspecific comparisons (4.3–14.7 %) (Table 4). The lowest value was obtained for the sister lineages 2 and 3 of the '*D. mergi*' complex. The single isolate of *D. mergi* Lineage 4 exhibited lower divergence values in comparisons with isolates of Lineages 2 and 3 (3.7–3.9 and 2.4 %, respectively). A total of 13 ITS1-5.8S-ITS2 sequences (975 nt) was generated from isolates sub-sampled within the seven

Table 2 Prevalence of *Diplostomum* spp. in the distinct samples (n ≥ 15) of the three lymnaeid snail hosts examined

Reservoir	Hengsteysee			Sorpetalsperre
Snail species	*R. auricularia*	*L. stagnalis*	*S. palustris*	*R. auricularia*
D. parviventosum	3.1–7.1 (2)			
'*D. mergi* Lineage 2'	2.1–6.7 (4)			2.2–10.7 (2)
'*D. mergi* Lineage 3'	1.0–3.1 (3)			
D. mergi Lineage 4	1.0 (1)			
'*Diplostomum* sp. Clade Q'	3.6 (1)			
D. spathaceum	2.1–4.1 (4)			
D. pseudospathaceum		3.7–16.7 (6)	1.0–8.7 (5)	

Prevalence is calculated for sample size n ≥ 15 only; the number of samples is given in parentheses

Table 3 Summary data for 51 isolates of *Diplostomum* spp. used for generation of the *cox*1 and ITS1-5.8S-ITS2 sequences

Species	Isolate	Snail host	Reservoir	GenBank accession number (*cox*1/ITS1-5.8S-ITS2)
D. parviventosum	RaHe1	*R. auricularia*	Hengsteysee	KR149504
D. parviventosum	RaHe2	*R. auricularia*	Hengsteysee	KR149505
D. parviventosum	RaHe3	*R. auricularia*	Hengsteysee	KR149506
D. parviventosum	RaHe4	*R. auricularia*	Hengsteysee	KR149507
D. parviventosum	RaHe5	*R. auricularia*	Hengsteysee	KR149508
D. parviventosum	RaHe6	*R. auricularia*	Hengsteysee	KR149509/KR149490
D. parviventosum	RaHe7	*R. auricularia*	Hengsteysee	KR149510
D. parviventosum	RaHe8	*R. auricularia*	Hengsteysee	KR149511/KR149491
D. parviventosum	RaHe9	*R. auricularia*	Hengsteysee	KR149512/KR149492
'*D. mergi* Lineage 2'	RaBa1	*R. auricularia*	Baldeneysee	KR149513/KR149493
'*D. mergi* Lineage 2'	RaHe10	*R. auricularia*	Hengsteysee	KR149514
'*D. mergi* Lineage 2'	RaHe11	*R. auricularia*	Hengsteysee	KR149515
'*D. mergi* Lineage 2'	RaHe12	*R. auricularia*	Hengsteysee	KR149516
'*D. mergi* Lineage 2'	RaHe13	*R. auricularia*	Hengsteysee	KR149517
'*D. mergi* Lineage 2'	RaHe14	*R. auricularia*	Hengsteysee	KR149518
'*D. mergi* Lineage 2'	RaHe15	*R. auricularia*	Hengsteysee	KR149519/KR149494
'*D. mergi* Lineage 2'	RaSo1	*R. auricularia*	Sorpetalsperre	KR149520
'*D. mergi* Lineage 2'	RaSo2	*R. auricularia*	Sorpetalsperre	KR149521/KR149495
'*D. mergi* Lineage 2'	RaSo3	*R. auricularia*	Sorpetalsperre	KR149522
'*D. mergi* Lineage 2'	RaSo4	*R. auricularia*	Sorpetalsperre	KR149523
'*D. mergi* Lineage 3'	RaHe16	*R. auricularia*	Hengsteysee	KR149524/KR149496
'*D. mergi* Lineage 3'	RaHe17	*R. auricularia*	Hengsteysee	KR149525/KR149497
'*D. mergi* Lineage 3'	RaHe18	*R. auricularia*	Hengsteysee	KR149526/KR149498
'*D. mergi* Lineage 3'	RaHe19	*R. auricularia*	Hengsteysee	KR149527
D. mergi Lineage 4	RaHe20	*R. auricularia*	Hengsteysee	KR149528/KR149499
D. pseudospathaceum	LsBa1	*L. stagnalis*	Baldeneysee	KR149529
D. pseudospathaceum	LsBa2	*L. stagnalis*	Baldeneysee	KR149530
D. pseudospathaceum	LsHe1	*L. stagnalis*	Hengsteysee	KR149531
D. pseudospathaceum	LsHe2	*L. stagnalis*	Hengsteysee	KR149532/KR149500
D. pseudospathaceum	LsHe3	*L. stagnalis*	Hengsteysee	KR149533/KR149501
D. pseudospathaceum	LsHe4	*L. stagnalis*	Hengsteysee	KR149534
D. pseudospathaceum	LsHe5	*L. stagnalis*	Hengsteysee	KR149535
D. pseudospathaceum	LsHe6	*L. stagnalis*	Hengsteysee	KR149536
D. pseudospathaceum	SpBa1	*S. palustris*	Baldeneysee	KR149537
D. pseudospathaceum	SpHe1	*S. palustris*	Hengsteysee	KR149538
D. pseudospathaceum	SpHe2	*S. palustris*	Hengsteysee	KR149539
D. pseudospathaceum	SpHe3	*S. palustris*	Hengsteysee	KR149540
D. pseudospathaceum	SpHe4	*S. palustris*	Hengsteysee	KR149541
D. pseudospathaceum	SpHe5	*S. palustris*	Hengsteysee	KR149542
D. pseudospathaceum	SpHe6	*S. palustris*	Hengsteysee	KR149543
D. pseudospathaceum	SpHe7	*S. palustris*	Hengsteysee	KR149544
D. pseudospathaceum	SpHe8	*S. palustris*	Hengsteysee	KR149545
D. pseudospathaceum	SpHe9	*S. palustris*	Hengsteysee	KR149546

Table 3 Summary data for 51 isolates of *Diplostomum* spp. used for generation of the *cox*1 and ITS1-5.8S-ITS2 sequences *(Continued)*

D. spathaceum	RaHe21	*R. auricularia*	Hengsteysee	KR149547
D. spathaceum	RaHe22	*R. auricularia*	Hengsteysee	KR149548
D. spathaceum	RaHe23	*R. auricularia*	Hengsteysee	KR149549
D. spathaceum	RaHe24	*R. auricularia*	Hengsteysee	KR149550
D. spathaceum	RaHe25	*R. auricularia*	Hengsteysee	KR149551/KR149502
D. spathaceum	RaHe26	*R. auricularia*	Hengsteysee	KR149552
D. spathaceum	RaHe27	*R. auricularia*	Hengsteysee	KR149553
'*Diplostomum* sp. Clade Q'	RaHe28	*R. auricularia*	Hengsteysee	KR149554/KR149503

*cox*1 lineages of newly-collected *Diplostomum* spp. The analysis of the ITS data supported the molecular identification of these isolates from *cox*1 gene trees except for *D. mergi* Lineage 4 which clustered within the isolates of '*D. mergi* Lineage 2' (Fig. 4).

Detailed morphological assessment of the isolates following the identification of independent evolutionary lineages confirmed their distinct status. Using the new set of morphological characters defined for each lineage, all isolates were assigned to lineage. Taken together, the results

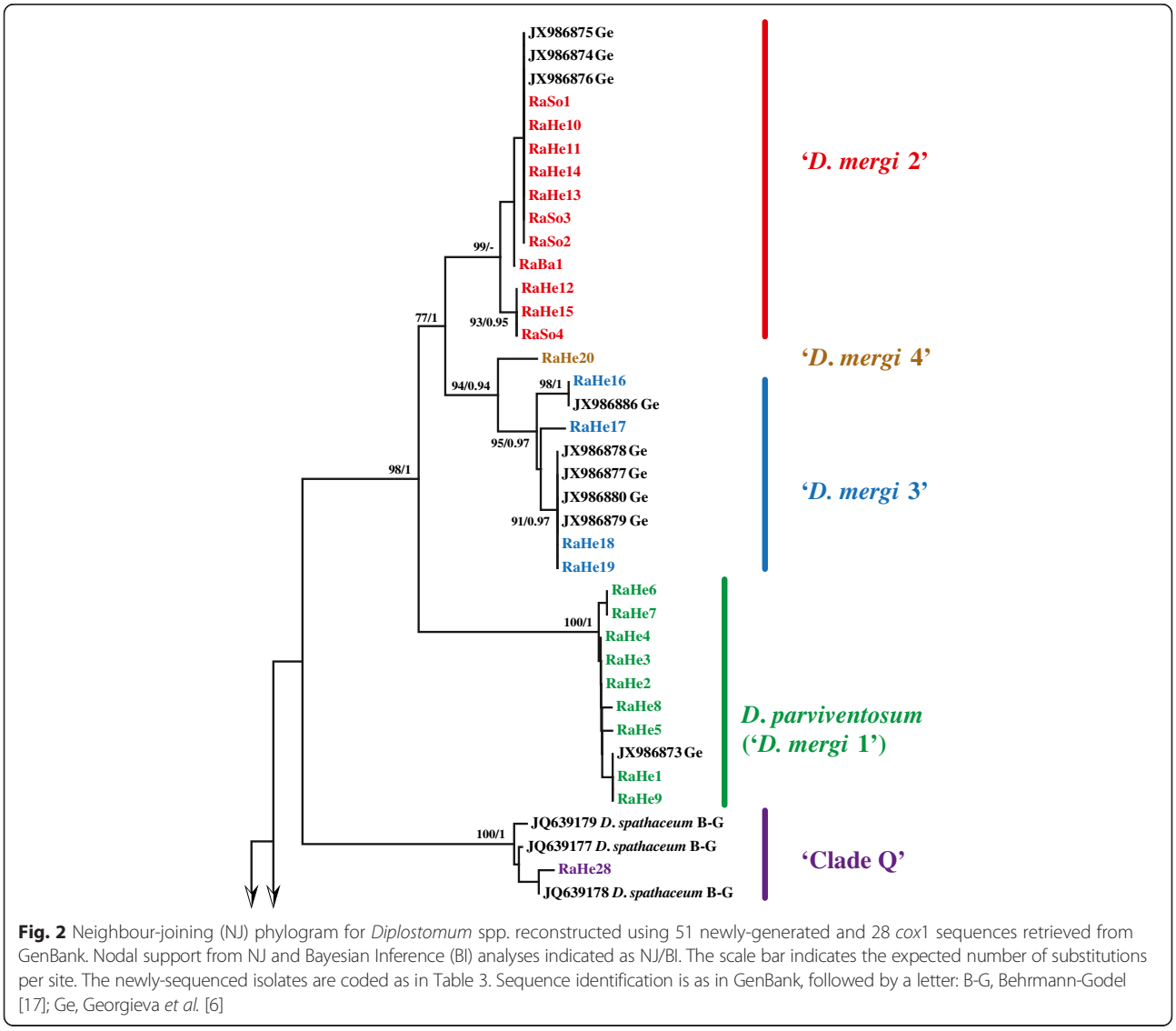

Fig. 2 Neighbour-joining (NJ) phylogram for *Diplostomum* spp. reconstructed using 51 newly-generated and 28 *cox*1 sequences retrieved from GenBank. Nodal support from NJ and Bayesian Inference (BI) analyses indicated as NJ/BI. The scale bar indicates the expected number of substitutions per site. The newly-sequenced isolates are coded as in Table 3. Sequence identification is as in GenBank, followed by a letter: B-G, Behrmann-Godel [17]; Ge, Georgieva *et al.* [6]

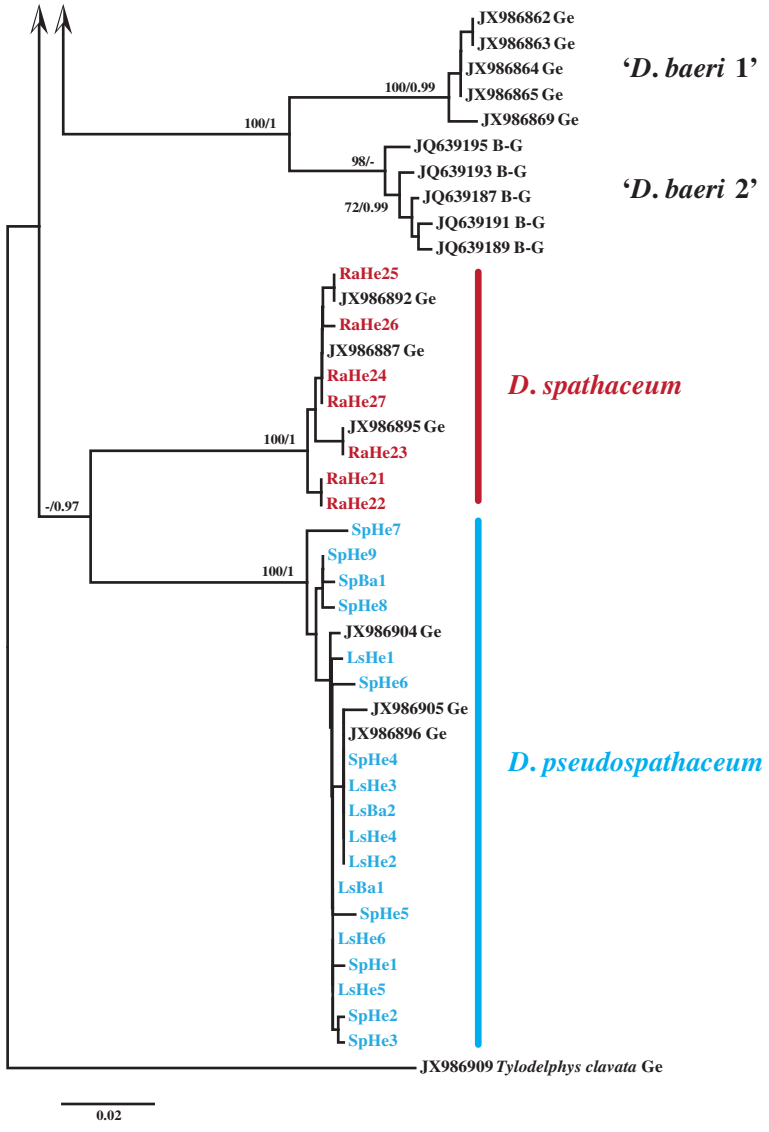

Fig. 3 Neighbour-joining (NJ) phylogram for *Diplostomum* spp. reconstructed using 51 newly-generated and 28 *cox*1 sequences retrieved from GenBank; continuation of Fig. 2. Nodal support from NJ and Bayesian Inference (BI) analyses indicated as NJ/BI. The scale bar indicates the expected number of substitutions per site. Codes for the newly-sequenced isolates are provided in Table 3. Sequence identification is as in GenBank, followed by a letter: B-G, Behrmann-Godel [17]; Ge, Georgieva *et al.* [6]

from the molecular and morphological analyses suggest that isolates sampled in lymnaeid snails from Germany represent three named species and four distinct lineages of *Diplostomum* spp. Descriptions of the cercariae of *D. parviventosum*, *D. pseudospathaceum*, *D. spathaceum* and of the three novel lineages of the '*D. mergi*' species complex plus '*Diplostomum* sp. Clade Q' are provided below.

Descriptions of the cercariae of *Diplostomum* spp. based on the molecular voucher material

Diplostomum parviventosum Dubois, 1932

First intermediate host: *Radix auricularia* (Linnaeus).
Locality: Hengsteysee, Germany.

[Figure 5 and Additional file 3: Figure S2A, Additional file 4: Figure S3A, Additional file 5: Figure S4A. Measurements of formalin-fixed specimens are provided in Table 5.] Body elongate-oval, 127 – 164 × 53 – 86 (146 × 62), shorter than tail stem [BL/TSL = 0.7 – 0.9 (0.8)], with aggregations of yellow pigment in parenchyma on both sides of terminal organ and around ventral sucker (Fig. 5a, b). Anterior organ elongate-oval, with posterior margin reaching to mid-length of forebody, 42 – 67 × 23 – 40 (51 × 32). Ventral sucker spherical, small, somewhat post-equatorial, 31 – 44 × 30 – 48 (37 × 37), with fine undulating membrane (3 high) (Fig. 5d); width slightly exceeds width of anterior organ [VSW/AOW = 1.1 – 1.3

Table 4 Mean divergence (uncorrected p-distance in %) estimated for the *cox*1 sequence pairs within- (diagonal) and among species and lineages of *Diplostomum*

		1	2	3	4	5	6	7	8
1	'*Diplostomum* sp. Clade Q'	0.56							
2	*D. baeri* (trout) 1	13.5	0.44						
3	*D. baeri* (perch) 2	13.3	6.5	0.95					
4	*D. parviventosum**	11.1	13.9	13.0	0.30				
5	'*D. mergi* Lineage 2'	10.9	13.8	13.8	6.7	0.30			
6	'*D. mergi* Lineage 3'	11.0	14.4	14.7	6.9	4.3	0.65		
7	*D. pseudospathaceum*	12.0	14.7	13.2	13.9	12.0	12.9	0.66	
8	*D. spathaceum*	12.1	14.6	12.4	11.2	10.7	11.6	10.0	0.53

*'*D. mergi* Lineage 1' of Georgieva *et al.* [6]

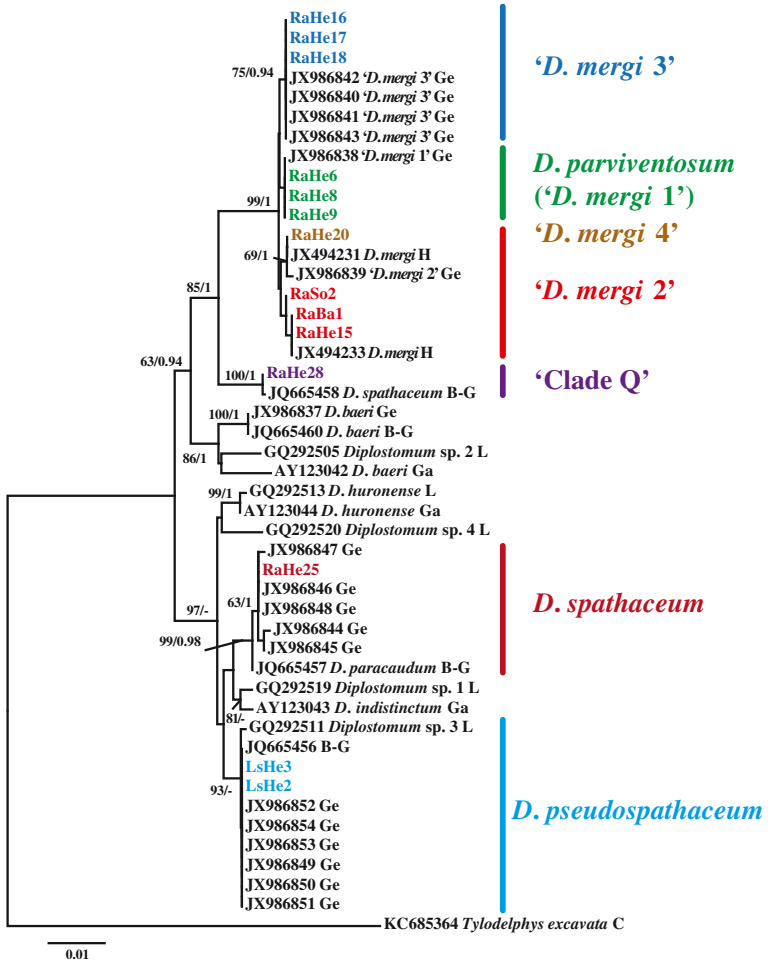

Fig. 4 Neighbour-joining (NJ) phylogram for *Diplostomum* spp. reconstructed using 13 newly-generated and 32 ITS1-5.8S-ITS2 sequences retrieved from GenBank. Outgroup: *Tylodelphys excavata*. Nodal support from NJ and Bayesian Inference (BI) analyses are indicated as NJ/BI. The scale bar indicates the expected number of substitutions per site. Codes for the newly-sequenced isolates are provided in Table 3. Sequence identification is as in GenBank, followed by a letter: B-G, Behrmann-Godel [17]; Ge, Georgieva *et al.* [6]; Ga, Galazzo *et al.* [14]; H, Haarder *et al.* [18]; L, Locke *et al.* [5]

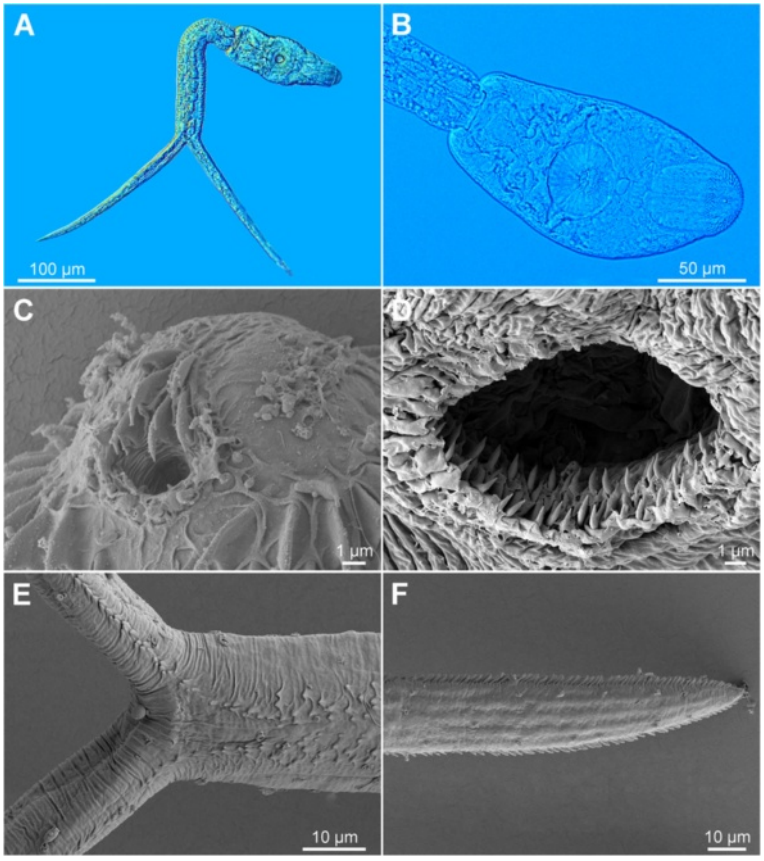

Fig. 5 Cercaria of *Diplostomum parviventosum* ex *Radix auricularia* (light and scanning electron microscopy, SEM). **a**, Resting position; **b**, Body; **c**, Anterior organ, apical view (SEM); **d**, Ventral sucker (SEM); **e**, Tail stem and furcae (SEM); **f**, Furcae (SEM)

Table 5 Comparative metrical data for cercariae of the *Diplostomum* '*mergi*' species complex

Species	*D. mergi*	'*D. mergi* Lineage 2'	'*D. mergi* Lineage 3'	*D. parviventosum*	
Source	Niewiadomska & Kiselienė [10]	Present study	Present study	Niewiadomska & Kiselienė [10]	Present study
Fixation method	Heat-killed in water	Formalin	Formalin	Heat-killed in water	Formalin
BL	161–204 (182)	157–179 (168)	171–191 (184)	162–185 (176)	115–151 (134)
BW	51–68 (59)	62–67 (64)	44–54 (49)	50–70 (55)	50–61 (54)
AOL	51–68 (58)	43–53 (49)	42–59 (48)	55–60 (56)	43–53 (47)
AOW	25–30 (28)	24–36 (33)	29–35 (32)	29–32 (31)	26–33 (30)
VSL	34–51 (44)	43–49 (46)	28–33 (31)	34–38 (36)	29–39 (33)
VSW	34–51 (44)	50–56 (53)	28–35 (32)	34–37 (36)	30–46 (37)
TSL	187–229 (207)	146–180 (168)	196–224 (212)	203–242 (228)	153–176 (165)
TSW	34–43 (36)	27–32 (29)	30–36 (33)	31–40 (35)	26–36 (30)
FL	187–229 (210)	203–227 (216)	210–245 (225)	191–218 (206)	178–217 (204)
VSW/AOW	(1.6)	1.5–2.1 (1.6)	0.9–1.1 (1.0)	(1.1)	1.0–1.4 (1.2)
BL/TSL	(0.9)	1.0–1.1 (1.0)	0.8–1.0 (0.9)	(0.8)	0.7–0.9 (0.8)
TSL/FL	(1.0)	0.7–0.8 (0.8)	0.9–1.0 (0.9)	(1.1)	0.8–0.9 (0.8)

Data are presented as the range followed by the mean in parentheses. See Methods and Additional file 1: Figure S1 for description and illustration of the metrical features

(1.2)]. Penetration gland-cells 2 pairs, relatively small, with fine granular content, posterior to ventral sucker, overlap caeca partially, posterior pair not reaching extremities of caeca; ducts open antero-laterally to mouth. Tail stem 148 – 208 (173) long, 27 – 40 (33) wide at base, slightly shorter than furcae [TSL/FL = 0.7 – 1.0 (0.8)], contains 10 – 12 pairs of caudal bodies, irregularly shaped but with smooth contours. Furcae 199 – 234 (216) long, with fish-fin like fin-folds.

Body armature: Pre-oral spines arranged in median group of 6 – 7 spines in 3 rows; lateral groups absent (Fig. 5c, Additional file 3: Figure S2A). Post-oral spines more robust than spines on body, in 7 – 8 alternate rows; rows 1 – 2 with median interruption; first 4 spines in row 1 on both sides of median interruption largest; spines in row 1 larger than remaining spines, all of similar size. Wide zone of smaller, less dense, irregularly dispersed spines present posterior to post-oral spines, followed by narrow spineless area and 11 transverse rows of spines extending to mid-level of ventral sucker. Rows 1 – 4 complete (*i.e.* encircling body); rows 5 – 8 discontinuous dorsally; rows 9 – 11 discontinuous ventrally and dorsally; rows 1 and 10 with additional spines laterally. Two ventro-lateral fields of smaller spines (0.8–1.2) present posterior to ventral sucker; fields converge close to posterior extremity of body. Ventral sucker armed with 2 rows of spines (*c.*40 per row; range 77 – 87; mean 81) (Fig. 5d, Additional file 4: Figure S3A). Tail stem and furcae with scale-like spines (Fig. 5e); spines on tail stem in 4 medio-lateral bands (2 ventral and 2 dorsal), each consisting of 2 – 3 scale-like spines, increasing in size posteriorly (0.6 – 2), plus a median row of minute spines; bands continue along furcal margins as rows of 2 spines anteriorly and single spine posteriorly; all spines on furcae enveloped by tegumental membrane forming fish-fin like fin-fold (Fig. 5f, Additional file 5: Figure S4A).

Resting position: Tail stem bent at < 90° (45–67°).

'Diplostomum mergi Lineage 2' of Georgieva *et al.* [6]

First intermediate host: *Radix auricularia* (Linnaeus).
Locality: Hengsteysee, Sorpetalsperre, Germany.

[Figure 6 and Additional file 3: Figure S2C, Additional file 4: Figure S3C, Additional file 5: Figure S4C. Measurements from formalin-fixed specimens are provided in Table 5.] Body elongate-oval, 154 – 179 × 60 – 66 (167 × 63), slightly shorter than tail stem [BL/TSL = 0.8 – 0.9 (0.9)], with aggregations of yellow pigment in parenchyma on both sides of terminal organ and around ventral sucker, most prominently anterior to ventral sucker (Fig. 6a, b). Anterior organ elongate-oval, with posterior margin reaching to mid-length of forebody, 50 – 62 × 26 – 30 (56 × 28). Ventral sucker subspherical, large, somewhat post-equatorial, 47 – 53 × 42 – 52 (50 × 47), with fine undulating membrane (3 – 4 high) (Fig. 6d); width exceeds width of anterior organ [VSW/AOW = 1.5 – 2.0 (1.7)]. Penetration gland-cells 2 pairs, large, with fine granular content, posterior to ventral sucker, overlap caeca partially, posterior pair not reaching extremities of caeca; ducts open antero-laterally to mouth. Tail stem 185 – 207 (195) long, 31 – 36 (33) wide at base, shorter than furcae [TSL/FL = 0.8 – 0.9 (0.8)], contains 36 – 40 caudal bodies; individual caudal bodies with smooth contours. Furcae 221 – 273 (247) long, with fish-fin like fin-folds.

Body armature: Pre-oral spines arranged in median group of 5 – 6 spines in 2 rows with one median spine very large (Fig. 6c, Additional file 3: Figure S2C); one additional very small spine may be present; lateral groups absent. Post-oral spines more robust than spines on body, in 11 alternate rows (one additional median row may be present); rows 1 – 2 with median interruption; first 4 spines in row 1 and first 3 spines in row 2 on both sides of median interruption largest; spines in row 1 larger than remaining spines, all of similar size. Wide zone of smaller, less dense, irregularly dispersed spines present posterior to post-oral spines, followed by narrow spineless area and 10 transverse rows of spines extending to mid-level of ventral sucker; row 10 with smaller and fewer spines. Rows 1 – 5 complete (*i.e.* encircling body); rows 6 – 10 discontinuous ventrally and dorsally; rows 1 – 4 with additional spines laterally; rows 5 and 6 with few additional spines laterally. Two ventro-lateral fields of smaller spines (1.0–1.5) present posterior to ventral sucker; fields converge close to posterior extremity of body. Ventral sucker armed with 2 rows of spines (*c.*57 per row; range 110 – 120; mean 114) (Fig. 6d, Additional file 4: Figure S3C). Tail stem and furcae with scale-like spines (Fig. 6e); spines on tail stem in 4 medio-lateral bands (2 ventral and 2 dorsal), starting from second quarter of tail, each consisting of 2 – 3 scale-like spines, increasing in size posteriorly (0.6 – 2.5); bands continue along furcal margins as rows of 2 spines anteriorly and 1 spine posteriorly; all spines on furcae enveloped by tegumental membrane forming fish-fin like fin-fold (Fig. 6f, Additional file 5: Figure S4C).

Resting position: Tail stem bent at < 90° (64 – 85°).

'Diplostomum mergi Lineage 3' of Georgieva *et al. [6]*

First intermediate host: *Radix auricularia* (Linnaeus).
Locality: Hengsteysee, Germany.

[Figure 7 and Additional file 3: Figure S2D, Additional file 4: Figure S3D, Additional file 5: Figure S4D. Measurements of formalin-fixed specimens are provided in Table 5.] Body elongate-oval, 158 – 169 × 50 – 57 (162 × 53), slightly shorter than tail stem [BL/TSL = 0.9 – 1.0 (0.9)], with aggregations of yellow pigment in parenchyma on both sides of ventral sucker (Fig. 7a, b). Anterior organ

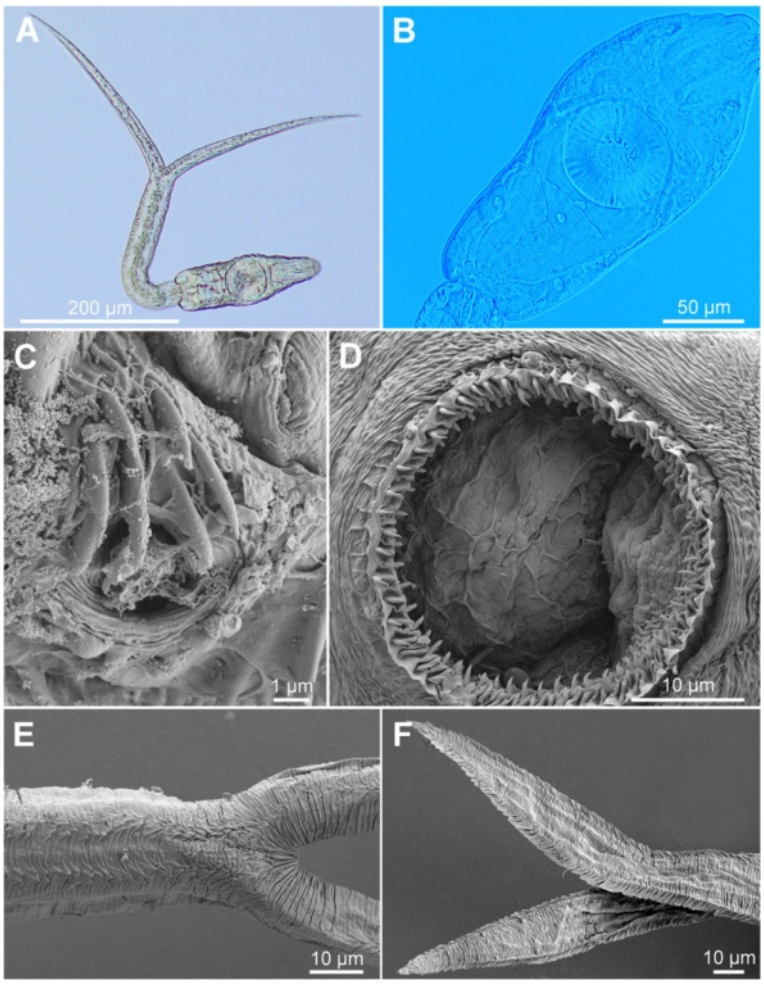

Fig. 6 Cercaria of '*Diplostomum mergi* Lineage 2' of Georgieva *et al.* [6]) ex *Radix auricularia* (light and scanning electron microscopy, SEM). **a**, Resting position; **b**, Body; **c**, Anterior organ, apical view (SEM); **d**, Ventral sucker (SEM); **e**, Tail stem and furcae (SEM); **f**, Furcae (SEM)

elongate-oval, with posterior margin reaching to mid-length of forebody, 50 – 57 × 26 – 29 (54 × 28). Ventral sucker spherical, small, somewhat post-equatorial, 29 – 34 × 28 – 32 (31 × 31), with fine undulating membrane (2 high) (Fig. 7d); width slightly exceeds width of anterior organ [VSW/AOW = 1.0 – 1.2 (1.1)]. Penetration gland-cells 2 pairs, medium-sized, with fine granular content, posterior to ventral sucker, overlap caeca partially, posterior pair not reaching extremities of caeca, ducts open antero-laterally to mouth. Tail stem 167 – 197 (179) long, 31 – 33 (32) wide at base, shorter than furcae [TSL/FL = 0.7 – 0.8 (0.8)], contains numerous (impossible to count) caudal bodies with incised contours. Furcae 215 – 240 (227) long, with fish-fin like fin-folds.

Body armature: Pre-oral spines arranged in median group of 7 spines in 3 rows with one median spine slightly larger (Fig 7c, Additional file 3: Figure S2D); lateral groups absent. Post-oral spines more robust than spines on body, in 10 alternate rows; rows 1 – 2 with a median interruption; first 4 spines in row 1 on both sides of median interruption largest; spines in rows 1 – 2 larger than remaining spines, all of similar size. Wide zone of smaller, less dense, irregularly dispersed spines present posterior to post-oral spines, followed by narrow spineless area and 11 transverse rows of spines extending to mid-level of ventral sucker; row 11 with smaller spines. Rows 1 – 3 complete (*i.e.* encircling body); rows 4 – 11 discontinuous ventrally and dorsally; row 1 doubled; rows 2 – 3 with additional spines laterally. Two ventro-lateral non-converging fields of smaller spines (0.7–1.0) present posterior to ventral sucker. Ventral sucker armed with 2 rows of spines (*c.*45 per row; range 90 – 92; mean 90) (Fig. 7d, Additional file 4: Figure S3D). Tail stem and furcae with scale-like spines (Fig. 7e); spines on tail stem in 4 medio-lateral bands (2 ventral and 2 dorsal), each consisting of 2 – 3 scale-like spines, increasing in size posteriorly (0.8 – 3.0); bands continue along furcal margins as rows of 2 spines anteriorly and 1

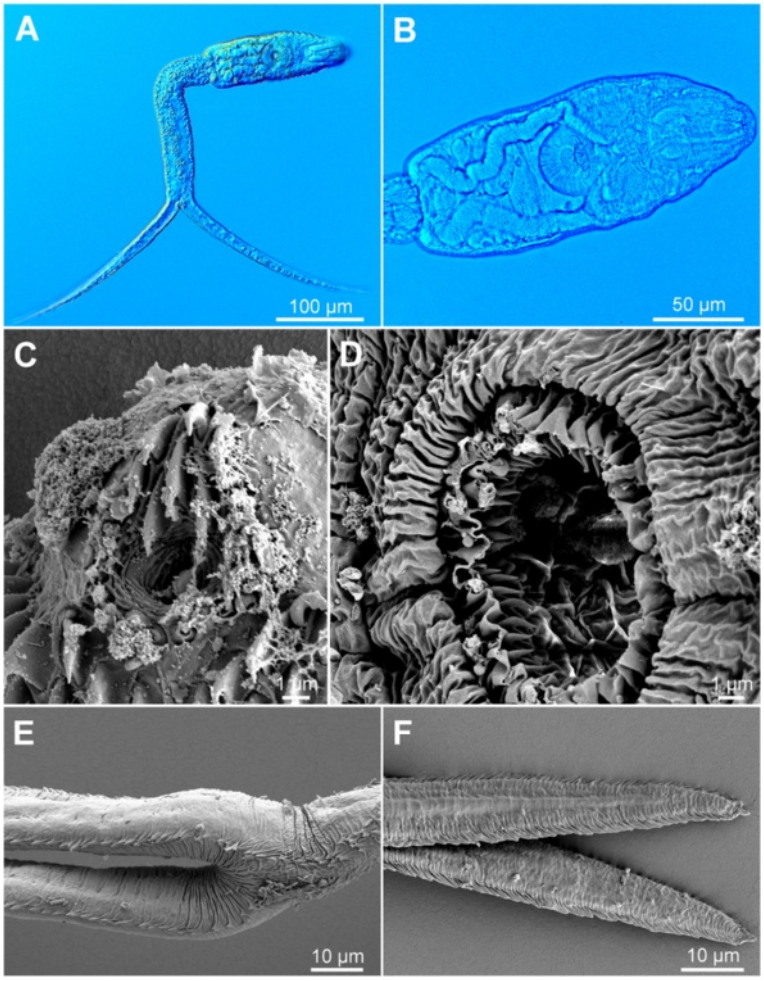

Fig. 7 Cercaria of '*Diplostomum mergi* Lineage 3' of Georgieva *et al.* [6] ex *Radix auricularia* (light and scanning electron microscopy, SEM). **a**, Resting position; **b**, Body; **c**, Anterior organ, apical view (SEM); **d**, Ventral sucker (SEM); **e**, Tail stem and furcae (SEM); **f**, Furcae (SEM)

spine posteriorly; all spines on furcae enveloped by tegumental membrane forming fin-fold (Fig. 7f, Additional file 5: Figure S4D).

Resting position: Tail stem bent at ≤ 90° (77 – 91°).

Diplostomum mergi Lineage 4

First intermediate host: *Radix auricularia* (Linnaeus).
Locality: Hengsteysee, Germany.

[Figure 8 and Additional file 3: Figure S2B, Additional file 4: Figure S3B, Additional file 5: Figure S4B.] Body elongate-oval, 180 – 186 × 94 – 115 (184 × 105), shorter than tail stem (BL/TSL = 0.7), with aggregations of yellow pigment in parenchyma on both sides of terminal organ and around ventral sucker (Fig. 8a, b). Anterior organ elongate-oval, with posterior margin reaching to mid-length of forebody, 54 – 74 × 38 – 51 (63 × 44). Ventral sucker transversely oval, large, somewhat post-equatorial, 58 – 79 × 65 – 78 (63 × 71), with fine undulating membrane (3 – 5 high) (Fig. 8d); width exceeds width of anterior organ [VSW/AOW = 1.5 – 1.7 (1.6)]. Penetration gland-cells 3 pairs (anterior pair small, posterior pairs large), with fine granular content, posterior to ventral sucker, overlap caeca partially, posterior pair not reaching extremities of caeca; ducts open antero-laterally to mouth. Tail stem 261 – 263 (262) long, 41 – 47 (43) wide at base, of equal length as furcae (TSL/FL = 1.0), with 36 – 40 caudal bodies; individual caudal bodies irregularly shaped with smooth contours. Furcae 269 long, with fish-fin like fin-folds.

Body armature: Pre-oral spines arranged in median group of 7 spines in 3 rows with one median spine very large (Fig. 8c, Additional file 3: Figure S2B); lateral groups absent. Post-oral spines more robust than spines on body, in 11 alternate rows; rows 1 – 2 with median interruption, rows 10 – 11 interrupted dorsally; first 4 spines in row 1 and first 3 spines in row 2 on both sides of median interruption largest; spines in first 2 rows larger than remaining spines, all of similar size. Wide zone of smaller, less dense, irregularly dispersed spines

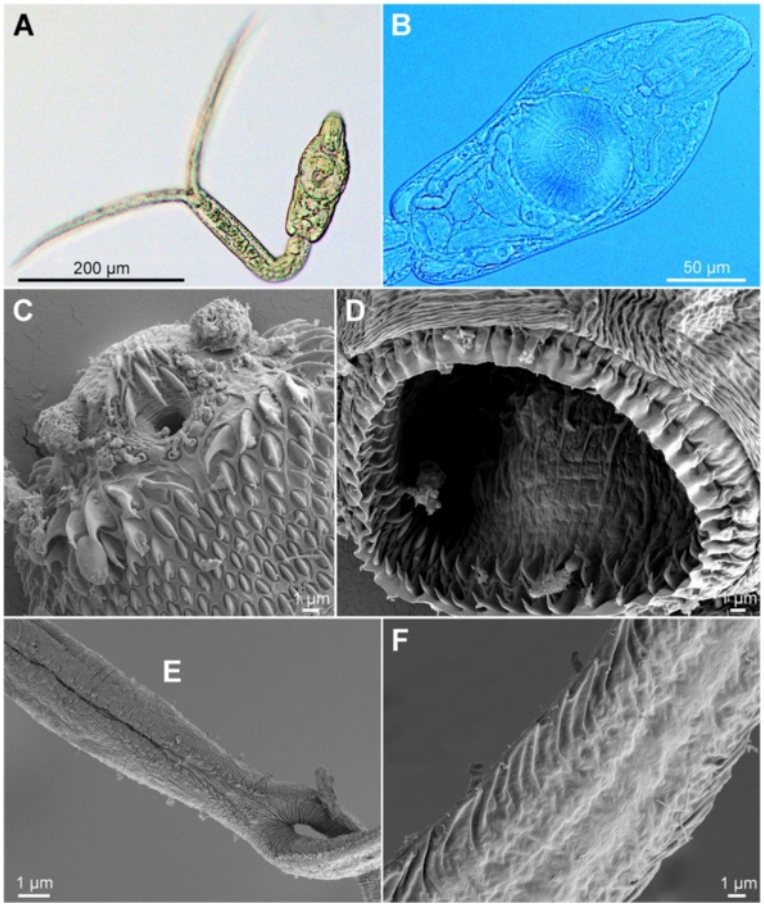

Fig. 8 Cercaria of *Diplostomum mergi* Lineage 4 ex *Radix auricularia* (light and scanning electron microscopy, SEM). **a**, Resting position; **b**, Body; **c**, Anterior organ, apical view (SEM); **d**, Ventral sucker (SEM); **e**, Tail stem and furcae (SEM); **f**, Furcae (SEM)

present posterior to post-oral spines, followed by narrow spineless area and 10 transverse rows of spines extending to mid-level of ventral sucker. Rows 1 – 4 complete (*i.e.* encircling body); rows 5 – 7 discontinuous dorsally; rows 8 – 10 discontinuous ventrally and dorsally. Two ventro-lateral fields of smaller spines (0.5–1.0) present posterior to ventral sucker; fields converge close to posterior extremity of body. Ventral sucker armed with 2 rows of spines (*c.*56 per row; range 112 – 114; mean 113) (Fig. 8d, Additional file 4: Figure S3B). Tail stem and furcae with scale-like spines (Fig. 8e); spines on tail stem in 4 medio-lateral bands (2 ventral and 2 dorsal), each consisting of 1 – 2 scale-like spines, increasing in size posteriorly (1 – 3); bands continue along furcal margins as single rows of spines; all spines on furcae enveloped by tegumental membrane forming fish-fin like fin-fold (Fig. 8f, Additional file 5: Figure S4B).

Resting position: Tail stem bent at < 90° (66°).

'Diplostomum* sp. Clade Q' of Georgieva *et al. [6]
First intermediate host: *Radix auricularia* (Linnaeus).
Locality: Hengsteysee, Germany.

[Figure 9 and Additional file 4: Figure S3E, Additional file 5: Figure S4E.] Body elongate-oval, 215 – 239 × 87 – 101 (224 × 96), shorter than tail stem (BL/TSL = 0.8), with aggregations of yellow pigment in parenchyma on both sides of terminal organ, around ventral sucker (Fig. 9a) and in furcae. Anterior organ elongate-oval, with posterior margin reaching to mid-length of forebody, 70 – 88 × 46 – 48 (80 × 47). Ventral sucker transversely oval, large, somewhat post-equatorial, 51 – 60 × 57 – 70 (56 × 65), with fine undulating membrane (5 high) (Fig. 9d); width exceeds width of anterior organ [VSW/AOW = 1.2 – 1.4 (1.3)]. Penetration gland-cells 2 pairs, large, with fine granular content, posterior to ventral sucker, overlap caeca partially, posterior pair not reaching extremities of caeca; ducts open antero-laterally to mouth. Tail stem 266 long, 43 – 48 (46) wide at base, of equal length to furcae (TSL/FL = 1.0), contains 10 pairs of caudal bodies with incised contours. Furcae 280 long, with fish-fin like fin-folds.

Body armature: Pre-oral spines arranged in median group of 9 spines in 3 rows (Fig. 9c); lateral groups absent. Post-oral spines more robust than spines on body,

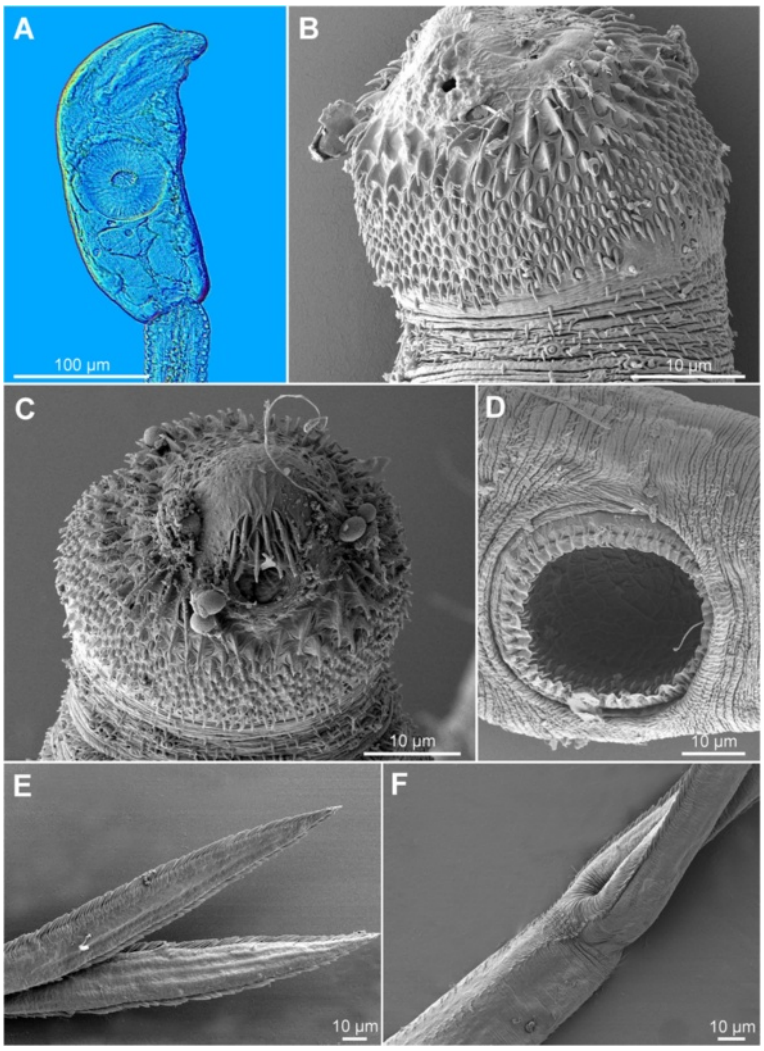

Fig. 9 Cercaria of '*Diplostomum* sp. Clade Q' of Georgieva *et al.* [6] ex *Radix auricularia* (light and scanning electron microscopy, SEM). **a**, Body; **b**, Anterior organ, lateral view (SEM); **c**, Anterior organ, apical view (SEM); **d**, Ventral sucker (SEM); **e**, Furcae (SEM); **f**, Tail stem and furcae (SEM)

in 12 alternate rows; first row with median interruption, rows 11–12 interrupted laterally; first 5 spines in row 1 on both sides of median interruption largest; remaining spines of different sizes, spines in rows 1–4 distinctly larger than those in remaining rows, spines in rows 5–10 small, spines in rows 10–12 medium-sized (Fig. 9b). Wide zone of smaller, less dense, irregularly dispersed spines present posterior to post-oral spines (spines sparser dorsally), followed by narrow spineless area and 10 transverse rows of spines extending to mid-level of ventral sucker. Rows 1 – 4 complete (*i.e.* encircling body); rows 5 – 10 discontinuous ventrally and dorsally; row 1 doubled, rows 2 – 3 with additional spines laterally. Two ventro-lateral non-converging fields of smaller spines (0.7–1.3) present posterior to ventral sucker. Ventral sucker armed with 2 rows of spines (*c.*57 per row; range 112 – 116; mean 114) (Fig. 9d, Additional file 4: Figure S3E). Tail stem and furcae with scale-like spines (Fig. 9f); spines on tail stem in 4 medio-lateral bands (2 ventral and 2 dorsal), each consisting of 1 – 2 scale-like spines anteriorly and of 3 spines posteriorly close to bifurcation; spines increase in size posteriorly (0.6 – 3.0); bands continue along furcal margins as single rows of spines; all spines on furcae enveloped by tegumental membrane forming fish-fin like fin-folds (Fig. 9e, Additional file 5: Figure S4E).

Resting position not observed.

***Diplostomum spathaceum* (Rudolphi, 1819)**

First intermediate host: *Radix auricularia* (Linnaeus).

Locality: Hengsteysee, Germany.

[Figure 10 and Additional file 3: Figure S2G, Additional file 4: Figure S3G, Additional file 5: Figure S4G. Measurements of formalin-fixed specimens are provided in Table 6.] Body elongate-oval, 159 – 178 × 46 – 60 (172 ×

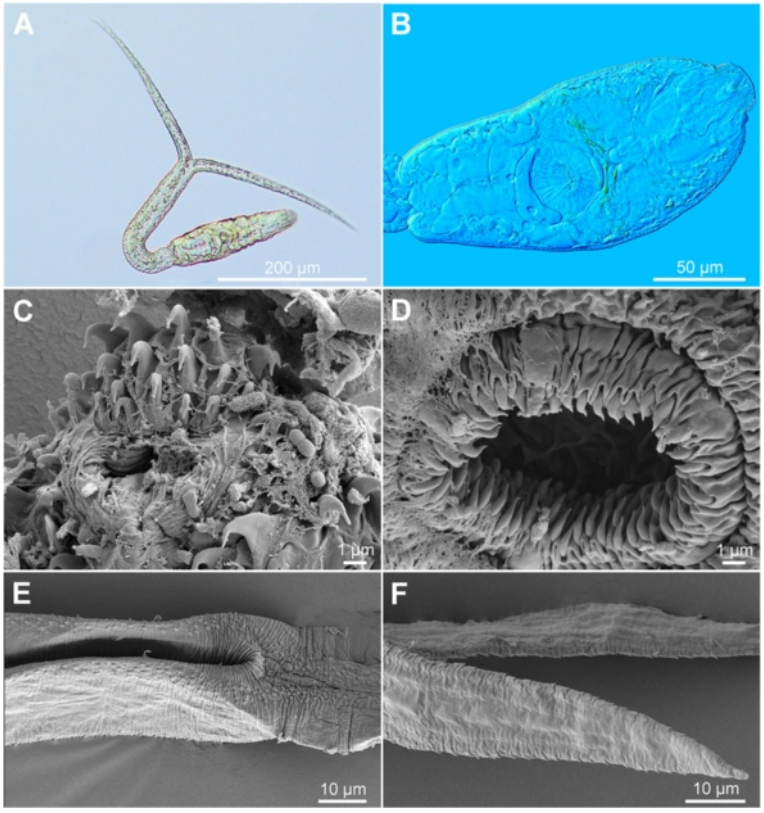

Fig. 10 Cercaria of *Diplostomum spathaceum* ex *Radix auricularia* (light and scanning electron microscopy, SEM). **a**, Resting position; **b**, Body; **c**, Anterior organ, apical view (SEM); **d**, Ventral sucker (SEM); **e**, Tail stem and furcae (SEM); **f**, Furcae (SEM)

51), shorter than tail stem [BL/TSL = 0.7 – 0.9 (0.8)], with aggregations of yellow pigment in parenchyma concentrated on both sides of anterior organ and above ventral sucker (Fig. 10a, b). Anterior organ elongate-oval, with posterior margin reaching to mid-length of forebody, 39 – 56 × 24 – 28 (50 × 26). Ventral sucker spherical, small, somewhat post-equatorial, 26 – 35 × 27 – 33 (30 × 30), with fine undulating membrane (2 – 3.5 high) (Fig. 10d); width slightly exceeds width of anterior organ [VSW/AOW = 1.0 – 1.3 (1.1)]. Penetration gland-cells 2 pairs, large, with fine granular content, posterior to ventral sucker, overlap caeca partially, posterior pair not reaching extremities of caeca. Tail stem 197 – 233 (219) long, 29 – 32 (31) wide at base, shorter than furcae [TSL/FL = 0.8 – 0.9 (0.8)], contains 56 – 60 caudal bodies; individual caudal bodies irregularly shaped with both incised and smooth contours. Furcae 250 – 267 (260) long, without fin-fold.

Body armature: Pre-oral spines arranged in median group of 18 – 19 spines in 3 rows (Fig. 10c, Additional file 3: Figure S2G); spines in most anterior row largest, gradually decreasing in size in remaining rows; 2 lateral groups with 1 small spine each present. Post-oral spines more robust than spines on body, in 9 alternate rows; row 1 with median interruption; row 9 interrupted laterally; spines in row 1 larger than remaining spines, all of similar size. Wide zone of smaller, less dense, irregularly dispersed spines present posterior to post-oral spines, followed by narrow spineless area and 10 transverse rows of spines extending to mid-level of ventral sucker. Rows 1 – 8 complete (*i.e.* encircling body); rows 9 – 10 discontinuous ventrally; rows 1 – 2 doubled ventrally; row 3 with additional spines laterally. Two ventrolateral fields of smaller spines (1.0–1.5) present posterior to ventral sucker; fields reach up to margin of ventral sucker and transverse row 10 laterally and dorsally and converge posterior to ventral sucker and close to posterior extremity of body. Ventral sucker armed with 3 rows of irregularly positioned spines (range 103 – 119; mean 110) (Fig. 10d, Additional file 4: Figure S3G). Tail stem and furcae with scale-like spines; spines on tail stem in 4 medio-lateral bands (2 ventral and 2 dorsal), each consisting of 3 scale-like spines (<1.0) (Fig. 10e); bands continue along furcal margins as rows of 4 spines anteriorly and single rows of spines posteriorly (Fig. 10f, Additional file 5: Figure S4G).

Resting position: Tail stem bent at < 45° (39°).

Table 6 Comparative metrical data for cercariae of *Diplostomum pseudospathaceum*, *D. spathaceum* and *D. paracaudum*

Species	*D. pseudospathaceum*		*D. spathaceum*	*D. paracaudum*	*D. spathaceum*
Source	Niewiadomska [27]; Niewiadomska & Kiselienė [10]	Present study	Niewiadomska [27]; Niewiadomska & Kiselienė [10]	Niewiadomska [28]; Niewiadomska & Kiselienė [10]	Present study
Fixation method	Heat-killed in water	Formalin	Heat-killed in water	Heat-killed in water	Formalin
BL	170–199 (185)	150–172 (162)	222–288 (235)	185–199 (189)	155–181 (170)
BW	44–51 (45)	57–64 (59)	66–81 (73)	44–51 (46)	50–58 (53)
AOL	40–51 (49)	43–51 (47)	61–95 (68)	51–54 (51)	33–50 (45)
AOW	25–27 (27)	28–34 (31)	30–40 (34)	23–30 (27)	28–33 (30)
VSL	23–30 (29)	19–31 (28)	47–68 (53)	23–30 (25)	29–31 (31)
VSW	23–34 (28)	26–33 (30)	51–68 (59)	27–30 (28)	25–31 (27)
TSL	199–214 (206)	203–221 (213)	244–303 (273)	185–222 (211)	241–260 (250)
TW	29–31 (29)	21–32 (29)	40–44 (44)	37	30–34 (32)
FL	185–222 (201)	212–246 (232)	251–296 (273)	185–222 (208)	237–261 (252)
VSW/AOW	(1.0)	0.8–1.1 (1.0)	(1.7)	(1.0)	0.8–1.1 (0.9)
BL/TSL	(0.9)	0.7–0.8 (0.8)	(0.9)	(0.9)	0.6–0.7 (0.7)
TSL/FL	(1.0)	0.9–1.0 (0.9)	(1.0)	(1.0)	0.9–1.0 (1.0)

Data are presented as the range followed by the mean in parentheses. See Methods and Additional file 1: Figure S1 for description and illustration of the metrical features

Diplostomum pseudospathaceum Niewiadomska, 1984

First intermediate hosts: *Lymnaea stagnalis* (Linnaeus); *Stagnicola palustris* (Müller).

Localities: Baldeneysee, Hengsteysee, Germany.

[Figure 11 and Additional file 3: Figure S2E, F, Additional file 4: Figure S3F, Additional file 5: Figure S4F. Measurements of formalin-fixed specimens are provided in Table 6.] Body elongate-oval, 152 – 183 × 46 – 54 (166 × 50), slightly shorter than tail stem [BL/TSL = 0.8 – 1.0 (0.9)] (Fig. 11a, b), with aggregations of yellow pigment in the parenchyma of whole body, concentrated on both sides of anterior organ, above ventral sucker and in tail stem and furcae. Anterior organ elongate-oval, with posterior margin reaching to mid-length of forebody, 41 – 58 × 22 – 28 (50 × 25). Ventral sucker spherical, small, somewhat post-equatorial, 24 – 32 × 27 – 32 (29 × 30), with fine undulating membrane (2 – 3 high) (Fig. 11d); width exceeds width of anterior organ [VSW/AOW = 1.1 – 1.4 (1.2)]. Penetration gland-cells 2 pairs, large, with fine granular content, posterior to ventral sucker, overlap caeca partially, posterior pair not reaching extremities of caeca. Tail stem 176 – 203 (187) long, 27 – 30 (29) wide at base, shorter than furcae [TSL/FL = 0.8 – 0.8 (0.8)], contains 35 – 45 caudal bodies; individual caudal bodies irregularly shaped with smooth contours. Furcae 219 – 253 (234) long, without fin-fold.

Body armature: Pre-oral spines arranged in median group of 10 – 11 spines in 3 rows; spines in anterior row largest, remaining spines of similar size; 2 lateral groups with 3 small spines each present (Fig. 11c, Additional file 3: Figure S2E, F). Post-oral spines more robust than spines on body, in 9 alternate rows; rows 1 – 2 with median interruption; row 9 interrupted laterally; first 2 spines in row 1 on both sides of median interruption largest; spines in row 1 larger than remaining spines, all of similar size. Wide zone of smaller, less dense, irregularly dispersed spines present posterior to post-oral spines, followed by narrow spineless area and 11 transverse rows of spines extending to mid-level of ventral sucker. Rows 1 – 8 complete (*i.e.* encircling body); row 9 discontinuous ventrally, rows 10 – 11 discontinuous ventrally and dorsally; rows 1 – 2 doubled ventrally; rows 3 – 7 with additional spines laterally. Two ventro-lateral fields of smaller spines (1.0–1.5) present posterior to ventral sucker; fields reach up to margin of ventral sucker and transverse row 11 laterally and dorsally and converge posterior of ventral sucker and close to posterior extremity of body. Ventral sucker armed with 2 rows of spines (*c.*42 per row; range 70 – 100; mean 84); third row may be partially formed (Fig. 11d, Additional file 4: Figure S3F). Tail stem and furcae with scale-like spines (Fig. 11e); spines on tail stem in 4 medio-lateral bands (2 ventral and 2 dorsal), each consisting of 1 – 2 scale-like spines anteriorly, 2 – 3 spines posteriorly, increasing in size posteriorly (0.5 – 1.3 μm); bands continue along furcal margins as rows of 3 spines anteriorly and 2 spines posteriorly (Fig. 11f, Additional file 5: Figure S4F).

Resting position: Tail stem bent at < 45° (29 – 38°).

Discussion

To the best of our knowledge, this study provides the first combined morphological and molecular characterisation of *Diplostomum* spp. in natural lymnaeid snail populations in central Europe and is the first to apply

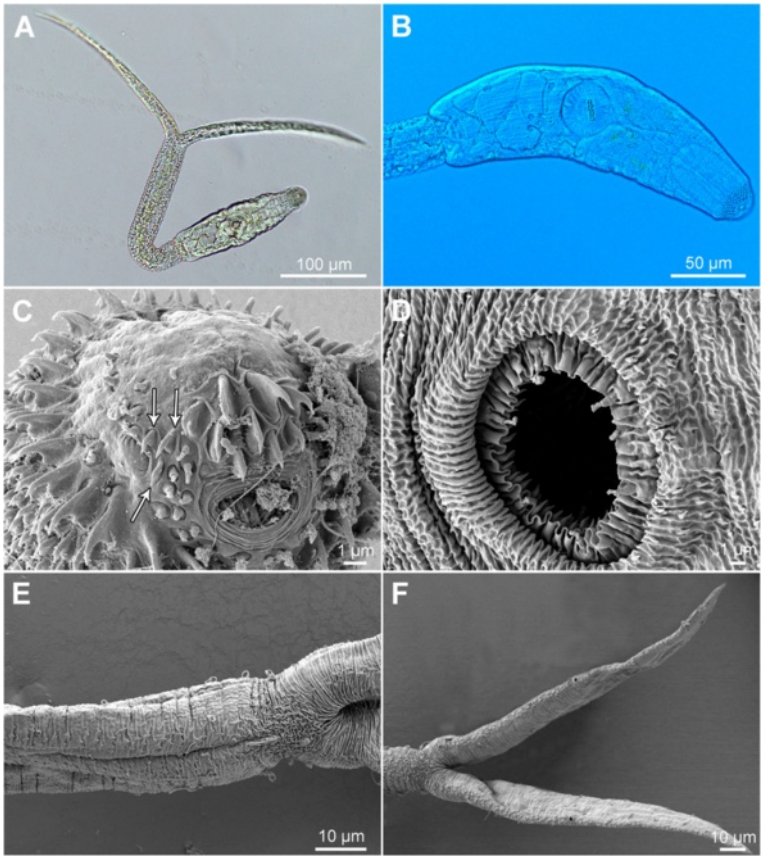

Fig. 11 Cercaria of *Diplostomum pseudospathaceum* ex *Lymnaea stagnalis* (light and scanning electron microscopy, SEM). **a**, Resting position; **b**, Body; **c**, Anterior organ, apical view, arrows indicate group of lateral pre-oral spines (SEM); **d**, Ventral sucker (SEM); **e**, Tail stem and furcae (SEM); **f**, Furcae (SEM)

thorough SEM analysis of species-specific features of the cercariae that can be used for species identification and delineation. This integrative approach allowed us to (i) provide evidence for morphological and molecular differentiation within the '*D. mergi*' species complex, including a previously undetected lineage; (ii) clarify that '*D. mergi* Lineage 1' of Georgieva *et al.* [6] represents *D. parviventosum*; (iii) partially elucidate the life-cycle of '*D. mergi* Lineage 3' and '*Diplostomum* sp. Clade Q' of Georgieva *et al.* [6]; (iv) expand the *cox*1 database for the European species *D. pseudospathaceum* and *D. spathaceum* in association with descriptions based on sequenced isolates; and (vii) assess the first intermediate host-specificity of *D. pseudospathaceum*.

Morphologically, the cercaria corresponding genetically to '*D. mergi* Lineage 1' of Georgieva *et al.* [6] keyed down to *D. parviventosum* in the key of Niewiadomska & Kiselienė [10] due to the presence of 6–7 pre-oral spines, 11 transverse rows of spines on the body, 10–12 pairs of caudal bodies in the tail, ventral sucker slightly exceeding the width of the anterior organ [VSW/AOW 1.0–1.4 (1.2)], fin-folds on the furcae, and small penetration gland-cells which do not cover ends of caeca (see Additional file 6: Table S2). As described for *D. parviventosum* by Niewiadomska & Kiselienė [10], the present cercaria also shows a characteristic resting position with the tail stem bent at about 45° but differs in having 11 transverse rows of spines on the body (*vs* 10); of these rows 9–11 are interrupted ventrally and dorsally and rows 5–8 are interrupted dorsally (*vs* rows 6–10 with ventral and dorsal interruption) (Additional file 6: Table S2). Comparisons of the metrical data for fixed cercariae revealed differences probably due to the fixation method (Table 5). The present cercariae (both live and formalin-fixed) exhibit lower ranges and means for the length of the body, anterior organ and tail stem, the latter resulting in a lower range for the ratio TSL/FL (mean 0.8 *vs* 1.1), and higher upper limits for the width of the ventral sucker (46 *vs* 37 µm). However, the ratios VSW/AOW and BL/TSL are similar (see means in Table 5). Furthermore, the number of rows of post-oral spines (7–8 alternate rows) and the number of spines on the ventral sucker (77–87 in two rows) agree well with the description by Niewiadomska & Kiselienė [10], *i.e.* 6–8 rows of post-oral spines and 80–88 spines on ventral

sucker in two rows (Additional file 6: Table S2). Our study thus provides the first detailed morphological description of the cercaria of *D. parviventosum* based on both light and scanning electron microscopy. Furthermore, the concordance of the morphological and molecular data clearly suggests that the isolates of '*D. mergi* Lineage 1' of Georgieva *et al.* [6] actually belong to *D. parviventosum*. However, the ITS sequences for this species generated by us formed a reciprocally monophyletic lineage within the '*D. mergi*' species complex instead of joining the cluster of sequences ('Clade Q' *sensu* Georgieva *et al.* [6]) representative for *D. parviventosum* according to Niewiadomska & Laskowski [23] (see also comment below).

Cercariae of the remaining three lineages of the '*D. mergi*' species complex discovered in this study differ from the description of *D. mergi* by Niewiadomska & Kiselienė [10] in four unique qualitative features, *i.e.* the presence of scale-like spines on the tail stem and furcae and of yellow pigment in the forebody, the greater number of rows of post-oral spines (10–11 *vs* 6–9) and in the different number of transverse rows with additional spines laterally (Additional file 6: Table S2). Cercariae of Lineages 2 and 3 of '*D. mergi*' can be further differentiated from *D. mergi* of Niewiadomska & Kiselienė [10]) by having furcae longer than tail stem (*vs* equal); the cercaria of '*D. mergi* Lineage 3' also possesses 11 transverse rows of spines on body (*vs* 10) and a smaller number of spines on ventral sucker (90–92 *vs* 94–130) and the cercaria of *D. mergi* Lineage 4 is unique in having three pairs of penetration gland-cells (Additional file 6: Table S2). Morphometric comparisons revealed that the cercariae of *D. mergi* Lineage 4 exhibit much higher upper ranges and means for all morphometric characters than those of the two other lineages (Lineage 1 and 2; both live and fixed samples) and those in the description of *D. mergi* by Niewiadomska & Kiselienė [10] (Table 5). Fixed cercariae of Lineages 2 and 3 both differ from those of *D. mergi* as described by Niewiadomska & Kiselienė [10] in having generally shorter (mean length 48–49 *vs* 58 μm) and wider (mean width 32–33 *vs* 28 μm) anterior organs, narrower tail stems (mean 29–33 *vs* 36 μm), longer furcae (mean 216–225 *vs* 210 μm) that are also longer than tails (mean TSL/FL 0.8–0.9 *vs* 1.0), and shorter ('*D. mergi* Lineage 2')/longer ('*D. mergi* Lineage 3') tail stems (means 160 and 212 *vs* 207 μm, respectively). Further, compared with the cercaria of *D. mergi* described by Niewiadomska & Kiselienė [10], the cercaria of '*D. mergi* Lineage 2' has a shorter body (mean 168 *vs* 182 μm), a wider ventral sucker (mean 53 *vs* 44 μm) and a greater ratio BL/TSL (mean 1.0 *vs* 0.9), and the cercaria of '*D. mergi* Lineage 3' has a narrower body (mean 49 *vs* 59 μm) and a smaller ventral sucker (mean 31 × 32 *vs* 44 × 44 μm) that is also narrower in relation to the anterior organ (mean VSW/AOW 1.0 *vs* 1.6).

Overall, the isolates of the three lineages (Lineages 2–4) of the '*D. mergi*' species complex described here exhibit a number of unique differentiating features (five for Lineages 2 and 4 and nine for Lineage 3; see Additional file 6: Table S2). In addition to the consistent differences in the morphometric characters and ratios (Table 5) the live cercariae of the single isolate of *D. mergi* Lineage 4 differ from those of both Lineage 2 and 3 in the relation BL < TSL = FL (*vs* BL ≤ TSL < FL and BL ≤ TSL < FL, respectively), in the pattern of incomplete transverse rows of spines on the body (ventral interruption in rows 8–10 *vs* 6–10 and 4–11, respectively; dorsal interruption in rows 5–10 *vs* 6–10 and 4–11, respectively), and in the lack of transverse rows with additional lateral spines.

Both live and fixed cercariae of '*D. mergi* Lineage 2' differ from those of '*D. mergi* Lineage 3' in having wider bodies (mean 63 and 64 *vs* 53 and 49 μm, respectively), larger ventral suckers (50 × 47 *vs* 31 × 31 μm and 46 × 53 *vs* 31 × 32 μm, respectively) that are also distinctly wider than anterior organs [ratio VSW/OSW 1.5–2.0 (1.7) *vs* 1.0–1.2 (1.1) and 1.5–2.1 (1.6) *vs* 0.9–1.1 (1.0), respectively]. The cercaria of '*D. mergi* Lineage 2' further differs from the cercaria of '*D. mergi* Lineage 3' in having 5–6 pre-oral spines of the median group located in 2 rows (*vs* 7 in 3 rows) with one spine very large, 11 rows of post-oral spines (*vs* 10) with spines in the first row larger than the remaining (*vs* spines in the first two rows), 10 transverse rows of spines (*vs* 11), as well as in the lack of double transverse rows (*vs* row 1), in the pattern of incomplete transverse rows and in having distinctly more spines on the ventral sucker (110–120 *vs* 90–92) (Additional file 6: Table S2). All these differences, in association with the molecular evidence, justify the distinct status of the four lineages of the '*D. mergi*' species complex examined by us. However, it is difficult to decide whether the description of Niewiadomska & Kiselienė [10] (see Additional file 6: Table S2) corresponds to one of these due to the different level of detail provided in the early description of *D. mergi* and the consistent differences outlined above.

The detailed morphological and molecular data provided here further advance our knowledge of the '*D. mergi*' species complex in several aspects. First, we have clarified that '*D. mergi* Lineage 1' of Georgieva *et al.* [6] in fact represents *D. parviventosum*. These authors provided sequence data for a single isolate ex *R. auricularia* from Hengsteysee thus making decisions of its relationships difficult, whereas our study provides ample evidence for the distinct status of this lineage, its identification to the species level and the detection of its relatively high prevalence in *R. auricularia* in Hengsteysee, and probably elsewhere in Europe. Our study further expands the number of isolates of '*D. mergi* Lineage 2' ex *R. auricularia* and its distribution in Baldeneysee, Hengsteysee and Sorpetalsperre. Finally, we provide the first link between

sequences for '*D. mergi* Lineage 3' from isolates of metacercariae in the second intermediate host (*Salmo trutta fario* and *Gobio gobio* from the River Ruhr; see Georgieva *et al.* [6]) and a number of isolates from the first intermediate hosts (*R. auricularia* from Hengsteysee) thus partially elucidating the life-cycle of this lineage (arguably species). Further efforts should be focused on the discovery of the adult stages and formal descriptions of the three novel lineages (Lineages 2, 3 and 4) of the '*D. mergi*' species complex.

Two ITS1-5.8S-ITS2 sequences for *D. mergi* (*sensu lato*) have been published recently by Haarder *et al.* [18] from cercarial isolates ex *Radix balthica* (L.) in Denmark. These authors have shown experimentally that the cercariae infect *Oncorhynchus mykiss* (Walbaum). Faltýnková *et al.* [8] suggested, based on analysis of ITS1 only, that one of the isolates (JX494231) may belong to '*D. mergi* Lineage 2' whereas the second (JX494233) appeared associated with '*D. mergi* Lineage 3'. In our analyses based on the entire ITS gene cluster one of the isolates (JX494231) clustered together with the single isolate of *D. mergi* Lineage 4 (however with low support) and the other clustered with isolates of '*D. mergi* Lineage 2'. Analysis of *cox*1 sequences for these two isolates would help reveal their actual assignment.

Our study expanded the *cox*1 database for European *D. pseudospathaceum* and *D. spathaceum* (18 and 7 isolates, respectively). The new isolates of both species clustered together with the isolates reported previously by Georgieva *et al.* [6] with high support. Based on all sequence data available to date, we can confidently suggest that *D. pseudospathaceum* completes its life-cycle using only *L. stagnalis* and *S. palustris* as first intermediate hosts and that the latter two hosts are infected only with this species. The two isolated records of *D. pseudospathaceum* ex *R. auricularia* (see [24, 25]) most probably represent misidentifications. The lack of infections with *D. pseudospathaceum* in more than 3,500 *R. auricularia* examined in the River Ruhr drainage ([26]; present study) provides further support for this suggestion. Morphologically, the cercarial isolates sequenced here generally (excluding the number of caudal bodies) key down to *D. pseudospathaceum* in the key of Niewiadomska & Kiselienė [10]. However, our detailed description (including SEM examination) of the cercaria revealed some differences compared with the data provided by these authors that generally show a wider range of variation: 10–11 pre-oral spines in the median group (*vs* 8–14); 3 pre-oral spines in each lateral group (*vs* 1–4); 9 post-oral rows of spines (*vs* 6–8); 11 transverse rows of spines on body (*vs* 10); transverse rows 3–7 with additional spines laterally (*vs* rows 3–4); spines present on entire tail stem (*vs* present at distal end of the tail stem); and resting position with tail stem bent at < 45° (*vs* at 90°) (see Additional file 6: Table S3). Morphometric comparisons revealed that both live and formalin-fixed cercariae described here possess shorter and wider bodies (means 166 × 50 and 162 × 59 μm, respectively, *vs* 185 × 45 μm), longer furcae (means 234 and 232 μm, respectively, *vs* 201 μm), the latter resulting in somewhat lower TSL/FL ratios (means 0.8 and 0.9, respectively, *vs* 1.0). Fixed cercariae described by us further exhibit greater width of the anterior organ (mean 31 *vs* 27 μm) and length of the tail stem (mean 213 *vs* 206 μm) and a lower BL/TSL ratio (mean 0.8 *vs* 0.9) (Table 6). These data indicate that SEM examination and adequate fixation should be considered for identification of the cercariae of *D. pseudospathaceum* in future studies.

This study is the first to provide a description of molecularly identified cercarial isolates of *D. spathaceum*. Both live and formalin-fixed isolates of *D. spathaceum* studied by us exhibit smaller dimensions for the size of the body, tail and all organs compared with the description of the cercaria of *D. spathaceum* by Niewiadomska [27] (the same data from 10 heat-fixed specimens were reiterated by Niewiadomska & Kiselienė [10]) (Table 6). Qualitative comparisons revealed that our isolates possess a slightly greater number of pre-oral spines (18–19 *vs* 8–16 in the median group and 1 in each lateral group *vs* no lateral spines), a smaller number of post-oral spines (9 *vs* 10–14), three spine rows on ventral sucker (*vs* 2), spined tail stem and furcae (*vs* unspined) and a smaller angle of bending of the tail stem in resting position (<45° *vs* 90°) (see Additional file 6: Table S3).

The cercaria of *D. spathaceum* described above exhibits similarities with the description of *D. paracaudum* by Niewiadomska [28] reiterated by Niewiadomska & Kiselienė [10] such as: an overlap in the number of the pre-oral spines (18–19 *vs* 15–20 spines in the median group; 1 *vs* 1–2 pre-oral spines in each lateral group) and the presence of 10 transverse rows of spines on the body, three (incomplete) spine rows on the ventral sucker and large penetration gland-cells that do not cover ends of the caeca (Additional file 6: Table S3). However, the present cercaria possesses yellow pigment in the body, a ventral sucker slightly wider than the anterior organ, nine (*vs* 6–7) rows of post-oral spines and a different pattern of spines in the transverse rows of spines on the body [rows 1–2 double ventrally only (*vs* row 1); rows 9–10 with ventral interruption (*vs* rows 5–10 with both ventral and dorsal interruption); row 3 with additional spines laterally (*vs* anteriormost rows)]. Further differences include the lower ranges of the number of spines on the ventral sucker (103–119 *vs* 116–141), the presence of spines on the tail stem and furcae (*vs* absent) and the much smaller angle of bending of the tail stem in resting position (<45° *vs* 90°) (Additional file 6: Table S3). Comparisons of the morphometric data revealed

that both live and formalin-fixed isolates of *D. spathaceum* studied by us exhibit shorter and wider bodies (means 172 × 51 and 170 × 53 μm, respectively, *vs* 189 × 46 μm), longer ventral suckers (means 30 and 31 μm, respectively, *vs* 25 μm), longer (means 219 and 250 μm, respectively, *vs* 211 μm) but narrower tail stems (means 31 and 32 μm, respectively, *vs* 37 μm), much longer furcae (means 260 and 252 μm, respectively, *vs* 208 μm) and lower BL/TSL ratios (means 0.8 and 0.7, respectively, *vs* 0.9) (Table 6). The above comparisons indicate that the morphology of the cercaria of *D. spathaceum* characterised molecularly in the present study departs from the single limited descriptions of cercariae of both *D. spathaceum sensu* Niewiadomska [27] and *D. paracaudum sensu* Niewiadomska [28]. It is unfortunate that the morphologies described by Niewidomska have not been confirmed for nearly 30 years.

Georgieva *et al.* [6] denoted as 'Clade Q' (questionable) a single genotype representing two cercarial isolates ex *R. ovata* identified as *D. spathaceum* (AF419275; AF419276) and two for a cercarial isolate ex *R. ovata* identified as *D. parviventosum* (AF419277; AF419278) by Niewiadomska & Laskowski [23]; one metacercarial isolate ex *R. rutilus* submitted to GenBank as *D.* cf. *parviventosum*/*spathaceum* (JF775727) by Rellstab *et al.* [29]; and one cercarial isolate ex *R. auricularia* (JQ665458; isolate RA97) annotated as *D. mergi* in GenBank but published as *D. spathaceum* by Behrmann-Godel [17]. One additional metacercarial isolate belonging to 'Clade Q' has been recently sequenced and described by Pérez-del-Olmo *et al.* [9]. Georgieva *et al.* [6] also suggested to use temporarily the name *D. parviventosum* as a label for the four identical sequences (AF419275–AF419278) of Niewiadomska & Laskowski [23]. One of the important results of our integrative taxonomic approach is the clarification of the distinct status of *D. parviventosum* and its close relationship with the species/lineages of the '*D. mergi*' species complex (see above). However, the species identification of the sequences within 'Clade Q' *sensu* Georgieva *et al.* [6] is still questionable. The fact that new isolates are being added to this clade ([9, 17]; present study) indicates that this lineage has a wider distribution and still requires taxonomic scrutiny. It is worth noting that the metacercaria of '*Diplostomum* sp. Clade Q' ex *Cyprinus carpio* L. from the Ebro Delta in Spain described and sequenced by Pérez-del-Olmo *et al.* [9] was shown to possess a smaller oral sucker and a shorter holdfast organ compared with the Spanish and Polish (see Niewiadomska [27]) isolates of *D. spathaceum* plus a distinctly lower number of excretory granules in the secondary excretory system than the metacercariae of *D. spathaceum sensu* Niewiadomska [27]. The cercaria of the single isolate of '*Diplostomum* sp. Clade Q' sequenced and described here keys down to *D. spathaceum* in the key by Niewiadomska & Kiselienė [10] and agrees with their description in many aspects. However, the present cercaria differs in having fewer pre-oral spines in the median group (9 *vs* 8–16), 12 post-oral rows of spines (*vs* 10–14), 10 pairs of caudal bodies (*vs* 11–12 pairs), as well as in the presence of two non-converging fields of dispersed spines in the hindbody (*vs* two fields converging ventrally) and of bands of scale-like spines on the tail stem and furcae (*vs* spines absent); spines on the latter enveloped by tegumental membrane forming a specific fish-fin like fin-fold (Additional file 6: Table S3). Although the metrical data for our isolate (live cercariae measured only) are not directly comparable with those by Niewiadomska [27] the former exhibits much lower values for the length of body (mean 224 *vs* 235 μm) and the ratios VSW/AOW (mean 1.3 *vs* 1.7) and BL/TSL (mean 0.8 *vs* 0.9) and much greater values for body width (mean 96 *vs* 73 μm) and for the size of the anterior organ (mean 80 × 47 *vs* 68 × 34 μm) and ventral sucker (mean 56 × 65 *vs* 53 × 59 μm). The above comparisons indicate that the cercariae and metacercariae of '*Diplostomum* sp. Clade Q' possess distinctive morphological characteristics that do not allow their identification as *D. spathaceum sensu* Niewiadomska [27]. The solution for the taxonomic status of this clade should await morphological and molecular data for the adult stages.

Conclusion

The integration of molecular and morphological evidence for *Diplostomum* spp. achieved in this study will serve as a baseline for species identification of these important parasites of snail and fish populations and thus advance further studies on the distribution of *Diplostomum* spp. in Europe.

Additional files

Additional file 1: Figure S1. Schematic illustration of a cercaria of *Diplostomum* spp. showing the metrical features used. *Abbreviations*: BL, body length; BW, maximum body width; AOL, anterior organ length; AOW, anterior organ maximum width; VSL, ventral sucker length; VSW, ventral sucker width; TSL, tail stem length; TSW, tail stem width (at base); FL, furca length.

Additional file 2: Table S1. Summary data for *cox*1 sequences for *Diplostomum* spp. retrieved from GenBank.

Additional file 3: Figure S2. Cercariae of *Diplostomum* spp. Pre- and post-oral spines (light microscopy). A, *Diplostomum parviventosum*; B, *Diplostomum mergi* Lineage 4; C, '*Diplostomum mergi* Lineage 2'; D, '*Diplostomum mergi* Lineage 3'; E, F, *Diplostomum pseudospathaceum* (arrows indicate lateral spines); G. *Diplostomum spathaceum*.

Additional file 4: Figure S3. Cercariae of *Diplostomum* spp. Ventral sucker (light microscopy). A, *Diplostomum parviventosum*; B, *Diplostomum mergi* Lineage 4; C, '*Diplostomum mergi* Lineage 2'; D, '*Diplostomum mergi* Lineage 3'; E, '*Diplostomum* sp. Clade Q'; F, *Diplostomum pseudospathaceum*; G. *Diplostomum spathaceum*.

Additional file 5: Figure S4. Cercariae of *Diplostomum* spp. Tail furcae (light microscopy). A, *Diplostomum parviventosum*; B, *Diplostomum mergi* Lineage 4; C, '*Diplostomum mergi* Lineage 2'; D, '*Diplostomum mergi*

Lineage 3'; E, '*Diplostomum* sp. Clade Q'; F, *Diplostomum pseudospathaceum*; G. *Diplostomum spathaceum*.

Additional file 6: Table S2. Comparative qualitative and meristic data for cercariae of the *Diplostomum 'mergi'* species complex. **Table S3** Comparative qualitative and meristic data for cercariae of *Diplostomum spathaceum*, *D. pseudospathaceum*, *D. paracaudum* and '*Diplostomum* sp. Clade Q' of Georgieva *et al.* [6].

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
CS and MS: obtained samples, undertook the identification and morphological characterisation of the isolates and prepared the first draft of the manuscript. CS and SG: carried out the sequencing. SG: performed the phylogenetic analyses and drafted the correponding parts of the MS. BS and AK: conceived the study, discussed the results and helped draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements
We thank Jana Köchling, Verena Altmann, Jessica Schwelm (University of Duisburg-Essen) for their assistance in sampling, Blanka Škoríková (Institute of Parasitology) for her kind help with figure preparations and Jiří Vaneček, Martina Tesařová and Petra Masařová (Laboratory of Electron Microscopy, Biology Centre, Czech Academy of Sciences) for their expert help in the SEM study. We thank two anonymous reviewers for their constructive comments which helped improve the manuscript. This study was partially funded by the Czech Science Foundation (15-14198S) and the 'Sichere Ruhr' project as part of the Bundesministerium für Bildung und Forschung (BMBF) program 'Sustainable Water Management' (grant 02WRS1283). CS benefited from a Deutsche Bundesstiftung Umwelt (DBU) PhD fellowship and a research grant by the Faculty of Biology of the University of Duisburg-Essen.

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Received: 11 March 2015 Accepted: 18 May 2015
Published online: 03 June 2015

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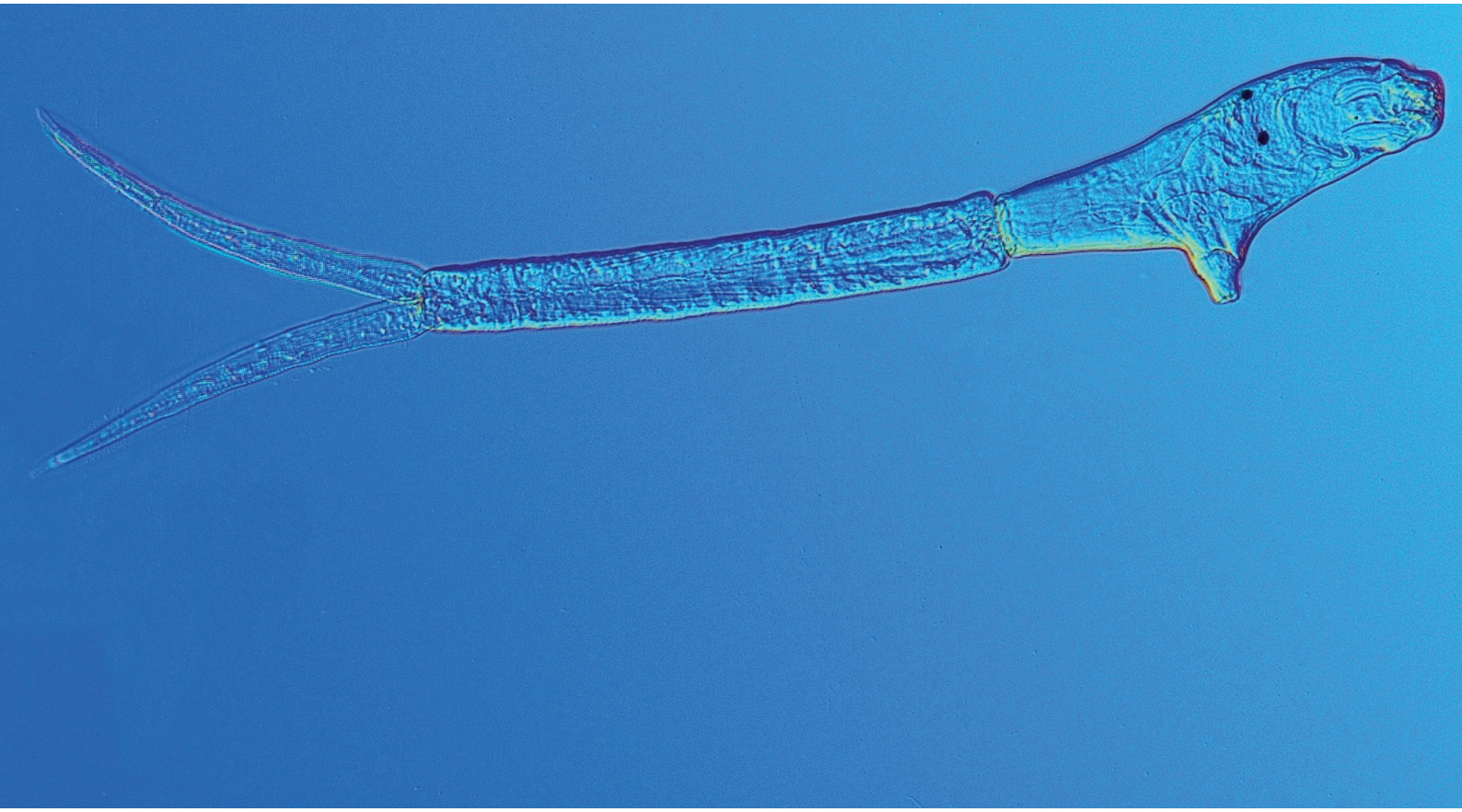

6. Chapter II

Functional aspects of trematodes – temporal release patterns and productivity of cercariae

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Functional aspects of trematodes – temporal release patterns and productivity of cercariae

This chapter contains the following study that investigates the productivity and emergence of cercariae, using the example of *Trichobilharzia szidati* cercariae from *Lymnaea stagnalis*, by determining (i) temporal cercarial emission patterns and (ii) the average daily output rate of cercariae per snail that will allow accurate estimations of the biomass of cercariae released into the ecosystem

6.1 Soldánová, M.*, Selbach, C.* & Sures, B. (2015). The early worm catches the bird? Productivity and patterns of *Trichobilharzia szidati* cercarial emission from *Lymnaea stagnalis.* (Submitted to PLOS ONE). *Authors contributed equally

The early worm catches the bird? Productivity and patterns of *Trichobilharzia szidati* cercarial emission from *Lymnaea stagnalis*

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Abstract

Digenean trematodes are common and abundant in aquatic habitats and their free-living larvae, the cercariae, have recently been recognized as important components of ecosystems in terms of comprising a significant proportion of biomass and in having a strong potential influence on food web dynamics. One strategy to enhance their transmission success is to produce high numbers of cercariae which are released during the activity peak of the next host. In laboratory experiments with 13 *Lymnaea stagnalis* snails infected with *Trichobilharzia szidati* the average daily emergence rate per snail was determined as 2,621 cercariae, with a maximum of 29,560. During a snail's lifetime this summed up to a mass equivalent to or even exceeding the snail's own body mass. Extrapolated for the eutrophic pond where the snails were collected, annual *T. szidati* biomass may reach 4.65 tons, a value equivalent to a large Asian elephant. Emission peaks were observed after the onset of illumination, indicating emission synchronizing with the high morning activities of the definitive hosts, ducks. However, high cercarial emission is possible throughout the day under favorable lightning conditions. Therefore, although bird schistosomes, such as *T. szidati* constitute only a fraction of the diverse trematode communities in the studied aquatic ecosystem, their cercariae can still pose a considerable risk for humans of getting cercarial dermatitis (swimmer's itch), due to the high number of cercariae emitted from infected snails.

Introduction

Except for their role as pathogens, parasites have long been considered negligible components of ecosystems. Recent studies elucidating the patterns of parasite biomass, species abundance and interactions in food webs have revealed that parasites actually represent important ecological players in the dynamics of natural systems (e.g. Lafferty et al. 2006, 2008, Kuris et al. 2008, Amundsen et al. 2009, Preston et al. 2012). Especially free-swimming larvae of trematodes, the cercariae, which emerge from the molluscan first intermediate hosts as a result of asexual reproduction, represent essential components of ecosystems subsuming a substantial fraction of biomass in marine (Kuris et al. 2008, Thieltges et al. 2008a) and freshwater ecosystems (Preston et al. 2012, Preston et al. 2013) and may exert strong influence on the structure, dynamics and function of food webs (Lafferty et al. 2006, 2008, Hechinger et al. 2005, 2007).

The cercarial stage is crucial in the life cycle of trematodes as it plays an inevitable role in the transmission to the next target hosts. After emerging from an infected snail host, the principal task of cercariae is to disperse in the environments, locate and infect as many hosts as possible. However, only a small fraction of the emitted cercariae successfully reaches its target because cercariae are short-lived and directly exposed to and affected by external biotic and abiotic environmental factors, often leading to a failure in infecting the next host (Pietrock et al. 2003, Thieltges et al. 2008b). Accordingly, many trematode species produce high numbers of cercariae, whose shedding peak is assumed to be synchronized with the behavior of the next host (Combes et al. 1994) and is often triggered by factors such as the photoperiod and light exposure or water temperature (Poulin 2006, Morley & Lewis 2013).

Bird schistosomes of the genus *Trichobilharzia*, which utilize freshwater snails and waterfowl in their life cycle, are the most common representatives of this group and their cercariae are important agents of swimmer's itch in humans in Europe (Soldánová et al. 2013) and North America (Brant & Loker 2013). Although complex information about various aspects of the biology of *Trichobilharzia* spp. has been gathered in past decades (e.g. Horák et al. 2002, 2015, Horák & Kolářová 2001, 2005, 2011), available data on the patterns of cercarial emission are still rather fragmentary and virtually no reliable data exist on the total number of cercariae entering the ecosystem. Early observations and estimations of cercarial productivity of *Trichobilharzia* are rather broad, ranging from several thousand cercariae during a snail's lifetime (Neuhaus 1952) to several thousand larvae during a few days or weeks (Anderson et al. 1976, Sluiters et al. 1980). Likewise, temporal emergence patterns have been studied for a variety of trematode species (e.g. Combes et al. 1994), but there are only few studies investigating the temporal patterns for *Trichobilharzia* sp. cercariae in detail (e.g. Anderson et al. 1976) that allow to assess these patterns on an ecosystem level.

Therefore, the main aim of the present study was to examine cercarial emergence of the model organism of bird schistosomes, *Trichobilharzia szidati*, under laboratory conditions in order to determine (i) daily output rates per individual snail; (ii) peaks in cercarial emission under controlled and natural light conditions; (iii) output variation among conditions and experiments; and (iv) direct contribution of the parasite larvae to ecosystem energetics using estimates for cercarial abundance and biomass. First, we investigated cercarial emergence in a series of laboratory experiments under different conditions using 13 naturally infected *Lymnaea stagnalis* with *T. szidati* over a 72-hour period during each experiment. Secondly, we quantified cercarial biomass using data on the mean daily output rates and metrical data of living cercariae, and finally we estimated the total biomass of cercariae for the life span of an individual snail and for the snail population within a typical freshwater ecosystem in Europe using data from the literature on snail abundance and *Trichobilharzia* spp. prevalence.

Materials and Methods

Experimental design

In our experimental study we selected *Trichobilharzia szidati* Neuhaus, 1952 as a model organism because knowledge on exact total or average numbers of cercariae released from a snail host is rather ambiguous and quantitative estimates on its cercariae productivity is entirely lacking, although it is probably the most studied model bird schistosome (Horák et al. 2002, 2014). Cercariae of *T. szidati* were obtained from naturally infected *Lymnaea stagnalis* (L.) which were sampled from a fishpond in the Czech Republic in June 2012 (Vlkovský pond: 49°08'56"N, 14°43'51"E). A total of 168 snails was collected along the pond shore from aquatic vegetation, transported to the laboratory and screened for patent infections by placing them individually into beakers with a small amount of filtered lake water under the light source for 24 hours. To avoid the possible influence of different snail host sizes on the cercarial emergence when larger snails produce higher daily outputs (e.g. Thieltges et al. 2008b, Poulin 2006, Studer & Poulin 2013), sampling effort was focused on snails of similar size cohorts between 40-50 mm in shell height only. Moreover, in this size/age *L. stagnalis* can be found with higher percentage of infection (Żbikowska et al. 2013), assuring a higher probability of encountering the desired trematode species. In total, 13 snails with *T. szidati* infections were found (7.74% of 168 snails examined). Because several stress factors such transportation of snails and their subsequent handling under different temperatures and illumination scenarios in the laboratory might negatively affect both host and parasites leading to distorted rhythms of cercarial emergence (e.g. Morley & Lewis 2013, Anderson et al. 1976, Brassard et al. 1982, Taskinen 1998), all snails infected with *T. szidati* were maintained in one aerated aquarium at room temperature ranging from 19°C to 21°C and under

natural photoperiod for 14 days for acclimatization. Snails were stopped feeding on lettuce five days prior to emission experiments and were measured before each experiment. As ambient temperature is considered the most important factor influencing daily cycles of cercarial emergence (Poulin 2006) all experiments were designed under stable thermal conditions and both air and water temperature were monitored throughout the entire course of the study.

Cercarial emergence of *T. szidati* from 13 naturally infected *L. stagnalis* was investigated under different conditions in a series of two types of laboratory experiments, each performed over three consecutive days in July and September (i.e. for 72 hours). A "daily output experiment" was designed to obtain data on the total and mean number of cercariae released per snail and day. Cercarial counts were carried out every four hours from 8:00 to 20:00 over a period of 72 hours. Because examination of cercariae for the state of degradation did not reveal any decomposition during this period, cercariae produced during the night intervals were counted once in the morning after 12-hours (from 20:00 to 8:00). Additionally, "peak output experiments" were performed aiming at assessing the chronobiological variation in a cercarial release during 72 hours, and to determine the day period with the highest emission rates. Cercarial counts were performed every two hours over a period of 72 hours. Each type of experiment was conducted under two different laboratory conditions, a natural photoperiod regime (sunrise at 5:30 and sunset at 21:50 in July; i.e. 16:20 h of light and 8:40 h of darkness) in the laboratory at room temperature (range from 19.6°C to 20.4°C; mean 20.2°C), and 12:12 light-dark cycle under standard conditions in a climate chamber with the temperature set to 20°C. In the climate chamber illumination was provided by an overhead halogen lamp; in the laboratory, snails were exposed to the natural photoperiod regime by placing them next to the window. In total, we investigated variation in cercarial output in four consecutive experiments in July: one "daily output experiment" at room temperature (further DJ-L) and one in a climate chamber (DJ-C), further one "peak output experiment" at room temperature in the laboratory (further PJ-L) and one in a climate chamber (PJ-C) (Table 1). Altogether, the four experiments in July lasted 15 days with an interval of one free day in between experiments. In September, only one "peak output experiment" in the climate chamber (PS-C) was performed (Table 1). Light dark circles in the climate chamber were set from 6:00 to 18:00 light and 18:00 to 6:00 dark in July and 8:00 to 20:00 light and 20:00 to 8:00 dark in September. No specific permissions were required for these locations/activities and our field study does not involve endangered or protected species.

Table 1 Numbers of cercariae of *Trichobilharzia szidati* released from naturally infected *Lymnaea stagnalis* per snail.

	July												September		
Snail code	**Daily output experiment in laboratory (DJ-L; n=13)**			**Peak output experiment in laboratory (PJ-L; n=11)**			**Peak output experiment in climate chamber (PJ-C; n=11)**			**Daily output experiment in climate chamber (DJ-C; n=10)**			**Peak output experiment in climate chamber (PS-C; n=9)**		
	Range	**Mean±SD**	**Total**	**Range**	**Mean±SD**	**Total**	**Range**	**Mean±SD**	**Total**	**Range**	**Mean±SD**	**Total**	**Range**	**Mean±SD**	**Total**
L1	510-2,740	1,483±1,142	4,450	1,290-2,520	1,700±710	5,100	1,650-3,230	2,190±901	6,570	400-2,320	1,603±1,009	4,810	9,550-24,070	15,213±7,769	45,640
L2	1,310-1,500	1,390±98	4,170	1,890-2,510	2,160±318	6,480	1,870-3,390	2,393±864	7,180	560-2080	1,107±845	3,320	7,530-21,430[b]	13,003±7,406	39,010
L3	360-620	460±140	1,380	810-1,760	1,190±503	3,570	430-1,100	667±376	2,000	390-1,000	703±305	2,110	2,520-7,920[b]	4,947±2,741	14,840
L4	330-1,020	597±371	1,790	740-1,680	1,300±495	3,900	520-730	590±121	1,770	40-260	173±117	520	5,970-19,950	12,783±6,997	38,350
L5	980-1,480	1,150±286	3,450	810-990	903±90	2,710	910-1,600	1,147±393	3,440	260-620	453±181	1,360	2,620-14,750[b]	10,470±6,808	31,410
L6	600-1,480	1,143±475	3,430	860-2,590	1,647±876	4,940	1,580-2,940	2,083±746	6,250	700-1,880	1,093±681	3,280	6,510-29,560	17,947±11,526	53,840
L7	380-1,490	963±557	2,890	1,360-2,600	1,887±641	5,660	240-2,230	960±1,103	2,880	100-140	120±20	360	2,690-4,720[b]	3,707±1,015	11,120
L8	540-1,200	880±330	2,640	880-1,450	1,197±290	3,590	350-780	553±216	1,660	-[a]	-	-	-[a]		
L9	780-1,190	1,043±229	3,130	1,160-1,350	1,277±102	3,830	910-2,060	1,457±577	4,370	560-1,820	1,117±643	3,350	5,290-10,730	7,993±2,720	23,980
L10	550-1,620	1,203±573	3,610	-[a]	-	-	-[a]	-	-	-	-	-	-[a]		
L11	300-690	490±195	1,470	610-1,590	1,007±516	3,020	390-2,130	1,057±939	3,170	330-1,640	1,070±671	3,210	2,840-8,730[b]	5,197±3,116	15,590
L12	940-3,000	1,740±1,104	5,220	-[a]	-	-	-[a]	-	-	-	-	-	-[a]		
L13	580-4,560	1,927±2,281	5,780	600-1,800	1,267±611	3,800	360-1,940	987±839	2,960	220-2,700	1,120±1,373	3,360	-[a]		

Range, means (± standard deviation, SD) and total numbers of emerged cercariae are given per snail pooled across three days (i.e. 72 hours) of each experiment. In September only one experiment was performed. Number of snails used in a given experiment is indicated by "n" in parentheses.

[a]Snail died during the experiment.

[b]Snail with patent double infection.

Determining cercarial numbers

The emission experiment of cercariae from individual snail replicates was carried out in plastic cups with 100 ml of lake water. Prior to use, the water was filtered in order to avoid contamination and placed in the relevant conditions corresponding to each type of experiment to balance potential differences in temperature. After each emission period, ten homogenized subsamples of one ml each were taken with a micropipette from each replicate while vigorously mixing the water containing swimming larvae and transferred into cell-well plates to count cercariae. Drops of vital stain (Natural Red) were added to make cercariae immobile and more visible, allowing precise counts under a dissection microscope. After each emission time unit interval (two or four and 12 hours depending on the type of experiment), snails were transferred to new clean plastic cups with fresh pond water. To avoid contamination of the sample with cercariae of other replicates, the spoon used was washed and dried thoroughly. Raw data (i.e. counts per snail per unit time) for each replicate were converted into daily output rates (i.e. number of cercariae emitted $snail^{-1}$ day^{-1}) as follows: the numbers of cercariae found in 1 ml after each emission interval were summed over 10 subsamples and means were calculated. Averages were multiplied by 100 (water volume) and pooled across all emission intervals from one day, resulting in an estimate of the total number of cercariae emitted from a single snail during a day.

Estimation of cercarial biomass

In order to assess the direct contributions of *T. szidati* cercariae to the energy flow in ecosystems, its cercarial biomass was quantified using data on the mean daily output rates which we acquired from emission experiments, metrical data of live cercariae and data from the literature on snail abundance and parasite prevalence. First, we obtained metrical data of 11 unflattened live cercariae by measuring length and width of cercarial body, tail stem and furcae from photographs taken with an Olympus UC30 digital camera fitted on an Olympus BX51 microscope. Measurements were taken with the program ImageJ 1.47v (Abràmoff et al. 2004). Based on formulas provided by Koehler et al. (2012) we calculated the total cercarial volume (in mm^3) as the sum of volumes calculated for the cercarial body (equation for ellipsoid), tail stem (equation for cylinder) and furcae (equation for cone), and estimated mass (in mg) of an individual cercaria by multiplying the total cercarial volume by a tissue density of 1.1 g/ml (Kuris et al. 2008). Thereafter, we estimated parasite productivity for the life span of an individual snail (2 years for *L. stagnalis*) (Glöer 2002), considering the mean cercarial emission per snail and day (pooled across all 5 experiments) and the hibernation period of *L. stagnalis* in moderate climate of Central Europe. Given that development of many trematode species in snails throughout the winter period is arrested and no cercarial emergence occurs due to the decreased metabolic activity of both host and parasite (Galaktionov & Dobrovolskij 2003), we assume that the ongoing uninterrupted

cercarial emission of *T. szidati* persists from April to October in two subsequent years (428 days). The total mass of *T. szidati* emitted from one infected snail during its life span was calculated by multiplying cercarial mass by mean daily cercarial output per snail by 428 days. The cercarial mass estimated to be emitted from a single snail individual was afterwards compared with the tissue mass of 30 uninfected snails of similar size (mean of 45.1 mm) to those 13 snails infected with *T.szidati* used in the experiments (mean of 42.2 for snails entering experiments in July and 47.5 mm for snails in September), which we obtained by weighing the snail body deprived of their shells. Furthermore, we were interested in the total annual biomass of *T. szidati* cercariae entering an ecosystem. Accordingly, we used data from the literature on density of *L. stagnalis* (10 snails/m^2) (Jurkiewicz-Karnkowska 2008) to estimate the total biomass of cercariae in the pond from which the snails were collected. We assessed the snail population size within the ecosystem by multiplying the snail density of 10 snails/m^2 by surface area (m^2) of the water body (47.09 ha for Vlkovský pond). Considering the usual prevalence of *T. szidati* in snail populations in Europe (5%) (e.g. Soldánová et al. 2013) we estimated the total number of infected snails within the water body and calculated the total biomass of cercariae released by these snails during one year from April to October (214 days). Since infection levels of *T. szidati* in eutrophic ponds might exceed 20% and occasionally reach more than 40% (Soldánová et al. 2013) we calculated a biomass of cercariae in the pond estimating a prevalence of 41.5% (Soldánová et al. 2011, 2013).

Data analyses

We used the non-parametric Spearman's correlation coefficient (r_s) to statistically assess the effect of host size (shell height) on cercarial emergence. Correlation was tested for total numbers of cercariae pooled across three days (i.e. 72 hours) and by each day of all five experiments separately due to unequal numbers of replicates. Due to the one “peak emission experiment” conducted in September (PS-C), cercarial numbers were compared with only two experiments of equal treatment in July, i.e. PJ-L and PS-C; and PJ-C and PS-C. Furthermore, five snails in the September experiment showed patent double infections with other trematode species (four with *Diplostomum pseudospathaceum* and one with *Plagiorchis elegans*). Therefore, Student’s t-test was applied to detect significant difference in total numbers of *T. szidati* cercariae (pooled across three days) emitted from singly and doubly infected snails.

To assess output variation among experiments in different conditions we carried out a comparative statistical assessment on daily cercarial emergence using a general linear model (GLM) repeated measures ANOVA (RM-ANOVA) with cercarial numbers as dependent variable and “experiment” and “day” as within-subjects factors. Further, we analyzed whether there are significant differences in cercarial output between all three “peak emission output” experiments (PJ-L, PJ-C and

PS-C) in relation to time unit interval of two hours and day of experiment ("hour" and "day" as within-subjects factors). Post hoc Tukey HSD tests were performed where appropriate. Data on cercarial counts were ln (x+1)-transformed in order to meet the assumption of normality. A probability value of $p<0.05$ was considered to represent a significant difference in all comparisons. Statistical analyses were performed using Statistica 7.0 software package (StatSoft, Inc., Tulsa, OK, USA).

Results

Emergence and biomass of cercariae

Cercarial emergence of *T. szidati* was circadian with high levels in the light period in both types of experiments and laboratory conditions, although being variable for individual replicates (Table 1; Fig. 1A-C of "peak emission experiments" in different conditions). The mean daily emergence rate was 1,117 cercariae snail^{-1} day^{-1} in the four experiments performed in July, with a maximum of 4,560 cercariae per day. Cercarial productivity in the single experiment carried out in September was much higher in all snails and the mean emission rate was 10,140 cercariae snail^{-1} day^{-1}, reaching a maximum of 29,560 cercariae per day. The mean daily emergence pooled across all five experiments in both months was 2,621 cercariae snail^{-1} day^{-1}. Total and mean numbers of cercariae emitted for each type and day of a given experiment are shown in Table 2. Four snails died during the course of the experiments in July or before the experiments in September, resulting in a reduced number of replicates in the latter setup. Comparison of cercarial outputs recorded of snails with single and double infections in September did not detect any significant difference, although the emission from doubly infected snails appeared to be lower ($t=2.13$; $p=0.07$).

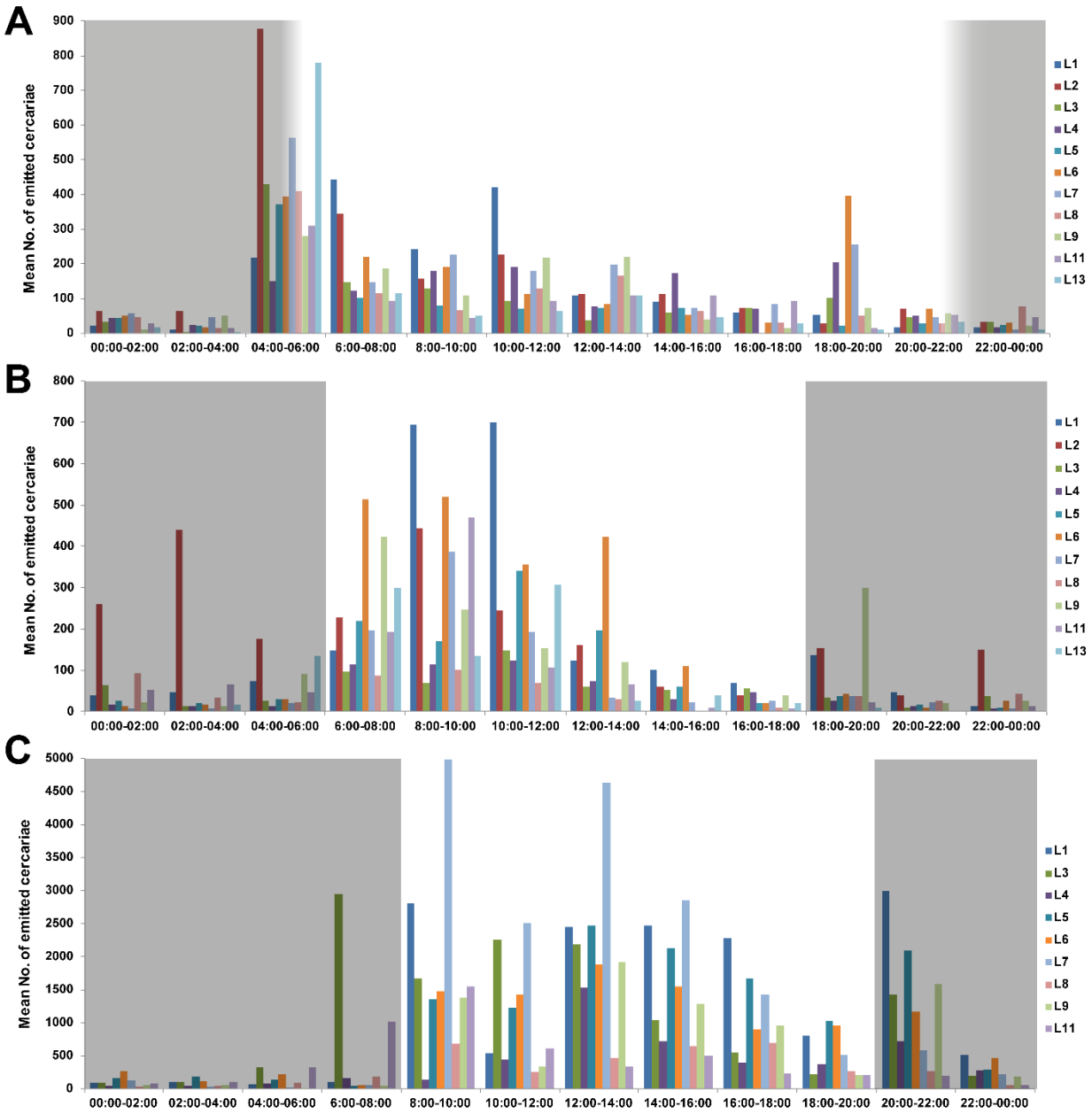

Fig 1. Daily output rates and emergence patterns of *Trichobilharzia szidati* cercariae from *Lymnaea stagnalis* individuals.

Plots show circadian cercarial emission with high levels in the light part of the day and variability across individual snail replicates (L1 – 13). Data are pooled across three days of each experiment. **(A)** Mean number of emitted cercariae from 11 naturally infected snails used in a "peak emission experiment" (PJ-L) in July under laboratory conditions at room temperature (range 19.6°C-20.4°C) and natural photoperiod regime (light dark cycle of 16:8 h). **(B)** Mean number of emitted cercariae from 11 naturally infected snails used in a "peak emission experiment" (PJ-C) in July under standard thermal conditions in a climate chamber (20°C) and controlled light dark cycle of 12:12 h. **(C)** Mean number of emitted cercariae from 9 naturally infected snails used in a "peak emission experiment" (PS-C) in September under standard thermal conditions in a climate chamber (20°C) and controlled light dark cycle of 12:12 h.

Table 2 Numbers of cercariae of *Trichobilharzia szidati* released from naturally infected *Lymnaea stagnalis* per day.

Month of experiment	Type of experiment	Day of experiment	Range	Mean±SD	Total
July	Daily output experiment	Day 1	330-4,560	1,193±1,083	15,510
	in laboratory	Day 2	300-1,440	808±397	10,500
	(DJ-L; n=13)	Day 3	360-3,000	1,338±789	17,400
	Total				**43,410**
	Peak output experiment	Day 1	600-2,520	1,181±614	12,990
	in laboratory	Day 2	820-2,510	1,436±473	15,800
	(PJ-L; n=11)	Day 3	810-2,600	1,619±587	17,810
	Total				**46,600**
	Peak output experiment	Day 1	530-3,230	1,849±881	18,490
	in climate chamber	Day 2	350-1,650	777±487	7,770
	(PJ-C; n=11)	Day 3	240-1,730	881±543	8,810
	Total				**35,070**
	Daily output experiment	Day 1	140-2,700	1,238±892	12,380
	in climate chamber	Day 2	120-2,320	894±761	8,940
	(DJ-C; n=10)	Day 3	40-970	436±285	4,360
	Total				**25,680**
	Pooled data (mean)			**1,117**	
September	Peak output experiment	Day 1	4,720-29,560	13,588±7,618	122,290
	in climate chamber	Day 2	3,710-24,070	11,494±7,669	103,450
	(PS-C; n=9)	Day 3	2,520-10,050	5,338±2,964	48,040
	Total				**273,780**
	Pooled data (mean)			**10,140**	
July & September	**Pooled data (mean)**			**2,621**	

Range and total numbers of cercariae are given for each day of experiment and pooled across snail individuals (number of snails used in a given experiment is indicated by "n" in parentheses). Mean (± standard deviation, SD) represents numbers of emerged cercariae $snail^{-1}day^{-1}$. In September, only one experiment was performed.

Based on our live photographs we calculated the total volume of an individual cercaria of *T. szidati* to 0.0039 mm^3 (summing volume of cercarial body: 0.0022 mm^3, tail stem: 0.0015 mm^3, and furcae: 0.0002 mm^3) and estimated its mass to 0.0043 mg. Applying data on average output rates from our emission experiments pooled across all experiments conducted in both months (2,621 cercariae $snail^{-1}day^{-1}$) resulted in an estimate of 4.8 g of cercariae of *T. szidati*, which are emitted into an ecosystem during the life span of an individual snail, a value equivalent to or even exceeding the weight of the snail's own body (range of soft tissue snail mass of 2.0-4.5 g, mean across 30 uninfected snails of 2.9 g). Based on snail density data and a *T. szidati* prevalence of 5%, the total annual parasite biomass in the large fishpond (47.09 ha) sums up to 561 kg. Since prevalence of *Trichobilharzia* spp. in these highly eutrophic ponds occasionally reaches more than 40% (Soldánová et al. 2011, 2013), we calculated a possible cercarial biomass in the pond of up to 4.65 tons per year.

Chronobiology

Although there were variations in the cercarial emission between replicates and days of experiments, clear peaks were observed in the morning hours after the onset of illumination (Table 3, Figs. 1 and 2). These morning peaks were confirmed by the "daily output experiments" with cercarial counts carried out every four hours. Again, the highest numbers of emitted cercariae occurred during an interval from 8:00 to 12:00. In addition to determining emission peaks, the "peak output experiments" revealed a complex intraspecific variation in cercarial emergence depending on experimental conditions. Under natural photoperiodic regime (light dark cycle of 16:8 h in the experiment PJ-L) the emission increased rapidly with sunrise around 5:30 (Fig. 2A). While the highest proportion of cercariae was released in a single two-hour interval from 4:00 to 6:00 across all replicates and three experimental days, i.e. on average 30% (range of 34-43% with a maximum of 74%) of the total number emerged within 24 hours, cercariae emitted in the remaining time units did not exceed 14% during a day. Under controlled conditions in a climate chamber (light dark cycle of 12:12 h in the experiment PJ-C) the initial peak appeared after the light was switched on at 6:00 (Fig. 2B). The highest proportion of emitted cercariae under these controlled laboratory conditions was observed for two experimental days between 6:00 and 10:00 with an average range of 24-28% of cercariae released from all snails (maximum range of 42-57%). In most of the remaining two-hour intervals mean numbers of emerged cercariae remained below 4%. The cercarial output in the experiment performed in September (PS-C) showed greater variability in relation to both time units and days of the experiment compared to the July experiment (PJ-C) under similar laboratory conditions (Fig. 2C). Altogether, on average 18% of cercariae (pooled across replicates and days) were released between 12:00 and 14:00 (11-21% range with maximum of 38%). Moreover, means of 22% (38% maximum) from 8:00 to 10:00 and 27% (65% maximum) from 20:00-22:00 were emitted on day one and day three, respectively.

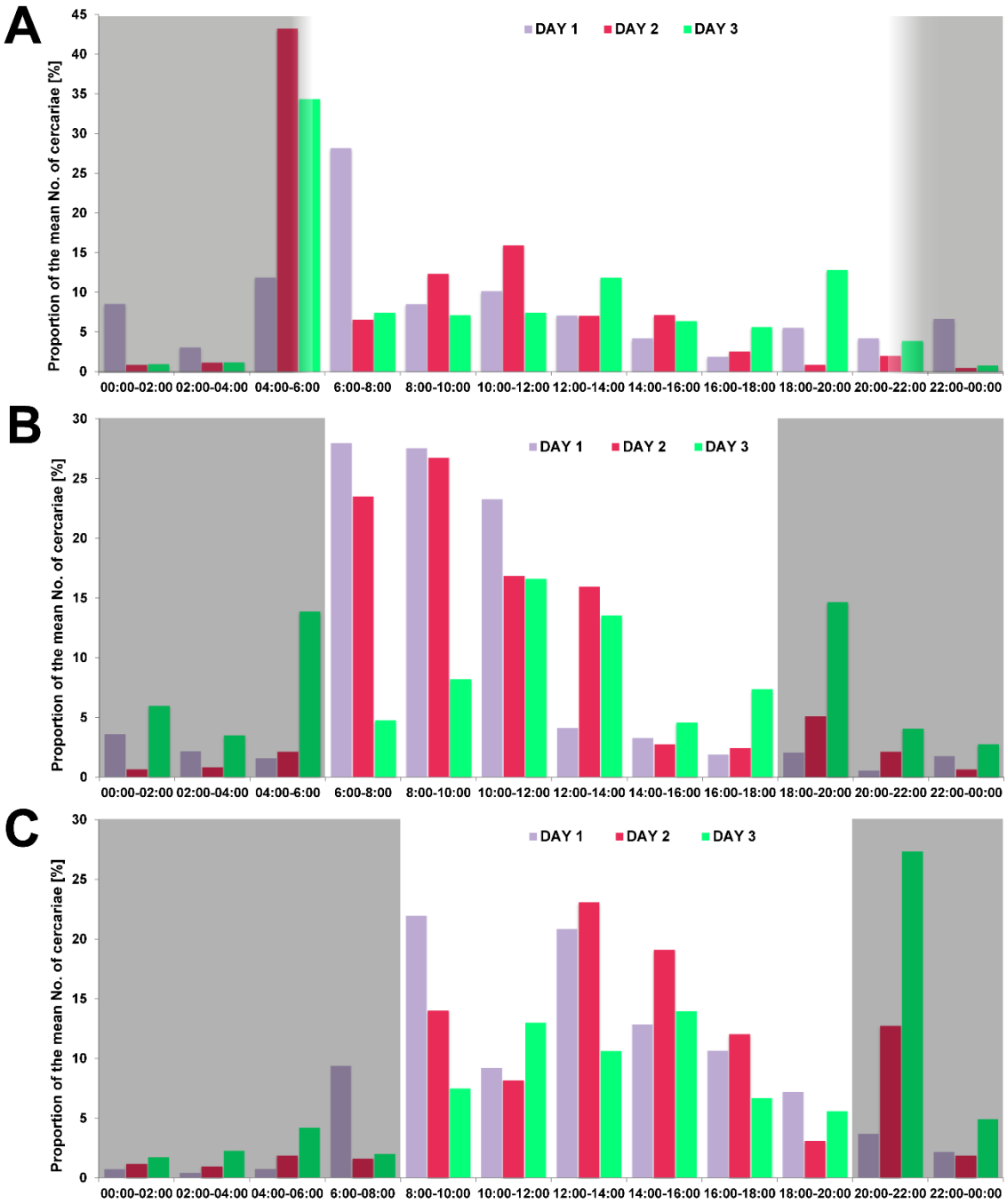

Fig 2. Proportions of emerged cercariae of *Trichobilharzia szidati* from *Lymnaea stagnalis* during two-hour-intervals.

Plots showing the emerged cercariae for each day as averaged proportions of the total numbers emerged within 24 hours across all replicate snails. **(A)** The "peak emission experiment" (PJ-L) in July under laboratory conditions at room temperature (19.6°C-20.4°C) and natural photoperiod regime (light dark cycle of 16:8 h) with highest cercarial emission corresponding to sunrise at 5:30. **(B)** The "peak emission experiment" (PJ-C) in July under standard thermal conditions in a climate chamber (20°C) and light dark cycle of 12:12 h showing high cercarial emission after onset of illumination at 6:00. **(C)** The "peak emission experiment" (PJ-C) in September under standard thermal conditions in a climate chamber (20°C) and light dark cycle of 12:12 h showing high emission rates after the onset of illumination at 8:00 but high variability in the emergence patterns during a day.

Table 3 Numbers of cercariae of *Trichobilharzia szidati* released from *Lymnaea stagnalis* per two-hour interval.

Month	Type of experiment	Day of experiment	Time/Number (mean) of emerged cercariae per two-hour interval											
			00:00-02:00	**02:00-04:00**	**04:00-06:00**	**06:00-08:00**	**08:00-10:00**	**10:00-12:00**	**12:00-14:00**	**14:00-16:00**	**16:00-18:00**	**18:00-20:00**	**20:00-22:00**	**04:00-06:00**
July	**Peak output experiment in laboratory (PJ-L; n=11)**	Day 1	950 (86)	390 (35)	1,430 (130)	3,750 (341)	1,230 (112)	1,670 (152)	870 (79)	500 (45)	210 (19)	780 (71)	450 (41)	1,430 (130)
		Day 2	120 (11)	180 (16)	7,070 (643)	1,040 (95)	1,860 (169)	2,400 (218)	1,140 (104)	1,050 (95)	420 (38)	150 (14)	320 (29)	7,070 (643)
		Day 3	160 (15)	220 (20)	5,840 (530)	1,330 (121)	1,340 (122)	1,320 (120)	1,880 (171)	1,140 (104)	1,030 (94)	2,690 (245)	720 (65)	5,840 (530)
		Total	**1,230 (112)**	**790 (72)**	**14,340 (1,304)**	**6,120 (556)**	**4,430 (403)**	**5,390 (490)**	**3,890 (354)**	**2,690 (245)**	**1,660 (151)**	**3,620 (329)**	**1,490 (136)**	**14,340 (1,304)**
	Peak output experiment in climate chamber (PJ-C; n=11)	Day 1	500 (50)	400 (40)	220 (22)	5,120 (512)	5,830 (583)	4,200 (420)	760 (76)	540 (54)	240 (24)	290 (29)	90 (9)	220 (22)
		Day 2	50 (5)	50 (5)	160 (16)	1,400 (140)	2,220 (222)	1,640 (164)	1,130 (113)	310 (31)	250 (25)	370 (37)	140 (14)	160 (16)
		Day 3	460 (46)	290 (29)	1,080 (180)	350 (35)	660 (66)	1,650 (165)	1,570 (157)	430 (43)	460 (46)	1,390 (139)	270 (27)	1,080 (180)
		Total	**1,010 (92)**	**740 (67)**	**1,460 (133)**	**6,870 (625)**	**8,710 (792)**	**7,490 (681)**	**3,460 (315)**	**1,280 (116)**	**950 (86)**	**2,050 (186)**	**500 (70)**	**1,460 (133)**
September	**Peak output experiment in climate chamber (PS-C; n=9)**	Day 1	810 (90)	530 (59)	880 (98)	12,180 (1,353)	28,060 (3,118)	14,020 (1,558)	25,470 (2,830)	15,260 (1,696)	11,110 (1,234)	7,510 (834)	4,230 (470)	880 (98)
		Day 2	1,080 (120)	950 (106)	1,140 (127)	1,100 (122)	16,600 (1,844)	9,400 (1,044)	22,810 (2,534)	17,650 (1,961)	12,810 (1,423)	3,160 (351)	14,800 (1,644)	1,140 (127)
		Day 3	880 (98)	830 (92)	1,780 (198)	570 (63)	3,430 (381)	5,420 (602)	5,300 (589)	6,670 (741)	3,380 (376)	3,060 (340)	14,090 (1,566)	1,780 (198)
		Total	**2,770 (308)**	**2,310 (257)**	**3,800 (422)**	**13,850 (1,593)**	**48,090 (5,343)**	**28,540 (3,204)**	**53,580 (5,953)**	**39,580 (4,398)**	**27,300 (3,033)**	**13,730 (1,526)**	**33,120 (3,680)**	**3,800 (422)**

Total and mean (in parenthesis) numbers of cercariae released by a given number of snails used in each experiment (indicated by "n" in parentheses) per day for each two-hour interval. In September only one experiment was performed.

Following Spearman's rank correlation analysis carried out separately for each experiment, no significant correlation between cercarial output and snail shell height (ln-transformed; mean of 42.2 mm for snails entering experiments in July and 47.5 mm for snails in September) was detected (all $p>0.05$). A statistical assessment of cercarial emission rates between experiments revealed significant differences between the four experiments and in relation to day of experiment, which were performed in July and one experiment in September with distinctively higher cercarial emission rates in September reaching 29,560 cercariae per snail per a day (REP-ANOVA $F_{(8, 64)}=7.86$, $p<10^{-4}$) (Tables 1 and 2, Fig. 3). There was a striking nine-fold increase in cercarial emission of *T. szidati* in September. Moreover, within experiments carried out in July, cercarial numbers significantly differed between DJ-C and the two "peak output experiments" ($p=0.001$ for PJ-L vs DJ-C; and $p=0.02$ for PJ-C vs DJ-C), probably due to the low numbers of replicate snails (Tables 1 and 2, Fig. 3). Furthermore, we found significant differences in cercarial emergence in different time intervals and between days of experiment when comparing cercarial output between all three "peak emission output" experiments (PJ-L, PJ-C and PS-C). While different light and laboratory conditions of the two "peak" experiments in July (PJ-L and PJ-C) did not affect the overall cercarial emission rates (Fig. 1), significant differences were detected in relation to two-hours intervals between PJ-L and PJ-C (REP-ANOVA $F_{112, 209)}=4.16$, $p<10^{-4}$) and also between all three "peak experiments" (REP-ANOVA $F_{(22, 297)}=11.31$, $p<10^{-4}$) (Table 3). However, no effect of day of experiment was detected between PJ-L and PJ-C in July (REP-ANOVA $F_{(2, 38)}=1.08$, $p=0.35$), indicating that cercarial emergence followed similar patterns between days in both experiments. When comparing all three "peak experiments", emission significantly differed among days due to the distinctly higher rates and longer release throughout the day in September (PS-C) (REP-ANOVA $F_{(4, 54)}=5.01$, $p=0.002$).

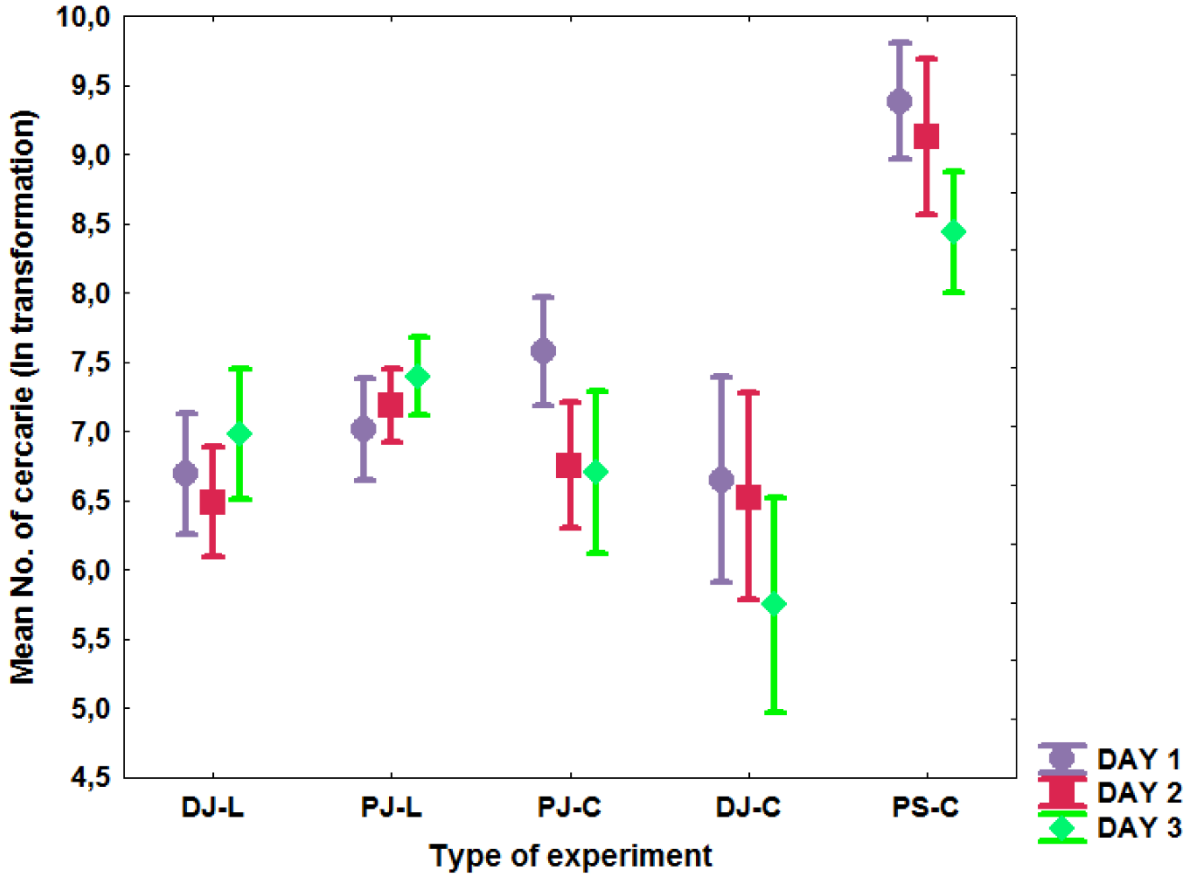

Fig 3. Mean number of cercariae of *Trichobilharzia szidati* (ln-transformed) from *Lymnaea stagnalis* in five emission experiments.
Statistical comparison using Repeated Measures ANOVA showing significant differences in cercarial emission rates between four experiments performed in July (DJ-L, PJ-L, PJ-C, DJ-C) and one experiment in September (PS-C), with significantly higher cercarial emission rates in September reaching up to 29,560 cercariae per snail and day (mean 10,140 $snail^{-1}day^{-1}$). Cercarial emission followed similar pattern among days of experiments. Vertical bars denote 0.95 confidence intervals.

Discussion

The present experimental study is the first providing comprehensive insights into the total production and patterns in cercarial emergence of the most common causative agent of swimmer's itch in Europe, bird schistosomes of the genus *Trichobilharzia*, and allows a better understanding of the parasites' ecological relevance and epidemiological consequences. Although it is often noted that *Trichobilharzia* cercariae are produced in high quantities (e.g. Horák et al. 2002), only few studies concern the total or average numbers of cercariae released from individual snail hosts. However, these data are rather ambiguous and often not easily comparable. In the first description of *T. szidati*, Neuhaus (Neuhaus 1952) estimated the number of *T. szidati* cercariae released from infected *L. stagnalis* over a period of 16 to 19 days to be 8,000 to 10,000, whereas Żbikowska et al. (2001) and Sluiters (1980) describe much

higher productions of *T. ocellata* (syn. *T. szidati*; see Rudolfová et al. 2005) cercariae from *L. stagnalis*, ranging from 7,500 to 10,700 per week. The most accurate descriptions from experiments on the emergence pattern of *T. ocellata* cercariae from *L. stagnalis* show a variation of 0 – 3,069 cercariae released during a 36-hour period and identify strong peak activities in the first hours after light exposure (Anderson et al. 1976).

Our results reveal a high variability in cercarial productivity of *T. szidati* between individual replicates, days, two-hour intervals, type of experiment and between the different months of the experiments. The mean daily emergence rate of 1,117 cercariae snail^{-1} day^{-1} recorded in the experiments in July falls into the lower range of the weekly emission numbers described by Sluiters (1980) and Żbikowska (2001). However, the cercarial productivity of individual snails was highly variable and ranged from 40 to 4,560 cercariae, which matches the observations of Anderson et al. (1976). However, the extremely high emission rates in September (mean 10,140 snail^{-1} day^{-1}, maximum of 29,560) have not been reported before and allow more precise estimates of the parasites' contribution to ecosystem biomass, and better assessments of swimmer's itch infection risks.

Temperature is a major factor influencing the development of trematodes in their snail host and the production of cercariae (e.g. Morley & Lewis 2013, Studer & Poulin 2013), but room temperature was highly similar across all experiments (20°C in the climate chamber and 19.6 – 20.4°C in the air-conditioned laboratory) and does not play a role in our results. The main difference between the experiments in the climate chamber in July and September was the shift in the light regime from 6:00 – 18:00 (July) to 8:00 – 20:00 (September). However, this does not account for the higher emission in the latter experiment, since Anderson et al. (1976) found no differences in emergence patterns, even if the lightning regime was reversed.

Since experimental setup and all other conditions in the climate chamber (handling, length of illumination, water and air temperature) were the same in the peak experiments, the nine-fold increase in cercarial emission of *T. szidati* in September can probably be best explained by intra-molluscan development cycles of sporocyst microhemipopulations in which sporocysts producing cercariae alternate with sporocysts producing daughter sprocysts which have been described to take 35-40 days in schistosomes (Théron 1981a, b). Sluiters et al. (1980) describe two peaks in cercarial production of *T. ocellata* ca. 40 days apart. In our case there were 64 days between experiments in July and September and it is possible that we hit a production peak during that time after the newly formed sporocysts matured. Since all snails were sampled at the same small water body at the same time and are of similar size/age, it is likely that they were infected at about the same time, explaining the similar pattern across all snails.

Furthermore, five snails in the September experiment showed patent double infections with other trematode species (four snails with *Diplostomum pseudospathaceum* and one snail with

Plagiorchis elegans), both are being frequently found in *Lymnaea stagnalis* (Selbach et al. 2015). Although some of these snails showed lower *T. szidati* emissions (see Table 1), there was no significant difference in overall productivity or emergence patterns. Double infections, and the subsequent elimination of less dominant or subordinate trematode species as a result of interspecific competition, may be common in natural waters, especially in small eutrophic ponds with high trematode prevalence and colonization rates (Soldánová et al. 2011b, 2012). The occurrence of double infections should, therefore, make the results more comparable to natural conditions and no snails with double infections were excluded. Variations in cercarial production among snails can also be due to a number of reasons, ranging from slight differences in the intra-molluscan development cycles described above to individual snail metabolism (see Poulin & George-Nascimento 2007, Hechinger 2013) or the initial miracidial dose (Sluiters et al. 1980).

Parasites are usually much smaller than their hosts. While this is certainly true at the level of the individual, the combined biomass of parasites contributes significantly to the total biomass in an ecosystem (2008). Only few studies quantified trematode biomass in an attempt to assess the potential function and contribution of free-living stages of parasites to the energy flow in ecosystems. Kuris et al. (Kuris et al. 2008) estimated that the annual production of trematode cercariae in the Carpinteria Salt Marsh represented biomass greater than that of other parasites and even of birds, the top predators in this system. They concluded that the presence and abundance of a certain type of organism in a given territory may be dependent on the activity of trematodes, thus stressing their importance in structuring aquatic food webs and energy transfer. Thieltges et al. (2008b) used published data on the cercarial output rates in marine systems and calculated an annual production of cercariae comparable to the biomass estimated for free-living benthic marine invertebrates. Most recently, in freshwater pond ecosystems in California, the trematode productivity and cumulative biomass, which was equal or exceeded the biomass of the most abundant insect groups, was comparable to those estimated for marine and estuarine systems (Preston et al. 2013).

Based on the information from our cercarial emission experiments, we can make sound estimations of the biomass productivity of *T. szidati* cercariae at two levels, i) the cercarial productivity of an individual snail during its lifetime, and ii) the annual contribution of *T. szidati* cercariae to an ecosystem's biomass. The cercarial production of 4.8 g of a single snail during its lifetime underlines the ecological importance of these trematodes at the host-parasite level. By relocating the snail's reproductive resources, and thereby castrating the host (Brant & Loker 2013), the parasites are able to transfer a substantial amount of biomass into cercarial production. Besides being "hands inside of a puppet" that take up a large amount of the host's soft tissue mass (on average around 20%; Hechinger et al. 2009), trematodes are able to produce a substantial amount of biomass outside the host that equals or even exceeds the weight of the soft tissue mass of the snail host during its life span.

On an ecosystem level, our results show that a single trematode species contributes a considerable amount of cercarial mass to an aquatic ecosystem during the parasite's active period in the summer months of a year. Using a conservative estimation of a *T. szidai* prevalence of 5%, we calculated an annual biomass contribution of 561 kg into the small fishpond. However, since prevalence of *Trichobilharzia* sp. in this pond of more than 40% have been reported (Soldánová et al. 2011a), we end up with a possible annual cercarial production of 4.6 tons. This would equal the weight of a large Asian elephant, an illustrative figure and comparison already used to describe parasite contributions to the biomass in estuarine systems (Hudson 2005). While both the elephant and the reproduction of the snail's individual weight are certainly impressive, we have taken care to follow a rather conservative approach in our calculations. *L. stagnalis* can live up to three years (Finch & Roth 1999) and may thus produce even more cercariae than estimated for the two-year lifetime we assumed. Furthermore, the body volume we calculated for *T. szidati* based on our measurements of live cercariae is considerably smaller than the one calculated for *T. szidati* by Koehler et al. (Koehler et al. 2012) based on literature data (0,0039 mm^3 vs. 0,0068 mm^3, respectively), resulting in possibly higher actual contribution of *T. szidati* to an ecosystem's biomass than our calculations. Moreover, the mean number of cercariae (2,621 $snail^{-1}day^{-1}$) across all our experiments is low compared to the possible peaks of 29,560 cercariae per snail and day detected in September. If such peaks occur frequently, the total number of cercariae and their biomass contribution may turn out higher still.

In Central European freshwater ecosystem bird schistosomes, such as *T. szidati*, only constitute a fraction of the diverse trematode communities which comprise a multitude of species (e.g. Soldánová et al. 2010, 2011b, Loy & Haas 2001), all of which contribute to the ecosystem's biomass. Depending on the transmission strategies of the parasites, daily cercarial emission can be significantly higher than in bird schistosomes, e.g. in *Diplostomum* spp. with productions of up to 60,000 cercariae $snail^{-1}day^{-1}$ (Lyholt & Buchmann 1996, Karvonen et al. 2004), and well beyond up to 500,000 cercariae per snail and day for some species (Haas 2003). It is therefore safe to assume that the overall cercarial biomass emitted into these systems is comparable to the impressive numbers recently calculated for marine (Thieltges et al. 2008b), estuary (Kuris et al. 2008) and freshwater ecosystems (Preston et al. 2013). Since the majority of produced cercariae are not able to successfully infect a suitable target host and end up as food for predators (Johnson et al. 2010, Morley 2012) or contribute to the ecosystem's detritus, the free living parasite stages, along with the life cycle stages within the hosts, contribute significantly to the energy flow in aquatic systems (Combes et al 1994, Thieltges et al. 2013).

Emergence of *Trichobilharzia* cercariae has been shown to occur in the first hours of light exposure (Anderson et al. 1976) and was determined to peak early in the morning hours between 9 and 11am (Sluiters et al. 1980). Our results clearly confirm the strong peak activities in cercarial emission after the onset of light exposure described by Anderson et al. (1976), both in controlled

environments and under natural conditions with gradually increasing daylight. In all experiments, high emission rates were observed immediately at the beginning of the light phase, following a low-emission dark regime. These patterns suggest a synchronization of cercarial emission with the daily activity patterns of the definitive hosts, ducks that also show strong diurnal patterns with the highest activities around sunrise and sunset (Sauter et al. 2011). Such a synchronization of cercarial release with host-time can be explained as an adaptive behavior enhancing the probability of transmission success of the short-lived cercariae to their hosts (Combes et al 1994). This is also a common feature with mammalian schistosomes in which cercarial emergence varies on a circadian cycle and is associated with definitive host availability (Théron et al. 1997, Pagess & Théron 1990, N'goran et al. 1997).

The morning emission of cercariae was most prominent in the peak experiment conducted in the laboratory with natural lightning conditions (Figs. 1A and 2A). The experiments conducted in the climate chamber showed longer windows of continuous emission during the light period (Figs. 1B-C, 2B-C). This may be due to the exposure to a direct light source (halogen lamp) in the climate chambers in contrast to the changing intensity of illumination in the laboratory throughout the day. Since the windows in the laboratory were facing north, snails were mostly exposed to indirect sunlight, whereas the climate chamber experiments simulate conditions similar to an unshaded area during a summer day, possibly explaining the longer emission patterns. Furthermore, we observed small emission peaks in the climate chamber experiments during the dark phases (see Figs. 1B-C, 2B-C). It is possible that snails were accidentally exposed to indirect light when the door to the climate chamber was briefly opened during the handing of the snails. This would suggest that only very brief light impulses are sufficient to trigger cercarial emission and show a high flexibility of the parasites to react to environmental changes, e.g. short sunny phases in cloudy weather.

Besides the biomass contribution of *T. szidati* and ecological importance of these parasites, e.g. in food webs, our results may help to give better estimations of the risks of swimmer's itch that can be caused by bird schistosomes. The exceptionally high emission rates of cercariae in the September experiment highlight why infections in humans are typical, even in regions where prevalence of bird schistosomes is very low. A single infected *L. stagnalis* snail appears to be enough to create a potential 'infection hot spot' of swimmer's itch, e.g. if about 29,500 cercariae are released at a shallow area frequented by many swimmers during a day.

Conclusions

The results of our study show the large cumulative biomass of *T. szidati* cercariae both on the individual host and the ecosystem level. We can confirm strong peak activities in cercarial emission as a result of illumination but were able to show that emission patterns can be flexible and large quantities of cercariae can be released throughout the course of a day, if the snails are exposed to light. Therefore, while the early worm catches the bird, it tries to do so many times. Although bird schistosomes constitute only a fraction of the diverse trematode communities in the studied aquatic ecosystems, their infective stages, the cercariae, can still pose a considerable risk of swimmer's itch due to the high number of cercariae emitted from infected snails.

Acknowledgments

We are grateful to Jana Köchling, Verena Altmann and Jessica Schwelm (University of Duisburg-Essen) for their tireless help during performing experiments. We also thank Aneta Kostadinova (Biology Centre of the Czech Academy of Sciences) for supplying the *Lymnaea stagnalis* snails used in this work, suggestions and discussion of results, Simon Kresmann (University of Duisburg-Essen) for spontaneous night work and Andrea Bednářová (Biology Centre of the Czech Academy of Sciences) for her assistance with dissections of snails.

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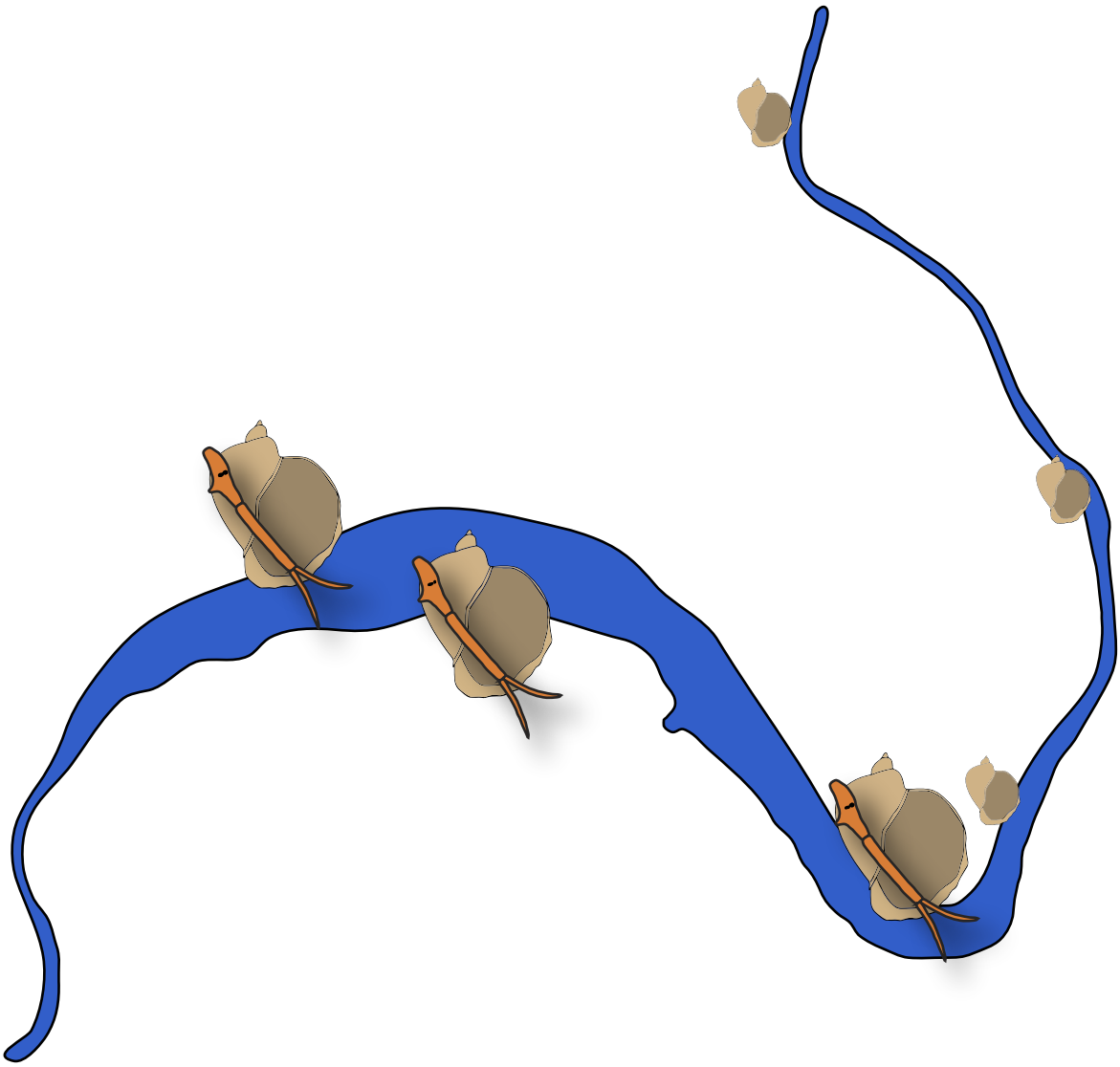

7. Chapter III

Trematodes and public health – bird schistosomes and swimmer's itch

7. Chapter III

Trematodes and public health – bird schistosomes and swimmer’s itch

This chapter contains the following study on the occurrence of the main causative agents of simmer’s itch, *Trichobilharzia* spp., in Lake Baldeney (Baldeneysee) and estimations of risk factors based on parasite and host biology and the given local situations.

7.1 Selbach, C., Soldánová, M. & Sures, B. (2015). Estimating the risk of swimmer's itch in surface waters - a case study from Lake Baldeney, River Ruhr. *International Journal of Hygiene and Environmental Health*, (In Press).

Contents lists available at ScienceDirect

International Journal of Hygiene and Environmental Health

journal homepage: www.elsevier.com/locate/ijheh

Estimating the risk of swimmer's itch in surface waters – A case study from Lake Baldeney, River Ruhr

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ARTICLE INFO

Article history:
Received 10 January 2015
Received in revised form 20 March 2015
Accepted 21 March 2015

Keywords:
Parasite
Trichobilharzia
Swimmer's itch
Cercarial dermatitis
Risk factors
Prevention

ABSTRACT

Swimmer's itch is a zoonotic disease caused by certain digenean trematodes, in Europe most noticeably by bird schistosomes of the genus *Trichobilharzia*. These parasites require waterfowl and aquatic snails as final and intermediate hosts, respectively, to complete their life cycle. Swimmer's itch occurs when the free-swimming larvae emitted from snails, the cercariae, accidentally infect humans. Here the parasites cannot complete their life cycle but can cause allergic inflammatory responses of the skin. In the context of the joint BMBF project 'Sichere Ruhr' (Safe Ruhr), which evaluates the Ruhr River as a potential bathing water, the occurrence of the causative agents of swimmer's itch in Lake Baldeney was studied. A total of 1741 snails was examined for the presence of trematode infections, including bird schistosomes. Snails infected with *Trichobilharzia* spp. were found at three sampling locations but showed low overall prevalences (0.6–3.0%). Based on parasite and host biology, risk factors were evaluated and discussed in the context of the potential use of Lake Baldeney as a bathing water. Although bird schistosomes only constitute a fraction of the trematode diversity occurring in natural snail populations and show low prevalence, they still pose an infection risk due to the high emission rates of cercariae from individual snail hosts. A wide variety of often interacting biotic and abiotic factors, as well as personal behaviour have an effect on the likelihood and severity of a human infection. Based on these risk factors, a number of possible preventive actions aiming at the disruption of the life cycle, or personal protective measures can be suggested. While absolute protection is impossible (unless swimming in natural waters is altogether avoided) some preventive measures can reduce the risk of human infections.

Introduction

Swimmer's itch or cercarial dermatitis is a water-borne parasitic disease that results from repeated infections with the free-swimming larval stages of schistosome trematodes which cause allergic inflammatory responses of the skin (Kolářová et al., 2010), an observation first made by Cort (1928) in Lake Michigan, USA. Swimmer's itch is considered a re-emerging disease that occurs world-wide and the spread of bird schistosomes and reported cases in Europe (Soldánová et al., 2013) highlight the wide geographical distribution of these parasites.

Cercarial dermatitis can be caused by a number of schistosomatid flukes, including human schistosomes (Kolářová et al., 2013). The most important agents of swimmer's itch however are bird schistosomes, in Europe most noticeably species of the genus *Trichobilharzia* that utilise lymnaeid snails (freshwater pulmonate molluscs) as intermediate hosts and waterfowl as definitive hosts to complete their life cycle (Fig. 1; for a full review see Horák et al., 2002 and references therein). Waterfowl, mostly ducks, serve as definitive hosts in which the adult parasites mate and produce eggs that are (i) either released into the water via the host's faeces, where a free-swimming larvae, the miracidium, hatches from each egg (visceral schistosomes), or (ii) placed in the host's nasal cavity, where the miracidium hatches and leaves upon contact with water (*Trichobilharzia regenti*, a nasal schistosome). In the water the miracidia actively seek out and infect suitable snail intermediate host species in which asexual reproduction via mother and daughter sporocysts takes place and free-swimming larvae, the cercariae (Fig. 2A), are produced. Cercariae are released and dispersed into the aquatic environment in large quantities and orientate themselves towards the light and the water surface (Fig. 2B)

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http://dx.doi.org/10.1016/j.ijheh.2015.03.012
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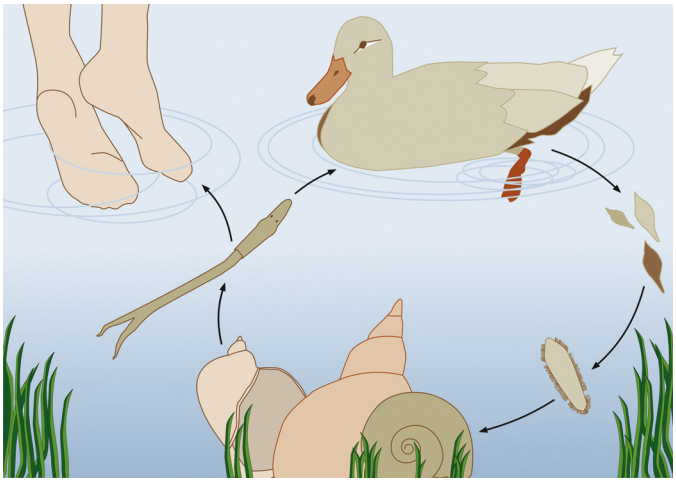

Fig. 1. *Trichobilharzia* spp. life cycle (from Soldánová et al., 2013, with permission).

to increase chances of host contact (Hertel et al., 2006). In the water, the short-lived cercariae (1–3 days; Neuhaus, 1952) show complex swimming behaviours and are sensitive to a range of chemical and tactile stimuli that allow the location and penetration into a potential definitive host (Haas, 1992; Haas and Haberl, 1997). Once inside a suitable bird host, the cercaria develops into a juvenile worm, the schistosomulum, and migrates to the preferred site of infection, either via the circulatory system (visceral schistosomes) or through nervous tissue (nasal schistosomes), where it matures, mates and reproduces.

Swimmer's itch occurs when the cercariae accidentally penetrate into humans. Here the parasites cannot complete their life cycle but become entrapped in the skin and die soon after penetration. While primary infections of accidental hosts usually show no or only mild reactions, repeated contact with the parasites lead to host sensitisation and immune responses that result in allergic inflammatory reactions of the skin (Fig. 2C; Kouřilová et al., 2004; Horák and Kolářová, 2005). Although unpleasantly itching, infections are usually harmless and recede after a few days, but can be more serious in repeatedly sensitised individuals (including symptoms such as oedema and fever; Horák and Kolářová, 2001). Although the disease technically represents an infestation, since the parasites cannot complete their life cycle and do not multiply in the human accidental host, the term 'infection' is more commonly used with reference to cases of swimmer's itch. Data from experimentally infected animal models indicate that bird schistosome cercariae may escape immune response and entrapment in the skin in unsensitised mammals and migrate within the body, where they can cause haemorrhages or neurological disorders (Horák and Kolářová, 2001). It remains unclear however, whether these symptoms can occur in humans and no such cases have been reported yet (Horák et al., 2015).

Considered a benign skin disease (Caumes et al., 2003), infections with bird schistosomes and swimmer's itch are not part of the European Union's directive concerning the management of bathing water quality (Directive 2006/7/EC, European Union, 2006) and thus not legally binding for the monitoring and classification of bathing water quality. They are of considerable public interest however, as news reports following individual cases of infection regularly show. Furthermore, parasites carry a negative connotation, and the idea of parasites burrowing into one's skin whilst going for a swim usually provokes a rather strong emotional reaction to the point that many bathers are willing to reduce or stop their bathing activities (Chamot et al., 1998). Whilst a much greater danger (one of the six leading causes of deaths for people under 24 in Europe, WHO, 2014), the risk of drowning seems to evoke a much milder reaction and appears to be much more 'acceptable'. Consequently, repeated cases or occasional but rare mass outbreaks of swimmer's itch (e.g. Allgöwer, 1995) can be of considerable economic importance, especially in regions with tourism based on recreational water activities. In this context, epidemiological studies, risk assessments or preventive measures have been undertaken

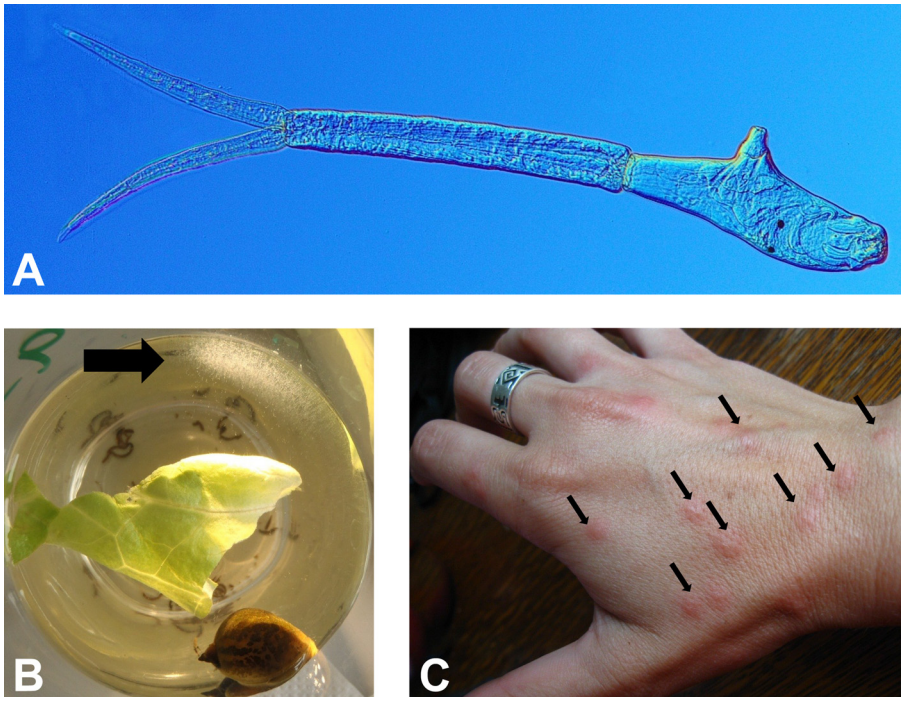

Fig. 2. (A) *Trichobilharzia franki* cercaria ex *Radix auricularia*. (B) *T. franki* cercariae released from infected *R. auricularia* orientating towards light source (indicated by arrow). (C) Case of swimmer's itch in one of the authors after collecting snails in one of the Ruhr reservoirs (inflammatory skin reaction around points of penetration by cercariae, indicated by arrows).

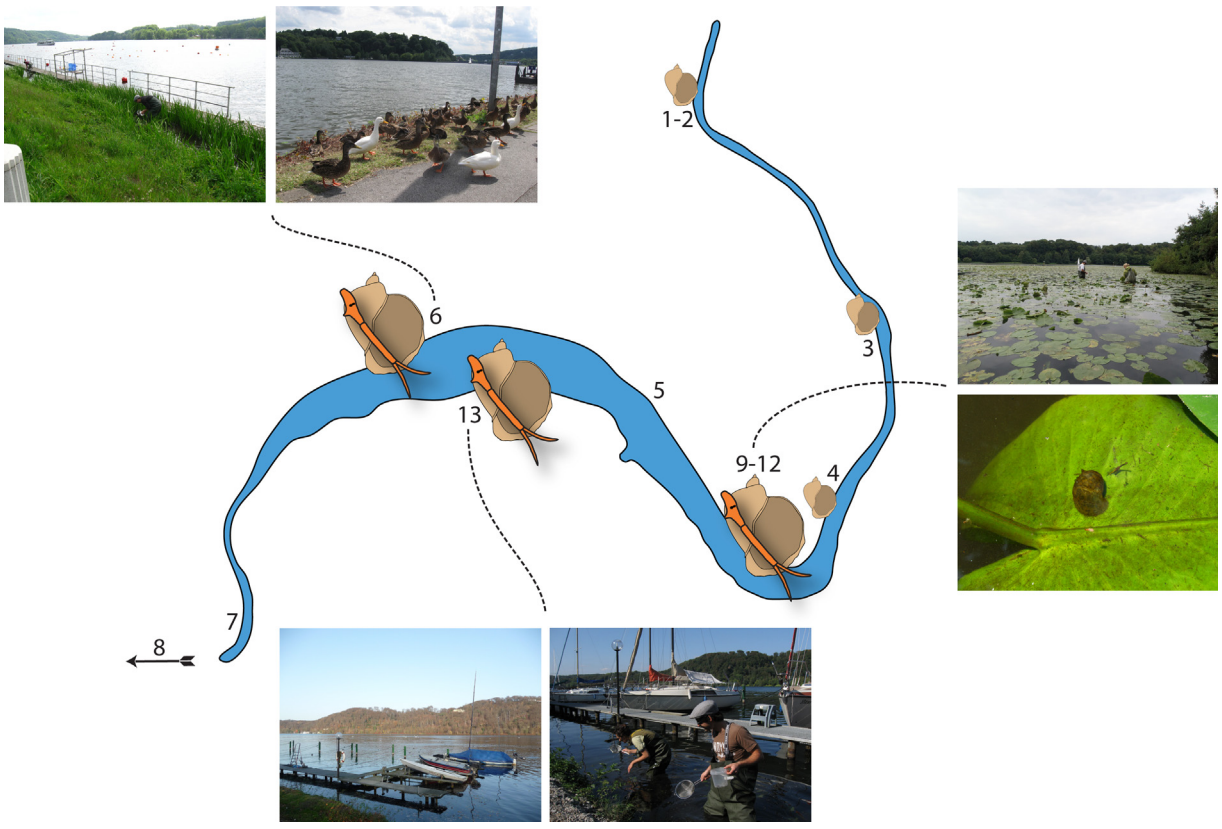

Fig. 3. Map of Lake Baldeney indicating the occurrence and size of lymnaeid snail populations and the findings of *Trichobilharzia* spp. in 2009 and 2012. Individual sampling sites numbered, site 8 located further downstream. Large snail symbols indicate lymnaeid populations; small snail symbols indicate scattered occurrence of individual lymnaeid snails; cercaria symbols indicate *Trichobilharzia* spp. findings.

in various lakes with histories of swimmer's itch outbreaks, e.g. in Germany (Lake Constance, Fiedler et al., 2005), France (Lake Annecy, Caumes et al., 2003), Switzerland (Lake Geneva, Chamot et al., 1998), Canada (Cultus Lake, Leighton et al., 2000) or the USA (Lake Michigan, Blankespoor and Reimink, 1991; Douglas Lake, Verbrugge et al., 2004).

In the context of the joint BMBF project 'Sichere Ruhr', which evaluates the Ruhr River as a potential bathing water, the occurrence of the causative agents of swimmer's itch in Lake Baldeney (Baldeneysee) was studied in order to estimate risk factors based on the parasites' and hosts' biology and the local situations. Individual cases of swimmer's itch have been reported from the Ruhr river system (AWWR and Ruhrverband, 2008), indicating the presence of bird schistosomes.

Materials and methods

In order to assess the occurrence of bird schistosomes and to estimate the infection risk of swimmer's itch in Lake Baldeney (51°24′20.08″N, 7°2′22.47″E), freshwater snails were sampled at various localities along the lake's shoreline (Fig. 3). In addition to the sampling sites of the Safe Ruhr project (sites 1–8), four sites in a bird reserve were selected (*Heisinger Bogen*, sites 9–12). Every site was sampled six times during the months May to September 2012. Snails were collected by hand from floating vegetation and/or stones along the shoreline of the lake. All snails were taken to the laboratory, placed in individual cups with lake water and exposed to a light source for two to five days to induce cercarial shedding. Snails that did not emit any cercariae during that time were dissected and checked for prepatent infections. Live trematodes were photographed with a digital camera through an Olympus BX51 light microscope and identified based on the keys by Faltýnková et al. (2007, 2008). Molecular identification of bird schistosomes was carried out for specimens found in 2012 (based on sequences of the internal transcribed spacer (ITS) regions of rDNA; see Dvořák et al., 2002), since cercariae of one schistosome species can be found in closely related intermediate hosts (Kock, 2001; Picard and Jousson, 2001), and vice versa, a single snail species may serve as a host for more than one schistosomatid (Rudolfová et al., 2005; Jouet et al., 2008). Furthermore, caution is required due to the complicated identification of *Radix* spp. and the presence of *Trichobilharzia* species complexes that require further taxonomic investigation (see Jouet et al., 2010).

Although all occurring snail species were checked for trematode infections, the focus of the study was on the lymnaeid snail species *Lymnaea stagnalis* and *Radix auricularia* present in Lake Baldeney. Both are known to serve as intermediate hosts for two of the most important agents of swimmer's itch in Europe, *T. szidati* and *T. franki*, respectively. A further species, *Trichobilharzia regenti*, utilises *Radix peregra*, *R. lagotis* and *R. labiata* (Huňová et al., 2012), but these hosts were not found in Lake Baldeney. Altogether, 1437 snails were collected in Lake Baldeney in 2012. Besides *L. stagnalis* and *R. auricularia*, 14 additional snail species were collected and examined for trematode infections. Additionally, 135 *L. stagnalis* and 169 *R. auricularia* from a sampling campaign during the summer months of 2009 (published in Soldánová et al., 2010) were added to our data, including an additional sampling site on the south bank of the reservoir (site 13, Fig. 3). Table 1 provides an overview of all collected snails by year. This combined dataset of 1741 snails provides a comprehensive insight into the lake's trematode fauna and shall allow estimating the relative importance of bird schistosomes among the trematode community.

Table 1
Overview of snail species sampled in 2009 and 2012, overall trematode prevalence and *Trichobilharzia* spp. prevalence (i.e. *Trichobilharzia szidati* ex *Lymnaea stagnalis* and *T. franki* ex *Radix auricularia*).

	Snail species	2009[a]			2012		
		Number of examined snails	Overall prevalence (%)	*Trichobilharzia* spp. prevalence (%)	Number of examined snails	Overall prevalence (%)	*Trichobilharzia* spp. prevalence (%)
1	*Acroloxus lacustris*				81	0	
2	*Ancylus fluviatilis*				157	2.6	
3	*Anisus vortex*				165	0.6	
4	*Bathyomphalus contortus*				176	2.3	
5	*Bithynia tentaculata*				131	16.8	
6	*Gyraulus albus*				5	0	
7	***Lymnaea stagnalis***	**135**	**14.1**	**2.96**	**28**	**17.9**	
8	*Physa fontinalis*				247	0.4	
9	*Physella acuta*				1	0	
10	*Planorbarius corneus*				119	1.7	
11	*Planorbis planorbis*				3	0	
12	*Potamopyrgus antipodarum*				2	0	
13	***Radix auricularia***	**169**	**18.3**	**0.59**	**275**	**16.7**	**0.73**
14	*Segmentina nitida*				15	0	
15	*Sphaerium* sp.				3	(33.3)[b]	
16	*Stagnicola palustris*				29	10.3	
	Total	304			1,437		

[a] Data from Soldánová et al. (2010).
[b] Sample size small.

Results

A total of 10 snail species out of 16 was found to be infected with trematode species. Trematode prevalences in the individual snail species from Lake Baldeney ranged from 0.4% in *Physa fontinalis* (2012) to 18.3% in *R. auricularia* (2009). Table 1 shows overall prevalences in all snail species collected in 2009 and 2012. However, due to the low number of specimens of some snail species (e.g. only five *Gyraulus albus* were found), trematode infections in these species may have evaded detection. A total of 17 trematodes was identified, 14 to species and three to genus level. Additionally, five trematode infections were found but not identified further than the family level, mostly from *Bithynia tentaculata* (aquatic prosobranch mollusc) for which no sufficient trematode keys exist. Since these comprised no bird schistosomes, no further molecular identification was carried out in these cases. Table 2 gives an overview of the trematode species found in Lake Baldeney. In both snail species that serve as potential hosts for *Trichobilharzia* spp. overall trematode prevalence was high in both years: *R. auricularia* 18.3% (2009) and 16.7% (2012); *L. stagnalis* 14.1% (2009) and 17.9% (2012).

Trichobilharzia spp. infections could be found at different sites in Lake Baldeney in 2009 and 2012. *Trichobilharzia franki* ex *R. auricularia* was found twice in May and September 2012 at a sampling site in the bird reserve *Heisinger Bogen* (site 11). In 2009 *T. franki* was found at a location of the reservoir's south bank (site 13). *Trichobilharzia szidati* ex *L. stagnalis* was discovered at one locality in 2009 (site 6), but could not be detected in the summer months of 2012, despite the presence of suitable lymnaeid snail populations at the site. In Fig. 3, all *Trichobilharzia* findings and the occurrence of lymnaeid snail populations at the individual sampling sites are highlighted. Prevalences of *Trichobilharzia* spp. in the pooled samples ranged from 0.59% (2009) and 0.73% (2012) in *R. auricularia* to 2.96% (2009) in *L. stagnalis* (Table 1).

Table 2
Overview of trematode species and their snail hosts found in Lake Baldeney in 2009 (Soldánová et al., 2010) and 2012.

	Trematode species	Snail host[a]
1	*Opisthioglyphe ranae*	RA, SP
2	*Plagiorchis elegans*	LS
3	*Echinoparyphium recurvatum*	RA, LS
4	*Echinostoma revolutum*	LS
5	*Echinostoma* sp. IG	RA
6	*Paryphostomum radiatum*	RA
7	*Isthmiophora melis*	AF
8	***Trichobilharzia franki***	**RA**
9	***Trichobilharzia szidati***	**LS**
10	*Tylodelphys clavata*	RA, LS, SP
11	*Notocotylus attenuatus*	RA
12	*Australapatemon burti*	AV, BC
13	*Diplostomum pseudospathaceum*	LS, SP
14	*Diplostomum spathaceum*	RA
15	*Notocotylus* sp.	BT
16	*Cotylurus* sp.	PC, BC
17	*Cyclocoelium* sp.	RA, SP
18	Echinostomatid rediae	BT, AF
19	Family Gorgoderidae	Ssp
20	Family Psilostomatidae	BT
21	unidentified rediae	BT
22	unidentified xiphidiocercariae	PF, BT

[a] Abbreviations for snail hosts: AF: *Ancylus fluviatilis*; AV: *Anisus vortex*; BC: *Bathyomphalus contortus*; BT: *Bithynia tentaculata*; LS: *Lymnaea stagnalis*; PC: *Planorbarius corneus*; PF: *Physa fontinalis*; RA: *Radix auricularia*; SP: *Stagnicola palustris*; Ssp: *Sphaerium* sp.

Discussion: Risk factors, diagnosis and preventive measures

Bird schistosomes are present in Lake Baldeney and could be detected at several sampling sites in the years 2009 and 2012. The low prevalences of *Trichobilharzia* spp. of 0.6–3% are typical for bird schistosomes and are quite common in areas where swimmer's itch occurs in humans (Loy and Haas, 2001; Zbikowska, 2004). However, these low prevalences make a precise risk assessment of swimmer's itch problematic, since the detection of the parasites is difficult and requires extensive screening of snail populations; and cases of cercarial dermatitis may still occur in water bodies where no infected snail can be found (Schets et al., 2010). Furthermore, the identification of cercariae by microscopy is labour intensive and requires specific parasitological expertise, especially since the trematode fauna of freshwater ecosystems is diverse and comprises a multitude of species, of which some share similar morphological features (Fig. 4). The recent discovery of new cryptic species and yet unrecognised molecular diversity in several trematode genera in the Ruhr area further highlight the taxonomic complexity of these parasites, e.g. *Echinostoma* spp. (Georgieva et al., 2013a), *Diplostomum* spp. (Georgieva et al., 2013b) or *Petasiger* spp. (Selbach et al., 2014).

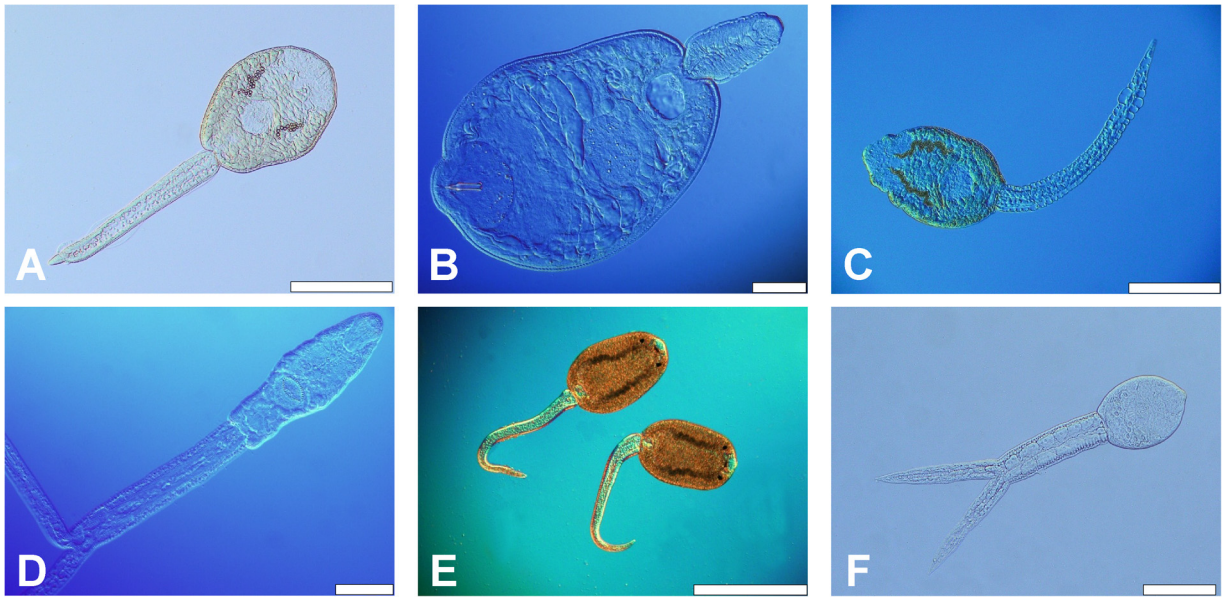

Fig. 4. Cercariae of selected trematode species found in Lake Baldeney. (A) *Echinostoma revolutum*. (B) *Plagiorchis elegans*. (C) *Paryphostomum radiatum*. (D) *Tylodelphys clavata*. (E) *Notocotylus attenuatus*. (F) *Australapatemon burti*. Scale bars: (A) and (C) 200 μm; (B) (D) and (F) 50 μm; (E) 500 μm.

Due to the difficult detection of bird schistosomes, the occurrence of suitable snail host populations and the potential dispersal of cercariae should be given main consideration to risk assessments. In the summer of 2012, constant lymnaeid snail populations in Lake Baldeney were found on floating vegetation (Nymphaeaceae) in the area of the bird reserve *Heisinger Bogen* at sites 9 and 11 (see Fig. 3), as well as on littoral aquatic vegetation at sampling site 6, a former and potential future bathing site (*Seaside Beach*). At sampling sites 1–3 only individual lymnaeid snails were found. These sites are further upstream along a more narrow section of the Ruhr with stronger water currents and more sparse floating vegetation, thus providing less suitable habitats for large lymnaeids. Although at a wider section of the reservoir and richer in vegetation, site 4 showed no abundant lymnaeid snail populations throughout 2012. At sites 5, 7 and 8 no suitable lymnaeid snail populations were found during the samplings. Site 5 is characterised by steep bank reinforcements with stones and hardly any vegetation, sites 7 and 8 are further downstream where water currents are stronger. In 2009, suitable snail populations were found on aquatic vegetation at site 13.

In accordance with the presence of abundant lymnaeid snail populations, infections with *Trichobilharzia* spp. were found at sampling sites 6, 11 and 13. Sites 6 and 11 are also characterised by the occurrence of waterfowl: the bird reserve *Heisinger Bogen* (site 11) provides a large protected area as breeding grounds for a multitude of bird species and the area surrounding the *Seaside Beach* (site 6) is a popular local recreation spot, where people can often be seen feeding birds, mainly anatids. These sites therefore provide ideal conditions for bird schistosomes to complete their life cycle.

Since the parasites have a complex life cycle, their occurrence is dependent on the availability of all hosts and is subject to a wide variety of environmental factors (reviewed in Soldánová et al., 2013). In order to assess the risk of a human infection, it is necessary to take these environmental factors, as well as personal aspects into consideration. Although infections of *Trichobilharzia* spp. were detected at different sampling sites, they only occurred in individual snails. However, despite the typically low prevalence of *Trichobilharzia* spp., human infestations are possible due to the high number of cercariae that can be released from individual infected snail hosts in short periods of time (Zbikowska, 2004; Fig. 2B). In general, localities with rich aquatic vegetation, usually along the shore, are considered more risky, as they provide suitable habitats for lymnaeid snails (Lévesque et al., 2002), which coincides with our findings of snail populations and *Trichobilharzia* infections in Lake Baldeney. However, lymnaeid snails can also occur in deeper waters and at sites free of vegetation (Fiedler et al., 2005). Furthermore, although the cercariae are rather short-lived, they are good swimmers and may be able to actively disperse within a radius of 100 m (Fiedler et al., 2005). Moreover, water currents may passively carry infective cercariae over distances of several kilometres into other areas where they may pose an infection risk (Leighton et al., 2000; Fiedler et al., 2005). In Lake Baldeney, human infections may therefore also occur at sections downstream from localities with lymnaeid snail populations, although they are less likely than directly at these sites.

Most cases of cercarial dermatitis in humans occur during the summer months when both cercarial emergence of bird schistosomes and human bathing activities peak (Lévesque et al., 2002; Valdovinos and Balboa, 2008). Besides season, time of day plays an important role in the risk of an infection, as most cercariae are released from their snail hosts during the early morning hours, leading to higher infection risks for swimmers in the morning (Lindblade, 1998; Verbrugge et al., 2004). As stated above however, cercariae remain infective for a couple of hours and can be transported over large distances, and the risk of infection is not necessarily restricted to proximity of time and place to cercarial release.

Temperature as well as sunshine play important roles for the parasites' development and have been shown to correspond with higher infection risks. Cercarial output is elevated in warm water (Valdovinos and Balboa, 2008) and on sunny days, especially after overcast periods (Horák et al., 2002). Moreover, high temperatures increase chances of encountering the parasites and becoming infected due to increased bathing activities, i.e. more time spent in the water (Chamot et al., 1998). Due to the parasites' seasonal and temperature-dependent development (Horák et al., 2002), climate change and global warming are considered important risk factors, especially since increased temperatures can provide favourable conditions for both snail hosts and overwintering migratory birds, allowing for longer host-parasite transmission windows and subsequently higher parasite prevalence in lakes (Mas-Coma et al., 2009).

Furthermore, eutrophication is an ecological key factor for swimmer's itch that leads to higher host and parasite abundances and increased risks of human infections (reviewed in Soldánová et al., 2013). In contrast to the typically low prevalences of less than 5%, exceptionally high infection rates with prevalences exceeding 40% can occur in snails in eutrophic systems (Soldánová et al., 2011). Consequently, most cases of swimmer's itch in Europe are registered in eutrophic lakes and manmade water bodies (Soldánová et al., 2013). Therefore, although presently not detected, higher bird schistosome prevalences are well possible in a shallow and eutrophic reservoir such as Lake Baldeney under certain conditions, e.g. local infection hot-spots at sites of high nutrient input (cf. Lake Hengstey; Soldánová et al., 2010).

Besides these environmental influences, personal factors have significant effects on the risk of swimmer's itch infections. Since cercarial dermatitis develops as a consequence of immune responses in already sensitised people (Kouřilová et al., 2004; Horák et al., 2008), the individual history of previous contact with the parasites plays an important role in the likelihood and severity of swimmer's itch (Chamot et al., 1998).

Personal bathing behaviour and bathing time have significant effects on the chances of encountering bird schistosomes. People engaged in immersed activities, such as swimming or wading are much more likely to become infected than people engaged in surface activities, such as water skiing or wind surfing (Lindblade, 1998). Likewise, the frequency of bathing activities and the amount of time spent in water is positively correlated with the risk of an infection due to the increased chance of contact with the parasites (Verbrugge et al., 2004; Schets et al., 2008). The fact that young children have been reported to show a higher risk of cercarial dermatitis (Lindblade, 1998) can possibly best be explained by their individual bathing habits, i.e. more frequent visits and more time spent in warm, shallow water along the shore (Lévesque et al., 2002). Therefore, besides the biotic and abiotic environmental factors that affect the parasites' occurrence, it is how people interact with the lake that determines the risk of encountering cercarial dermatitis (Verbrugge et al., 2004).

Based on the parasites' biology and the ecological factors that drive their occurrence, a number of preventive measures are possible (for a full review see Soldánová et al., 2013; Horák et al., 2015 and references therein). Many possible measures directly aim at disrupting the parasitic life cycle at some stage, mainly either by targeting the free-swimming larval stages or by removing suitable host populations. Most of these actions are however rather difficult, expensive or labour-intense (e.g. biological control of parasites or hosts, manual removal of snails) and their effectiveness unsure, or come at high ecological costs (e.g. use of chemical molluscicides or mechanical habitat destruction). However, simple measures such as encouraging people not to feed waterfowl (e.g. by putting up information boards) may help and provide the opportunity to inform the public about infection risks.

Since personal behaviour plays an important role on the likeliness of encountering the parasites, personal preventive actions can help reducing the risk of an infection. Avoiding shallow waters close to the shoreline (e.g. by entering the water via a bathing jetty) and sites with aquatic vegetation, as well as avoiding swimming during the morning hours have been suggested (Lindblade, 1998; Lévesque et al., 2002). Although free of ecological side effects, these actions cannot guarantee absolute safety from infections (Fiedler et al., 2005). Furthermore, briskly rubbing the skin with a towel after bathing has been suggested to kill off invading cercariae (Baird and Wear, 1987). However, since penetration of cercariae of *Trichobilharzia* spp. into human skin can be fast (as fast as 83 s, average 4 min; Haas and van de Roemer, 1998), this only offers some protection immediately after contact and seems not feasible for swimmers. Cream formulations that inhibit cercarial skin penetration have been tested and proven successful (Wulff et al., 2007), but would need to be applied extensively before bathing.

Altogether, while these actions may lower the risk of human infection, they cannot guarantee total safety. Bird schistosomes, along with the other trematode species, are integral parts of the ecosystem and the risk of swimmer's itch comes with the use of freshwater lakes as bathing waters. Therefore, although not legally binding or dangerous, these risks should be included in the evaluation of the Ruhr River as a potential bathing water and should be communicated to the public.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgements

We thank Jana Köchling, Verena Altmann, Jessica Schwelm, Ana Pérez-del-Olmo and Aneta Kostadinova for their assistance in sampling and in the laboratory, and three anonymous reviewers for their comments. The study was supported by the 'Sichere Ruhr' project as part of the Federal Ministry of Education and Research (BMBF) program 'Sustainable Water Management' (BS, grant 02WRS1283). MS received partial support of a Czech Science Foundation grant (P505/10/1562). CS benefited from a Deutsche Bundesstiftung Umwelt (DBU) PhD fellowship and a research grant by the Faculty of Biology of the University of Duisburg-Essen.

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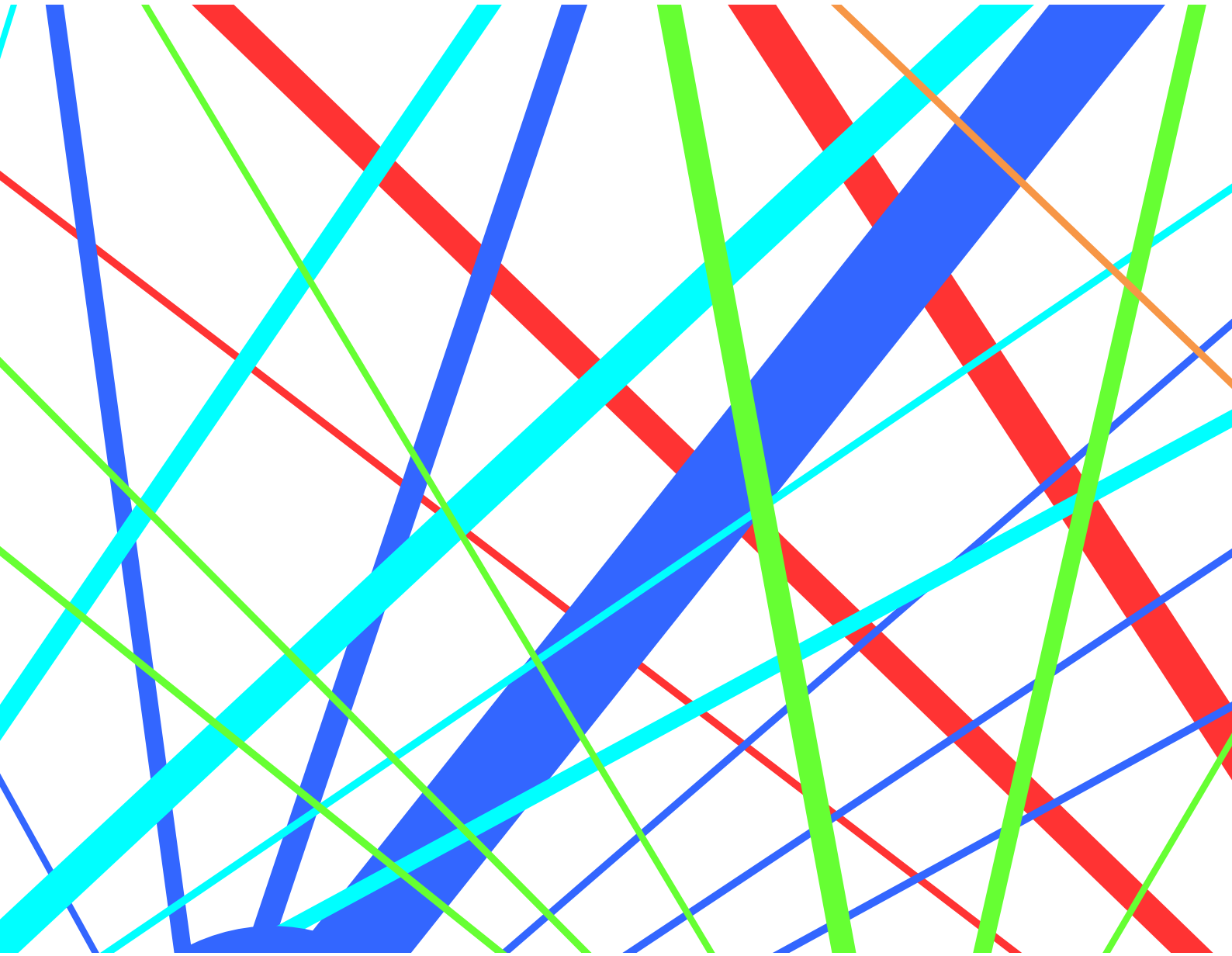

8. Chapter IV

Trematode ecology- biodiversity of trematodes in the Ruhr

8. Chapter IV

Trematode ecology – biodiversity of trematodes in the Ruhr

This chapter contains the following manuscript on the overall diversity and structure of trematode assemblages and communities in snails in the Ruhr reservoirs.

8.1 Selbach, C., Soldánová, M., & Sures, B. Hidden diversity on our doorstep – trematode assemblages and communities in lymnaeid and planorbid snails in a Central European reservoir system.

Hidden diversity on our doorstep: trematode assemblages and communities in lymnaeid and planorbid snails in a Central European reservoir system

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Introduction

Except for their role as disease agents, parasites have long been ignored and considered unimportant in the context of most ecological studies. However, the last 25 to 30 years have seen a slow but steady advancement of our understanding of parasites as important and integral elements of healthy and functioning ecosystems (Poulin 1999, Hudson et al. 2006) and it is impossible to fully understand ecosystems without considering parasites (Lafferty et al. 2006). In this context, parasites have been shown to be important structuring forces in ecological food webs (Lafferty et al. 2008) that make up a large proportion of an ecosystem's biomass (Kuris et al. 2008, Soldánová et al. submitted) and thus considerably contribute to the energy flow within ecosystems (Thieltges et al. 2008). Furthermore, parasites provide viable 'ecosystem services' (Dobson et al. 2008), such as regulation of host abundance or even concentrations of pollutants (Sures 2003). Moreover, based on their often complex life cycles and strong interaction in ecosystems, parasites may serve as useful bioindicators to assess environmental conditions and changes (Lafferty 1997, Vidal-Martínez et al. 2010, Nachev & Sures, 2015).

One fundamental question in science that has been debated throughout the years is the total number of species on Earth, but with about 90% of the estimated 8.7 million species still awaiting description (Mora et al. 2011), we are a long way from a full inventory of our planet's biodiversity. Likewise, the question how many parasite species there are on Earth has been a major point of discussion with estimations ranging from one third to over half the diversity on the planet (reviewed in Poulin 2014). These are very broad and rather imprecise estimations and we still know only little of the true diversity of parasites and are far from a total knowledge of the diversity of parasite taxa (Poulin & Morand 2004), if such knowledge is possible at all. However, as Poulin (2014) points out, knowing the exact number of parasite species may currently not be as important as understanding

other more pressing issues, such as cases of parasite extinctions, identification of parasite diversity hot spots or emerging zoonotic diseases caused by parasites.

Since our environment is rapidly changing due to anthropogenic pressures (e.g. global warming, population growth, increased needs of resources and pollution), we need to better understand these changes and their often complex effects on the health of humans, livestock and ecosystems. Especially climate change and global warming are regarded to have major impacts on parasites with many, often still unforeseeable, consequences on parasite transmission patterns, life-history traits, virulence, and on entire ecosystems (Marcogliese 2001, 2008). However, examples have shown how even more small-scale local environmental alterations, such as eutrophication of water bodies due to agriculture, industrialization or urbanization, can affect parasite communities and trigger complex and often drastic changes that can restructure whole ecosystems. For example local extinctions of amphibian populations in North American fresh waters were caused by increased prevalence of the trematode *Riberioa ondatrae* as a result of anthropogenic nutrient enrichment in these ecosystems that provided suitable conditions for snail intermediate hosts and the parasites (Johnson & Chase 2004, Johnson et al. 2007). However, although a world-wide decline of amphibian populations had been observed and possible reasons discussed before (Kiesecker et al. 2001), parasites were not considered at first, despite their crucial role in this development. It is, therefore, essential that we understand parasite assemblages and local distribution patterns at the ecosystem level and thoroughly study individual host-parasite systems.

There are some exceptionally well-studied aquatic ecosystems (e.g. Kuris et al. 2008, Preston et al. 2013, Thieltges et al. 2013, Lagrue et al. 2015) that focus on the roles of parasites in these systems and allow estimations of the effects of environmental changes. However, these systems are geographically far apart and highly different (marine, intertidal and freshwater). On the other hand, our knowledge of host-parasite interaction in man-made waterbodies, such as impounded lakes and reservoirs, is still very limited and large-scale studies focussing on the diversity and distribution of parasites in such systems are lacking. This is especially critical, since such waterbodies fulfil vital roles as drinking water storages, recreational areas and important biodiversity hot spots, especially in urban areas. Studies on parasite diversity and community structure from man-made waterbodies would, therefore, be crucial to advance our understanding of such ecosystems and to allow better comparisons between different habitats, geographical regions and host-parasite systems.

Trematodes are a diverse group of ubiquitous and cosmopolitan parasites that have complex life cycles, involving molluscs as first intermediate and a wide range of invertebrate and vertebrate second intermediate and definitive hosts. Due to these complex life cycles with snails serving as common hosts, trematode communities in snails reflect the richness and abundance of free-living

assemblages and are suitable bionindicators of free-living diversity (Hechinger et al. 2007) that lend themselves to studying the complex interactions within ecosystems.

In a preliminary study, we were able to show that the Ruhr river system in Germany offers ideal conditions to study trematode community structure in snails in a man-made reservoir system (Soldánová et al. 2010). Such reservoir systems are typical for man-made waterbodies in Central Europe and fulfil viable functions in densely populated areas. Future anthropogenic and environmental changes will have impacts on these ecosystems and its parasitic and free-living organisms that we will not be able to assess without a thorough understanding of the parasite communities therein. In two consecutive sampling campaigns during the summer months of 2012 and 2013 we have built an extensive dataset on the snail-trematode assemblages from five interconnected reservoirs of the Ruhr river and its tributaries. Our knowledge of the trematode diversity in these Ruhr reservoirs includes 'cryptic' diversity, since questionable taxonomic groups were studied in detail by applying integrative approaches combining morphological and molecular tools that have revealed new and yet unknown species lineages in several genera (see Georgieva et al. 2013, Selbach et al. 2014, Selbach et al. 2015). This information on the diversity of trematodes, including taxonomically controversial trematode groups, allows an accurate assessment of the diversity and distribution of trematodes in snails in the Ruhr system. Based on this extensive dataset we are able to address the overall diversity of trematode assemblages in snails and analyse how the parasites are distributed at small scales in the man-made Ruhr reservoir system. We are interested whether trematode assemblages in snails are equally distributed among the interconnected reservoirs or whether we can identify patterns and structure in the trematode communities. Furthermore, we want to find out what information the potential trematode component community structure and the parasites' transmission pathways between their hosts can reveal about the ecosystem.

The aims of the study are (i) to assess structure and composition of trematode assemblages and component communities across the interconnected reservoirs in the Ruhr system, (ii) to study the role of lymnaeid and planorbid snails as first intermediate hosts for digenean trematodes, (iii) to identify the diversity of trematode assemblages in the individual snail hosts and in the different reservoirs, (iv) to identify the most common and dominant trematode species, and based on the knowledge of the parasites' life cycles (v) to identify transmission pathways between the hosts that can reveal information on final host occurrence and trophic interactions in the ecosystems.

Materials and Methods

Sampling

In order to assess the diversity and distribution patterns of trematode communities, we collected snails at several sampling sites in five reservoirs of the Ruhr river catchment area, Germany: Baldeneysee (51°24′ 20.08″N, 7°2′22.47″E), Hengsteysee (51°24′52.17″N, 7° 27′42.55″E), Hennetalsperre (51°19′50.97″N, 8°15′46.82″E), Sorpetalsperre (51°20′15.01″N, 7°56′46.18″E) and Versetalsperre (51°10′55.71″N, 7°40′57.12″E) (Figure 1). All waterbodies were constructed during the first half of the 20th century along the Ruhr river and its tributaries as drinking water reservoirs, natural river water treatment plants and to regulate the water flow of the river system. Table 1 provides additional information on the individual reservoirs.

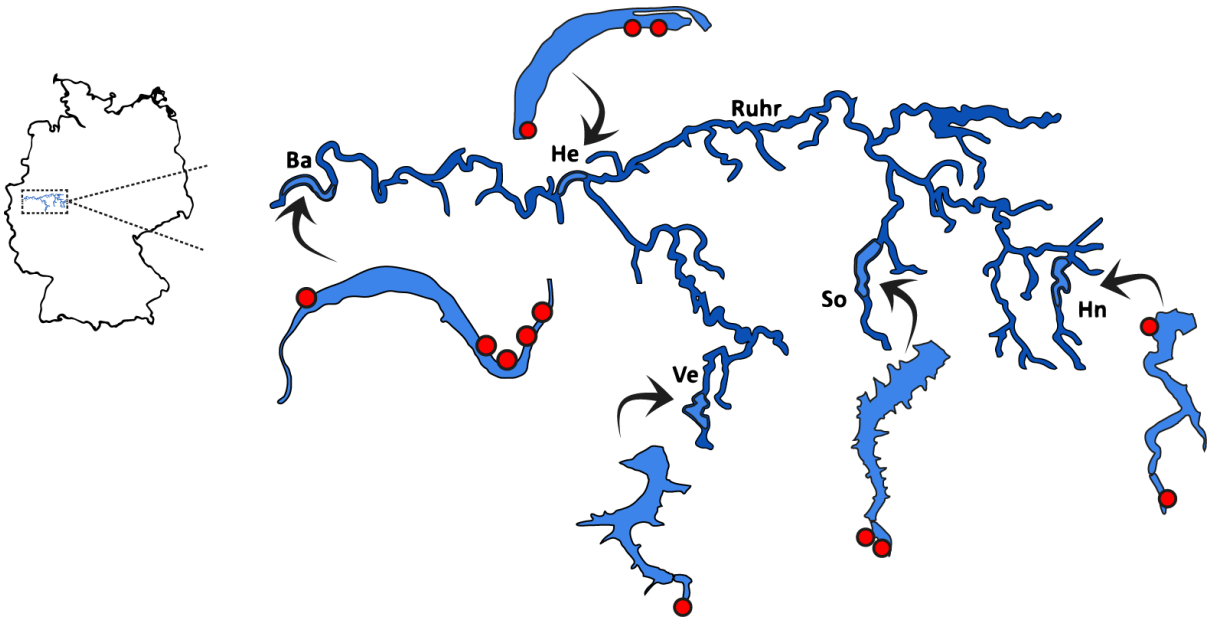

Figure 1 Map of the Ruhr area and the reservoir system studied. Individual sampling sites are indicated by red dots. *Abbreviations*: Ba, Baldeneysee; He, Hengsteysee; So, Sorpetalsperre; Hn, Hennetalsperre; Ve, Versetalsperre.

Table 1 General characteristics of the study reservoirs and water quality data

Reservoir data	Baldeneysee	Hengsteysee	Sorpetalsperre	Hennetalsperre	Versetalsperre
Construction (year)[a]	1931-1933	1917-1929	1926-1935	1901-1905 (1950-1955)	1929-1951
Surface area (km²)[a]	2.6	1.4	3.3	2.1	1.8
Depth (m)[a]	3.1 (mean)	1.9 (mean)	up to 57	up to 51	up to 52
Volume (Mio. m³)[a]	7.6	3.3	70	38.4	32.8
Eutrophication status[b]	eutrophic	eutrophic	mesotrophic–oligotrophic	mesotrophic	oligotrophic

[a] Ruhrverband (2015)
[b] AWWR & Ruhrverband (2013)

Each sampling site was visited repeatedly (11 times) during the summer months (May – September) in two consecutive years, 2012 and 2013. At each site snails were collected by hand or with the aid of hand-nets from stones, sediment and aquatic vegetation. Sampling was mostly focused on snails belonging to two families, Lymnaeidae and Planorbidae, since they proved to harbour the most diverse trematode fauna in Europe (Faltýnková & Haas 2006, Soldánová et al. 2011, Brown et al. 2011). In total, 3,171 lymnaeid snails belonging to four species [1,909 *Radix auricularia* (L.), 668 *Stagnicola palustris* (Müller), 349 *R. peregra* (Müller) and 245 *Lymnaea stagnalis* (L.)] and 2,176 planorbid snails belonging to two species [1,981 *Gyraulus albus* (Müller) and 195 *Segmentina nitida* (Müller)] were collected and screened for trematode infections. Although sampling was not quantitative, the sample sizes reflected the abundance of the individual hosts at the sampling sites, since the sampling effort (i.e. time spent at each locality) was comparable at each site.

In the laboratory, all snails were placed in individual cups with lake water and exposed to a light source for two to five days to induce cercarial shedding. Snails that did not emit cercariae during that time were dissected and screened for prepatent infections. Trematode stages were identified alive under an Olympus BX51 microscope with the help of appropriate identification keys or other relevant primary sources (e.g. Faltýnková et al. 2007, 2008, Niewiadomska 1986, Niewiadomska & Kiseliene 1994) and documented with an Olympus UC30 digital camera. For further investigation of taxonomically problematic groups, trematode material was fixed in molecular grade ethanol and 4% formaldehyde solution for molecular and morphological studies. Trematode genera that are known to be taxonomically problematic or groups that showed conspicuous morphological features were subjected to thorough morphological and molecular studies to reveal potential cryptic diversity (see Georgieva et al. 2013, Selbach et al. 2014, Selbach et al. 2015).

Data Analysis

Following Poulin & Morand (2004), we distinguished between different hierarchical levels at which we studied parasite biodiversity, the *component community* level and *parasite assemblages*. A *component community* is defined as all parasite species exploiting a host population at a given point in time (i.e. all parasite species found in one snail population at a certain locality during one sampling trip). All other entities are referred to as *parasite assemblages*, i.e. all parasite species found within one specified subdivision of a given scale (e.g. *trematode assemblage per snail species*: all parasites found in one host species; or *trematode assemblage per lake*: all parasites found in one lake; or *trematode assemblage per snail species and lake*: all parasites found within one host species in one lake). In order to identify the most common final hosts, we grouped parasite species in guilds, following the broad definition of Polis et al. (1989) as "all taxa in a community that use similar resources (food or space) and thus may compete, regardless of differences in the tactics of resource acquisition".

Parasite prevalence (P) was calculated for parasite assemblages in hosts and lakes as well as for each component community as the proportion of infected host individuals in relation to total number of host individuals in a population ($P = n_{inf} / N * 100$, where n_{inf} is the number of infected snails and N are all snails in a population). In order to compare the diversity of trematode assemblages between the different snail species and among the different lakes, we calculated the following diversity indices: species richness S (the total number of parasite species in a community or assemblage), Shannon diversity index H ($H = -\sum (P_i * \ln P_i$, where P_i is the proportion of infections with species i relative to the total number of infections), Shannon evenness or equitability (J) as a measure of evenness with which individuals are divided among the taxa (Shannon diversity divided by the logarithm of number of taxa), and the Berger-Parker dominance d ($d = N_{max} / N$, where N_{max} is the number of infections of the most abundant species, and N is the total number of infections in the sample). Furthermore, we calculated the average taxonomic diversity Δ (i.e. the average taxonomic distance of every pair of individual infections in a sample, Warwick & Clarke, 1995) to take into account the phylogenetic diversity up to the family level. All calculations were performed with PAST 3.08 (Hammer et al. 2003), except for the average taxonomic diversity Δ which was calculated using PRIMER v6 (Clarke & Gorley 2006). Trematodes that could not be identified because they were immature were used for the overall prevalence calculations but were excluded from the diversity analyses. Double infections were rare throughout our samplings and treated as two individual infections for the diversity calculations.

Non-parametric tests (Spearman's rank order correlations) were carried out with Statistica v.7 (StatSoft Inc.) to identify possible correlations between trematode species richness and overall sampling size of the snail host species (pooled number of all snails of a species as a measure for overall snail abundance), as well as between trematode species richness and size of the different snail hosts, reflecting the resources each host group represents to parasites. Snail hosts were categorized according to length and width measurements given in the literature (Glöer 2002) and ranked from small to large: 1. *S. nitid*a, 2. *G. albus*, 3. *S. palustris*, 4. *R. peregra*, 5. *R. auricularia* and 6. *L. stagnalis*. These categories match the data from our measurements of sampled snails. Furthermore, possible correlations between overall prevalence and sample size as well as snail host size were analysed.

In order to identify patterns and structures at the trematode component community level, component community composition analyses, i.e. non-metric multi-dimensional scaling (MDS) ordination and randomization tests on similarity matrices (ANOSIM) based on Bray-Curtis index values, were performed with PRIMER v6. In the MDS plots each parasite component community is represented by a symbol; the more similar the parasite communities are to each other, the closer the respective symbols will group together. Different characteristics of the component communities can be visualised (e.g. snail host or reservoir of origin) in order to analyse the structure of trematode communities. To reduce the bias due to small sample size, only data from distinct samples ($n > 14$ snails) were used at

the component community level (n = 75). In order to identify the most dominant species, we considered trematode species with prevalence higher than 10% in at least one component community dominant.

Most component community data was available from *R. auricularia* (48 component communities), allowing us to test for community structures within this host species in detail by comparing component communities between different lakes, seasons and years. We tested whether snail abundance (indicated by sample size) was correlated with overall prevalence and species richness (Spearman's rank order correlations). Mean snail size (snail shell length) in the individual *R. auricularia* component communities varied considerably (9.6 mm – 22.5 mm, mean 14.1 mm). Therefore, we tested the possible relationship of mean snail size with overall prevalence and species richness (Spearman's rank order correlations). If the test showed significant positive correlation between snail size and overall prevalence, suggesting higher rates of infection in communities with larger snails, we used more appropriate statistical test to evaluate the effect of the variables 'lake', 'season' and 'year' on the overall prevalence by two-way analyses of covariance (ANCOVA), while controlling the effect of snail size (entered as covariate). For the factor 'season' samples were grouped as follows: spring (May), summer (June – August) and autumn (September). For these analyses we used two datasets: for the analysis of variation between lakes and years, five samples from Hennetalsperre were excluded, since they were available from one year only (2012) and would not have allowed comparisons between the years. For the analysis of seasonal variation, the samples from spring (n = 7) were excluded, since they were from one year (2012) only. Prepatent infections, which were identified as sporocysts or rediae and could not be assigned to any trematode species due to their immature stage were excluded from the analyses. Similarly, rare trematode species which occurred in less than three component communities were not included in the analyses. This resulted in the 15 most common species (out of 23, 8 were excluded) which were entered into analyses testing the infection levels variation in space and time. To improve the fit of the normal distribution, data on sample size and snail size (mean length) were log10 (x) transformed, prevalence data (expressed as proportions) were arcsin square-root-transformed, and species richness data log10 (x+1) transformed. All tests were carried out with Statistica v.7 (StatSoft Inc.).

Results

Of the 5,347 snails sampled in both years, 2012 and 2013, 1,049 showed patent or prepatent infections with trematodes, resulting in a total prevalence of 19.6%. Overall prevalence was highly different among the individual snail species, ranging from 2.6% in *Segmentina nitida* to 31.7% in *Radix auricularia*. Table 2 provides an overview of the total number of snails sampled in the five reservoirs in 2012 and 2013 and the overall prevalence of trematode infections for each reservoir and year. In

total 37 different trematode species belonging to nine families were found. Table 3 shows the individual prevalence of larval trematode species in the six snail hosts per reservoir. Trematode species richness varied considerably between the different hosts assemblages and ranged from only three species in *S. nitida* to 23 species in *R. auricularia*. Figure 2 shows a graphical representation of the sizes of the individual snail host populations and the number of trematodes found therein.

Spearman's rank order correlations for the pooled trematode assemblages in snails revealed a significant positive correlation between overall trematode species richness and sample size of each host species (r_s = 0.943, p = 0.005) but did not show any significant correlation between snail host size and trematode species richness (r_s = 0.2, p = 0.7), showing that snail species that were more abundant harboured more species rich trematode assemblages, but trematode assemblage species richness was not correlated with the size of the host species. Overall prevalence in the different host species was not correlated with either sample size or host size.

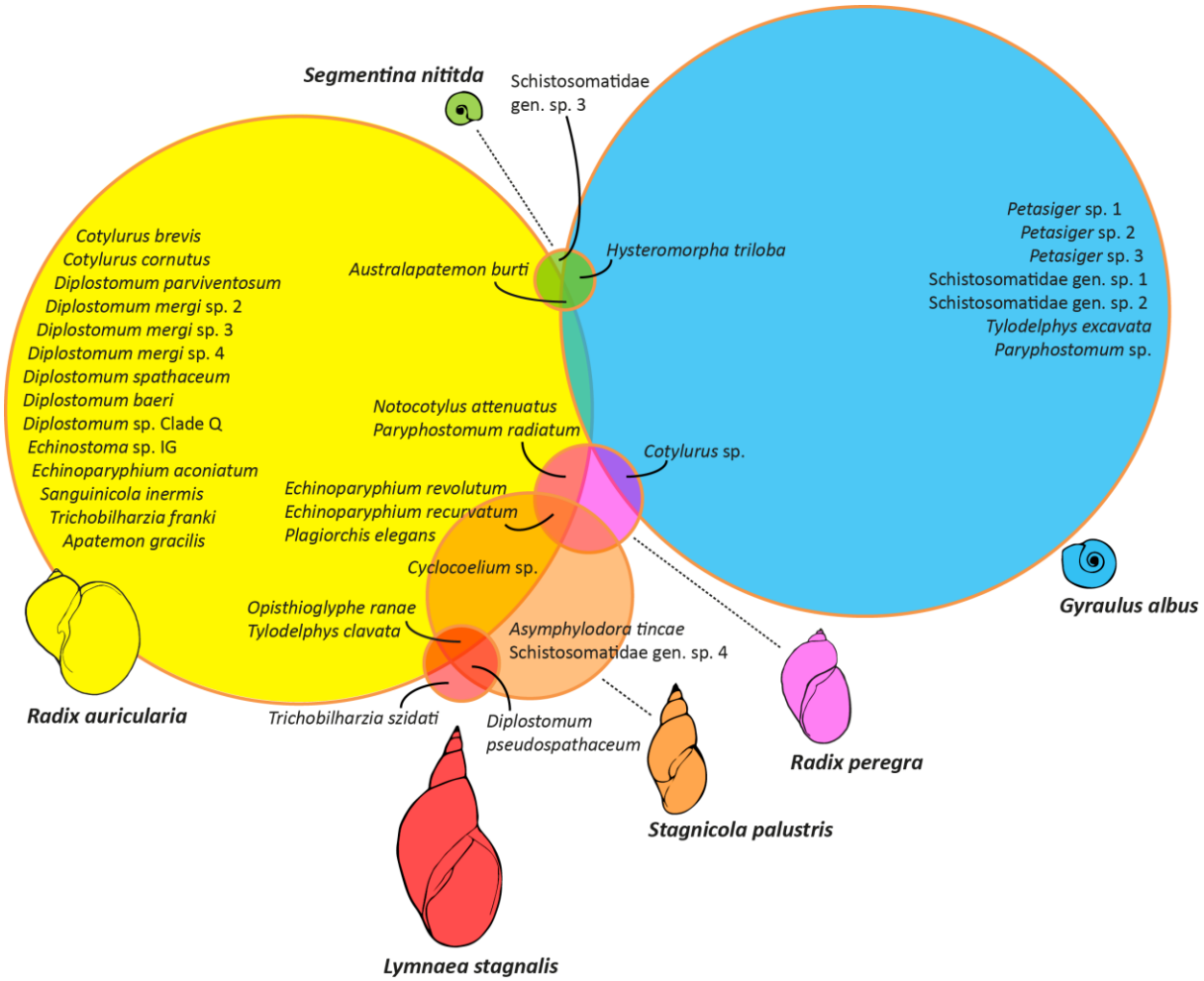

Figure 2 Graphical representation of the snail species sampled during the study and their trematode fauna in the Ruhr reservoirs. Each circle represents one host snail species, the size of each circle corresponds to the number of snails sampled during the study, trematode species are indicated in the respective circles; overlaps show shared trematode species.

Table 2 Total numbers of the six snail species sampled in the five reservoirs in 2012 and 2013, overall prevalence of trematode infections (% of infected snails in the pooled samples) and number of samples used in the component community analyses

		Baldeneysee			Hengsteysee			Sorpetalsperre			Hennetalsperre			Versetalsperre			Total
		2012	2013	Both	2012	2013	Both	2012	2013	Both	2012	2013	Both	2012	2013	Both	
Radix auricularia	No. of snails	275	91	366	596	353	949	248	90	338	220	36	256				1.909
	No. of infections	46	15	61	178	132	310	99	56	155	70	10	80				606
	Prevalence	16.7	16.5	16.7	29.9	37.4	32.7	39.9	62.2	45.9	31.8	27.8	31.3				31.7
	Distinct samples[a]	10 (8)	3 (3)	13 (11)	12 (11)	11 (9)	23 (20)	9 (9)	3 (3)	12 (12)	5 (5)	0	5 (5)				
Radix peregra	No. of snails										16	20	36	294	19	313	349
	No. of infections										12	7	19	7	1	8	27
	Prevalence										75.0	35.0	52.8	2.4	5.5	2.6	7.7
	Distinct samples[a]										1 (1)	0	1 (1)	4 (3)	0	4 (3)	
Lymnaea stagnalis	No. of snails	28	8	36	100	108	208		1	1							245
	No. of infections	5	2	7	12	21	33		0	0							40
	Prevalence	17.9	25.0[b]	19.4	12.0	19.4	15.9		0	0							16.3
	Distinct samples[a]	0	0	0	2 (2)	4 (4)	6 (6)		0	0							
Stagnicola palustris	No. of snails	29	6	35	530	85	615	15	3	18							668
	No. of infections	3	3	6	75	6	81	1	1	2							89
	Prevalence	10.3	50.0[b]	17.1	14.5	7.1	13.2	6.7	33.3[b]	11.1							13.3
	Distinct samples[a]	0	0	0	7 (6)	2 (1)	9 (7)	0	0	0							
Gyraulus albus	No. of snails	5	14	19	2	26	28				1.098	830	1.928	1	5	6	1.981
	No. of infections	0	8	8	0	2	2				157	114	271	1	0	1	282
	Prevalence	0	57.1	42.1	0	7.7	7.1				14.3	13.7	14.1	100.0[b]	0	16.7[b]	14.2
	Distinct samples[a]	0	0	0	0	0	1 (0)				6 (6)	5 (4)	11 (10)	0	0	0	
Segmentina nitida	No. of snails	15		15							127	53	180				195
	No. of infections	0		0							2	3	5				5
	Prevalence	0		0							1.6	5.7	2.8				2.6
	Distinct samples[a]	0		0							4 (1)	1 (1)	5 (2)				

[a] Distinct samples only n ≤ 14 used in component community analyses; numbers in brackets show samples with trematode infections

[b] Sample size small (n ≤ 14)

Table 3 Overall prevalence of individual larval trematode species (% of infected snails in the pooled samples from 2012 and 2013) in the six snail species and final hosts of the trematodes. *Abbreviations*: WAT, waterfowl; F-B, fish-eating birds; RAL, Rallidae; AMP, amphibians; B, birds; MAM, mammals; CYP, cyprinids; STO, storks.

Snail species	Trematode family	Trematode species	Final host	Reservoir Versetalsperre	Sorpetalsperre	Hennetalsperre	Hengsteysee	Baldeneysee	TOTAL
Radix auricularia	Echinostomatidae	*Echinoparyphium aconiatum**	WAT		0.3	3.5	0.2		0.6
		*Echinoparyphium recurvatum**	WAT		13.0	3.9	1.5	0.3	3.6
		*Echinostoma revolutum**	WAT			2.3			0.3
		Echinostoma sp. IG	WAT		0.3			0.3	0.1
		*Paryphostomum radiatum**	F-B		11.5	6.6	9.9	12.0	10.2
	Notocotylidae	*Notocotylus attenuatus**	WAT		4.4	4.7	2.2	0.8	2.7
	Cyclocoelidae	*Cyclocoelum* sp.[a]*	RAL		0.9		4.1	0.3	2.3
	Telorchiidae	*Opisthioglyphe ranae*	AMP			0.4	0.3	0.8	0.4
	Plagiorchiidae	*Plagiorchis elegans**	B, MAM		4.7	4.7	7.0		4.9
	Schistosomatidae	*Trichobilharzia franki **	WAT		5.0	0.4	0.4	0.6	1.3
	Diplostomidae	*Diplostomum parviventosum*	F-B				1.0		0.5
		Diplostomum mergi 2*	F-B		1.2		0.7	0.3	0.6
		Diplostomum mergi 3	F-B				0.4		0.2
		Diplostomum mergi 4	F-B				0.1		0.1
		Diplostomum sp. Clade Q	F-B				0.1		0.1
		Diplostomum spathaceum	F-B				0.7		0.4
		Diplostomum baeri	F-B				0.1		0.1
		*Tylodelphys clavata**	F-B		1.8	3.1	1.2	1.1	1.5
	Strigeidae	*Australapatemon burti*	WAT		0.9		1.0	0.3	0.7
		*Cotylurus cornutus**	WAT		1.2		0.6		0.5
		Cotylurus brevis	WAT			0.8			0.1
		Apatemon gracilis	F-B				0.1		0.1
	Sanguinicolidae	*Sanguinicola inermis*	CYP		1.2				0.2
Radix peregra	Echinostomatidae	*Echinostoma revolutum**	WAT			16.7			1.7
		*Echinoparyphium recurvatum**	WAT	1.6		13.9			2.9
		Paryphostomum radiatum	F-B			11.1			1.2
	Notocotylidae	*Notocotylus attenuatus*	WAT			2.8			0.3
	Plagiorchiidae	*Plagiorchis elegans*	B, MAM	0.6		2.8			0.9
	Strigeidae	*Cotylurus* sp.	WAT			2.8			0.3

[a] Metacercariae only

[b] Sample size small (n < 14)

*Species dominant (prevalence ≥ 10 %) in at least one component community

Table 3 Continued.

Snail species	Trematode family	Trematode species	Final host	Reservoir					
				Versetalsperre	Sorpetalsperre	Hennetalsperre	Hengsteysee	Baldeneysee	TOTAL
Lymnaea stagnalis	Schistosomatidae	*Trichobilharzia szidati*	WAT				1.0		0.8
	Diplostomidae	*Diplostomum pseudospathaceum**	F-B				10.6	13.9	11.0
		Tylodelphys clavata	F-B					5.6	0.8
	Telorchiidae	*Opisthioglyphe ranae*	AMP				1.4	2.8	1.6
Stagnicola palustris	Echinostomatidae	*Echinoparyphium recurvatum*	WAT				0.2		0.2
		Echinostoma revolutum	WAT				0.2		0.2
	Cyclocoelidae	*Cyclocoelum* sp. [a]*	RAL				8.9		8.2
	Telorchiidae	*Opisthioglyphe ranae*	AMP				0.3	8.6	0.8
	Plagiorchiidae	*Plagiorchis elegans*	B, MAM		5.6		0.3		0.5
	Diplostomidae	*Diplostomum pseudospathaceum**	F-B				2.6	5.7	2.7
		Tylodelphys clavata	F-B				0.2	2.9	0.3
	Lissorchiidae	*Asymphylodora tincae*	CYP				0.2		0.2
	Schistosomatidae	Schistosomatidae gen. sp.4	WAT				0.2		0.2
Gyraulus albus	Echinostomatidae	*Paryphostomum* sp.*	F-B			2.2		26.3	2.4
		Petasiger sp. 1	F-B			0.2			0.2
		Petasiger sp. 2	F-B			0.1			0.1
		Petasiger sp. 3	F-B			0.1	3.6		0.2
	Diplostomidae	*Tylodelphis excavata*	STO					5.3	0.1
	Strigeidae	*Hysteromorpha triloba*	F-B			3.3	3.6		3.3
		*Australapatemon burti**	WAT	16.7 [b]		7.5			7.3
		Cotylurus sp.	WAT					10.5	0.1
	Schistosomatidae	Schistosomatidae gen. sp. 1	WAT			0.1			0.1
		Schistosomatidae gen. sp. 2	WAT			0.2			0.2
Segmentina nititda	Strigeidae	*Hysteromorpha triloba*	F-B			0.6			0.5
		Australapatemon burti	WAT			1.1			1.0
	Schistosomatidae	Schistosomatidae gen. sp. 3	WAT			0.6			0.5

[a] Metacercariae only

[b] Sample size small (n < 14)

*Species dominant (prevalence ≥ 10 %) in at least one component community

Trematode assemblages in *Radix auricularia*

A total of 31.7% (606 out of 1,909) of the collected *R. auricularia* harboured infections with larval trematodes. The most abundant *R. auricularia* population was found in Hengsteysee (949 snails), followed by Baldeneysee (366), Sorpetalsperre (338) and Hennetalsperre (256); no *R. auricularia* were found at Versetalsperre. Trematode prevalences was highest in Sorpetalsperre (45.9%), followed by Hengsteysee and Hennetalsperre (32.7% and 31.3% respectively), and was lowest in Baldeneysee (16.7%) (Table 2).

In total, *R. auricularia* housed 23 different trematode species, more than half of them belonging to the families Diplostomidae (8 species) and Echinostomatidae (5 species), including cryptic and novel species (Georgieva et al. 2013, Selbach et al. 2015) that contribute to the diversity in these groups (Table 3). The remaining species belong to a further seven families (Table 3). Altogether, *R. auricularia* showed by far the highest species richness of all sampled host species. Accordingly, trematode assemblages in *R. auricularia* showed the highest Shannon diversity (H = 2.3) and average taxonomic diversity (Δ = 79.8) as well as the lowest Berger-Parker dominance (d = 0.3; Table 4, Figure 3), indicating a high trematode diversity in this host. Coinciding with the most abundant snail population in Hengsteysee, the highest trematode species richness and diversity occurred in this waterbody (19 species, H = 2.2), followed by Sorpetalsperre (13 species, H = 2.0), Hennetalsperre and Baldeneysee (10 species each). Interestingly, the latter reservoir showed a far lower diversity and Shannon evenness compared with the other reservoirs, even when having the same species richness (H = 1.2, J = 0.5 in Baldeneysee vs. H = 2.1, J = 0.9 in Hennetalsperre), since the trematode assemblage in Baldeneysee was clearly dominated by one species, *Paryphostomum radiatum* (overall prevalence 12.0%).

Individual component communities in *R. auricularia* (n=48) comprised 1 – 13 trematode species, were generally species rich (mean S = 3.7) and were dominated by 1 – 4 species (prevalence ≥ 10%). In total, 11 species were considered dominant in one or more of the component communities. Most noticeably, *Paryphostomum radiatum* occurred in 31 component communities and was dominant in 15 communities, with individual prevalence up to 67.9%. Other dominant species were *Plagiorchis elegans* (occurred 27 times, dominant 6 times), *Notocotylus attenuatus* (occurred 22 times, dominant 2 times) or *Echinoparyphium recurvatum* (occurred 19 times, dominant 7 times).

With two exceptions, all trematode species found in *R. auricularia* utilise birds as definitive hosts (21 species), and the majority of trematodes fall either within the fish-eating guild that uses fish and fish-eating birds as second intermediate and definitive host (10 species), or within an anatid generalist guild that utilise waterfowl (9 species). The remaining species utilize birds of the family Rallidae, amphibians, cyprinids or mammals and various birds as definitive host (Table 3).

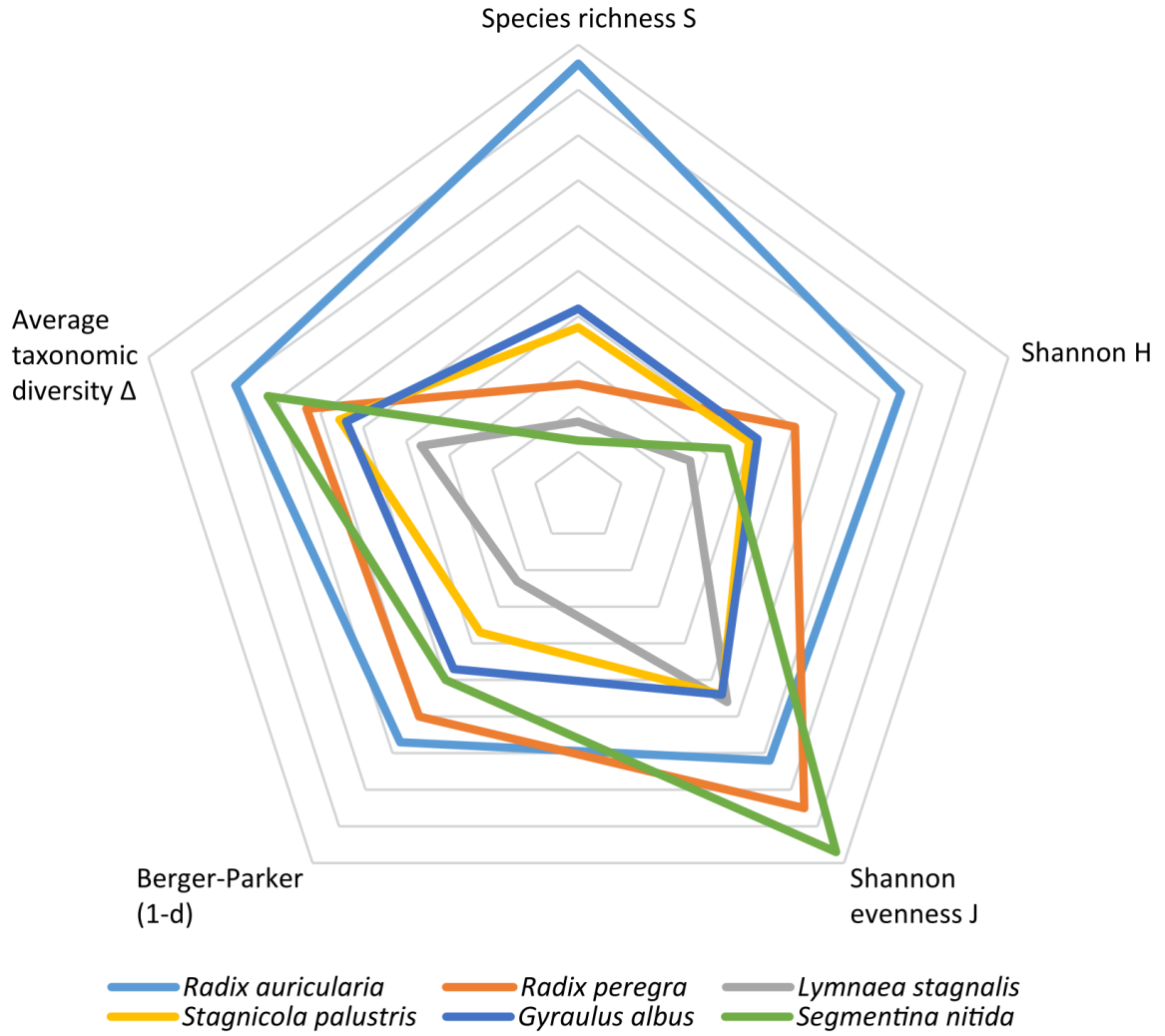

Figure 3 Schematic representation of the diversity indices of trematode assemblages in the six snail hosts. Berger-Parker dominance is inverted (1-d), so the further the lines extend to the margins, the higher the diversity and evenness in the trematode assemblages. (Possible maximum values of the individual indices: Species richness = 24; Shannon H = 3; Shannon evenness and 1 - Berger-Parker = 1; Average taxonomic distinctness = 100).

Trematode assemblages in *Radix peregra*

A total of 27 out of the 349 collected *R. peregra* were infected with larval trematodes (7.7%). An abundant *R. peregra* population was only found in Versetalsperre (313 snails), a further few snails (36) were found at one locality at Hennetalsperre. Trematode prevalence was low at Versetalsperre (2.6%) but very high at Hennetalsperre (52.8%), probably due to the small sampling size at the latter reservoir (Table 2).

In total *R. peregra* harboured six different trematode species of four families (Table 3), all of which were present at Hennetalsperre and two at Versetalsperre. Half of the species belonged to the family Echinostomatidae and showed high prevalence in Hennetalsperre (11.1% – 16.7%). Despite the low sample size at Hennetalsperre, species richness, diversity and average taxonomic diversity were

relatively high (H = 1.5; Δ = 63.6) and Berger-Parker dominance was low (d = 0.3) in this system, especially compared to the low diversity (H = 0.6), taxonomic diversity (Δ = 47.6) and high Berger-Parker dominance (d = 0.7) in Versetalsperre (Table 4), indicating a diverse trematode fauna in Hennetalsperre, despite the small *R. peregra* population.

Individual component communities in *R. peregra* (n = 4) comprised 1 – 4 trematode species and showed a mean species richness of S = 2.0. Two trematode species of the family Echinostomatidae were considered dominant (*Echinoparyphium recurvatum* and *Echinostoma revolutum*) and appeared in the same component community with very high prevalence (25.0% and 37.5%, respectively), most likely due to the rather small same size of 16 snails in this sample.

Of the six species found in *R. peregra*, all species use birds as definitive host, with the majority belonging to the anatid generalist guild that utilises waterfowl (4 species), one species using fish and fish-eating birds as second intermediate and definitive hosts, and one generalist species using mammals or various birds as definitive host (Table 3).

Trematode assemblages in *Lymnaea stagnalis*

Of the 245 *L. stagnalis* sampled, 40 were infected with larval trematodes, resulting in an overall prevalence of 16.3%, the second highest after *R. auricularia*. The only abundant *L. stagnalis* population was found in Hengsteysee (208 snails), whereas at Baldeneysee and Sorpetalsperre only individual snails were found (36 and 1, respectively); no *L. stagnalis* were found at the other reservoirs. Trematode prevalence was generally high in both lakes, 15.9% in Hengsteysee and 19.4% in Baldeneysee, the latter probably resulting from the rather small sample size (Table 2).

In total, *L. stagnalis* harboured four trematode species belonging to three families (Table 3). Altogether, trematode assemblages in *L. stagnalis* showed the second lowest species richness and by far the lowest overall diversity (H = 0.8), average taxonomic diversity (Δ = 36.6) and highest Berger-Parker dominance (d = 0.8) of all host assemblages (Table 4), due to the domination of trematode assemblages in both reservoirs by one species, *Diplostomum pseudospathaceum*. Altogether, with 11.0% *D. pseudospathaceum* from *L. stagnalis* showed the highest overall prevalence of all trematode species.

Individual component communities in *L. stagnalis* (n = 6) comprised 1 – 3 trematode species and were generally species poor (mean S = 1.3). Only one species, *D. pseudospathaceum*, was dominant, and dominated the majority of the individual samples with prevalence ranging from 13.3% – 18.0%. In communities where *D. pseudospathaceum* was present but not dominant, this species showed lower prevalence (4% – 9%).

Of the four species found in *L. stagnalis*, two belong the fish-eating guild that uses fish and fish-eating birds as second intermediate and definitive host, and one species each utilises anatid birds and amphibians as definitive host (Table 3).

Trematode assemblages in *Stagnicola palustris*

A total of 89 of the 668 collected *S. palustris* (13.3%) was infected with larval trematodes. The most abundant *S. palustris* population occurred in Hengsteysee, where the majority of snails was found (615 snails). Only few snails were found in Baldeneysee and Sorpetalsperre (35 and 18 snails, respectively). Overall trematode prevalence was high in all reservoirs, 13.2% in Hengsteysee, 17.1% in Baldeneysee and 11.1% in Sorpetalsperre, the high values in the latter two are due to the rather small samples from these lakes (Table 2). *Stagnicola palustris* harboured nine trematode species belonging to seven families (Table 2). The trematode assemblage in this host showed mid-range values for Shannon diversity (H = 1.2), average taxonomic diversity (Δ = 55.5), Shannon evenness (J = 0.5) and Berger-Parker dominance index (d = 0.6) (Table 3). Coinciding with the most abundant snail population in Hengsteysee, the highest trematode species richness occurred in this waterbody (9 species), compared with Baldeneysee (3 species) and Sorpetalsperre (1 species). Although showing equal Shannon diversity values (H = 1.0 vs. H = 1.0), average taxonomic diversity (Δ = 68.9 vs. Δ = 49.0) was considerably higher and Shannon evenness much lower (J = 0.5 vs. J = 0.9) in Hengsteysee compared to Baldeneysee, respectively (Table 4), due to the higher number of species from different families in Hengsteysee.

Individual component communities in *S. palustris* (n = 7) comprised 1 – 4 trematode species and were rather species poor (mean S = 1.9). In two component communities dominant species occurred, *D. speudospathaceum* (13.0%) and *Cyclocoelum* sp. (41.0%). The exceedingly high prevalence of the latter occurred in one sample from Hengsteysee, where it dominated the entire trematode community.

The nine trematode species found in *S. palustris* use a wide range of different hosts; two species utilize fish and fish-eating birds, three species waterfowl. The remaining species use amphibians, cyprinids, birds of the family Rallidae, or a wide group of mammals and birds as definitive host (Table 3).

Trematode assemblages in *Gyraulus albus*

A total of 14.2% (282 out of 1,981) of the collected *G. albus* had infections with larval trematodes. With 1,981 sampled snails, *G. albus* was the most abundant snail species in our system. The vast majority of snails comes from a population at one locality at Hennetalsperre (1,928 snails), whereas only few snails were found at Baldeneysee (19), Hengsteysee (28) and Versetalsperre (6). Prevalence in the small samples ranged from a fairly low 7.1% at Hengsteysee to 16.7% in Versetalsperre and very high 42.1% in Baldeneysee, caused by the small samples size. The largest population in Hennetalsperre showed a prevalence of 14.1% (Table 2).

In total *G. albus* harboured 10 different trematode species belonging to four families (Table 3). Most species belonged to the families Strigeidae (3 species) and Echinostomatidae (4 species), the latter comprising three different species of the genus *Petasiger* (Selbach et al. 2015). For *G. albus*

overall Shannon diversity (H = 1.3) and average taxonomic diversity (Δ = 54.0) as well as Berger-Parker dominance index (d = 0.5) were in the mid-range (Table 4). Overall Shannon evenness (J = 0.5) was low compared with other hosts, due to the dominant role of a few trematode species.

Individual component communities in *G. albus* (n = 10) comprised 1 – 6 trematode species and were generally species rich (mean S = 3.4) and were dominated by 1 – 2 species (prevalence ≥ 10%). In total, three species, *Australapatemon burti*, *Hysteromorpha triloba* and *Paryphostomum* sp., were considered dominant in one or more of the component communities; these species were present in the majority of the *G*. *albus* samples (10, 5 and 8 times, respectively).

Of the 10 different species found in *G. albus,* five belong the fish-eating guild that uses fish and fish-eating birds as second intermediate and definitive host, four species each utilise anatid birds, and one species uses storks (*Ciconia* sp.) as definitive hosts (Table 3).

Trematode assemblages in *Segmentina nititda*

The majority of *S. nititda* (180 out of 195) originated from the same locality at Hennetalsperre where *G. albus* was also abundant; a further few individual snails were found in Baldeneysee (15). Trematode prevalence in *S. nititda* was by far the lowest of all snail hosts (2.6%; 5 out of 195) and all infections were recorded in Hennetalsperre (Table 2). In total *S. nitida* harboured three different trematode species belonging to two families (Table 3). Overall Shannon diversity was low (H = 1.0) but average taxonomic diversity was rather high (Δ = 72.2) and Shannon evenness was the highest recorded in all assemblages (J = 1.0); the Berger Parker index was median (d = 0.5) (Table 4). However, due to the low number of infections, these diversity indices are not very conclusive.

Component communities in *S. nitida* (n = 2) comprised only one trematode species each. The two species recorded in the component communities showed low prevalence. Due to the low number of distinctive *S. nititda* communities with only one infection each, they were excluded from the component community analysis.

The three species found in *S. nitida* utilize either anatid birds as final host (2 species), or fish and fish-eating birds as second intermediate and definitive host (1 species; Table 3).

Table 4 Trematode diversity in the six snail species. *Abbreviations*: Ba, Baldeneysee; He, Hengsteysee; So, Sorpetalsperre; Hn, Hennetalsperre; Ve, Versetalsperre.

			Radix auricularia	***Radix peregra***	***Lymnaea stagnalis***	***Stagnicola palustris***	***Gyraulus albus***	***Segmentina nitida***
Overall		S	23	6	4	9	10	3
		N_{INF}	596	25	35	87	273	4
		Shannon H	2.3	1.5	0.8	1.2	1.3	1.0
		Shannon evenness J	0.7	0.9	0.6	0.5	0.5	1.0
		Berger-Parker dominance d	0.3	0.4	0.8	0.6	0.5	0.5
		Average taxonomic diversity Δ	79.8	63.2	36.6	55.5	54.0	72.2
Lake	Ba	S	10		3	3	3	
		N_{INF}	61		8	6	8	
		Shannon H	1.2		0.9	1.0	0.9	
		Shannon evenness J	0.5		0.8	0.9	0.8	
		Berger-Parker dominance d	0.7		0.6	0.5	0.6	
		Average taxonomic diversity Δ	45.9		48.8	68.9	60.7	
	He	S	19	6	3	9	2	
		N_{INF}	300	18	27	80	2	
		Shannon H	2.2	1.5	0.6	1.0	0.7	
		Shannon evenness J	0.7	0.9	0.6	0.5	1	
		Berger-Parker dominance d	0.3	0.3	0.8	0.7	0.5	
		Average taxonomic diversity Δ	80.7	63.6	33.1	49.0	100.00	
	So	S	13			1*		
		N_{INF}	157			1*		
		Shannon H	2.0			-*		
		Shannon evenness J	0.8			-*		
		Berger-Parker dominance d	0.3			- *		
		Average taxonomic diversity Δ	77.6			-*		
	Hn	S	10				8	3
		N_{INF}	78				262	4
		Shannon H	2.1				1.2	1.0
		Shannon evenness J	0.9				0.6	2.8
		Berger-Parker dominance d	0.2				0.6	0.5
		Average taxonomic diversity Δ	78.9				52.0	72.2
	Ve	S		2			1†	
		N_{INF}		7			1†	
		Shannon H		0.6			-†	
		Shannon evenness J		0.9			-†	
		Berger-Parker dominance d		0.7			-†	
		Average taxonomic diversity Δ		47.6			-†	

*Only one infection in sample

† Sample size small (< 14 snails), only one infection in sample

Component community structure

For the analysis of individual trematode component communities only distinct samples comprising more than 14 snails were used, resulting in a total of 75 distinct samples with trematode communities (48 samples of *R. auricularia*, 10 samples of *G. albus*, 7 samples of *S. palustris*, 6 samples of *L. stagnalis* and 4 samples of *R. peregra*).

The non-metric MDS ordination revealed differences in composition and structure of trematode component communities. Although the ANOSIM test revealed a significant difference for the factor 'reservoir' (but with a low R = 0.189, p = 0.001), no overall structure for this factor was visible in the MDS ordination (Figure 4A, stress value 0.1), and the differences are rather due to the two isolated groups of samples from Hennetalsperre and Hengsteysee. A clear structuring pattern became obvious for the factor 'snail host' that revealed clear differences in composition and structure of trematode communities in *Radix auricularia*, *Radix peregra*, *Stagnicola palustris*, *Lymnaea stagnalis* and *Gyraulus albus* (Figure 4B, stress value 0.1). This was supported by the results of the ANOSIM with a high R = 0.637 (p = 0.001). The distinctive groups in Hennetalsperre and Hengsteysee are clearly due to the different snail hosts of these communities.

Temporal and spatial variation of trematode communities in *Radix auricularia*

Spearman's rank order correlations of *Radix auricularia* communities showed significant positive correlations between sample size and overall prevalence (r_s = 0.322, p = 0.02) as well as species richness (r_s = 0.473, p = 0.0003), indicating more species rich and prevalent trematode communities in localities with abundant *R. auricularia* populations. Mean snail size in the individual host populations was positively correlated with overall prevalence (r_s = 0.338, p = 0.01) but not with species richness (r_s = 0.23, p = 0.1), revealing higher infections levels but not more parasite species in populations with larger, i.e. older snails.

Correlations showed that overall prevalence is probably affected by the size of snails (mean length tested). In order to avoid this effects while evaluating possible differences in overall prevalence between lakes, seasons and years to examine spatial and temporal variation in overall prevalence, we performed sets of ANCOVAs using 'season', 'lake' and 'year' as factors and 'mean snail length' as a covariate to control for the effect of snail size. After the effect of snail size was accounted for, no significant differences in overall prevalence was detected between lakes, seasons and years, indicating spatially and temporarily stable communities in *R. auricularia* in the Ruhr ecosystem.

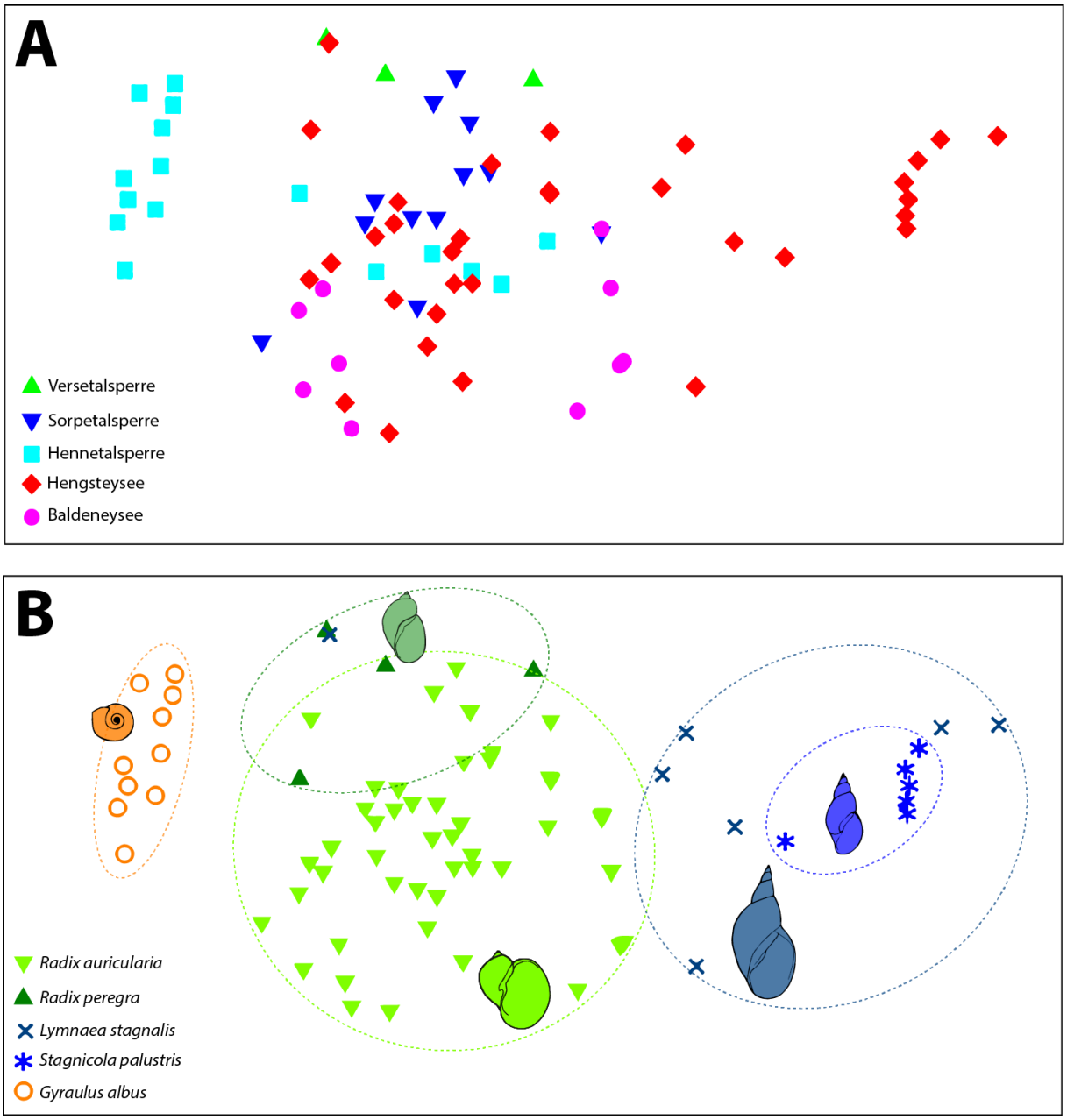

Figure 4 Two-dimensional MDS ordination plot of 75 trematode component communities based on the similarity in trematode component community structure (Bray-Curtis index). A: Ordination plot for the factor 'reservoir'; B: Ordination plot for the factor 'snail host' (stress value = 0.1).

Discussion

The 5,347 snails belonging to six species revealed a species-rich and diverse trematode fauna in the Ruhr reservoirs with a total of 37 different trematode species belonging to nine families. This is considerably higher than the trematode species richness in snail intermediate hosts described from most other well-studied ecosystems. In their extensive study of the parasite contribution to the biomass in an estuarine saltmarsh system, Kuris and colleagues (2008) examined 14,000 snails belonging to 11 species and found 18 recognised trematode 'castrator' species. In studies from the same ecosystem, the most abundant snail species in the saltmarshes, the California horn snail *Cerithidea californica* (see Huspeni & Lafferty 2004), showed high trematode prevalence (30.1%, 926 out of 3,079) and revealed a species-rich trematode fauna with 16 species (Hechinger et al. 2007). Altogether, this provides a comparable situation to our system, with respect to one snail host species, in our case *Radix auricularia*, harbouring the most species-rich trematode assemblages. However, the species richness of the trematode assemblage in *R. auricularia* (23 species), as well as the overall species richness (37) in the Ruhr reservoir system are much higher than the 16 species in *C. californica* and the total of 18 species described in the extensive sampling from the Californian estuary system, despite the much larger number of potential host snail species studied in the latter. Thieltges et al. (2006) studied the macroparasite community in molluscs in the Wadden Sea and found 26 trematode species from 10 gastropod and bivalve host species, again a much lower species richness than in the Ruhr reservoirs, despite the wider host range. Likewise, Lagrue & Poulin (2015) investigated the entire community of free-living and parasitic metazoans from the littoral zone of four lakes in New Zealand, yet only 11 trematode species that utilize a single snail species, *Potamopyrgus antipodarum*, as first intermediate host were found. An extensive study of freshwater ponds in North America investigated 10,821 snails of one species, *Helisoma trivolis* (Richgels et al. 2013), and revealed six trematode groups that were, however, not identified beyond the genus level, making a precise diversity assessment impossible, but staying under the diversity found in the Ruhr system.

Comparable studies from European freshwaters are mostly from small inland freshwaters. Faltýnková & Haas (2006) sampled 6,403 molluscs of 28 species, of which 15 harboured a total of 31 trematode species. However, these data are pooled across a variety of different habitats all over Southeast Germany including rivers, ponds and lakes, and include a much wider intermediate snail host range. Similarly, ponds in the Czech Republic harboured 28 larval trematode species from 12 species of molluscs (Faltýnková 2005). Most comparably to our study, six snail species (10,581 snails) studied from Polish reservoirs were shown to be parasitized by 25 species of digeneans, most of which were recorded from *L. stagnalis* (Żbikowska 2007). Likewise, the study by Loy & Haas (2001) describes a much more species-rich trematode fauna for *L. stagnalis* (18 species) than found in the same host in this study in the Ruhr area (4 species). However, the study summarizes data from 43,000 snails

sampled from 174 ponds over a period of 20 years, making a direct comparison to local host-parasite interactions not feasible. Community-based approaches from fishponds in the Czech Republic revealed a similarly diverse trematode fauna with 14 species found in 7,600 *L. stagnalis* (Soldánová et al. 2012). A first study from the Ruhr area (Soldánová et al. 2010) found *L. stagnalis* to be infected with six species, three of which we did not encounter in our sampling, resulting in a total of seven trematode species described from this host in the Ruhr region so far. This is still considerably less than in the above mentioned studies and the 23 trematode species described in this host from Central Europe (Brown et al. 2011 and references therein).

Radix auricularia on the other hand harboured the most species-rich and diverse trematode fauna of all studied hosts in the Ruhr system (Figure 3), and by far outnumbers the 12 species initially found in this system (Soldánová et al. 2010). This high species richness is in stark contrast to what is described from this snail species in the literature. Initial compilations of the cercariae species recorded in lymnaeid snails counted six species in *R. auricularia* (Bargues et al. 2001 and references therein) and sampling data from Southeast Germany (4 trematode species, Faltýnková & Haas 2006), the Czech Republic (3 species, Faltýnková 2005) and Poland (1 species, Żbikowska 2007) supported the low number of trematode species in this host. The highest species richness recorded are eleven species of cercariae recovered from *R. auricularia* in a gravel pit in the United Kingdom (Adam & Lewis 1993).

The remarkably high trematode species richness in *R. auricularia* in the Ruhr system supports the assumption that *R. auricularia* plays the most important role in the life cycle in large reservoirs and lakes, in contrast to the more dominant role of *L. stagnalis* in small pond systems (see Soldánová et al. 2010 and reference therein). The only snail species that was equally abundant in the Ruhr was *G. albus,* but the trematode richness and diversity were far lower in this snail host (see Figure 2; Figure 3). *G. albus* was only abundant at one locality where it dominated the snail community. The other planorbid snail species, *S. nitida*, occurred at the same locality but was less abundant and harboured a less species-rich and diverse trematode assemblage, although with a higher evenness and higher taxonomic distinctness, most likely to the limited number of samples from this host (see Table 3). Interestingly, an overall survey of larval trematodes of planorbid snails showed *S. nitida* to be more abundant and with higher trematode prevalence (6.2%) compared to *G. albus* (0.9%) (Faltýnková et al. 2008). Since these data are from small ponds in Germany and the Czech Republic, it is well possible that the same reversed role that applies to *R. auricularia* and *L. stagnalis* is true for small planorbid snails in large reservoir systems. The data from the Ruhr, therefore, suggests a characteristic host-parasite dynamics in such large reservoir systems.

Host population density and host body size have been identified as two universal determinants of parasite species richness in hosts, since dense host populations are more likely to be colonised by parasite species and large bodied hosts provide greater space and other resources to the parasites

(Kamiya et al. 2014 and references therein). The positive correlations between trematode species richness and sample size (reflecting host population density) confirmed this for both the overall host level, as well as at the component community level for *R. auricularia*. However, for the studied host species in the Ruhr reservoirs, trematode species richness was not correlated with the overall size of the snail host species, since some small-bodied hosts harboured considerably more species-rich trematode assemblages than larger bodied hosts, i.e. the trematode assemblage in *G. albus* was more species-rich than in *S. palustris*, which in turn harboured more trematode species than *R. peregra*; and *R. auricularia* harboured almost six times the species richness of the larger *L. stagnalis*. Within the component communities of *R. auricularia*, significant positive correlations of both sample size and mean snail size with prevalence were revealed, indicating that snail populations with larger, older host individuals harbour more prevalent trematode communities, most likely due to longer potential recruitment period, and that abundant local populations of *R. auricularia* offer ideal conditions for the asexual reproduction of digenean trematodes. However, after the effect of snail size was accounted for, no significant differences in overall prevalence was detected between reservoirs, seasons and years, indicating spatially and temporarily stable trematode communities in *R. auricularia*.

The two-dimensional MDS ordination plot of 75 trematode component communities supported this and did not show a conclusive structure of the communities in different reservoirs (Figure 4A), although the ANOSIM test revealed a significant difference for the factor 'reservoir' (but with a low $R = 0.189$, $p = 0.001$). These differences are rather due to the two isolated groups of samples from Hennetalsperre and Hengsteysee. A clear structure in the trematode communities was revealed for the factor 'snail host' and clearly supported by the results of the ANOSIM with a high $R = 0.637$ ($p = 0.001$). It is, therefore, the structure of snail host populations in the waterbodies that shape the trematode community structure.

Almost all of the trematode species found in *R. auricularia* utilize birds as final hosts, mainly either anatid or fish-eating birds that overwinter in the Ruhr area, due to the abundant food resources available at the reservoirs. This continuous presence of final bird hosts that can migrate among the reservoirs and the presence of abundant snail host populations can explain the seasonal and spatial homogeneity in this system. Interestingly, this contrasts with the variable trematode communities in *L. stagnalis* described from smaller waterbodies in Central Europe (Soldánová et al. 2011). Altogether, the results highlight the important and integral role of digenean trematodes in the Ruhr reservoir system. From the parasites' perspective, the patchy snail populations represent habitable islands and within the reservoirs it is the availability of hosts in the ecosystem that shapes the distribution and structure of trematode communities.

Having identified the diversity and structure of trematode assemblages and communities in the Ruhr reservoirs, we may ask what information about the ecosystem this can reveal. The majority

of the 37 trematode species identified in the six snail intermediate host species use birds as definitive hosts to complete their life cycle, only two species require cyprinids and one species amphibians. Most of the species utilizing birds either fall within the large guild of parasites that use fish and fish-eating birds (mainly cormorants, gulls or grebes), or belong to the generalist guild parasitizing waterfowl (mostly anatids) as final hosts. The remaining species use either birds of the families Rallidae (rails) or Ciconiidae (storks), or a wider host spectrum of various birds or mammals.

Altogether, this information on the life cycle and required host species allows the reconstruction of the transmission pathways the individual parasite species take through the ecosystem (Figure 5). Since the infections of snails as intermediate hosts require final hosts to release eggs into the ecosystem, the transmission pathways provide accurate information on the presence of these hosts in the ecosystem. Especially in the case of highly mobile birds that can migrate some distances and may not be present at the sampling site at all times, the occurrence of trematode infections in snails can provide evidence of the local host distribution. Trematode infections, therefore, provide an integral of past final host presence at a locality (Hudson et al. 2006). This can be especially useful in the case of rare species, such as storks, where trematode infections in snails may be used to map host distribution, e.g. with regard to conservation biology. Such examples highlight practical applications where trematodes may serve as bionindicators of free-living diversity and species distribution (Hechinger et al. 2007).

Furthermore, all transmission strategies, except for the direct infection of final hosts by the cercariae (e.g. in bird schistosomes), involve trophic transmission to the final host and thus provide information on trophic interactions and energy flow within the ecosystem. This connectivity exposes to what extent intra-host stages of digenean trematodes are embedded in larger food webs. Moreover, trematode infections can have drastic effects on second intermediate hosts and lead to behavioural changes that make parasitized prey easier for predators to capture (Lafferty & Morris 1996). Therefore, more than mere ‘blind passengers’, parasites often directly or indirectly manipulate their hosts (Thomas et al. 2005) and thereby actively shape the structure of food webs through which they are transmitted, thus regulating host population dynamics and influencing the community structure of free-living species (Marcogliese 2004). The large percentage of trophically transmitted parasite species underlines their central structuring role in the food webs of the Ruhr reservoir ecosystem.

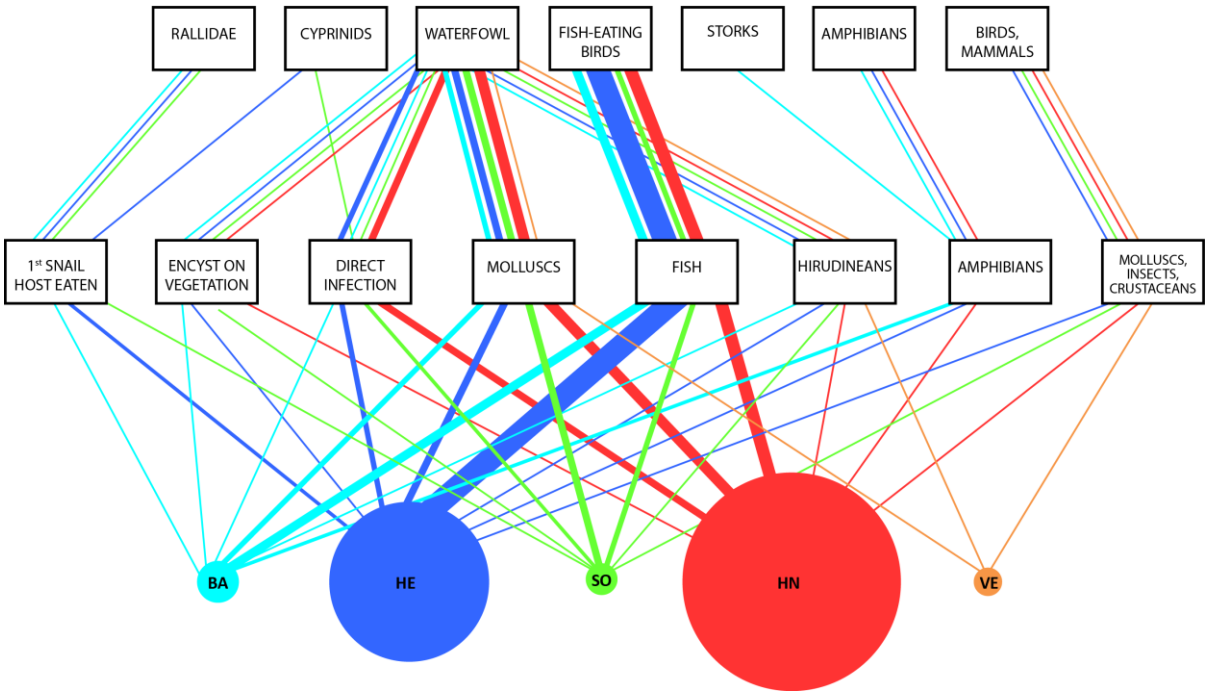

Figure 5 Scheme of transmission pathways of individual trematode species found in the snail populations in the five studied reservoirs. The size of the circles at the bottom is proportional to the size of the overall snail populations in each reservoir (pooled across snail species, sampling sites and years), boxes in the middle represent 2nd intermediate hosts, boxes at the top final host groups. The lines indicate trematode species utilising individual transmission pathways, thickness of the lines represents the number of trematode species utilising each transmission pathway. *Abbreviations*: BA, Baldeneysee; HE, Hengsteysee; SO, Sorpetalsperre; HN, Hennetalsperre; VE, Versetalsperre.

Environmental changes, such as local anthropogenic habitat alterations or long term climate changes and global warming will have severe effects on whole ecosystems, with many often unpredictable consequences for the free-living and parasitic species therein. Since parasites with complex life cycles may serve as early warning indicators of ecological changes to ecosystem health or environmental conditions (Marcogliese 2004 and references therein), and especially trematode diversity indices have been shown the provide prospective and valuable bionindicators (Shea et al. 2012), this dataset from the Ruhr reservoirs may serve as a baseline to assess and interpret possible future changes in this ecosystems. Furthermore, together with other well-studied host-parasite assemblages from other ecosystems, this work shall help to facilitate our understanding of parasite distribution and diversity, and shall allow us to better understand (and maybe foresee) how environmental changes will affect parasites and their hosts on a larger scale. Therefore, while the cumulative curve of known parasite species is nowhere near levelling off and we may not (yet) be able to precisely approximate the total number of parasite species in the world (see Poulin 2014), we can do so at a local ecosystem level to better understand the role and distribution of parasites. Hidden diversity is not restricted to the tropics and the deep-sea but might be sitting right on our doorstep, and it can provide valuable information on our environment.

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9. General discussion

9. General discussion

As outlined in the introduction, parasites have recently been recognised as important and integral elements of ecosystems in which they play crucial roles. However, while some very well-studied systems have provided insights into the multifaceted roles of parasites, data from freshwater reservoir systems in Europe is lacking, despite the vital function of such man-made waterbodies for human usage, e.g. as drinking water supplies, and as ecosystems that support biodiversity. By zooming in on various relevant aspects of trematode infections in snails, namely taxonomy, biomass productivity, human health relevance and their contribution to biodiversity and structure in ecosystems, the individual studies of this thesis illustrate to what extent digenean trematodes are embedded within the interconnected reservoir system of the Ruhr area. This holistic approach provides a detailed and comprehensive overview of the complexity and centrality of trematodes in typical reservoir systems in Europe. In the following, I will discuss the central findings of the individual studies and provide a brief outlook at further research questions and possible follow-up studies resulting from this work.

The morphological species identification of trematodes is often problematic, especially due to the presence of groups with morphologically similar cryptic species. The incorporation of molecular data has allowed unambiguous species identification and the re-evaluation of morphological identification criteria and thus considerably advanced our understanding of trematode taxonomy. Furthermore, molecular tools provide effective means of inferring complex trematode life cycles by matching sequences from different developmental stages that were sampled at different times and/or locations (Criscione et al. 2005, Pérez-Ponce de León & Nadler 2010). In order to identify the diversity of trematodes in the Ruhr, integrative approaches that combine both morphological and molecular methods were used to approach taxonomically questionable groups.

These combined morphological and DNA-based approaches allowed the delineation of two cryptic species of the '*revolutum*' group of the genus *Echinostoma* from larval stages found in *R. auricularia*, *R. peregra* and *S. palustris* (see Georgieva et al. 2013b, Chapter I). The inclusion of samples from Iceland showed that both species lineages were present in central and northern Europe, indicating a wide geographical distribution of the parasites with their final bird hosts. Furthermore, the analysis of comparable molecular data from North America provided evidence that these isolates previously identified as *E. revolutum* represent yet another cryptic species of the '*revolutum*' species complex and the suggested cosmopolitan distribution of this species (Detwiler et al. 2010) may actually result from overlooked cryptic variation within this genus. The results from this study highlight the hidden diversity in this well-studied group of parasites and the need for further integrative taxonomic

studies to advance our understanding of the complex relationships within the '*revolutum*' group of *Echinostoma*.

The abundant *Gyraulus albus* population found at one locality in Hennetalsperre harboured larval trematodes of the genus *Petasiger* that provided another taxonomically uncertain group, which required an in-depth taxonomical investigation (see Selbach et al. 2014, Chapter I). Comparative molecular and morphological analyses revealed the distinctive status of three species of *Petasiger* from *G. albus* that occurred in sympatry at one locality in Hennetalsperre. This highlights the high diversity of *Petasiger* spp. in one host species even at small spatial scales, whilst at the same time showing the suitability of the Ruhr reservoirs as a habitat for trematodes. Overall, the high diversity of *Petasiger* spp. at such a small scale suggests a much higher number of species in this genus than the seven species that have been recorded or described in Europe so far (see Faltýnková et al. 2008b).

A third group that required close attention was the genus *Diplostomum*, a major taxonomic group of trematodes that use lymnaeid snails, fish and fish-eating birds to complete their life cycle, and constitute important fish pathogens, especially in aquaculture (Overstreet & Curran 2004, Blasco-Costa et al. 2014). Recent molecular studies have discovered a much higher diversity within this group than previously expected in samples from North America (Locke et al. 2010) and Europe (Blasco-Costa et al. 2014, Faltýnková et al. 2014, Pérez-del-Olmo et al. 2014), including the Ruhr area (Georgieva et al. 2013a). The study from the Ruhr reservoirs by Georgieva and colleagues (2013a) showed the presence of six species, including three different lineages in the '*D. mergi*' species complex. However, since most of the samples from this study were from fish intermediate hosts and only few isolates from snails were available, the samples from lymnaeid snails obtained during this dissertation provided a chance to thoroughly assess the diversity of *Diplostomum* spp. from their snail intermediate hosts (*R. auricularia*, *R. peregra*, *L. stagnalis* and *S. palustris*) in this region for the first time. Altogether, seven different species of *Diplostomum* were found in the snail populations from the Ruhr reservoirs, three named species, *D. spathaceum*, *D. pseudospathaceum* and *D. parviventosum*, and four species-level lineages, '*Diplostomum* sp. Clade Q' and '*D. mergi*' Lineages 2-4 (see Selbach et al. 2015, Chapter I). This study is the first in-depth integrative approach of molecular and morphological methods to *Diplostomum* diversity in natural snail populations in Central Europe and provides the first description of molecularly identified cercariae of *D. spathaceum*, as well as providing evidence that the cercariae of '*D. mergi*' Lineage 1 of Georgieva et al. (2013a) are actually *D. parviventosum*. These findings therefore advance our knowledge of the complicated taxonomic situation of *Diplostomum* spp. in Europe and the molecular and morphological data will serve as a baseline for future studies of the diversity of this important group of parasites. Remarkably, *L. stagnalis* and *S. palustris* harboured only one species, *D. pseudospathaceum*, while *R. auricularia* populations revealed a highly diverse picture with six different species of *Diplostomum*. This provides a promising case to study the co-

evolutionary development of host-parasite specificity, especially since these lymnaeid snails share the same habitats and are widely distributed. Interestingly, with its Holarctic distribution, *L. stagnalis* has a wider geographical range than the Palearctic *R. auricularia* (Glöer 2002), and *L. stagnalis* is a much larger host than *R. auricularia*. Both host body size and host geographical range have been identified as key determinants of parasite species richness (Kamiya et al. 2014) and it remains to be investigated why the diversity of *Diplostomum* in these hosts presents such contrary situation.

Altogether, the taxonomical studies of these three trematode genera reveal a remarkably high diversity of these parasites in the Ruhr reservoir system. Future findings of *Diplostomum* spp., *Petasiger* spp. or *Echinostoma* spp. isolates from their respective second intermediate or definitive hosts would be desirable and could be matched via the molecular information provided in the publications to infer the complete parasite life cycles and fully describe these species. In order to be able to use trematodes as bioindicators of free-living diversity and to assess and evaluate habitat alterations and ecosystems changes (Lafferty 1997, Vidal-Martínez et al. 2010, Nachev & Sures 2015), we need to know which species are present and able to complete their life cycles in an ecosystem. A clear species identification and knowledge about the parasites' biology is a central requirement for the use of trematodes as bioindicators. The individual studies presented in Chapter I therefore provide the foundation for the subsequent ecological study of trematode diversity and distribution in the Ruhr reservoirs.

However, the knowledge of the sheer diversity of trematodes in snails does not yet allow us to assess their functional role within an ecosystem. Parasites with complex life cycles such as trematodes often include numerous asexually reproduced dispersal stages in order to facilitate transmission success to the next host. Therefore, despite their small size, the high production of free-living larval stages of trematodes in snails has been calculated to amount to a significant contribution to an ecosystem's biomass (Kuris et al. 2008, Thieltges et al. 2008, Preston et al. 2013, Lambden & Johnson 2013). However, no studies on the productivity and biomass contribution of trematode species commonly found in European freshwater systems were available.

In order to assess the contribution of trematode cercariae to the biomass in European freshwaters, cercarial emergence of the model organism *Trichobilharzia szidati* from naturally infected *Lymnaea stagnalis* was studied in a set of laboratory experiments (Soldánová et al. submitted, Chapter II). Furthermore, since bird schistosomes of the genus *Trichobilharzia*, which utilize freshwater snails and waterfowl in their life cycle, are the most important agents of swimmer's itch in humans in Europe (Soldánová et al. 2013, see Appendix II) and North America (Brant & Loker 2009, 2013), temporal emission patterns were observed to allow a better understanding of the parasites' ecological relevance and potential epidemiological consequences. The study revealed an average daily emergence rate of 2,621 cercariae per snail, but emissions could reach peaks of up to 29,560 cercariae per snail and day,

a magnitude that has never been reported before for bird schistosomes. Based on the mean emission rates and cercarial measurements, the cumulative biomass contribution of *T. szidati* larvae could be assessed. Calculated for an individual snail's lifetime this summed up to 4.8 g, a mass equivalent to or even exceeding the snail's own body mass. This illustrates the ecological importance of trematodes at the host-parasite level, since the parasites relocate a substantial amount of the host's resources into cercarial production. On an ecosystem level, the results show how a single trematode species contributes a considerable amount of cercarial mass to an aquatic ecosystem during the parasite's active period in the summer months of a year. While annual ecosystem level biomass contributions of 4.6 tons of cercariae were calculated for small eutrophic ponds where *T. szidati* prevalence of more than 40% have been reported (Soldánová et al. 2011), prevalence of *Trichobilharzia* was typically lower in the Ruhr system. However, bird schistosomes only constitute a small fraction of the species-rich and diverse trematode fauna in snails in the Ruhr reservoirs (see Chapter IV) and the productivity of other trematode species can even be significantly higher than in bird schistosomes, e.g. in *Diplostomum* spp. with productions of up to 60,000 cercariae per snail and day (Lyholt & Buchmann 1996, Karvonen et al. 2004), and well beyond up to 500,000 cercariae per snail and day for some species (Haas 2003). It is therefore safe to assume that the overall cercarial biomass emitted into the Ruhr reservoirs is comparable to the impressive numbers recently calculated for marine (Thieltges et al. 2008), estuary (Kuris et al. 2008) and North American freshwater ecosystems (Preston et al. 2013). The results of this study demonstrate how trematodes, despite their small individual size, contribute to the biotic productivity in the Ruhr freshwater system.

With respect to human infection risks, the occurrence of bird schistosomes in Baldeneysee (Lake Baldeney) was studied to evaluate the suitability of the Ruhr reservoirs as potential bathing waters (Selbach et al. 2015, Chapter III). Since swimmer's itch is considered a re-emerging disease in Europe (Soldánová et al. 2013) and individual cases of swimmer's itch have been reported from the Ruhr river system in the past (AWWR & Ruhrverband 2008), we assessed the disease risk factors in the Ruhr area based on the occurrence, distribution and biology of bird schistosomes. Two bird schistosome species, *Trichobilharzia franki* and *T. szidati,* could be detected at several sampling sites in Baldeneysee where abundant lymnaeid snail populations were present. Together with samples obtained during a preliminary study at the Ruhr reservoirs (Soldánová et al. 2010), the combined data provides a coherent overview of the distribution of bird schistosomes in the waterbody and allows a thorough risk assessment of human infection based on the parasites' and hosts' biology. Although *Trichobilharzia* spp. showed low prevalence at the sampling sites in Baldeneysee (0.6 – 3%), this is typical for bird schistosomes and rather common in areas where swimmer's itch occurs in humans (Loy & Haas 2001, Żbikowska 2004). Despite these low prevalence values, human infections are well possible due to the high numbers of cercariae that can be released from individual infected snail hosts

in short periods of time, as we were able to show in the emission studies (see Soldánová et al. submitted, Chapter II). Given that a single infected *L. stagnalis* is able to release nearly 30,000 cercariae per day under favourable conditions, a few snails with *Trichobilharzia* infections can create potential infection hot spots for humans, especially in shallow areas where cercariae may become densely concentrated. Besides the spatial localisation of the cercariae, temporal release patterns play an important role for the risk of an infection, as most infections of swimmer's itch usually occur in the morning hours (Lindblade 1998, Verbrugge et al. 2004). Accordingly, main cercarial emission peaks in our laboratory experiments (Chapter II) were observed after the onset of illumination, indicating a synchronized emission of cercariae with the main activities of the anatid definitive hosts. However, under favourable lightning conditions, high cercarial emission was shown to be possible at different times throughout the day, demonstrating that infections can occur at any time of day, especially since cercariae remain infective for several hours after being released into the environment. Altogether, the assessment of the Ruhr, by way of example of Lake Baldeney, revealed that *Trichobilharzia* spp. present a potential risk of human infections in these waterbodies. It therefore seems advisable to communicate such potential risks of swimming in surface waters to the public and provide adequate and understandable information at swimming sites, e.g. information boards on swimmer's itch. Such a raised awareness will not only help to better identify cases and thus facilitate our understanding of the disease's occurrence, but may also allow a factual discussion of the problem free of exaggerated alarmism (Soldánová et al. 2013). After all, bird schistosomes, along with the other trematode species, are integral and natural parts of the ecosystem and the risk of swimmer's itch comes with the use of freshwater lakes as bathing waters.

Having looked at the high taxonomic complexity, the fundamental contribution of trematode cercariae to biotic productivity, both on the individual host and the ecosystem level, as well as discussing the important role of some species as human pathogens, the last study of this thesis (Chapter IV) provides an overview of the overall diversity and structure of trematode assemblages and communities in the Ruhr reservoirs. The 37 different trematode species found in lymnaeid and planorbid snails reveal the important role of these snails as first intermediate hosts for digenean trematodes in the Ruhr reservoir system. Altogether the diversity in the Ruhr reservoir system is considerably higher than the trematode species richness in snail intermediate hosts described from most other well-studied ecosystems (e.g. Faltýnková 2005, Faltynkova & Haas 2006, Thieltges et al. 2006, Żbikowska 2007, Kuris et al. 2008, Lagrue & Poulin 2015). In the Ruhr reservoirs *Radix auricularia* harbours by far the most species-rich and diverse trematode fauna of all studied hosts, which supports the initial assumption that *R. auricularia* plays the most important role in the life cycle of trematodes in large reservoirs and lakes, in contrast to the more dominant role of *L. stagnalis* in small pond systems (Soldánová et al. 2010 and reference therein). Remarkably, the same seems to apply to planorbid

species in the large Ruhr reservoirs, where *G. albus* harbours more diverse and prevalent trematode communities compared to *S. nitida*, which contrasts with the situation observed for these two species in small ponds (Faltýnková et al. 2008a). The analysis of the data from the Ruhr, therefore, suggests a characteristic host-parasite dynamics in large reservoir systems.

The majority of the trematode species found in snail intermediate hosts in the Ruhr requires birds as definitive hosts to complete their life cycle, with most of the species either falling within a large guild of parasites that use fish and fish-eating birds (mainly cormorants, gulls or grebes), or belonging to a generalist guild parasitizing waterfowl (mostly anatids) as final hosts. Based on the knowledge of the required intermediate and final hosts, transmission strategies of the individual parasite species could be analysed. Except for the direct infection of final hosts by the cercariae of bird schistosomes, all transmission strategies involve trophic transmission of the parasites to their respective final host, which provides information on trophic interactions and energy flow in the ecosystem. Furthermore, since trematodes often directly or indirectly alter their host's behaviour in order to facilitate transmission success to the next host (see e.g. Lafferty & Morris 1996), they actively shape the structure of food webs through which they are transmitted. In addition to these life cycle stages within the hosts, the free living parasite larvae contribute significantly to the energy flow in aquatic systems, since the majority of produced cercariae are not able to successfully infect a suitable target host and end up as food for predators or contribute to the ecosystem's detritus (Johnson et al. 2010, Morley 2012). This illustrates how a single trematode species acts at many different levels in food webs at the same time. Therefore, the rich and abundant trematode fauna in the Ruhr freshwaters plays a highly complex role in the food web connectivity in the Ruhr reservoirs.

Based on the novel insights gained from the individual studies of this thesis, the important and central role of digenean trematodes in reservoirs becomes obvious (see Figure 4). Furthermore, we can obtain valuable information on inter-specific interactions in these systems by looking at the parasites' life cycles. However, does that imply that such an ecosystem is a healthy one? Being 'healthy' is a rather vague conception and often equated with being pristine or free of illness (Hudson 2006). Accordingly, one would certainly consider an ecosystem with such a rich and prevalent parasite fauna far from being healthy, and this study has not even included parasitic nematodes or cestodes. The health of an ecosystem, however, is not defined by the presence or absence of parasite species, but rather by the performance and sustainability of the whole system, i.e. its ability to be resilient and to maintain its organization (structure and biodiversity) and vigor (function and productivity) over time (Costanza & Mageau 1999). The studies of this thesis show how digenean trematodes significantly contribute to the biodiversity, food web connectivity and productivity in the Ruhr reservoir. Therefore, far from diminishing the ecosystem's health, the rich trematode fauna in the Ruhr contributes to key aspects that make this ecosystem more diverse, productive and stable, and thus healthy.

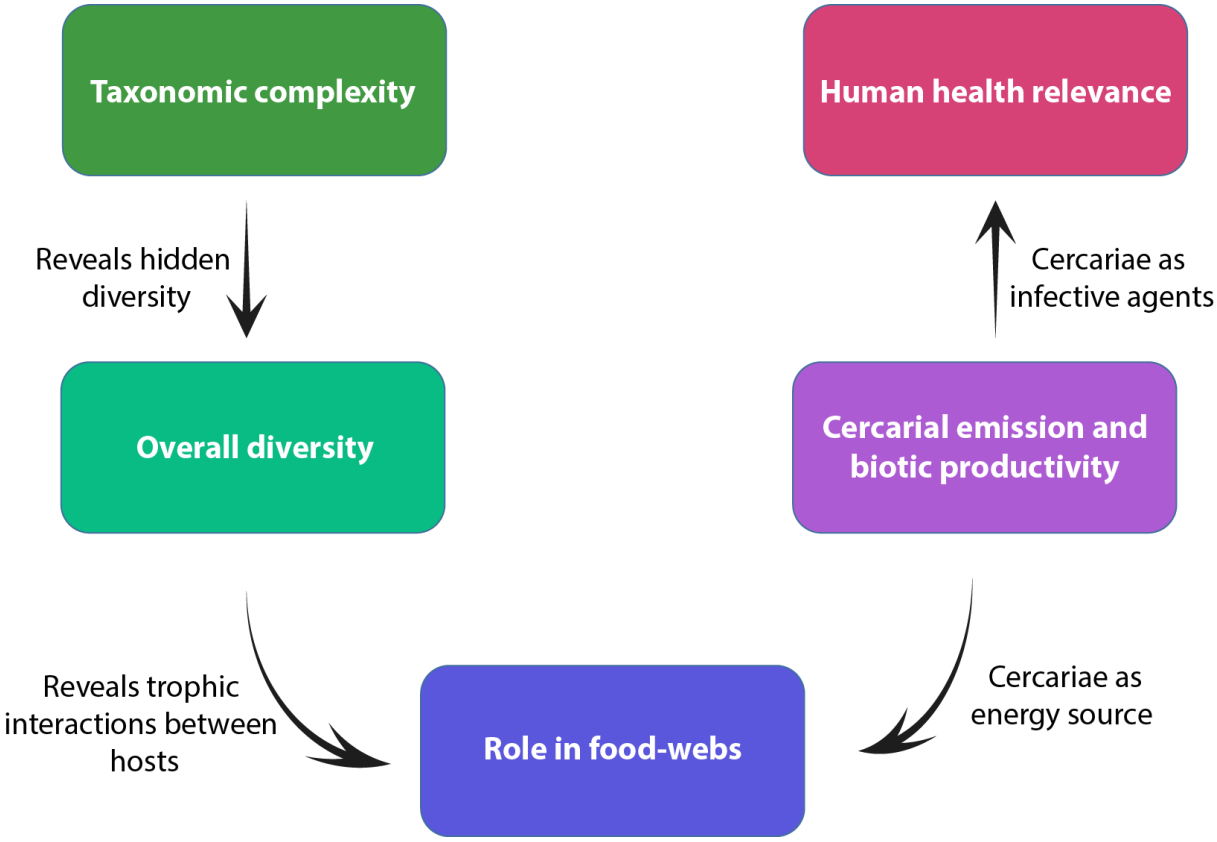

Figure 4 Overview of the main aspects of trematode infections in snail populations studied in the Ruhr reservoir system

The selected aspects of trematodes in the Ruhr river system studied in the context of this thesis give a broad and comprehensive overview of these parasites in a freshwater ecosystem that is typical for freshwater reservoirs in Europe. Of course, a thesis can never fully cover all aspects of a given topic, especially when it is such a complex one as the role of host-parasite systems in an ecosystem, and for every question we solve and new insight we gain, new interesting questions arise that are worth being studied. As discussed above, the dominant role of *R. auricularia* for such a diverse and species-rich trematode fauna in large reservoir freshwaters provides an interesting model system to study host-parasite co-evolution and interaction, comparable to *L. stagnalis* as a model organism in smaller water bodies. Furthermore, the dataset of this thesis may provide a basis to assess how parasite communities will change over time, which may reveal valuable information about the dynamics of the ecosystem, especially in the light of environmental changes, e.g. whether the food web structure in the system will remain stable.

In conclusion I may ask, what have the studies of this dissertation shown and contributed? When presenting results of this work, I have often used the, maybe somewhat obvious but certainly illustrative, analogy of an iceberg to show the hidden but fundamental nature of parasites in

ecosystems. On the surface, parasites are usually not directly visible in an ecosystem, while their hosts are naturally regarded to constitute the biota that inhabit an ecosystem. For this reason parasites have traditionally been omitted from the majority of ecological studies (Poulin 2007), as discussed in the introduction. This is illusive however, since it only shows the tip of the iceberg, i.e. a small fraction of the whole ecosystem. From the parasites' perspective the hosts themselves represent patchy habitats that offer resources and shelter. Beneath the surface, parasites are deeply embedded in and active elements of the ecological processes that shape and structure ecological communities, energy flow and the biodiversity of complex ecosystems. Therefore, rather than proverbially opening a can of worms that creates irresolvable problems and confusion, the individual studies of this thesis shall advance our understanding of the central and complex roles of trematodes in the Ruhr reservoir system. Coming full circle to the initially discussed dichotomy of classical ecology and parasitology, I hope this thesis can contribute to our overall understanding of the complexity of ecosystems. After all, regardless of the different labels, e.g. ecology, parasitology, evolutionary biology etc., the fundamental and unifying purpose and aim of science are simply to better understand the complex and fascinating world that surrounds us and that we live in.

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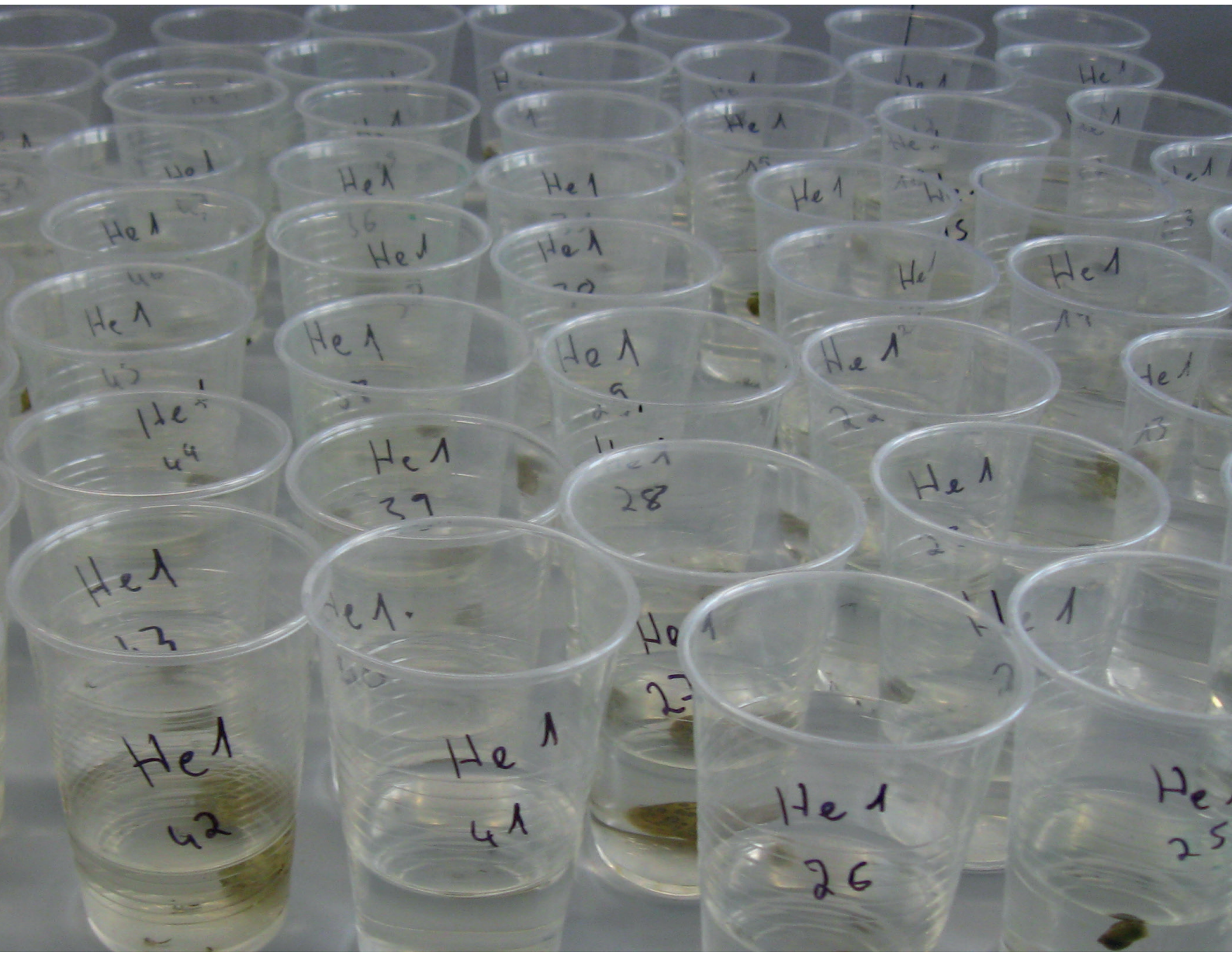
13. Abbreviations

28s rRNA	28S ribosomal ribonucleic acid
AF	*Ancylus fluviatilis*
AIC	Akaike information criteria
ANCOVA	analysis of covariance
ANOSIM	analysis of similarities
AOL	anterior organ length
AOW	anterior organ width
AV	*Anisus vortex*
Ba	Baldeneysee
BC	*Bathyomphalus contortus*
BI	Bayesian inference
BL	body length
BT	*Bithynia tentaculata*
BW	body width
C	cercaria
cox1	cytochrome c oxidase subunit 1
DJ-C	daily output experiment in the climate chamber in July
DJ-L	daily output experiment in the laboratory in July
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
FL	furca length
GLM	general linear model
He	Hengsteysee
HKY	Hasegawa-Kishino-Yano model
Hn	Hennetalsperre
ITS	internal transcribed spacer cluster
ITS1-5.8S-ITS2	see ITS
L.	Linnaeus
LS	*Lymnaea stagnalis*
M	metacercariae
MCMC	Markov chain Monte Carlo

ML	maximum likelihood
*nad*1	mitochondrial gene nicotinamide adenine dinucleotide dehydrogenase subunit 1
NJ	neighbour-joining
PC	*Planorbarius corneus*
PCR	polymerase chain reaction
PF	*Physa fontinalis*
PJ-C	peak output experiment in the climate chamber in July
PJ-L	peak output experiment in the laboratory in July
PS-C	peak output experiment in the climate chamber in September
RA	*Radix auricularia*
rDNA	ribosomal deoxyribonucleic acid
RM-ANOVA	repeated measures analysis of variance
rRNA	ribosomal ribonucleic acid
SEM	scanning electron microscope
So	Sorpetalsperre
SP	*Stagnicola palustris*
sp.	species
spp.	species pluralis
Ssp	*Sphaerium* sp.
TL	tail length
TSL	tail stem length
TSW	tail stem width
TW	tail width
Ve	Versetalsperre
VSL	ventral sucker length
VSW	ventral sucker width



14. Appendices

14.1 Appendix I
Additional files

This appendix contains the additional files to the following publication:

Selbach, C., Soldánová, M., Georgieva, S., Kostadinova, A., & Sures, B. (2015). Integrative taxonomic approach to the cryptic diversity of *Diplostomum* spp. in lymnaeid snails from Europe with a focus on the '*Diplostomum mergi*' species complex. *Parasites & Vectors, 8*, 300.

Additional file 1: Figure S1. Schematic illustration of a cercaria of *Diplostomum* spp. showing the metrical features used. *Abbreviations*: BL, body length; BW, maximum body width; AOL, anterior organ length; AOW, anterior organ maximum width; VSL, ventral sucker length; VSW, ventral sucker width; TSL, tail stem length; TSW, tail stem width (at base); FL, furca length.

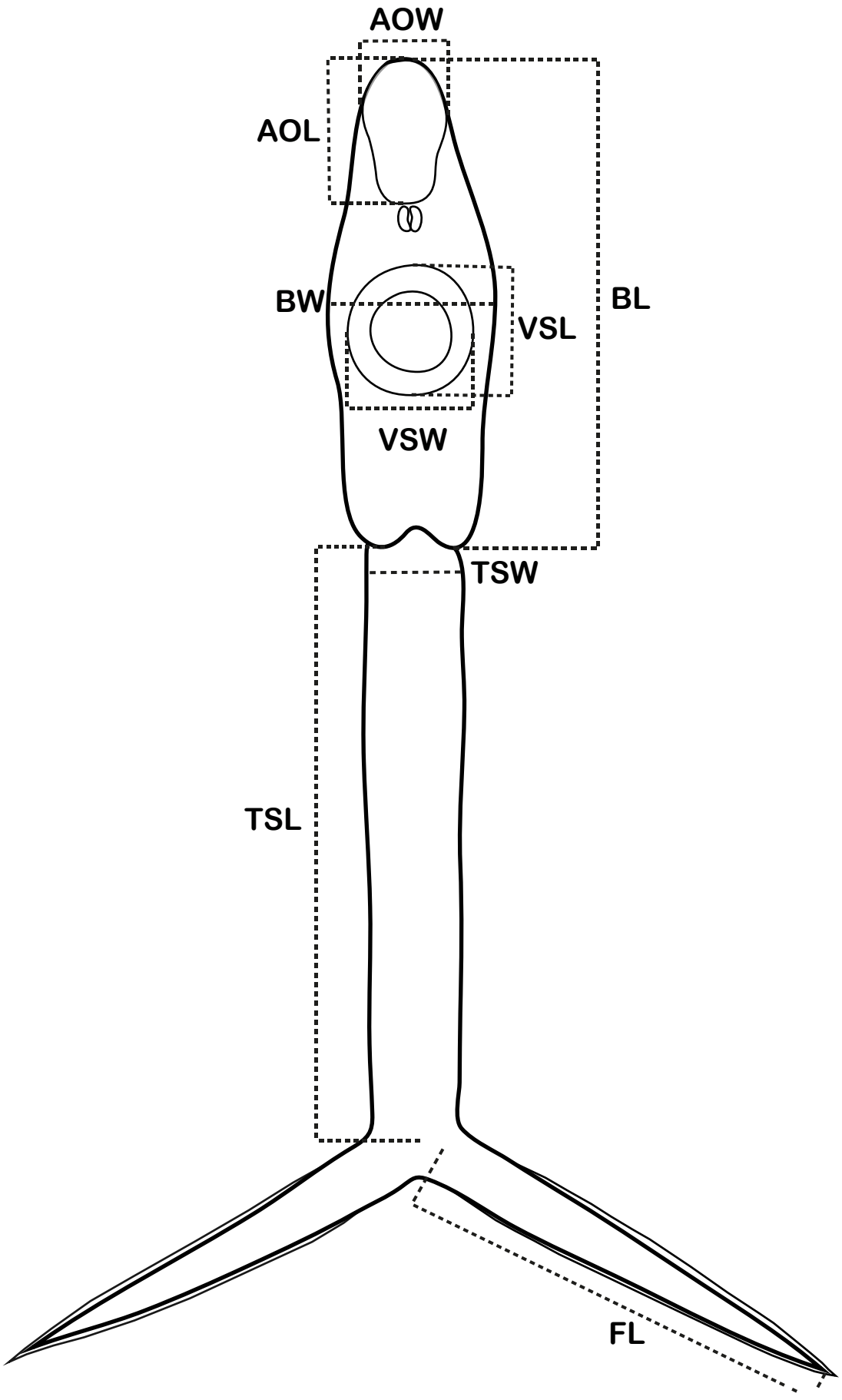
Additional file 2: Table S1. Summary data for *cox*1 sequences for *Diplostomum* spp. retrieved from GenBank.

Additional file 3: Figure S2. Cercariae of *Diplostomum* spp. Pre- and post-oral spines (light microscopy). A, *Diplostomum parviventosum*; B, *Diplostomum mergi* Lineage 4; C, '*Diplostomum mergi* Lineage 2'; D, '*Diplostomum mergi* Lineage 3'; E, F, *Diplostomum pseudospathaceum* (arrows indicate lateral spines); G. *Diplostomum spathaceum*.

Additional file 4: Figure S3. Cercariae of *Diplostomum* spp. Ventral sucker (light microscopy). A, *Diplostomum parviventosum*; B, *Diplostomum mergi* Lineage 4; C, '*Diplostomum mergi* Lineage 2'; D, '*Diplostomum mergi* Lineage 3'; E, '*Diplostomum* sp. Clade Q'; F, *Diplostomum pseudospathaceum*; G. *Diplostomum spathaceum*.

Additional file 5: Figure S4. Cercariae of *Diplostomum* spp. Tail furcae (light microscopy). A, *Diplostomum parviventosum*; B, *Diplostomum mergi* Lineage 4; C, '*Diplostomum mergi* Lineage 2'; D, '*Diplostomum mergi* Lineage 3'; E, '*Diplostomum* sp. Clade Q'; F, *Diplostomum pseudospathaceum*; G. *Diplostomum spathaceum*.

Additional file 6: Table S2. Comparative qualitative and meristic data for cercariae of the *Diplostomum* '*mergi*' species complex. **Table S3.** Comparative qualitative and meristic data for cercariae of *Diplostomum spathaceum*, *D. pseudospathaceum*, *D. paracaudum* and '*Diplostomum* sp. Clade Q' of Georgieva *et al.* (2013).



Supplementary Figure S1 Schematic illustration of a cercaria of *Diplostomum* spp. showing the metrical features used. *Abbreviations*: BL, body length; BW, maximum body width; AOL, anterior organ length; AOW, anterior organ maximum width; VSL, ventral sucker length; VSW, ventral sucker width; TSL, tail stem length; TSW, tail stem width (at base); FL, furca length.

Supplementary Table S1 Summary data for *cox* 1 sequences for *Diplostomum* spp. retrieved from GenBank

Species	Host	Locality	Isolate	Life-cycle stage	Tissue	GanBank accession numbers *cox* 1	ITS1+5.8S +ITS2	Reference
Diplostomum baeri	*Perca flavescens**	Canada	6DM25	metacercaria	eye vitreous humour/retina*		AY123042	Locke et al. (2010); Galazzo et al. (2002)
Diplostomum huronense	*Larus delawarensis**	Canada		adult (exp.)*			AY123044	Galazzo et al. (2002)
Diplostomum huronense	*Catostomus commersoni*	Canada	isolate D.LL.IVT.Cc.3F.1	metacercaria	eye lens		GQ292513	Locke et al. (2010)
Diplostomum indistinctum	*Larus delawarensis**	Canada		adult (exp.)*	eye lens*		AY123043	Galazzo et al. (2002)
'*Diplostomum mergi* 1'	*Radix auricularia*	Germany: Hengsteysee	RAH1	cercaria		JX986873	JX986838	Georgieva et al. (2013)
'*Diplostomum mergi* 2'	*Radix auricularia*	Germany: Hengsteysee	RAH3	cercaria		JX986875	JX986839	Georgieva et al. (2013)
'*Diplostomum mergi* 2'	*Radix auricularia*	Germany: Hengsteysee	RAH4	cercaria		JX986876		Georgieva et al. (2013)
'*Diplostomum mergi* 2'	*Radix auricularia*	Germany: Hengsteysee	RAH2	cercaria		JX986874		Georgieva et al. (2013)
'*Diplostomum mergi* 3'	*Gobio gobio*	Germany: River Ruhr (Henne)	GGR4	metacercaria	eye lens		JX986843	Georgieva et al. (2013)
'*Diplostomum mergi* 3'	*Gobio gobio*	Germany: River Ruhr (Henne)	GGR2	metacercaria	eye lens	JX986877	JX986840	Georgieva et al. (2013)
'*Diplostomum mergi* 3'	*Salmo trutta fario*	Germany: River Ruhr (Henne)	STR10	metacercaria	eye lens	JX986878	JX986841	Georgieva et al. (2013)
'*Diplostomum mergi* 3'	*Gobio gobio*	Germany: River Ruhr (Henne)	GGR3	metacercaria	eye lens		JX986842	Georgieva et al. (2013)
'*Diplostomum mergi* 3'	*Salmo trutta fario*	Germany: River Ruhr (Henne)	STR15	metacercaria	eye lens	JX986886		Georgieva et al. (2013)
'Diplostomum *mergi* 3'	*Salmo trutta fario*	Germany: River Ruhr (Henne)	STR12	metacercaria	eye lens	JX986880		Georgieva et al. (2013)
'*Diplostomum mergi* 3'	*Salmo trutta fario*	Germany: River Ruhr (Henne)	STR11	metacercaria	eye lens	JX986879		Georgieva et al. (2013)
Diplostomum mergi	*Radix balthica*	Denmark: Lake Fure	D14	cercaria			JX494231	Haarder et al. (2013)
Diplostomum mergi	*Radix balthica*	Denmark	D13	cercaria			JX494233	Haarder et al. (2013)
Diplostomum sp. 'Clade Q'	*Radix auricularia*	Germany: Lake Constance	RA97	cercaria		JQ639179	JQ665458	Behrmann-Godel (2013)
Diplostomum sp. 'Clade Q'	*Rutilus rutilus*	Germany: Lake Constance	RR43	metacercaria	eye lens	JQ639177		Behrmann-Godel (2013)
Diplostomum sp. 'Clade Q'	*Rutilus rutilus*	Germany: Lake Constance	RR45	metacercaria	eye lens	JQ639178		Behrmann-Godel (2013)
Diplostomum pseudospathaceum	*Larus cachinnans*	Czech Republic: near Tovacov	LCT3	adult		JX986896	JX986849	Georgieva et al. (2013)
Diplostomum pseudospathaceum	*Larus cachinnans*	Czech Republic: near Tovacov	LCT4	adult		JX986905	JX986854	Georgieva et al. (2013)
Diplostomum pseudospathaceum	*Gasterosteus aculeatus*	Germany: Hengsteysee	GAH6	metacercaria	eye lens		JX986852	Georgieva et al. (2013)
Diplostomum pseudospathaceum	*Lymnaea stagnalis*	Germany: Baldeneysee	LSB2	cercaria			JX986850	Georgieva et al. (2013)
Diplostomum pseudospathaceum	*Lymnaea stagnalis*	Germany: Harkortsee	LSH1	cercaria			JX986851	Georgieva et al. (2013)
Diplostomum pseudospathaceum	*Larus argentatus*	Poland: near Gdansk	LAG2	adult		JX986904		Georgieva et al. (2013)
Diplostomum pseudospathaceum	*Gymnocephalus cernuus*	Germany: Lake Constance	GC87	metacercaria	eye lens		JQ665456	Behrmann-Godel (2013)
Diplostomum pseudospathaceum	*Larus argentatus*	Poland: near Gdansk	LAG2	adult			JX986853	Georgieva et al. (2013)
Diplostomum spathaceum	*Larus cachinnans*	Czech Republic: near Tovacov	LCT2	adult		JX986895	JX986848	Georgieva et al. (2013)
Diplostomum spathaceum	*Larus argentatus*	Poland: near Gdansk	LAG1	adult		JX986892	JX986847	Georgieva et al. (2013)
Diplostomum spathaceum	*Radix auricularia*	Germany: Hengsteysee	RAH5	cercaria			JX986845	Georgieva et al. (2013)
Diplostomum spathaceum	*Radix auricularia*	Germany: Hengsteysee	RAH6	cercaria			JX986846	Georgieva et al. (2013)
Diplostomum spathaceum	*Larus cachinnans*	Czech Republic: near Tovacov	LCT1	adult		JX986887	JX986844	Georgieva et al. (2013)
Diplostomum spathaceum	*Coregonus lavaretus*	Germany: Lake Constance	CL100	metacercaria	eye lens		JQ665457	Behrmann-Godel (2013)
Diplostomum sp. 1	*Larus delawarensis*	Canada	D.IN.SSO.Ld.1F.27	adult				Locke et al. (2010)
Diplostomum sp. 1	*Larus delawarensis*	Canada	D.IN.SSO.Ld.2F.6	adult			GQ292519	Locke et al. (2010)
Diplostomum sp. 2	*Pimephales notatus*	Canada	D.BR.S.B.20.1	metacercaria	brain		GQ292505	Locke et al. (2010)
Diplostomum sp. 3	*Micropterus salmoides*	Canada	D.RL.B08.Ms.1F.1	metacercaria	eye lens		GQ292511	Locke et al. (2010)
Diplostomum sp. 4	*Larus delawarensis*	Canada	D.IN.SSO.Ld.2F.10	adult			GQ292520	Locke et al. (2010)
'*Diplostomum baeri* 1'	*Salmo trutta fario*	Germany: River Ruhr (Henne)	STR4	metacercaria	eye vitreous humour	JX986864		Georgieva et al. (2013)
'*Diplostomum baeri* 1'	*Salmo trutta fario*	Germany: River Ruhr (Henne)	STR7	metacercaria	eye vitreous humour	JX986869		Georgieva et al. (2013)
'*Diplostomum baeri* 1'	*Salmo trutta fario*	Germany: River Ruhr (Henne)	STR3	metacercaria	eye vitreous humour	JX986862	JX986837	Georgieva et al. (2013)
'*Diplostomum baeri* 1'	*Salmo trutta fario*	Germany: River Lenne	STL1	metacercaria	eye vitreous humour	JX986863		Georgieva et al. (2013)
'*Diplostomum baeri* 1'	*Salmo trutta fario*	Germany: River Lenne	STL2	metacercaria	eye vitreous humour	JX986865		Georgieva et al. (2013)
'*Diplostomum baeri* 2'	*Perca fluviatilis*	Germany: Lake Constance	PF6D3	metacercaria	eye vitreous humour	JQ639189		Behrmann-Godel (2013)
'*Diplostomum baeri* 2'	*Perca fluviatilis*	Germany: Lake Constance	PF15D4	metacercaria	eye vitreous humour	JQ639187		Behrmann-Godel (2013)
'*Diplostomum baeri* 2'	*Perca fluviatilis*	Germany: Lake Constance	PF8D7	metacercaria	eye vitreous humour	JQ639191		Behrmann-Godel (2013)
'*Diplostomum baeri* 2'	*Perca fluviatilis*	Germany: Lake Constance	PF15D9	metacercaria	eye vitreous humour	JQ639193		Behrmann-Godel (2013)
'*Diplostomum baeri* 2'	*Perca fluviatilis*	Germany: Lake Constance	PF5D3	metacercaria	eye vitreous humour	JQ639195		Behrmann-Godel (2013)
'*Diplostomum baeri* 2'	*Perca fluviatilis*	Germany: Lake Constance	PF4D3	metacercaria	eye vitreous humour		JQ665460	Behrmann-Godel (2013)

* No data in GenBank

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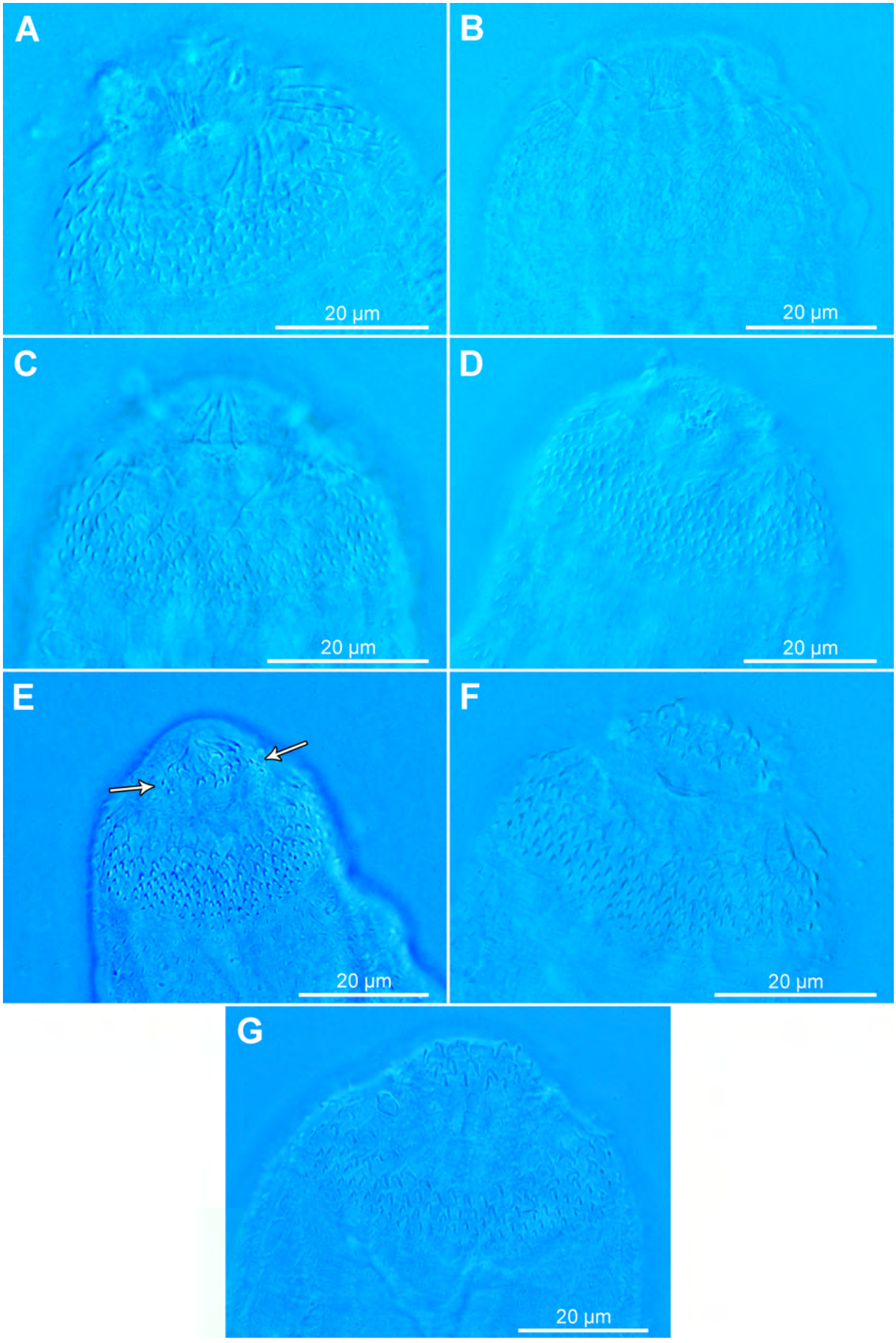
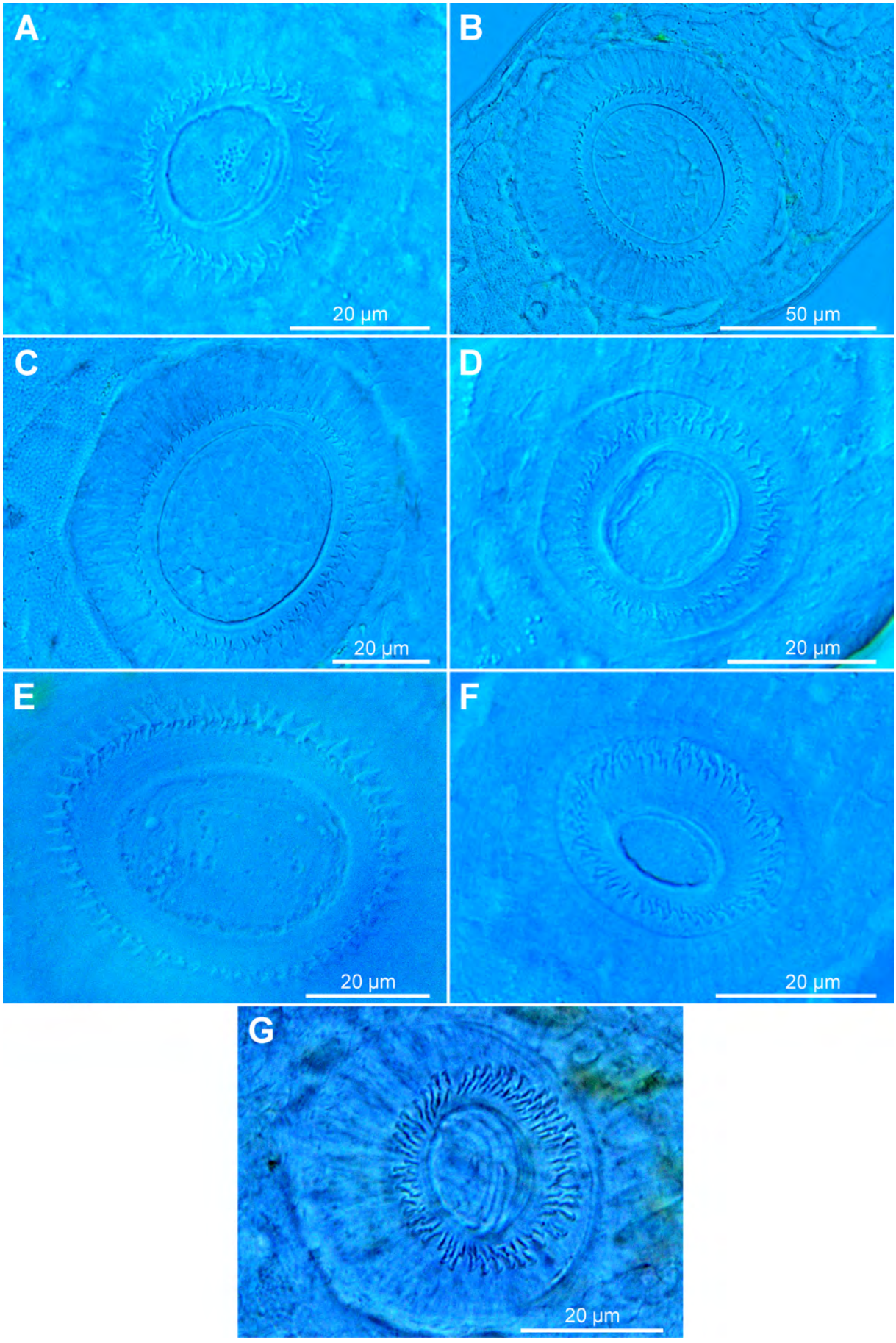
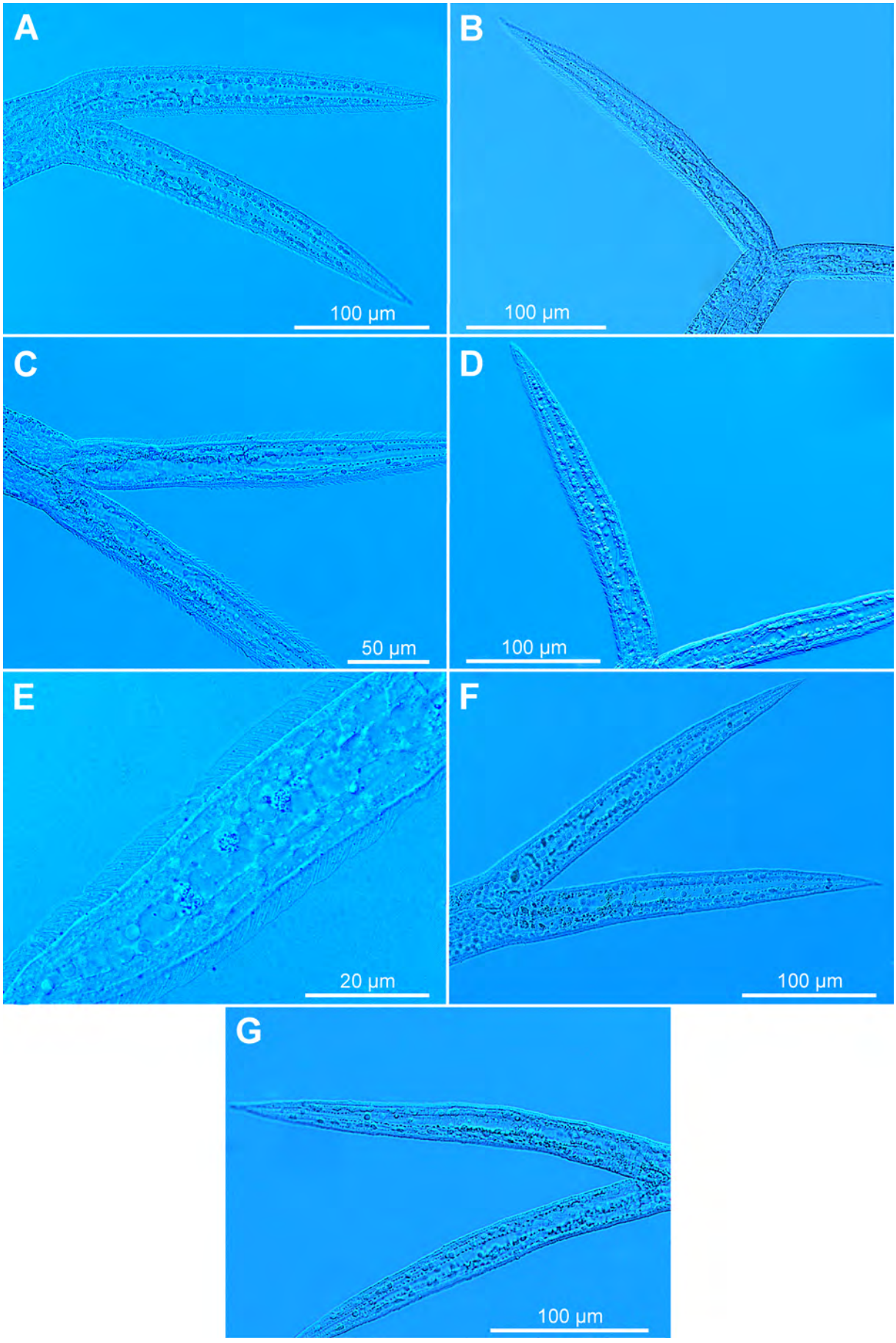


Supplementary Figure S2 Cercariae of *Diplostomum* spp. Pre- and post-oral spines (light microscopy). A, *Diplostomum parviventosum*; B, *Diplostomum mergi* Lineage 4; C, '*Diplostomum mergi* Lineage 2'; D, '*Diplostomum mergi* Lineage 3'; E, F, *Diplostomum pseudospathaceum* (arrows indicate lateral spines); G. *Diplostomum spathaceum*



Supplementary Figure S3 Cercariae of *Diplostomum* spp. Ventral sucker (light microscopy). A, *Diplostomum parviventosum*; B, *Diplostomum mergi* Lineage 4; C, '*Diplostomum mergi* Lineage 2'; D, '*Diplostomum mergi* Lineage 3'; E, '*Diplostomum* sp. Clade Q'; F, *Diplostomum pseudospathaceum*; G. *Diplostomum spathaceum*



Supplementary Figure S4 Cercariae of *Diplostomum* spp. Tail furcae (light microscopy). A, *Diplostomum parviventosum*; B, *Diplostomum mergi* Lineage 4; C, '*Diplostomum mergi* Lineage 2'; D, '*Diplostomum mergi* Lineage 3'; E, '*Diplostomum* sp. Clade Q'; F, *Diplostomum pseudospathaceum*; G. *Diplostomum spathaceum.*

Electronic supplementary material for

Integrative taxonomic approach to the cryptic diversity of *Diplostomum* spp. in lymnaeid snails from Europe with a focus on the '*Diplostomum mergi*' species complex

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Table S2 Comparative qualitative and meristic data for cercariae of the *Diplostomum* '*mergi*' species complex

Species	*Diplostomum parviventosum* Dubois, 1932		*Diplostomum mergi* Dubois, 1932	'*Diplostomum mergi* Lineage 2' of Georgieva *et al.* [6]	'*Diplostomum mergi* Lineage 3' of Georgieva *et al.* [6]	*Diplostomum mergi* Lineage 4
Hosts	*Radix auricularia*	*Radix ovata*	*R. ovata*	*R. auricularia*	*R. auricularia*	*R. auricularia*
Source	Present study	Niewiadomska & Kiseliene [10]	Niewiadomska & Kiseliene [10]	Present study	Present study	Present study
Yellow pigment in body	Present	na	Absent	Present	Present	Present
Relation BL-TSL-FL	BL<TSL<FL	BL<TSL>FL	BL<TSL=FL	Live: BL≤TSL<FL Fixed: BL=TSL<FL	Live: BL≤TSL<FL Fixed: BL<TSL≤FL	Live: BL<TSL=FL
Relation VSW-AOW	VSW>AOW	VSW>AOW	VSW>AOW	VSW>AOW	Live: VSW≥AOW Fixed: VSW=AOW	Live: VSW>AOW
No. of pre-oral spines (median group)	6–7 in 3 rows	5–7 in 2 rows	6–8 in 2 rows	5–6 in 2 rows	7 in 3 rows	7 in 3 rows
No. of pre-oral spines in each lateral group	No lateral group	No lateral group	No lateral group	No lateral group	No lateral group	No lateral group
No. of rows of post-oral spines	7–8	6–8	6–9	11 (an additional median row may be present)	10	11
Incomplete rows of post-oral spines	Rows 1–2 with median interruption	na	na	Rows 1–2 with median interruption	Rows 1–2 with median interruption	Rows 1–2 with median interruption, rows 10–11 interrupted dorsally
Size of post-oral spines	First 4 spines in row 1 on both sides of median interruption largest; spines in row 1 larger than remaining spines	na	na	First 4 spines in row 1 and first 3 spines in row 2 on both sides of median interruption largest; spines in row 1 larger than remaining spines	First 4 spines in row 1 on both sides of median interruption largest; spines in rows 1–2 larger than remaining spines	First 4 spines in row 1 and first 3 spines in row 2 on both sides of median interruption largest; spines in rows 1–2 larger than remaining spines
Zone of dispersed post-oral spines	Present (wide)	Present	Present	Present (wide)	Present (wide)	Present (wide)
Spineless area posterior to dispersed spines	Present (narrow)	Present	Present	Present (narrow)	Present (narrow)	Present (narrow)
Transverse rows of spines on body	11	10	10	10	11	10
Double transverse rows	None	na	na	None	Row 1	None
Incomplete transverse rows	Rows 9–11 discontinuous ventrally and dorsally; rows 5–8 discontinuous dorsally	Rows 6–10 discontinuous ventrally and dorsally	Last rows	Rows 6–10 discontinuous ventrally and dorsally	Rows 4–11 discontinuous ventrally and dorsally	Rows 5–7 discontinuous dorsally; rows 8–10 discontinuous ventrally and dorsally
Transverse rows with additional spines laterally	Rows 1 and 10	na	Rows 1–2	Rows 1–4 (rows 5–6 with 1–2 additional spines)	Rows 2–3	None

Species	*Diplostomum parviventosum* Dubois, 1932		*Diplostomum mergi* Dubois, 1932	'*Diplostomum mergi* Lineage 2' of Georgieva *et al.* [6]	'*Diplostomum mergi* Lineage 3' of Georgieva *et al.* [6]	*Diplostomum mergi* Lineage 4
Hosts	*Radix auricularia*	*Radix ovata*	*R. ovata*	*R. auricularia*	*R. auricularia*	*R. auricularia*
Source	Present study	Niewiadomska & Kiseliene [10]	Niewiadomska & Kiseliene [10]	Present study	Present study	Present study
Zone of dispersed spines in hind body	2 fields converging posteriorly	2 wide ventro-lateral bands converging posterior to VS and close to posterior extremity	na	2 fields converging posteriorly	2 wide, non-converging fields	2 fields converging posteriorly
No. of spine rows on ventral sucker	2	2	2	2	2	2
No. of spines on ventral sucker (mean)	77–87 (81)	80–88	94–130	110–120 (114)	90–92 (90)	112–114 (113)
Penetration gland-cells	2 pairs; small, do not cover ends of caeca	2 pairs; small, do not overpass caeca laterally	2 pairs; large, do not cover ends of caeca	2 pairs; large, do not cover ends of caeca	2 pairs; medium-sized, do not cover ends of caeca	3 pairs (anterior pair small, posterior 2 pairs large); do not cover ends of caeca
Spines on tail stem	Present (2 ventral and 2 dorsal bands)	Dispersed spines on ventral and dorsal surfaces	Absent	Present (2 ventral and 2 dorsal bands) start from second quarter of tail	Present (2 ventral and 2 dorsal bands)	Present (2 ventral and 2 dorsal bands)
Spines on furcae	Present	Present	Absent	Present	Present	Present
Fin-folds on furcae	Present (fish-fin like fin-folds)	Present	Present	Present (fish-fin like fin-folds)	Present (fish-fin like fin-folds)	Present (fishfin-like fin-fold)
No. of caudal bodies	10–12 pairs	10–11 pairs	10–11 pairs	36–40 individual caudal bodies	Individual caudal bodies impossible to count	36–40 individual caudal bodies
Shape of caudal bodies	With smooth contours	With incised contours	With incised contours	With smooth contours	With incised contours	With smooth contours
Resting position	tail stem bent at < 90° (45–67°)	Tail stem bent at *c.*45°	Tail stem bent at < 90°	Tail stem bent at < 90° (64–85°)	Tail stem bent at 90° (77–91°)	Tail stem bent at < 90° (66°)

Abbreviations: BL, body length; BW, maximum body width; AOW, anterior organ width; VS, ventral sucker; VSW, ventral sucker width; TSL, tail stem length; FL, furca length; na, no data available

Table S3 Comparative qualitative and meristic data for cercariae of *Diplostomum spathaceum*, *D. pseudospathaceum*, *D. paracaudum* and '*Diplostomum* sp. Clade Q' of Georgieva *et al.* [6]

Species	*D. spathaceum* (Rudolphi, 1819)	*D. paracaudum* (Iles, 1959)	'*Diplostomum* sp. Clade Q' of Georgieva *et al.* [6]	*Diplostomum spathaceum* (Rudolphi, 1819)	*Diplostomum pseudospathaceum* Niewiadomska, 1984	
Hosts	*R. auricularia*, *R. ovata*, *R. peregra*	*R. auricularia*, *R. ovata*, *S. palustris*	*R. auricularia*	*R. auricularia*	*L. stagnalis*, *S. palustris*	*L. stagnalis*, *S. palustris*
Source	Niewiadomska & Kiseliene [10]	Niewiadomska [28]; Niewiadomska & Kiseliene [10]	Present study	Present study	Niewiadomska & Kiseliene [10]	Present study
Yellow pigment in body	Present	Absent	Present	Present	Present	Present
Relation BL-TSL-FL	BL<TSL=FL	BL<TSL≥FL	Live: BL<TSL=FL	Live: BL<TSL<FL Fixed: BL<TSL=FL	BL<TSL=FL	Live: BL≤TSL<FL Fixed: BL<TSL≤FL
Relation VSW-AOW	VSW>AOW	VSW=AOW	Live: VSW>AOW	Live: VSW≥AOW Fixed: VSW≤AOW	VSW=AOW	Live: VSW>AOW Fixed: VSW=AOW
No. of pre-oral spines (median group)	8–16 in 3–4 rows	15–20 in 3 rows	9 in 3 rows	18–19 in 3 rows	8–14 in a triangle	10–11 in 3 rows
No. of pre-oral spines in each lateral group	No lateral groups	1–2	No lateral groups	1 small spine	1–4	3 small spines
No. of rows of post-oral spines	10–14	6–7	12	9	6–8	9
Incomplete rows of post-oral spines	na	na	Row 1 with median interruption, rows 11–12 interrupted laterally	Row 1 with median interruption; row 9 interrupted laterally	na	Rows 1–2 with median interruption; row 9 interrupted laterally
Size of post-oral spines	na	na	First 5 spines in row 1 on both sides of median interruption largest; remaining spines of different sizes	Spines in row 1 larger than remaining spines	na	Spines in row 1 larger than remaining spines
Zone of dispersed post-oral spines	Present	Present	Present (wide)	Present (wide)	Present	Present (wide)
Spineless area posterior to dispersed spines	Present	Present	Present (narrow)	Present (narrow)	Present	Present (narrow)
Transverse rows of spines on body	9–10	10	10	10	10	11
Double transverse rows	Rows 1–2	Row 1 (drawing)	Row 1	Rows 1–2 (only ventrally)	Rows 1–2	Rows 1–2 (only ventrally)
Incomplete transverse rows	Posterior rows discontinuous ventrally and dorsally	Rows 5–6 to 10 discontinuous ventrally and dorsally	Rows 5–10 discontinuous ventrally and dorsally	Rows 9–10 discontinuous ventrally	Last rows	Row 9 discontinuous ventrally; rows 10–11 discontinuous ventrally and dorsally

Species	*D. spathaceum* (Rudolphi, 1819)	*D. paracaudum* (Iles, 1959)	'*Diplostomum* sp. Clade Q' of Georgieva *et al.* [6]	*Diplostomum spathaceum* (Rudolphi, 1819)	*Diplostomum pseudospathaceum* Niewiadomska, 1984	
Hosts	*R. auricularia*, *R. ovata*, *R. peregra*	*R. auricularia*, *R. ovata*, *S. palustris*	*R. auricularia*	*R. auricularia*	*L. stagnalis*, *S. palustris*	*L. stagnalis*, *S. palustris*
Source	Niewiadomska & Kiseliene [10]	Niewiadomska [28]; Niewiadomska & Kiseliene [10]	Present study	Present study	Niewiadomska & Kiseliene [10]	Present study
Transverse rows with additional spines laterally	Rows 3–4	Anteriormost rows	Rows 2–3	Row 3	Rows 3–4	Rows 3–7
Zone of dispersed spines in hind body	2 fields converging ventrally	2 fields converging ventrally and dorsally	2 non-converging fields posterior to VS	2 fields converging posteriorly to VS and close to posterior extremity of body	2 lateral fields	2 fields converging posteriorly to VS and close to posterior extremity of body
No. of spine rows on ventral sucker	2	3	2	3	2 (3rd row may be partly formed)	2
No. of spines on ventral sucker (mean)	108–125	116–141	112–116 (114)	103–119 (110)	66–107	70–100 (84)
Penetration gland-cells	Large, do not cover ends of caeca	Large, do not cover ends of caeca	Large, do not cover ends of caeca	Large, do not cover ends of caeca	Large, do not cover ends of caeca	Large, do not cover ends of caeca
Spines on tail stem	Absent	Absent	Present (2 ventral and 2 dorsal bands)	Present (2 ventral and 2 dorsal bands)	Present near distal end	Present (2 ventral and 2 dorsal bands)
Spines on furcae	Absent	Absent	Present	Present	Present	Present
Fin-folds on furcae	Present	Absent	Present (fish-fin like fin-folds)	Absent	Absent	Absent
No. of caudal bodies	11–12 pairs	10–11 pairs	10 pairs	56–60 individual caudal bodies	10 pairs	35–45 individual caudal bodies
Shape of caudal bodies	With incised contours	With incised contours	With incised contours	With both incised and smooth contours	With incised contours	With smooth contours, irregular in shape and size
Resting position	Tail stem bent at 90°	Tail stem bent at 90°	na	Tail stem bent at < 45° (39°)	Tail stem bent at 90°	Tail stem bent at < 45° (29–38°)

Abbreviations: BL, body length; BW, maximum body width; AOW, anterior organ width; VS, ventral sucker; VSW, ventral sucker width; TSL, tail stem length; FL, furca length; na, no data available

14.2 Appendix II
Additional publication

This appendix contains the following publication:

Soldánová, M., Selbach, C., Kalbe, M., Kostadinova, A., & Sures, B. (2013). Swimmer's itch: etiology, impact, and risk factors in Europe. *Trends in Parasitology, 29*(2), 65–74.

Review

Swimmer's itch: etiology, impact, and risk factors in Europe

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This review summarizes current knowledge about the occurrence and distribution of swimmer's itch, with a focus on Europe. Although recent publications have reviewed the biology and systematics of bird schistosomes and their complex host–parasite interactions, the underlying ecological factors that create favorable conditions for the parasites and the way humans interact with infested water bodies require further attention. Relevant studies from the past decade were analyzed to reveal an almost complete set of ecological factors as a prerequisite for establishing the life cycle of bird schistosomes. Based on both records of the occurrence of the parasite infective agents, and epidemiological studies that investigate outbreaks of swimmer's itch, this review concentrates on the risk factors for humans engaged in recreational water activities.

Bird schistosomes as causative agents of swimmer's itch

Swimmer's itch is regarded as a re-emerging disease [1,2] in Europe and can lead to severe skin infections in humans engaged in recreational water activities. Although schistosomes infecting mammals are also known to cause swimmer's itch not only worldwide [3], and more recently also in Europe [4], the most common causative agents in Europe are the larval stages (cercariae) of the bird schistosomes of the genus *Trichobilharzia* that complete their life cycles in freshwater snails and waterfowl. Although cercariae may infect humans, they are not able to complete their life cycle within these accidental hosts, and most die soon after penetration [5,6]. Primary infection in unsensitized mammals usually show mild or no skin reaction, but parasites may escape from the skin and migrate to different organs ([1,3] and references therein). However, repeated exposure of humans to cercariae results in an inflammatory skin reaction termed cercarial dermatitis or swimmer's itch. This is caused by host immune response leading to destruction of the parasites entrapped in the skin. Swimmer's itch is typically not harmful but is very unpleasant due to intense itching; nevertheless, in repeatedly sensitized persons other symptoms such as fever, local lymph node swelling, and edema can occur [1]. Furthermore, bird schistosomes such as the neuropathogenic *Trichobilharzia regenti* may escape mammalian immune defense and migrate further in the accidental host, thus presenting risks of potential neurologic disorders [1,7].

Recent reviews have compiled much information about the current state of the taxonomy of the bird schistosomes (e.g., [8,9] and references therein for a focus on Europe), the present knowledge of their biology and the complex host–parasite associations [3,10], or estimated effects of climate change on the spread of trematodiases in general (e.g., [11,12]). However, the underlying ecological factors that create favorable conditions for bird schistosomes and the way humans interact with infested water bodies require more in-depth investigation. Increasing recreational use of freshwater habitats associated with the ongoing global changes such as climate warming, urbanization, and anthropogenic pressures on natural habitats, enhances the risks of acquiring swimmer's itch in the future.

Accordingly, our aim is to focus on the ecological conditions that drive parasite occurrence and disease to assess the risk factors for humans engaging in recreational activities in the aquatic environments in Europe. The records of parasite occurrence and concomitant epidemiological studies that investigate outbreaks of swimmer's itch are discussed. The available literature is used to predict the potential occurrence of *Trichobilharzia* spp., the most common agents of swimmer's itch in Europe [8], and to interpret the associated risks of infection at swimming sites into an integrated risk assessment.

The life cycle of bird schistosomes

Bird schistosomes are distributed worldwide [3,8]. They require two hosts from different trophic levels for the successful completion of their life cycles (Figure 1). The definitive vertebrate hosts are typically waterfowl, in which the schistosomes reach sexual maturity, mate, and produce eggs. Eggs containing fully developed larvae (miracidia) are deposited and released depending on the location of adult worms: (i) in the tissues of the visceral organs and associated blood vessels of the definitive host, and are released with the host's feces (visceral

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Keywords: bird schistosomes; *Trichobilharzia*; swimmer's itch; Lymnaeidae; ecological factors; risk factors; Europe.

1471-4922/$ – see front matter http://dx.doi.org/10.1016/j.pt.2012.12.002 Trends in Parasitology, February 2013, Vol. 29, No. 2

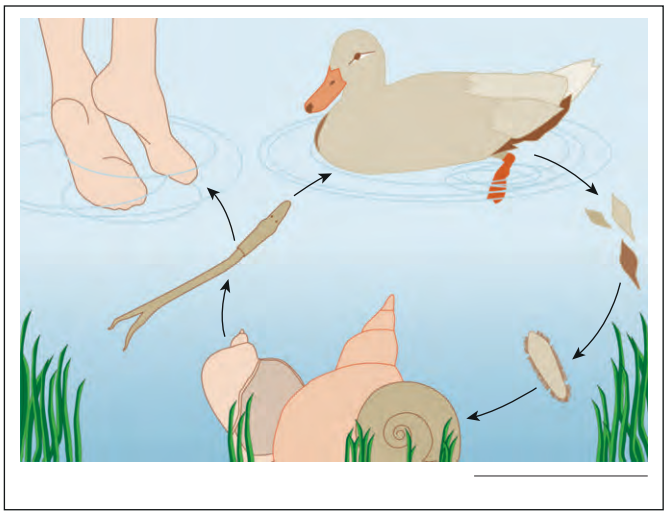

Figure 1. Generalized life-cycle of a bird schistosome. Adult trematodes reproduce in waterfowl, and eggs are released into the water. From eggs the first dispersive larval stages (miracidia) hatch and infect suitable snail intermediate hosts in which asexual reproduction occurs, resulting in the production of the second dispersive larval stages (ocellate furcocercariae). Cercariae emerge from infected snails and infect appropriate final hosts (birds) thus completing the life cycle. Swimmer's itch occurs when cercariae accidentally penetrate human skin.

schistosomes); or (ii) in the nasal mucosa where miracidia hatch directly in the soft tissues of the nasal cavity, and leave the host after its contact with water (nasal schistosomes) [8]. The free-swimming miracidia locate and penetrate suitable snail intermediate hosts, where asexual reproduction of the parasite takes place. Miracidia transform into mother sporocysts, which in turn give rise to daughter sporocysts, each forming numerous infective dispersive stages (ocellate furcocercariae). After their emergence from snails, cercariae disperse in the aquatic environment where they actively seek out and penetrate the skin of bird legs or the skin of humans accidentally. After successful penetration of a suitable bird host, cercariae transform to schistosomula (juvenile worms), which migrate to the preferred sites of infection. Migration routes of the visceral schistosomes are through the circulatory system, and those of the nasal *Trichobilharzia regenti* are through the peripheral nerves and central nervous system. Schistosomula migration, together with the presence of adults and eggs (in birds only), may result in severe damage of various organs and tissues and possibly neuromotor disorders; these have been documented in natural infections and experiments on bird and mammalian models (reviewed in [1,10]).

Both free-living dispersal stages, miracidia and cercariae, do not feed and possess limited energy supplies, resulting in very short lifespans of 1 day and up to 2.5 days, respectively (e.g., *T. szidati* [13]). Miracidia can locate the appropriate specific snail species according to differential miracidia-attracting glycoproteins (e.g., *T. szidati* [14,15] and references therein), whereas the negatively geotropic and positively phototactic cercariae use complex swimming behaviors stimulated by shadow, water turbulence. and tactile stimuli for definitive host-finding [16,17]. Recognition and responding to chemical cues of the host skin components such as ceramides, cholesterol, and fatty acids, trigger the attachment and penetration processes, respectively [18–20]. Cercarial emergence rates have been quantified as ca 400–500 cercariae snail^{-1}.day^{-1}, with an estimated total number of ca 25 000 cercariae snail^{-1} [13], and emergence peaks between 9 and 11 am [21].

Prevalence in snails and birds

Six genera of schistosomes utilize aquatic birds, mostly of the order Anseriformes (ducks, geese, and swans) as definitive hosts in Europe: *Allobilharzia*, *Bilharziella*, *Dendritobilharzia*, *Gigantobilharzia*, and *Trichobilharzia* [8,22]. Species of the genus *Ornithobilharzia* occur in charadriiform and procellariiform birds and marine gastropods [23,24]. Several new and previously undescribed species, mostly detected as larval stages in the snail hosts, have been recognized recently as a result of the application of molecular markers for species discrimination (e.g., [9,25–30]). Snail hosts supporting most of the bird schistosome diversity in Europe are freshwater pulmonates of the families Lymnaeidae and Planorbidae [8,30]. Bird schistosomes in snails are widespread throughout Europe (Figure 2; Table S1 in the supplementary material online) including cold areas at higher latitudes. The lymnaeids *Lymnaea stagnalis* and *Radix* spp. represent the most frequent, abundant, and widely distributed intermediate hosts for the most important and common bird schistosomes of the genus *Trichobilharzia* [8,10]. Notably, a recent molecular study revealed high schistosome species diversity in small planorbids, adding six previously undescribed species [30].

The specificity of host-snail recognition in *Trichobilharzia* spp. might be rather high (as shown for *T. szidati* [15]). Further research on host compatibility appears to be an area of importance for our understanding of local transmission patterns because recent findings suggest that bird schistosome specificity towards the snail hosts may be lower than previously known (compare snail and schistosome richness in Table 1).

Infection rates in snails may vary annually and/or seasonally with increases in the summer months [31]. Prevalence of bird schistosomes in snails in Europe show generally low levels (mostly ranging from 0.05% to 5.0%) compared to infection rates in birds (up to 74.5%) ([8,10] for details). Occasionally prevalence values in snails reach ca 25% [9,32–35] but may exceed 40%, especially in eutrophic environments [36] (Table 1).

Ecological factors driving bird schistosome and swimmer's itch occurrence

Table 1 summarizes the data extracted from the literature in an attempt to reveal patterns and derive predictions for the occurrence of swimmer's itch, snail intermediate hosts, and bird schistosomes in relation to ecological conditions of the aquatic habitats in Europe. We focused primarily on original records of swimmer's itch and/or bird schistosomes from studies providing data on sampling sites, snail and schistosome identification, and prevalence of infection. Of 69 studies (Table S1 and Reference List S1 in the supplementary material online), 10 contain both data on ecological conditions of the habitats and reports of swimmer's itch. Overall, the dataset comprised records of 30 parasite taxa (six identified species and 24 as yet undescribed species) in

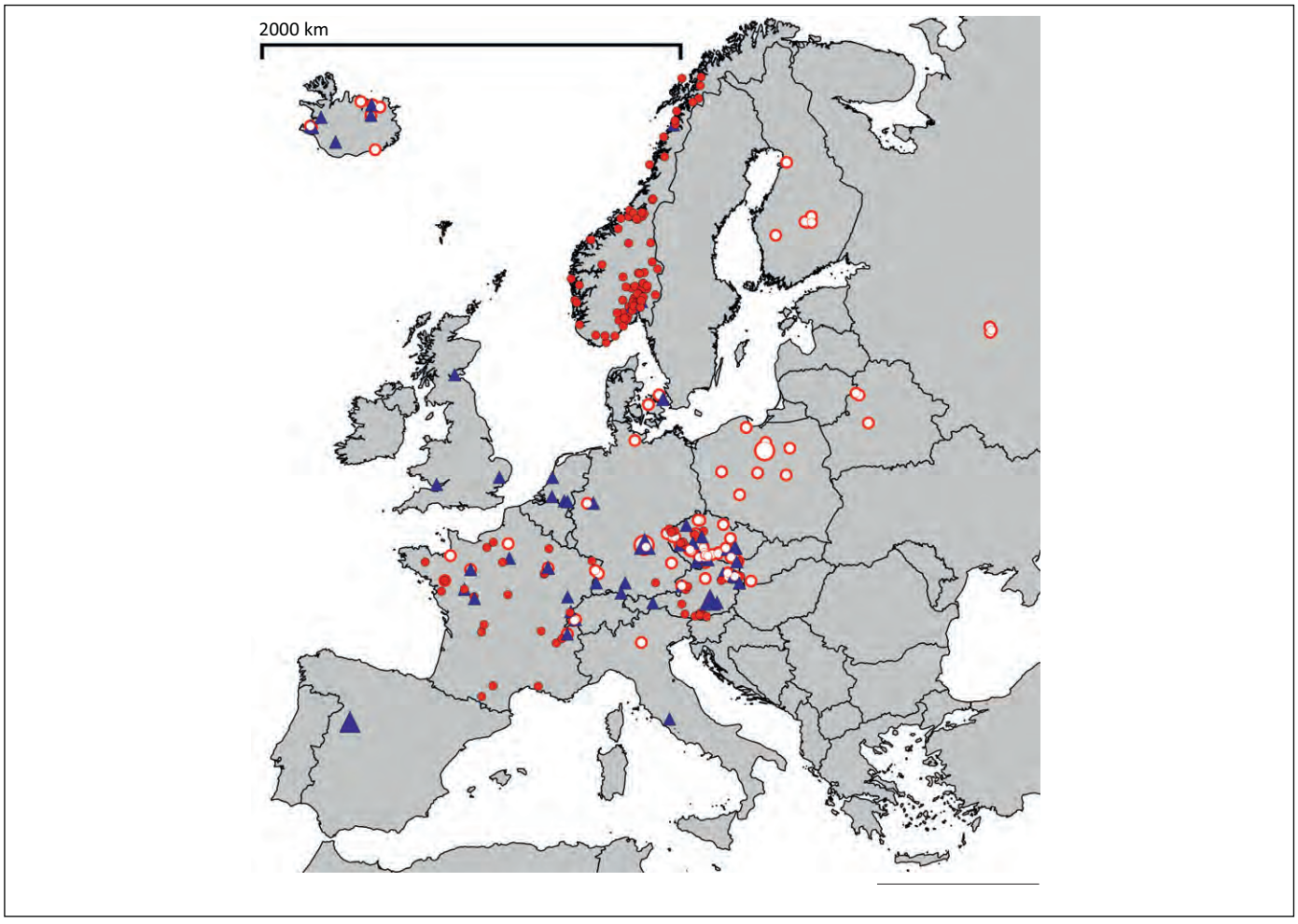

Figure 2. Occurrence of bird schistosomes and reported cases of swimmer's itch in Europe. Open red circles (○) show the confirmed occurrence of bird schistosomes, filled red circles (●) show reported cases of swimmer's itch, and blue triangles (▲) indicate both reported cases of swimmer's itch and confirmed occurrence of bird schistosomes in a body of water. Enlarged symbols indicate pooled data from several bodies of water in a region and/or across years. Only data from publications that clearly identify bodies of water with cases of swimmer's itch and/or bird schistosome occurrence are used. The clusters seen (e.g., in the Czech Republic, Austria, and Norway) are likely due to an increased regional research focus on bird schistosomes and/or swimmer's itch, rather than to the presence of infection hot-spots in these areas. Therefore, occurrence of bird schistosomes and consequently increased risks of swimmer's itch can also be expected in areas with no data currently available.

22 snail intermediate hosts from 17 European countries. Notably, recent molecular studies revealed a much higher diversity of the bird schistosomes than previously known (Table S1 in the supplementary material online). Most cases of swimmer's itch were registered in eutrophic lakes [61% and 55% of the records from large (>1000 ha) and smaller lakes (<1000 ha), respectively]. In addition, many cases were reported from pond systems (mostly in the Czech Republic and Germany), which are artificial man-made water bodies for intensive fish farming with eutrophic and even hypertrophic status. These results coincide with a higher species richness of both snail hosts and bird schistosomes under eutrophic conditions. Furthermore, natural reserves and protected zones, which provide suitable habitats for nesting and stop-over sites for migratory birds, appear to be hot-spots for high richness and infection levels of bird schistosomes (Table 1). The rarity of reports of swimmer's itch from oligotrophic and mesotrophic lakes might be related to snail distribution as evidenced by the low snail species diversity, which in turn results in low bird schistosome species richness. It can be expected that snail populations in less productive meso- and oligotrophic systems will be less abundant and patchy, and bird schistosomes will be also.

Anthropogenic eutrophication is a major stressor leading to changes in disease patterns (reviewed in [10,37,38]). Increases in nitrogen and phosphorus concentrations in freshwater bodies stimulate biomass growth of primary producers, which in turn provides ideal conditions for abundant snail populations. Due to high abundance of potential prey, eutrophication has been linked to increased colonization of the aquatic ecosystems by birds [10,39], which may result in higher input of trematode eggs into the habitat. Taken together, such dense intermediate and definitive host populations create ideal opportunities for rich and abundant trematode communities and enhanced parasite transmission [36–40].

Furthermore, nutrient-rich environments allow snails to grow faster, thus reaching a size-refuge in which they are invulnerable to predators [41]. Increased lifespan leads to higher infection risks for subsequent hosts in the schistosome life cycle because larger snails also support higher production of infective cercarial stages [10,40]. The idea of bird schistosome proliferation with increased productivity

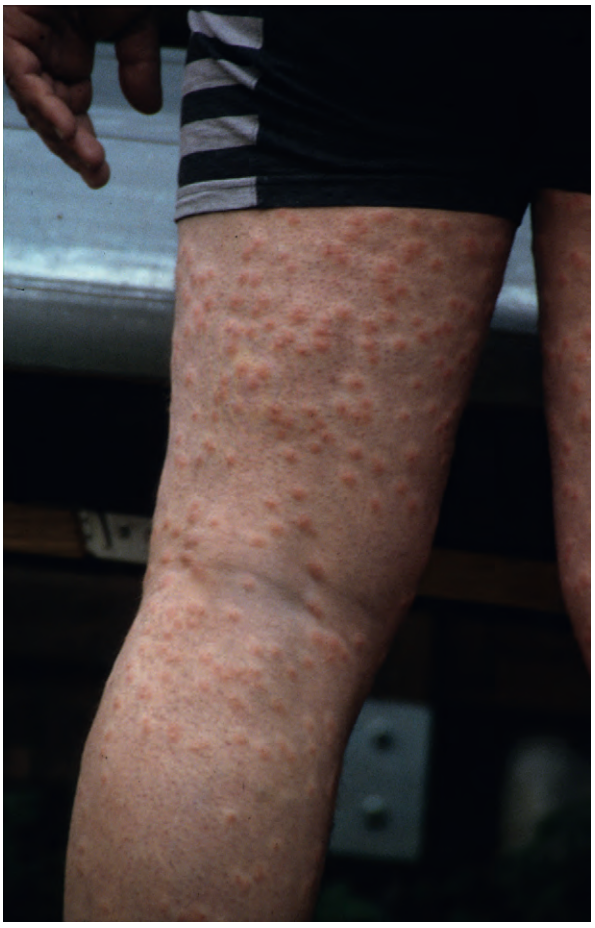

Figure 3. Swimmer's itch developed after sampling snails in shallow water for 2 h by one of the authors.

is supported by the demonstration of extraordinarily rapid rates of recruitment of *T. szidati* to the snail populations in eutrophic ponds in Europe [42].

Owing to high recruitment rates and trematode diversity, competition appears to play a major structuring role in larval trematode communities in eutrophic environments [43]. Species of *Trichobilharzia* and *Austrobilharzia* may be obligate secondary invaders that preferentially infect snails with compromised immunity due to prior trematode infection [44,45], or may persist for a long time in double infections with otherwise dominant species ([46] and references therein). This is consistent with the finding that *T. szidati* most frequently occurs in multiple infections, thus counteracting the effects of competition on transmission dynamics in the eutrophic environment [43].

Climate change is another key ecological process driving the emergence and increase in prevalence of parasitic diseases [47–49]. Temperature-mediated changes in parasite transmission dynamics in aquatic systems may result from either direct effects on the survival and infectivity of the free-living infective larvae [50,51], or indirectly via altering host distribution and abundance and the composition of local host communities [11,47]. Trematodes are extremely sensitive to changes in temperature [52] because cercarial production and emission rates are temperature-dependent. Field evidence from geothermally heated lakes and ponds in Iceland indicates acceleration of both snail and trematode development, such that young snail cohorts are already infected with bird schistosomes, and thus swimmer's itch cases are reported throughout the year irrespectively of season [9]. Usually, the occurrence of cercariae in aquatic habitats is limited seasonally when ambient temperature exceeds a minimum threshold for their emergence [53]. Highest cercarial output for *Trichobilharzia* spp. occurs at temperatures of 17–25 °C [54], and even small increases in temperature can speed up parasite development rates and transmission success, thus leading to a fivefold increase in cercarial emergence with a 10 °C increase in temperature (e.g., *Trichobilharzia* sp. [52]). Because more cercariae released into the aquatic environment exert a higher infection pressure on the definitive hosts, the likelihood of successfully completing the life cycle is enhanced, even though cercarial survival and infectivity are lower at higher temperatures [52]. Trematode transmission may be further facilitated by longer growing seasons that increase snail generations per year, and by mild winters that alter the population dynamics of the aquatic bird hosts [47]. Migratory waterfowl have therefore become sedentary and overwinter at many European lakes, allowing a longer host–parasite transmission window [10,55,56].

Although a range of additional ecological factors affect the occurrence and distribution of bird schistosomes, eutrophication and temperature are the most crucial. This is mirrored by the number of papers dealing with these aspects, and therefore justifies our focus on these factors.

The human perspective of swimmer's itch: impact, epidemiology, and risk factors

Although the connection between the infection and bird schistosome larvae was first made by Cort in 1928 [57], it is clear that the disease had been troubling people around the world long before because it had become linguistically established in different languages; for example 'koganbyo' or 'rice farmer dermatitis' in Japan, 'sawah itch' in Indonesia, 'puces de canard' in Switzerland or 'dermatite des nageurs' in France and Switzerland, 'Entenbilharziose' or 'Wasserhibbel' in Germany and Austria, or 'sedge pool itch' in the United States. This linguistic diversity not only reflects the long history of the disease but highlights its worldwide occurrence and impact on humans in different regions. Swimmer's itch can, therefore, be regarded as a re-emerging rather than a newly emerging disease [1,2]. The map (Figure 2) provides a graphical representation of both the confirmed occurrence of bird schistosomes and cases of swimmer's itch across Europe. The wide spread of the records across different regions highlights the importance of swimmer's itch in Europe. However, its occurrence is underestimated because the often benign pathology results in only a minority of victims consulting a physician [58,59] and many cases remain unreported. Furthermore, infections may be confused with insect bites or allergic reactions (Figure 3) [60] thus leaving even more cases unreported. In addition to the health risks for humans, especially with regard to the potential migration of both nasal [8,10] and visceral bird schistosomes [3,61] in mammals, swimmer's itch is an important economic factor in areas where

Table 1. Swimmer's itch and/or bird schistosome records in Europe[a]

Water body type (surface in ha)	Ecological conditions (trophic status, habitat description)[b,c]	Swimmer's itch reported	Snail species richness (no. of spp.)	Schistosome species richness (no. of spp.)	Bird presence and/or abundance reported	Prevalence (range, %)	Country[d]	Total no. of studies
Lakes (>1000)	E: 4	14 (3[d])	11	14	5	0.05–9.6	AUT, BLR, CHE, DEU, DNK, FRA, ITA, ISL, NOR, POL, UK	23
	M: 3	1	2	2	1	0.10–0.8	AUT, FIN	3
	O: 3	–	2	2	–	0.27–8.3	CZE, FIN	3
Lakes (<1000)	E: 5	11 (2[e])	6	5	6	0.76–26.7	AUT, BLR, DEU, FIN, FRA, ISL, NLD, NOR, POL, UK	20
	M: 1	–	1	1	–	24.1	DEU	1
	O: 3	1	2	2	–	17.1	FIN, ISL	3
Ponds (>30)	11	4 (1[e])	5	4	6	0.20–41.5	AUT, CZE, FRA	13
Ponds (<30)	15	8 (2[e])	11	15	8	0.01–9.7	AUT, BLR, CZE, DEU, FRA, ISL, RUS	25
Rivers, canals, wetlands	3	7 (2[e])	5	4	1	2.20–6.5	AUT, ESP, FRA, NOR	9
Regional surveys	6	4	8	5	2	0.04–22.0	DEU, CZE, POL, SVK	10
Natural reserves/ protected areas	12	5	6	6	8	0.05–41.5	BLR, CZE, DEU, FRA, ISL, ITA, POL	12

[a]Number of records in relation to ecological conditions, snail and bird schistosome species richness, prevalence in snails and geographical distribution in Europe broken down by water body type, size, and trophic status (Table S1 in the supplementary material online for details).

[b]Records providing information for the trophic status and/or sampling site descriptions (e.g., bottom substrate, aquatic vegetation).

[c]Abbreviations: E, eutrophic lake; M, mesotrophic lake; O, oligotrophic lake.

[d]Alpha-3 country codes; a list of codes is given in Table S2 in the supplementary material online.

[e]Studies reporting swimmer's itch only.

tourism depends on recreational water use, and health complaints may lead to severe economic losses [62,63]. As recent studies on bird schistosome diversity, for example, [30] suggest a much broader spectrum of potential infective agents, a potentially wider range of swimmer's itch cases than currently known can be expected.

Risk assessment of swimmer's itch is usually based on the detection of bird schistosomes in snails [24]. Congruent detection of parasites and cases of swimmer's itch can be difficult due to their low prevalence in snails [64]. Therefore, human infections may occur at sites where no parasitized snails can be found [65]. We have compiled data from epidemiological studies on swimmer's itch and field and laboratory studies on the biology of *Trichobilharzia* spp. for a comprehensive risk assessment that may help to understand the recent re-emergence of the disease, as well as help to identify potential infection hot-spots and create a basis for discussion of possible preventive measures.

A wide variety of often interacting factors, ranging from ecological (e.g., eutrophication) to weather influence and personal behavior have been shown to affect the risk of human infection (Table 2). All these considerations illustrate that a mono-causal risk assessment is impossible because a multitude of interacting biotic and abiotic as well as personal factors play an important role in the risk of encountering the disease. A thorough analysis of the local situation taking this variety of factors into consideration is therefore essential in a reliable risk assessment of swimmer's itch.

Possible prevention measures

Here we summarize preventive measures that have been suggested or applied to control the disease and discuss their effectiveness and ecological impacts. Preventive actions should be linked to parasite ecology and host-finding behavior, and aim at interrupting the trematode life cycle at some stage, preventing free-swimming dispersal larval stages (miracidia and cercariae) from entering their respective hosts, or at changing human bathing behaviors in a way that reduces the risk of encountering the infective agents (Table 3).

Adult worms as a target

Reducing the prevalence of bird schistosomes in their final hosts appears to be the most obvious measure for controlling the distribution of swimmer's itch, and reducing duck abundance has been repeatedly suggested. However, intensive hunting or even merely scaring away waterfowl is not well accepted in recreational areas by the public, and especially during the bathing season. Ducks are well known to escape hunting pressure and this approach is therefore not very effective. One way to prevent aggregation of potential bird hosts close to swimming sites is by placing prohibition signs asking people not to feed ducks in the area. This is also a good opportunity to inform visitors about the etiology of swimmer's itch, the causative agent and the ecological context, and particularly allay baseless public fears about the severity of exposure and concerns about the water quality. However, so far there is no

Table 2. Most important swimmer's itch risk factors

Risk factor		Effect	Refs
Personal factors and behavior	History of swimmer's itch	Individual history of swimmer's itch plays an important role, because repeated exposure and infection correspond with a higher chance of contracting cercarial dermatitis and severity of infection due to host sensitization.	[1,3,62]
	Age	Significantly higher risks of infection among children younger than 15 years are reported, most likely due to age-related bathing habits, i.e., children spending more time in shallow, warm water where snails and parasites accumulate. However, evidence is inconclusive because some studies observed no significant effect of the age factor.	[58,60,62, 90,91]
	Bathing activity	Bathing behavior has a significant influence on the chances of encountering bird schistosome cercariae. People engaged in immersed activities (swimming, wading) are at higher risk than those merely engaged in surface activities, such as surfing, water skiing, or boating, due to higher exposure to cercariae.	[90]
	Bathing time	The amount of time spent in water positively correlates with the risk and severity of an infection, because the longer exposure allows more possible cercarial penetrations.	[60,62,92]
	Frequency of visits	The frequency of visits to the water body corresponds with higher incidence of swimmer's itch, due to increased chances of encountering the parasites. Contrastingly, negative associations between days of exposure and disease severity were found, which probably indicates that sensitized people with a known history of swimmer's itch tend to reduce or avoid recreational use of potentially infested waters.	[60,92]
Bathing locality	Shallow waters/surface water	Spending time in shallow water is associated with high infection risks because snails accumulate in shallow waters where cercarial production is further enhanced by high water temperatures in summer. However, suitable hosts can also occur at greater depths and the risk of swimmer's itch can, therefore, not be restricted to shallow areas, although risk of an infection is highest there. Bird schistosome cercariae become concentrated in the surface. Therefore, contact with surface water at sites with abundant snail communities can be considered a risk factor.	[8,18,31,33,54, 58,60,76,78,90]
	Aquatic vegetation	Rich aquatic vegetation is often considered an indicator of potential high-risk sites because it provides ideal conditions for abundant snail communities. However, suitable snail hosts can also occur in areas free of vegetation.	[78,93]
Time	Season	Shows a significant association with human infections because most cases occur during warm summer months with prolonged daylight hours and increased water temperatures when prevalence in snails is highest, and both cercarial emergence and human bathing activities peak.	[58,93]
	Time of day	Both probability and severity of infection are highest in morning hours when cercarial emergence peaks. Infectivity of released free-swimming cercariae declines with time, and the free-swimming larval stages are subject to predation by a wide variety of animals, probably reducing the risk of human infection as the day progresses.	[60,62,76,90]
Weather	Air temperature	Higher temperatures correspond with higher infection risk, probably due to increased bathing activity (i.e., more time spent in water) and higher cercarial emergence rates.	[62]
	Water temperature	High water temperature is considered a risk factor because high abundances of infected snails can often be found in warm waters.	[93]
	Sunshine	Long periods of sunshine increase the number of recorded outbreaks of swimmer's itch due to increased bathing activities and elevated cercarial emission on sunny days, especially following overcast periods.	[8,94]
	Wind and water movement	Onshore winds and water currents correspond with increased infection risks because they can transport cercariae that become concentrated in the surface water layers towards bathing sites. Clouds of cercariae may be transported by winds and currents for several kilometers. Infections can, therefore, also occur in areas seemingly devoid of snails.	[60,76,78,95]
Other factors	Eutrophication	Key ecological factor that triggers a cascade of factors (high host abundances and high prevalence) ultimately leading to an increased risk of human infections.	[2,40,90,93–95]
	Global warming	Increased risk of swimmer's itch expected because overwintering migratory birds in central Europe allow a longer host–parasite transmission window, leading to higher parasite prevalence and higher densities of cercariae.	[11]

evidence whether this rather cheap measure has any effect on the frequency of swimmer's itch outbreaks. A direct reduction of adult schistosomes in birds has been successfully achieved in a field trial by treatment with praziquantel [66]. However, this cost- and labor-intensive action can be effective only in areas with low waterfowl migration. Furthermore, the possible ecological consequences need to be considered carefully; although the drug and its metabolites show only low toxicity on non-target organisms [67], mass treatment of waterfowl with praziquantel might cause species shifts in entire trematode communities in birds, with unpredictable effects – for example, on the fish fauna.

The miracidia

The larvae hatching from released bird schistosome eggs need to find and infect a suitable snail host within their

Table 3. Possible preventive measures to control swimmer's itch

Target	Action	Estimated effectiveness	Estimated ecological impact
Final hosts and adult worms as targets	Hunting and scaring away waterfowl	Low: waterfowl escape hunting pressure	High
	Avoiding feeding waterfowl	Probably low but may help to inform people	Low
	Treatment of birds with anthelminthics (praziquantel)	Medium: time-consuming and labor-intense; probably only works in areas with low density of migratory waterfowl	Medium: small dosages given to individual birds; total effects unpredictable
Miracidia as targets	Traps for miracidia	?[a] Promising, but difficult	Low
	Predators of miracidia	?[a]	Hard to predict
	Decoy snails (non-host snails)	Probably low	High
Snails as targets	Manual removal of snails	Probably low: labor-intense, collecting all snails is impossible	Low
	Mechanical destruction of snails/disturbance of habitat	Medium: only local reduction of snail density	Medium-high: local habitat disturbance
	Use of molluscicides	High	High: severe ecological side effects; not acceptable
	Predators of snails	?[a]	Hard to predict
	Removal of vegetation (indirect snail control)	Low: snails also in areas free of vegetation	High
Intra-molluscan stages as targets	Antagonistic trematode species	?[a] Promising, but difficult	Hard to predict
Cercariae as targets	Predators of cercariae	?[a]	Hard to predict
	Traps for cercariae	?[a] Promising, but difficult	Probably low
Personal preventive measures	Protective cream formulations	High: effectively inhibit cercarial penetration	None
	Avoiding shallow waters	Medium: cercariae can also be released from snails in deeper water	None
	Avoiding morning hours	Medium: risk highest in morning, but infections can occur throughout the day	None
	Prohibition of bathing	Absolute if restrictions are respected	None

[a]?, unknown.

lifespan of only a few hours. Considering that after the enormous intramolluscan multiplication a single miracidium can produce several thousands of cercariae, it is obvious that this stage is a promising target for any control measure of trematode diseases. This idea has fuelled numerous studies on miracidial host-finding behavior and the chemical nature of the snail-emitted cues. Owing to the sensitivity with which *T. szidati* miracidia recognize macromolecular glycoconjugates from their molluscan hosts [15], traps baited with a synthetic, super-attractive snail kairomone have been suggested [68]. However, miracidia of two *Trichobilharzia* spp. have also been found to be capable of distinguishing between susceptible and nonsusceptible snail host species [15,69,70], and there is no reason to assume that other bird schistosomes are less capable of recognizing exclusively their specific host snail. Therefore, it might be difficult to compose a universal miracidial attractant for traps for all the different *Trichobilharzia* species, but more research is needed.

Another approach for reducing infections of the specific snail hosts is the introduction of large numbers of non-host snails, which miracidia penetrate but then fail to develop further. However, this so-called decoy effect was demonstrated mainly in neotropical *Schistosoma mansoni* strains, which seems to be less capable of species-specific snail recognition [71,72]. Non-host snails were shown to be unattractive for miracidia of *T. szidati* under simulated field conditions [14]. Therefore, besides possible negative ecological effects, the introduction of decoy snails does not appear to be a promising measure to control swimmer's itch.

With respect to biological measures, typical filter feeders have a significant effect on free-swimming miracidia, such as the annelid *Chaetogaster limnaei*, an ecto-commensal living directly on the surface and in the mantle cavity of freshwater snails; the annelid feeds upon miracidia approaching the snail, as well as upon cercariae freshly emerged from the snail [38]. In fact, evidence from experimental [73] and field studies [74] suggests that an increased presence of *C. limnaei* negatively correlates with trematode infection in snails.

Snails and intramolluscan stages

The most obvious way to control bird schistosomes inside their intermediate hosts is the reduction of the host snail populations. For ecological reasons, the massive use of molluscicides such as niclosamide in recreational lakes, which often serve as wildlife sanctuaries, is definitely not acceptable. By contrast, collecting potential host snails by hand is a rather mild intervention. Although this was regarded as a suitable option locally in some places [75], it appears far too personnel-intensive and also not efficient enough as a reasonable control strategy. Mechanical

crushing of snails on a large scale by dragging heavy, harrow-like devices over shallow areas along the shore of swimming lakes has led to a significant reduction in the number of cases of swimmer's itch in two lakes in Canada and France [76,77]. However, this procedure requires good accessibility with heavy machines and an even and solid lake bottom substrate without larger obstacles. Furthermore, the effort and the expected effects should be carefully balanced against the serious ecological damage. Hence, this solution is probably applicable for bathing ponds or only very locally in frequently visited parts of larger lakes. Furthermore, the removal of aquatic vegetation has been suggested to destroy snail habitats [75]. Nevertheless, suitable snail hosts may also occur in areas lacking aquatic vegetation [78]. Biological control of snail populations by fish has also been discussed in schistosomiasis areas (e.g., [32,79]); however, conclusive field data are still lacking.

An elegant biological method to control schistosomes inside their snail hosts is the use of antagonistic trematodes. Contrary to schistosome intramolluscan sporocyst stages that take up nutrients solely via their surface, other digenean groups develop via rediae, larval stages with a mouth and a pharynx; these not only feed on the snail's hepatopancreas but also on other coinfecting trematodes. Laboratory and field studies have shown that echinostomatids outcompete and reduce/eliminate schistosomes in the snails [80–83]. However, as some echinostome metacercariae are a major pathogen, for example in amphibians [37], only indigenous, sympatric non-pathogenic echinostome species can be used for this purpose, which requires a thorough investigation of the natural trematode fauna to identify suitable candidate species in a given habitat.

The infective cercariae

Another auspicious target could be the actual causative agent of swimmer's itch, the furcocercariae emerging in large numbers from infected snails. Here again, control approaches for bird schistosomes could make use of the experience gained in research efforts towards control methods for *Schistosoma* spp. that are human pathogens.

Several aquatic organisms such as small fish [84], turbellarians [85], and planktonic crustaceans [51,86] have been shown to feed upon schistosome cercariae. However, manipulating the faunal composition of a natural water body to increase potential predators is probably not easy to achieve and always a mixed blessing: high densities of daphnids and copepods require eutrophic conditions from which the host snails benefit as well. High numbers of fish larvae feed not only on cercariae but also on daphnids and copepods. Furthermore, in contrast to *S. mansoni*, for example, the cercariae of *Trichobilharzia* spp. accumulate at the water surface [54] and are thus not accessible to several potential predators. The oligochaete *C. limnaei* is probably the most promising candidate, and is frequently associated with lymnaeid snails that serve as hosts for bird schistosomes in temperate zones [38], but so far there is nothing known about how its prevalence on snails can actually be increased under natural conditions.

Traps for *S. mansoni* cercariae have been successfully applied for monitoring cercarial densities, using polyunsaturated fatty acids as an attractant [87]. These compounds resemble the lipid composition of human skin and stimulate the cercariae to transform their tegument even without host contact, which kills them due to the loss of their osmotic protective surface [88,89]. Cercariae of *Trichobilharzia* spp. respond to similar fatty acids (the reason for the erroneous penetration of the human non-host) [18], which might have the same cercaricidal effect. Here more research on ecological non-hazardous derivates could provide another promising approach to tackle swimmer's itch [68].

In summary, there are several possibilities to interrupt the life cycle of the bird schistosomes causing swimmer's itch. Every measure against the different target stages has pros and cons (Table 3), and not all options suggested from laboratory experiments appear applicable or promising under natural conditions, in lakes and recreational areas in developed countries. Each strategy needs to be evaluated in relation to the specific characteristics of a given locality, and the economic effort and ecological interference should be weighed against the expected risk and the actual necessity to take action against an unpleasant and painful, but eventually not really dangerous disease. Of course, the situation in a sparsely visited, ecologically-sensitive conservation area differs completely from a bathing lake in a tourist hot-spot. A useful and very successful procedure in one place might appear as an economical and/or ecological disaster in another site. There is definitely no patent remedy for all affected water bodies, and none of the biological control methods reviewed here could guarantee a complete disappearance of swimmer's itch. Furthermore, the total elimination of parasites that belong to a natural ecosystem is highly questionable and may even be in conflict with the European Water Framework Directive [59]. Nevertheless, if carefully evaluated and competently organized, some of the approaches described might prove to be mild but powerful tools. Last but not least, applying biological methods for controlling bird schistosomes in wealthy developed countries offer not only interesting scientific possibilities but might equally serve for testing potential projects in developing countries where schistosomiasis in humans is a serious medical and socio-economic problem.

Personal preventive measures

In addition to the biological control and preventive measures, personal protective care and bathing behavior can have effects on the risks of an infection in humans. Based on the potential risk factors (Table 2), changes in bathing behavior (e.g., avoiding swimming during morning hours or in shallow waters) have been suggested to decrease the risk of an infection [58,90]. Although such measures have no negative ecological impact, their effectiveness is questionable because snail and trematode occurrence cannot be restricted to regional clusters or temporal windows [78]. Cream formulations have shown to inhibit cercarial penetration effectively, but current products are expensive and may not be widely available [63]. Although total avoidance of potentially infested waters appears the only effective measure to ensure absolute protection, bathing restrictions may not be respected [62], and infections will still occur. The personal protective measures can only help to reduce the risk of obtaining swimmer's itch but can never provide absolute protection. It is therefore important to

communicate the possible negative effects of swimming in surface waters to the public [59] and provide adequate and understandable information on the potential risks (e.g., information boards on swimmer's itch at swimming sites). This raised awareness will not only help to identify cases better, and thus facilitate our understanding of the disease's occurrence, but may also allow a factual discussion of the problem free of exaggerated alarmism.

Acknowledgments

We are grateful to Jana Köchling, Verena Altmann, and Jessica Schwelm for their invaluable help with our excel files, Sonja Kreft and Frank Bolz for their superb work on the illustrations, and the editor for the valuable input to a more concise piece of writing. We thank three anonymous reviewers for their comments and suggestions. We acknowledge the partial support of a Czech Science Foundation grant (P505/10/1562 to A.K. and M.S.), a PhD fellowship of the Deutsche Bundesstiftung Umwelt (DBU) to C.S., and partial funding of the 'Sichere Ruhr' project (grant 02WRS1283 to B.S.) as part of the Bundesministerium für Bildung und Forschung (BMBF) program 'Sustainable Water Management'.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pt.2012.12.002.

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15. Contributions

Georgieva, S.*, Selbach, C.*, Faltýnková, A., Soldánová, M., Sures, B., Skírnisson, K., Kostadinova, A. (2013). New cryptic species of the '*revolutum*' group of *Echinostoma* (Digenea: Echinostomatidae) revealed by molecular and morphological data. *Parasites & Vectors*, *6*(1), 64. * shared first authorship

Personal contributions: Together with MS and KS, I obtained the samples for this study. AF, MS, SG and I undertook the morphological species identifications and drafted the manuscript. KS, BS and AK conceived and coordinated the study. SG and I share the first authorship of this publication.

Selbach, C., Soldánová, M., Georgieva, S., Kostadinova, A., Kalbe, M., Sures, B. (2014). Morphological and molecular data for larval stages of four species of *Petasiger* Dietz, 1909 (Digenea: Echinostomatidae) with an updated key to the known cercariae from the Palaearctic. *Systematic Parasitology, 89*, 153–166.

Personal contributions: MS, MK and I obtained the samples, undertook the identification and morphological characterisation of the isolates and prepared the first draft of the manuscript. SG performed the phylogenetic analyses and drafted the corresponding parts of the manuscript. BS and AK conceived the study, discussed the results and helped draft the manuscript.

Selbach, C., Soldánová, M., Georgieva, S., Kostadinova, A., & Sures, B. (2015). Integrative taxonomic approach to the cryptic diversity of *Diplostomum* spp. in lymnaeid snails from Europe with a focus on the '*Diplostomum mergi*' species complex. *Parasites & Vectors, 8*(1), 300.

Personal contributions: MS and I obtained the samples, undertook the identification and morphological characterisation of the isolates and prepared the first draft of the manuscript. SG and I carried out the sequencing. SG performed the phylogenetic analyses and drafted the corresponding parts of the manuscript. BS and AK conceived the study, discussed the results and helped draft the manuscript.

Soldánová, M. *, Selbach, C.*, Sures, B. (2015). The early worm catches the bird? Productivity and patterns of *Trichobilharzia szidati* cercarial emission from *Lymnaea stagnalis.* (Submitted manuscript).* shared first authorship

Personal contributions: MS and I conducted the experiments, performed the analyses and wrote the manuscript. MS conceived the study. BS coordinated the study. MS and I share the first authorship of this manuscript.

Selbach, C., Soldánová, M., Sures, B. (2015). Hidden diversity on our doorstep - trematode assemblages and communities in lymnaeid and planorbid snails in a central European reservoir system.

Personal contributions: MS and I obtained the samples, identified the trematode species and analysed the data. I wrote the manuscript. BS oversaw the study.

Soldánová, M., Selbach, C., Kalbe, M., Kostadinova, A., Sures, B. (2013). Swimmer's itch: etiology, impact, and risk factors in Europe. *Trends in Parasitology*, *29*(2), 65–74.

Personal contributions: MS, BS and I conceived the review. MS and I did the literature review. MS, MK and I wrote the manuscript. AK and BS discussed the results and helped draft the manuscript.

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Hiermit bestätige ich die oben gemachten Angaben.

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16. Curriculum vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten

17. Declarations

Hiermit erkläre ich, gem. § 7 Abs. (2) d) + f) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient, bei der Abfassung der Dissertation nur die angegeben Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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