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Timing matters: exploring emergence patterns of two species of trematode furcocercariae from their snail hosts

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Abstract: Cercariae are motile infectious larval stages of digenetic trematodes that emerge from their molluscan first intermediate host to seek the next host in their life cycle. A crucial transmission strategy of trematodes involves releasing the maximum number of cercariae at times that coincide with the presence and activity of the next hosts, thereby increasing the likelihood of successful infection and continuation of the parasite's life cycle. We investigated the cercarial emergence of two furcocercous (with forked tail) trematodes *Tylodelphys clavata* (von Nordmann, 1832) and unidentified species of *Sanguinicola* Plehn, 1905 from naturally infected *Ampullaceana balthica* (Linnaeus) and *Radix auricularia* (Linnaeus) snails under natural light and constant temperature conditions. Both trematodes, which are important fish pathogens, showed distinct daily emergence rhythms influenced by light intensity, with emergence peaking at sunset and night for *T. clavata* and at night for *Sanguinicola* sp. The daily emergence rhythms of *T. clavata* cercariae were consistent in both summer and autumn, indicating adaptability to natural changes in seasonal photoperiods. The interspecific differences in emergence patterns are likely related to the behavioural patterns of upstream, i.e., next in the life cycle, fish hosts. Cercarial output also varied between trematode species and seasons, likely due to combined effects of snail size, intensity of trematode infection in snails and size of cercariae rather than seasonal temperatures. The trematodes were molecularly characterised using mitochondrial (*cox*1) and nuclear (28S rDNA and ITS1-5.8S-ITS2) regions to confirm their identity and facilitate future studies. This study highlights the importance of light-regulated and host-synchronised cercarial emergence rhythms for increased trematode transmission success and reveals significant variation in cercarial output influenced by environmental and biological factors, contributing to a deeper understanding of trematode ecology and disease management.

Keywords: snails, cercariae, *Tylodelphys clavata*, *Sanguinicola* sp., transmission, DNA

This article contains supporting files (Tables S1–S6 and Fig. S1) online at http://folia.paru.cas.cz/suppl/2025-72-008.pdf

The transmission strategies of heteroxenous parasites (i.e., infecting multiple hosts) exhibit a variety of sophisticated mechanisms aimed primarily at facilitating the completion of their life cycles (e.g., Thomas et al. 2002, Cornet et al. 2014, Wesołowska and Wesołowski 2014, Nezhybová et al. 2020, Faltýnková et al. 2023). These have evolved as adaptations to the risky process of host-to-host transmission mediated by their motile free-living stages in the environment (Kennedy 1975, Poulin et al. 2011, Whittington and Kearn 2011).

The probability of a parasite encountering a suitable host may be limited by the sparse distribution or density of the host (Kuris and Lafferty 2005, Fredensborg et al. 2006, Byers et al. 2008, Stien et al. 2010), predation on parasites (Anderson et al. 1978, Johnson and Thieltges 2010, Thieltges et al. 2013, Koprivnikar et al. 2023) and other environmental factors (Pietrock and Marcogliese 2003, Thieltges et al. 2008a). Therefore, the timing of the emergence of parasitic infective stages certainly matters, as the synchronisation of the maximum abundance of the parasite with the highest activity and aggregation of the next hosts represents a critical moment for parasite survival and reproduction (Kennedy 1975, Combes et al. 1994, Craig and Scott 2014, Rijo-Ferreira et al. 2020).

Trematodes are a shining example of complex life cycles and diverse adaptations to enhance successful transmission. They typically follow a three-host life cycle, including a definitive host for sexual reproduction, egg production and development of first free-living infective larvae, miracidia; a first intermediate molluscan host in which asexual reproduction takes place via sporocysts and/or rediae and second free-living infective larvae, cercariae, are produced; and a second intermediate host in which metacercariae (final dormant infective larval stages) await ingestion by a definitive host (Esch et al. 2002, Galaktionov and Dobrovolskij 2003). Molluscs play a crucial role in the development of trematodes.

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This intimate relationship is reflected in the behavioural, morphological and physiological patterns of both the parasite and the infected host to meet the parasite's needs for successful transmission. For example, trematode intramolluscan stages cause castration of snails (Sorensen and Minchella 2001, Fredensborg et al. 2005, Lafferty and Kuris 2009, Żbikowska 2011), gigantism (Mouritsen and Jensen 1994, Chapuis 2009), modifications in their shell shape (Levri et al. 2005, Hammoud et al. 2022), locomotory activity (Mouritsen and Jensen 1994, O'Dwyer et al. 2014) and behaviour related to thermal preferences (Żbikowska and Cichy 2012, Żbikowska and Marszewska 2018) or predation risk (Levri and Lively 1996, Levri 1998).

Trematode cercariae emerging from molluscs in active search for their next hosts also employ numerous transmission strategies to compensate for their short lifespan and infectivity (Morley 2012, Born-Torrijos et al. 2022). These include a variety of swimming and dispersal behaviours to position themselves in "host space and time" (Beuret and Pearson 1994, Combes et al. 1994, Haas 1994, 2003, Haas et al. 2008, Selbach and Poulin 2018, Faltýnková et al. 2023). However, the emergence patterns (daily rhythms) and output rates (daily production) of cercariae from the snail are arguably the most critical strategies to efficiently infect the next hosts and maintain the trematode life cycle.

The cercarial emergence is a diverse and complex process that largely depends on the trematode transmission pathways to their intermediate or definitive hosts (Théron 1984, Combes et al. 1994, Théron 2015) and environmental factors such as snail size (Poulin 2006, Morley et al. 2010), infection intensity (Le Clec'h et al. 2019), trematode genotypes (Berkhout et al. 2014), salinity (Mouritsen 2002, Koprivnikar and Poulin 2009, Born-Torrijos et al. 2014), nutrients (Johnson et al. 2007) and water level (Mouritsen 2002, Fingerut et al. 2003, Koprivnikar and Poulin 2009, Born-Torrijos et al. 2014), of which temperature and light intensity are the most important (e.g., Smyth and Halton 1983, Morley 2012, Théron 2015). In general, an increase in temperature and a change in light intensity favour transmission.

A higher temperature triggers the emergence of cercariae and promotes their production up to a certain optimum, which is usually followed by a rapid decline (e.g., Poulin 2006, Thieltges and Rick 2006, Morley et al. 2010, Morley and Lewis 2013, Khosravi et al. 2023). The effect of light intensity, on the other hand, is species-specific and more pronounced in daily emergence rhythms (McCarthy 1999, Prokofiev et al. 2016, Soldánová et al. 2016, Vyhlídalová and Soldánová 2020). Trematodes can emerge diurnally (during the daytime) or nocturnally (at night) with a circadian (one emergence peak over 24 hours), ultradian (two or more peaks) or infradian rhythm (without any periodicity) (Combes et al. 1994, Lo and Lee 1996, Hannon et al. 2018). This influence of light on the timing of cercarial emergence is highly variable in many trematode genera, being specific even at the species level (Théron 1989, Théron et al. 1997, Mouahid et al. 2012, Prokofiev et al. 2016, Vyhlídalová and Soldánová 2020).

Daily emergence peaks also often coincide with the presence and seasonal behavioural changes of a suitable host (Théron 1984, McCarthy et al. 2002, Kiatsopit et al. 2014, Prokofiev et al. 2016), but at the same time seem to be independent of fluctuations in the natural seasonal photoperiod. This means that the cercarial emergence rhythms show similar patterns regardless of the temporal shifts of the photoperiod during the year (Soldánová et al. 2016, Vyhlídalová and Soldánová 2020) or geographical areas (Anderson et al. 1976, Soldánová et al. 2016, Soldánová et al. 2022a). Nevertheless, the cercarial emergence of many trematode species is still poorly understood, despite their veterinary or medical importance and despite their ecological importance in ecosystems due to their enormous biomass and high potential to alter trophic links in food webs (e.g., Kuris et al. 2008, Soldánová et al. 2016, Koprivnikar et al. 2023).

Determination of emergence peaks of cercariae therefore provides valuable insights into the extent of parasite adaptations to transmission, an extremely relevant prerequisite for understanding parasite ecology, disease epidemiology and effective prevention and treatment measures. In addition, it can help to better understand the effects of environmental factors, such as the presence of filter-feeding bivalves that clear the water or eutrophication that reduces light penetration, on light intensity in aquatic ecosystems, which in turn could affect the cercarial emergence and transmission dynamics (Vyhlídalová and Soldánová 2020).

The main aim of this study was to investigate the cercarial emergence of *Tylodelphys clavata* (von Nordmann, 1832) (Diplostomidae) and an unidentified species of *Sanguinicola* Plehn, 1905 (Sanguinicolidae) from naturally infected small lymnaeids *Ampullaceana balthica* (Linnaeus) and *Radix auricularia* (Linnaeus) (Gastropoda, Lymnaeidae) under different natural light conditions and temperature-controlled laboratory conditions mimicking natural seasonal conditions. Both species have a similar morphology in the cercarial stage, characterised by a forked tail that facilitates movement in the water. They are important fish parasites, but differ in their mode of transmission. *Tylodelphys clavata* uses fish as a second intermediate host and fish-eating birds as a definitive host (Kozicka and Niewiadomska 1960), while species of *Sanguinicola* directly infect fish as a definitive host (Kirk 2012).

Although cercarial emergence is a basic transmission strategy for trematodes, there are little data on these important fish parasites. For *T. clavata*, any information is lacking, while for some species of *Sanguinicola* data are available (Martin and Vazquez 1984, Sommerville and Iqbal 1991, Kirk and Lewis 1993). In addition, only a few studies from Europe have addressed the production and emergence patterns of cercariae from small lymnaeid snails, particularly from *Myxas glutinosa* (Müller) (Karvonen et al. 2006), *Lymnaea peregra* (Müller) (syn. *Peregriana peregra*; nomenclature according to Aksenova et al. 2018) (McCarthy 1999, Morley et al. 2003, Morley et al. 2010), *Radix balthica* (Linnaeus) (syn. *Ampullaceana balthica*, nomenclature according to Aksenova et al. 2018) (Soldánová et al. 2022a) and *Radix lagotis* (Schrank) (syn. *Ampullaceana lagotis*, nomenclature according to Akseno-

va et al. 2018) (Vyhlídalová and Soldánová 2020), underlying the need for more detailed studies in this regard.

Therefore, we specifically aimed to (i) molecularly characterise the experimental trematodes and their snail hosts, (ii) explore and provide new data on the daily production and emergence rhythms of cercariae of *T. clavata* and *Sanguinicola* sp., (iii) evaluate seasonal differences in daily production and emergence rhythms as a function of different photo- and thermoperiods, and (iv) investigate the effects of biotic (snail size and infection intensity) and abiotic (temperature and light intensity) factors on the emergence of cercariae.

We expect distinct interspecific variations in the cercarial emergence due to temperature, parasite life cycle and life-history traits of trematodes, such that cercariae of experimental trematodes show higher production at higher temperatures, but different emergence rhythms depending on light intensity and behaviour on the next life cycle hosts. Furthermore, we assume a pronounced adaptability of the emergence patterns of cercariae to changing seasonal conditions, i.e., despite different thermal and light conditions, so that daily rhythms of the individual species are identical across seasons but shifted in time due to a seasonally specific photoperiod.

MATERIALS AND METHODS

Sampling and processing of snails

Lymnaeid snails *Ampullaceana balthica* and *Radix auricularia* were collected in the littoral zone of lakes Medard and Otakar in northern Bohemia, Czech Republic. Lake Medard (50.1789N, 12.5986E) is the largest man-made lake in the country with an area of 496 hectares and a maximum depth of 50 metres. It is located in a former coal mining area that ceased operations in 2000, followed by gradual flooding from 2011 to 2016 for landscape revitalisation and recreational purposes (Žižka et al. 2020). The fish fauna comprises 12 species from five families (Supplementary Table S1) and molluscan fauna ten species (Beran 2019), including four species of small lymnaeid snails: *Radix ampla* (Hartmann) (syn. *Ampullaceana ampla*), *Radix balthica* (syn. *Ampullaceana balthica*), *Galba truncatula* (Müller) and *R. auricularia* (nomenclature according to Aksenova et al. 2018).

Lake Otakar (50.6513N, 13.7397E) is a smaller and shallower waterbody (9 ha, max. depth 18 m), which was created by spontaneous flooding of a small surface coal mine in the 1980s (Přikryl and Havel 2010). Since then, the lake has been accessible to the public for various recreational activities and fishing. The fish fauna of Lake Otakar consists of five species belonging to five families (Supplementary Table S1) but is largely dominated by the common carp *Cyprinus carpio* Linnaeus (Czech Anglers Union, pers. comm.). Both lakes represent important ecosystems that promote remarkable animal biodiversity (Pešout et al. 2022), particularly renowned for their role as sanctuaries for birds (Bažant 2015, 2018, 2020), which serve as definitive hosts for numerous trematode species including one of the experimental trematodes *Tylodelphys clavata*.

A total of 819 *A. balthica* snails from Lake Medard and 117 *R. auricularia* snails from Lake Otakar were randomly handpicked from stones in the shallow littoral zone during July and September 2021 and July 2022 (Table 1). After transportation to the laboratory, the snails were preliminarily identified based on their morphology according to the keys of Glöer (2002, 2019), placed individually in transparent 40 ml plastic beakers filled with 30 ml of filtred lake water and exposed to a light source for 24 h to induce cercarial emergence. Snails were then examined under a stereomicroscope for patent infections (cercariae released into the water), and their shell length and width were measured using a digital caliper (MarCal 16 EWRi-V Digital Universal Caliper IP67, Mahr, Germany).

Cercariae were identified alive under an Olympus BX51 light microscope (Olympus Optical Co., Ltd., Tokyo, Japan) using the morphological keys of Našincová (1992) and Faltýnková et al. (2007) and photographed using Promicam 3-5CP digital camera (Promicra, Prague, Czech Republic) attached to the light microscope as described by Kundid et al. (2024). Snails with patent infections of *T. clavata* (ex *A. balthica*) and *Sanguinicola* sp. (ex *R. auricularia*) were transferred to separate aquaria with aerated lake water and fed *ad libitum* with lettuce (*Lactuca sativa*) until the start of the experiments. All infected snails had mature infections with both experimental trematodes and were actively shedding cercariae prior to the experiments. No double infections were observed before or during the experiments. The total body length of six cercariae of *T. clavata* and five cercariae of *Sanguinicola* sp. were measured using the programme ImageJ v.1.53e (Abràmoff et al. 2004).

Due to overlapping shell characteristics of the genera *Ampullaceana* Servain and *Radix* Montfort (Schniebs et al. 2011, Huňová et al. 2012), eight representative snails (two from each experiment) were molecularly analysed to confirm their identity. The prevalence of infection was determined by dividing the number of infected snails by the total number of snails examined in the population sample (Bush et al. 1997), and differences in prevalences of each species between seasons were compared using a chi-square test (χ^2), with results considered significant at $P < 0.05$ differences in prevalences of each species between seasons were compared using a chi-square test (χ^2), with results considered significant at $P < 0.05$.

Experimental trematodes

Trematodes *T. clavata* and *Sanguinicola* sp. were selected due to their relatively high abundance in snails in the study area (Table 1). Additionally, there is a lack of sufficient knowledge about their cercarial output rates and emergence patterns, as well as an overall scarcity of ecological and molecular data on the genus *Sanguinicola* (Zhokhov et al. 2021).

The cercariae of both species aim to infect different microhabitats in fish as the second intermediate host (*T. clavata*) and definitive host (*Sanguinicola* sp.). Metacercariae of *T. clavata* are fish pathogens most commonly found in perch (*Perca fluviatilis* Linnaeus) infecting the vitreous humour of the eye. Large numbers of accumulated metacercariae can impair the vision and foraging success of fish, causing substantial damage to aquaculture facilities (Vivas Muñoz et al. 2017, 2019, Heneberg and Sitko 2021, Unger et al. 2022). The cercariae of the blood fluke *Sanguinicola* sp. infect the definitive fish host, principally the Cyprinoidei, directly through the skin, gills, fins and opercular cavity, eventually migrating to the blood vessels (Kirk 2012, Zhokhov et al. 2021).

Infection with *Sanguinicola* sp. can cause significant damage to the fish gills and lead to respiratory distress, reduced growth and increased mortality, especially in fish fry (Kirk 2012).

Molecular identification of parasites and snails

To molecularly confirm the species identity, at least 20 cercariae per experimental snail were preserved in molecular-grade ethanol. Genomic DNA was isolated using the Monarch® Genomic DNA Purification Kit (New England Biolabs®, Ipswich, MA, USA). For molecular identification of *T. clavata*, the mitochondrial cytochrome c oxidase subunit 1 gene (*cox*1) was amplified using the primers Plat-diploCOX1F (5′-CGT TTR AAT TAT ACG GAT CC-3′) and Plat-diploCOX1R (5′-AGC ATA GTA ATM GCA GCA GC-3′) (Moszczynska et al. 2009) following the cycling conditions of Kudlai et al. (2017).

For a single isolate of *T. clavata*, the following nuclear markers were sequenced to provide additional molecular data: i) D1–D3 region of the large ribosomal subunit (28S rDNA) using the forward primer ZX-1 (5′-ACC CGC TGA ATT TAA GCA TAT-3′) and reverse primer 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) following the protocol described by Tkach et al. (2003), and ii) complete fragment of the ribosomal ITS1-5.8S-ITS2 region using the forward primer D1 (5′-AGG AAT TCC TGG TAA GTG CAA G-3′) and reverse D2 (5′-CGT TAC TGA GGG AAT CCT GGT-3′) as described in Galazzo et al. (2002).

To molecularly identify *Sanguinicola* sp., the D1–D3 region of the large ribosomal subunit (28S rDNA) was sequenced as previously described. Additional genetic mitochondrial *cox*1 data were obtained for one isolate of *Sanguinicola* sp. using the primers SchistoCox1-5′ (5′-TCT TTR GAT CAT AAG CG-3′) and SchistoCox1-3′ (5′-TAA TGC ATM GGA AAA AAA CA-3′) (Lockyer et al. 2003) under the cycling conditions of Reier et al. (2020).

Foot tissue samples from eight representative snails (two per experiment) were randomly selected for DNA isolation and sequencing. The ITS-2 rDNA region was amplified using the primers NEWS (5´-TGT GTC GAT GAA GAA CGC AG-3´) and RIXO (5´-TTC TAT GCT TAA ATT CAG GGG-3´) (Almeyda-Artigas et al. 2000) following the protocol described by Bargues et al. (2001).

PCR amplicons were purified using ExoSAP-IT™ Express PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA) and sequenced from both strands using the corresponding PCR primers. The following internal primers were additionally used for 28S: 300F (5'-CAA GTA CCG TGA GGG AAA GTT G-3') (Littlewood et al. 2000) and ECD2 (5'-CCT TGG TCC GTG TTT CAA GAC GGG-3') (Littlewood et al. 1997). Sequencing was carried out utilising ABI Big Dye™ v.3.1 chemistry on an AB3730 x 1 capillary sequencer at the commercial company SeqMe (Dobříš, Czech Republic). Newly generated sequences were assembled and edited in Geneious Prime® 2024.0.5. and deposited in GenBank.

Four alignments, including novel and previously published sequences, were built using MUSCLE implemented in Geneious Prime® 2024.0.5. Alignment 1 (365 nt long) included 55 *cox*1 sequences of *Tylodelphys* spp., with 19 sequences generated in the present study identified as *T. clavata.* A sequence of *Diplostomum spathaceum* (Rudolphi, 1819) (JX986887), a parasite of *Larus cachinnans* Pallas (Charadriiformes, Laridae) from the Czech Republic, was used as the outgroup based on the topology in the phylogenetic tree provided by Sereno-Uribe et al. (2019).

Alignment 2 (817 nt long) included 26 ITS1-5.8S-ITS2 sequences of *Tylodelphys* spp., with one sequence generated in the present study. A sequence of *Diplostomum pseudospathaceum* Niewiadomska, 1984 (JX986854), parasite of *L. cachinnans* from the Czech Republic, was used as the outgroup based on the topology in the phylogenetic tree provided by Sokolov et al. (2022).

Alignment 3 (1,144 nt long) included 15 28S rDNA sequences of *Tylodelphys* spp., with one sequence generated in the present study. A sequence of *D. pseudospathaceum* (KR269766), a parasite of *Chroicocephalus ridibundus* (Linnaeus) (Charadriiformes, Laridae) from the Czech Republic, was used as the outgroup based on the topology in the phylogenetic tree published by Sereno-Uribe et al. (2019).

Alignment 4 (1,128 nt long) included 37 28S rDNA sequences of species from the family Sanguinicolidae; 13 sequences of one species identified as *Sanguinicola* sp. were generated in the present study. Sequence of *Acipensericola glacialis* Warren et Bullard in Warren, Roberts, Arias, Koenigs et Bullard, 2017 (MF186851), a parasite of *Acipenser fulvescens* Rafinesque (Acipenseriformes: Acipenseridae) from the USA, was used as the outgroup based on the topology in the phylogenetic tree published by Outa and Avenant-Oldewage (2024). Pairwise genetic distances (uncorrected p-distance and number of differences) for the four datasets were calculated in MEGA v. 11.

Phylogenetic relationships of the taxa in all alignments were assessed using maximum likelihood (ML) and Bayesian inference (BI) analyses. The analyses were conducted using the GTR + I + G model for Alignment 1 and 4, and HKI + G + I for Alignment 2 and 3, which was predicted as the best model by the Akaike Information Criterion in jModelTest 2.1.4 (Darriba et al. 2012). BI analysis was performed using MrBayes software (version 3.2.3) through the CIPRES Science Gateway version 3.3 (Miller et al. 2010), accessed on 22 May 2024. Markov Chain Monte Carlo chains were run for 10,000,000 generations, log-likelihood scores were plotted, and only the final 75% of trees were used to build the consensus tree. ML analysis was performed using PhyML version 3.0 (Guindon et al. 2010) and run on Geneious with a non-parametric bootstrap value of 100 pseudoreplicates.

Experimental setup

To monitor cercarial emergence patterns and daily output rates, the experiments were conducted over a 72-hour period (three consecutive days) at four main daily intervals (sunrise, day, sunset and night) under natural light conditions and constant controlled temperature (see Table 2). The four main daily intervals were chosen to capture key environmental conditions (here light) that cyclically fluctuate throughout the day and are known to influence cercarial emergence. This design ensures that data are collected during biologically relevant phases of the diel (24-hour) cycle. Furthermore, it maximises resource efficiency while still providing high-quality, interpretable data that reflects the natural dynamics of cercarial emergence.

The experiments with *T. clavata* were conducted shortly after samplings, i.e., in August and September 2021, representing the summer and autumn seasons, and the experiments with *Sanguinicola* sp. took place in early August 2021 and late July 2022, representing the summer season. A different set of snails was used for each experiment, i.e., snails from field collections corresponding to the months of the experiments. When possible, snails with

similar shell sizes were selected to minimise a potential bias due to larger snails producing more cercariae (Loker 1983, Poulin 2006, Morley et al. 2010).

Infected snails were placed in 40 ml transparent plastic beakers filled with 30 ml of lake water and allowed to acclimatise for 24 hours before the start of the experiment. During the entire acclimatisation and experimental period, the snails were not fed according to Vyhlídalová and Soldánová (2020) and Soldánová et al. (2022a). One snail infected with *T. clavata* (1T-S) died during acclimatisation, and one snail infected with *Sanguinicola* sp. (1S-A) did not shed cercariae during the experiment, so they were excluded from the dataset.

Beakers containing infected snails were attached to a metal construction covered with a transparent plastic lid (allowing penetration of the natural light) to prevent the snails from escaping, and partially submerged in an aquarium filled with tempered water. The water temperature was set to the mean value of the seasonal temperature measured in the field with an aquarium heater (Tetra HT 25W, Melle, Germany). Based on field data, the water temperature was set to ~22° C in August 2021 and ~20° C in September 2021 (*T. clavata*) and ~22° C in August 2021 and ~22° C in July 2022 (*Sanguinicola* sp.).

Temperature and light intensity data were monitored during the experiments using data loggers (Onset HOBO UA-002-64 Pendant 64K; Onset, USA), data on these parameters are presented in Table 1, and data on the length of daily intervals in Table 2. The length of photoperiods (light : dark) were approximately 15 : 9 h and 13 : 11 h for *T. clavata* in August and September 2021, respectively, and 16 :8 h and 15 : 9 h for *Sanguinicola* sp. in August 2021 and July 2022, respectively. All snails were dissected either during (in case of premature snail death) or after the experiment to estimate the infection intensity by visual assessment of the proportion of hepatopancreas occupied by sporocysts.

Cercarial number estimation

At the start and end of each daily interval (sunrise, day, sunset and night), the snails were carefully transferred with a clean spoon into new transparent beakers containing fresh filtred lake water (tempered as described in *Experimental setup*) to avoid contamination. The water containing cercariae from a given interval was then thoroughly mixed and ten subsamples of 1 ml suspension were transferred to six-well plates for counting. A few drops of a 4% formaldehyde solution (Sigma-Aldrich, Prague, Czech Republic) were added to each well to immobilise the cercariae and facilitate counting.

The number of cercariae that emerged from each snail was estimated according to a previously described procedure (Soldánová et al. 2016, Vyhlídalová and Soldánová 2020). Briefly, for each daily interval, a mean value of the ten subsamples was calculated and multiplied by the total sample volume (30 ml). The mean values were then combined across all daily intervals to give an estimate of the average number of cercariae emerged per snail per day (cercarial output rates). To analyse cercarial emergence patterns, the mean number of cercariae that emerged during each daily interval (raw data in Table 2) was converted to a one-hour basis, taking into account the different durations of the daily intervals. This conversion was performed for each experimental species and each season.

The raw data showing the number of cercariae that emerged from each snail, experimental day and season are deposited in Mendeley Data – https://data.mendeley.com/datasets/kkbzn44dd2/1

Analysis of the emergence data

The emergence patterns of cercariae were analysed as follows: first, the data were inspected visually from each snail individual by plotting the number of emerged cercariae per hour for each experimental species to assess the consistency of emergence periodicity (Supplementary Fig. S1A–C). This dataset included all snails that survived at least one experimental day (24 h), i.e., ten snails for *T. clavata* in August, ten snails for *T. clavata* in September and 13 snails for *Sanguinicola* sp. (Supplementary Fig. SC1).

The second dataset included only the snails that survived all three experimental days, i.e., ten snails for *T. clavata* in August, nine snails for *T. clavata* in September and 12 snails for *Sanguinicola* sp. (see Supplementary Table S2 for details). Subsequent statistical analyses were performed on this second dataset. Repeated measures ANOVA (RMA) was performed to test for differences in emergence patterns among specific daily intervals. The number of cercariae that emerged during each interval (converted to 1 hour) was used as the dependent variable, while time (daily intervals) and day were considered within-subjects factors. Tukey's Honestly Significant Difference (HSD) *post-hoc* tests were performed to identify emergence peaks within daily intervals.

RMA analyses for *T. clavata* were performed separately for each season (August and September 2021). To compare the emergence patterns (daily rhythms) of *T. clavata* cercariae between seasons, a combined RMA was performed with season as a categorical variable (between-subjects factor). Separate analyses provide a clearer overview of daily emergence patterns within each season, while the combined analysis provides a more comprehensive comparison of seasonal effects. Due to the small number of experimental snails infected with *Sanguinicola* sp. in August 2021, the emergence data from August 2021 (ten snails) and July 2022 (two snails) were pooled and analysed as one dataset in the subsequent analyses. Another reason for pooling the data was the similar timing of seasonal infections with only minor differences in photoperiod length between years (mid-August 2021 and late July 2022, i.e., 18 days), and emergence patterns were comparable in both years (compare emergence from two snails 2S-A and 3S-A in August with those in September in Supplementary Fig. S1C). Following the RMAs, which confirmed specific daily peaks in emergence, the emergence data from experimental snails were pooled and plotted together for better visualisation (Fig. 6A–B).

To compare daily output rates (daily production) of *T. clavata* cercariae between seasons and daily cercarial output rates between trematode species and seasons, general linear models (GLM-ANOVA) were applied using three different datasets with the number of cercariae as the dependent variable and with snail length (as a measure of snail size) entered as a covariate where appropriate to control for its potential confounding effect. To assess the importance of snail size, a series of univariate analyses of variance (one-way) ANOVA were first conducted for each dataset to test for statistical differences in snail length between the groups studied.

Following the results of one-way ANOVAs, the three dataset were as follows: (i) Dataset 1 was used to test for differences in *T. clavata* cercariae output rates between seasons. Based on significantly larger snails detected in September compared to

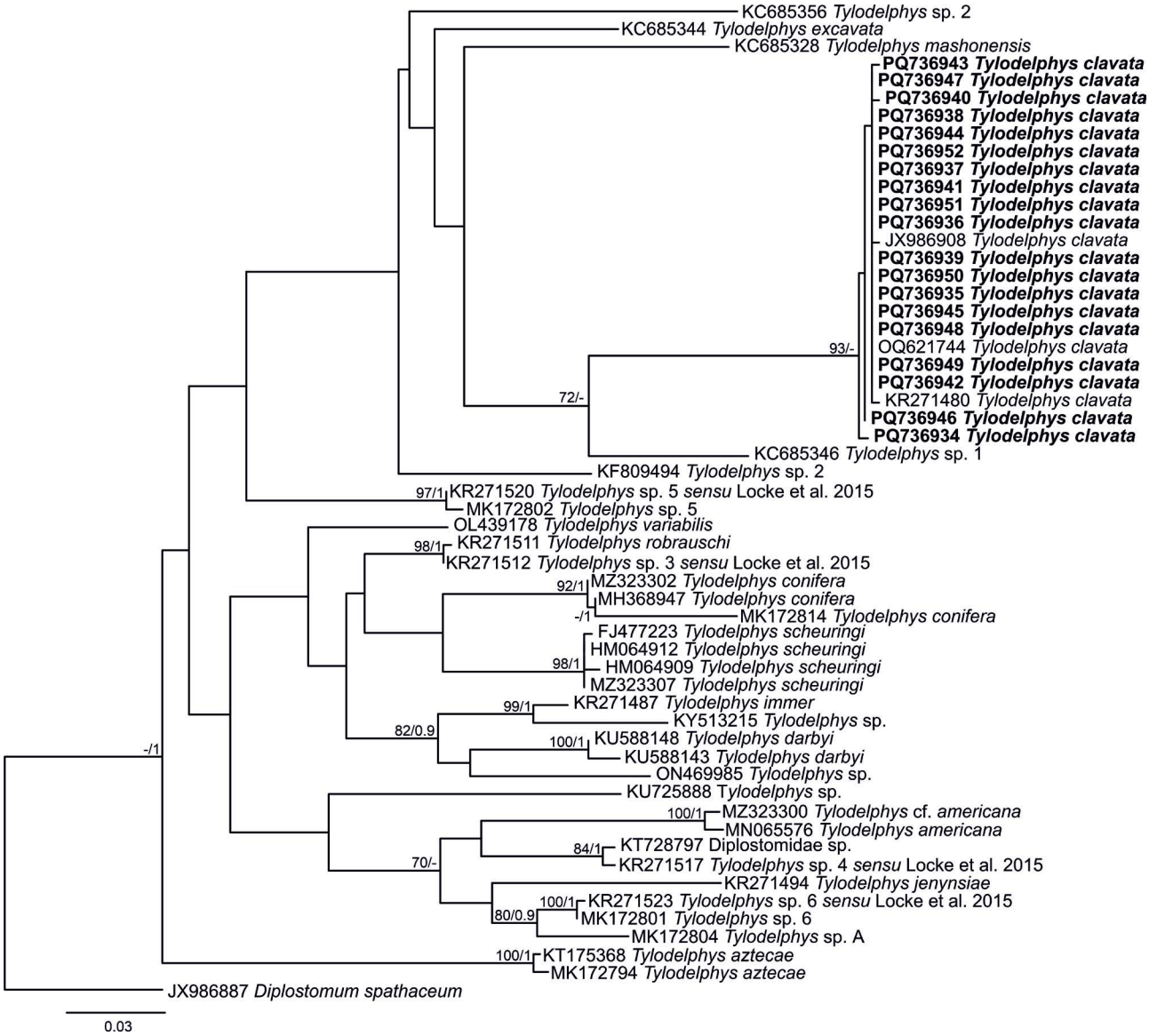

Fig. 1. The phylogenetic tree resulted from maximum likelihood (ML) analysis of the *cox*1 mtDNA sequences datasets of *Tylodelphys* spp., with nodal support values shown at the node as ML/BI (Bayesian inference). Support values < 70 (ML) and 0.90 (BI) are not shown. Sequences generated in the present study are highlighted in bold.

August (see Table 1 for mean shell lengths; one-way ANOVA, $F_{1, 17} = 12.65$, $P < 0.01$; *post-hoc* Tukey's HSD test with $P < 0.01$), a GLM-ANOVA was performed with snail size as a covariate, the factor season as a fixed-effect categorical variable, and snail identity (10 and 9 snails in August and September, respectively) as a random effect nested within season to differentiate set of different snail replicates in each season.

(ii) Dataset 2 was used to test for differences in the cercarial output rates in August between trematode species. Based on significantly smaller snails infected with *T. clavata* compared to snails infected with *Sanguinicola* sp. (mean shell length ± SD: 7.3 ± 1.3 mm *vs* 10.5 ± 1.5 mm, respectively; one-way ANOVA, $F_{1, 20} = 23.79$, $P < 0.001$; *post-hoc* Tukey's HSD test with $P < 0.001$), a GLM-ANOVA was performed with snail size as a covariate, the factor trematode species as a categorical variable and snail identity (10 and 12 snails infected with *T. clavata* and *Sanguinicola* sp., respectively) as a random effect nested within trematode species.

(iii) Dataset 3 was used to compare cercarial ouput rates of *T. clavata* in September with cercarial ouput rates of *Sanguinicola* sp. in August. Based on no significant differences in snail size between trematode species and seasons (mean shell length ± SD: 9.6 ± 1.2 mm *vs* 10.5 ± 1.5 mm, respectively; one-way ANOVA, $F_{1, 19} = 1.58$, $P = 0.22$), a GLM-ANOVA was performed with the factor trematode species as a categorical variable and snail identity (9 and 12 snails infected with *T. clavata* and *Sanguinicola* sp., respectively) as a random effect nested within trematode species.

Pearson's correlation analysis was used to examine the relationship between the number of emerged cercariae and the environmental factors tested, i.e., snail length as a measure of snail size, infection intensity, water temperature and light intensity, for each experimental day and pooled across days. Raw numbers of emerged cercariae were used for analysis concerning snail size and infection intensity, as these factors rather affect the cercarial output rates (Poulin 2006, Morley et al. 2010, Le Clec'h et al.

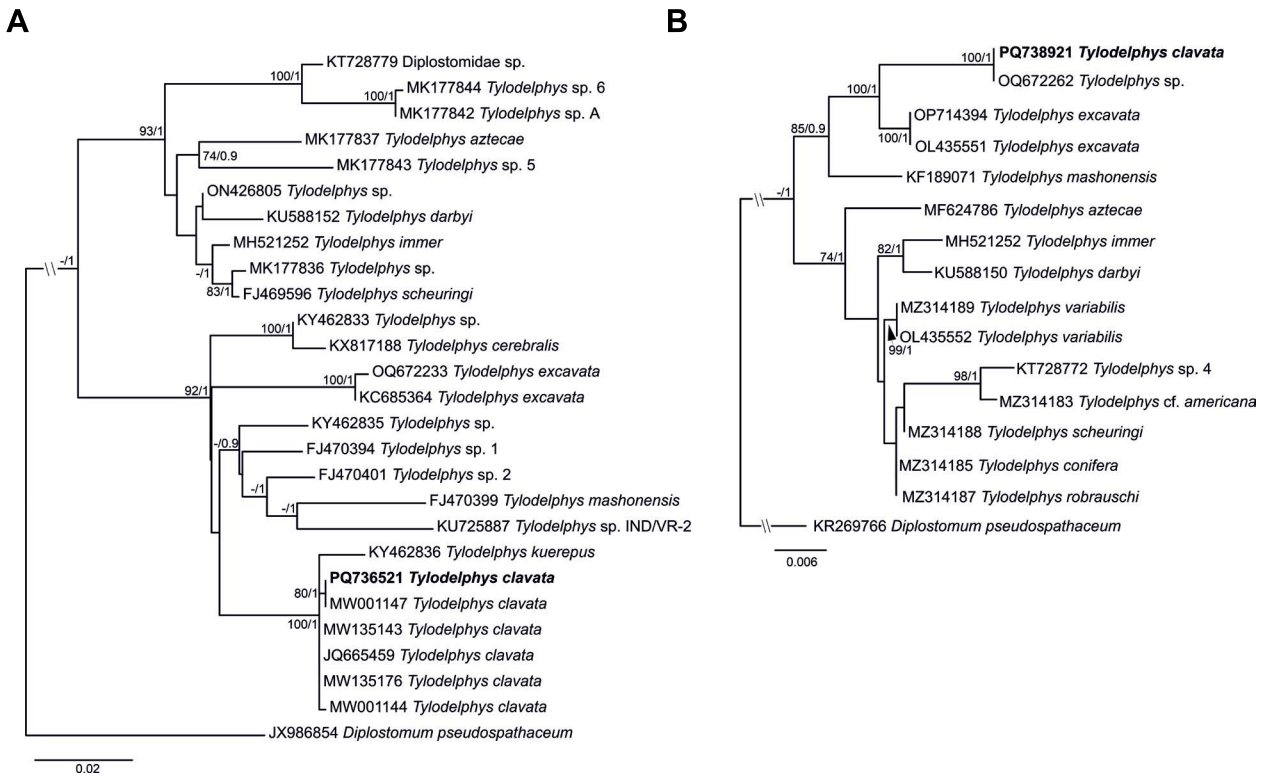

Fig. 2. The phylogenetic tree resulted from maximum likelihood (ML) analysis of the: **A** – ITS1-5.8S-ITS2 and **B** – 28S sequences datasets of *Tylodelphys* spp., with nodal support values shown at the node as ML/BI (Bayesian inference). Support values < 70 (ML) and 0.90 (BI) are not shown. Sequences generated in the present study are highlighted in bold.

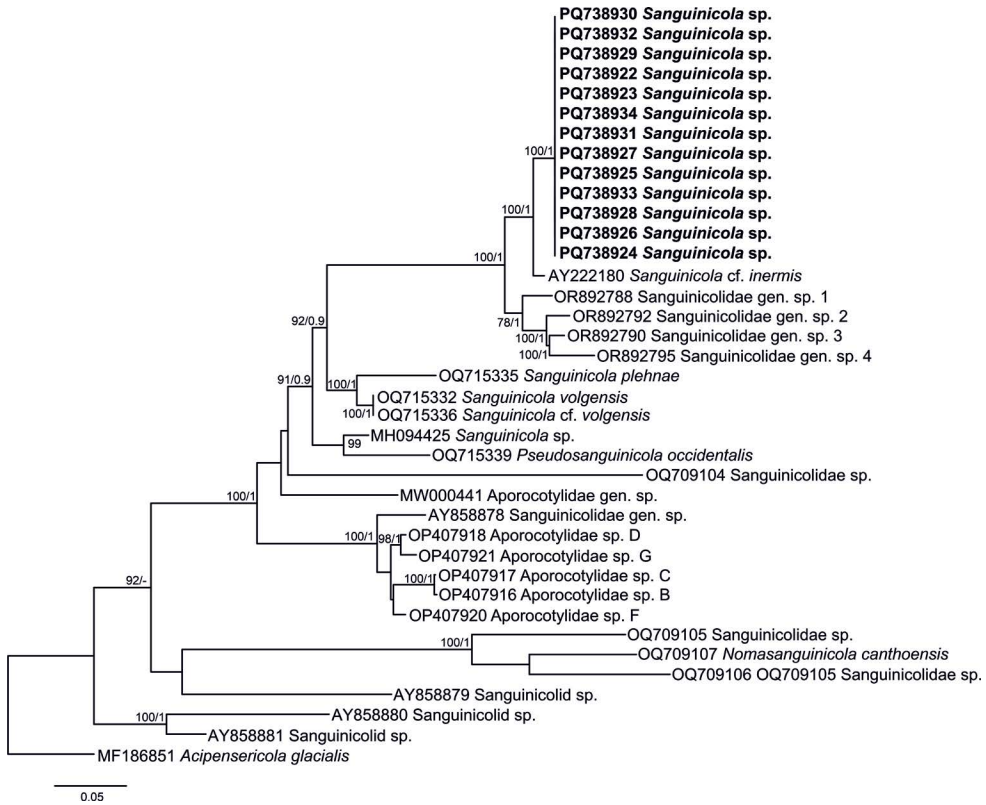

Fig. 3. The phylogenetic tree resulted from maximum likelihood (ML) analysis of the 28S sequences datasets of the Sanguinicolidae, with nodal support values shown at the node as ML/BI (Bayesian inference). Support values < 70 (ML) and 0.90 (BI) are not shown. Sequences generated in the present study are highlighted in bold.

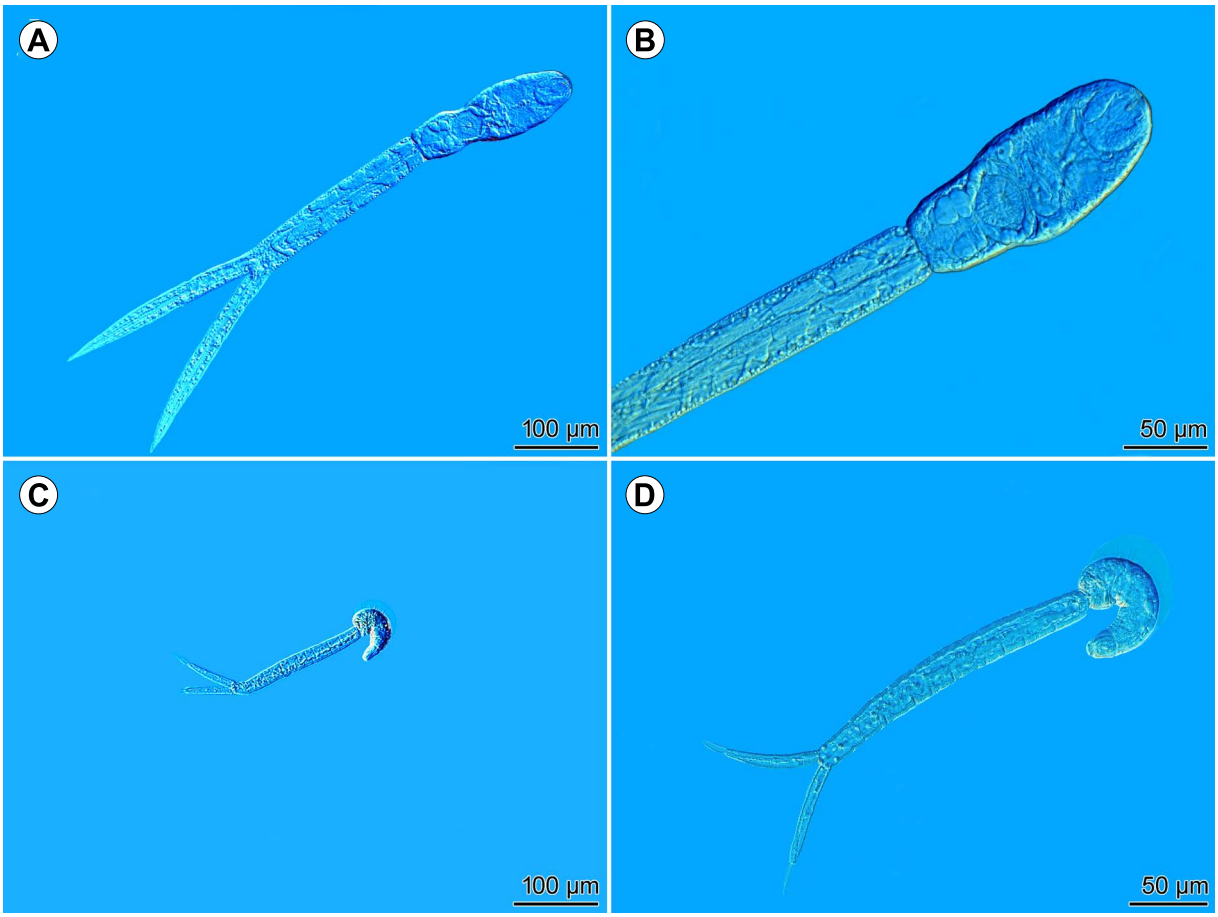

Fig. 4. Photomicrographs and size comparison of live cercariae of two experimental trematode species: **A**, **B** – *Tylodelphys clavata* (von Nordmann, 1832) and **C**, **D** – *Sanguinicola* sp.

Table 1. Samples of *Ampullaceana balthica* (Linnaeus) and *Radix auricularia* (Linnaeus), infections with *Tylodelphys clavata* (von Nordmann, 1832) and *Sanguinicola* sp. and data on the experimental setup.

	T. clavata **(ex *A. balthica*, Lake Medard)**			***Sanguinicola* sp.** **(ex *R. auricularia*, Lake Otakar)**		
	July (August) 2021[a]	September 2021	Total	July (August)[a] 2021	July 2022	Total
No. of examined snails	490	329	819	69	48	117
No. of all infected snails (prevalence, %)	57 (12)	47 (14)	104 (13)	8 (12)	16 (33)	24 (21)
No. of infected snails with experimental species (prevalence, %)	43 (9)	18 (6)	61 (8)	6 (9)	14 (30)	20 (17)
No. of experimental snails, initial/survived	11/10	11/9	22/19	2/2	11/10	13/12
Length of experimental snails mean ± SD (range, mm)	7.3 ± 1.3 (4.9–9.6)	9.6 ± 1.2 (7.9–11.6)	—	8.5 ± 0.5 (8.1–8.9)	10.8 ± 1.3 (7.8–12.9)	10.5 ± 1.5 (7.8–12.9)
Width of experimental snails mean ± SD (range, mm)	4.8 ± 0.7 (3.6–5.8)	5.9 ± 0.8 (4.6–6.9)	—	4.6 ± 0.6 (4.2–5.0)	6.5 ± 0.9 (4.3–8.1)	6.2 ± 1.1 (4.2–8.1)
Water temperature, mean ± SD (range, °C)	21.9 ± 1.1 (19.9–24.6)	20.8 ± 0.42 (19.9–21.8)	—	22.5 ± 0.5 (21.6–23.4)	21.4 ± 0.1 (21.2–21.6)	21.9 ± 0.8 (21.2–23.4)
Water illumination, mean (range, lx)	220 (0–990)	198 (0–699)	—	240 (0–1,033)	186 (0–840)	213 (0–1,033)
Infection intensity, mean (%)	51.7	74.2	—	75.0	94.6	91.2

[a]Snails sampled in July (experiments conducted in August)

2019), while the data converted to 1 h emergence were compared to temperature and light intensity data as the main factors influencing daily emergence patterns (Prokofiev et al. 2016, Vyhlídalová and Soldánová 2020).

Therefore, each factor was analysed separately to account for their distinct influences on output rates and daily patterns, ensuring a more accurate understanding of the relationships between these variables. In addition to snail size (see above), a one-way ANOVA and Tukey's *post-hoc* test were used to examine differences in infection intensity of *T. clavata* snails between the August and September experiments.

Prior to analyses, the number of emerged cercariae and snail size were transformed using the natural logarithm function ln (x+1) and ln (x), respectively, to improve the normality and homoscedasticity of the data. Statistical analyses were performed using the Statistica v.14.0.1 software package (StatSoft Inc., Tulsa, OK), with results considered significant at $P < 0.05$.

Table 2. Overview of total raw numbers (mean) (i.e., not recalculated to one-hour intervals) of emerged cercariae of *Tylodelphys clavata* (von Nordmann, 1832) and *Sanguinicola* sp. from *Ampullaceana balthica* (Linnaeus) and *Radix auricularia* (Linnaeus) snails during daily intervals over experimental seasons and days.

Trematode	Season	Year	Day	Number of emerged (mean) cercariae during daily intervals			
T. clavata	August (n^a = 10)	2021		**Sunrise**	**Day**	**Sunset**	**Night**
				5:15–5:50	5:50–20:30	20:30–21:10	21:10–5:15
			Day 1	75 (8)	722 (72)	315 (32)	1,368 (137)
			Day 2	45 (5)	696 (70)	76 (8)	1,104 (110)
			Day 3	54 (5)	834 (83)	96 (10)	834 (83)
	September (n = 9)	2021		**Sunrise**	**Day**	**Sunset**	**Night**
				6:10–6:45	6:45–19:10	19:10–19:45	19:45–6:10
			Day 1	231 (26)	4,398 (489)	912 (101)	6,615 (735)
			Day 2	306 (34)	4,272 (475)	1,059 (118)	7,635 (848)
			Day 3	215 (24)	4,116 (457)	729 (81)	9,324 (1,036)
***Sanguinicola* sp.**	August/July[b] (n = 2/10)	2021/2022		**Sunrise**	**Day**	**Sunset**	**Night**
				4:50–5:30 / 5:30–6:05[c]	5:30–20:50 / 6:05–20:10[c]	20:50–21:30 / 20:10–21:45[c]	21:30–4:50 / 20:45–5:30[c]
			Day 1	1,656 (138)	24,255 (2,021)	4,470 (373)	30,093 (2,508)
			Day 2	192 (16)	17,238 (1,437)	2,934 (245)	27,369 (2,281)
			Day 3	774 (65)	11,040 (920)	417 (35)	30,927 (2,577)

[a]Number of experimental snails; snails that died before or during the experiment are not included; [b]Pooled data from August 2021 and July 2022 considered as August; [c]Duration of daily interval in July 2022/August 2021

RESULTS

Sequence-based identification

Newly generated sequences from *Ampullaceana balthica* (E1–E19) clustered with sequences of *Tylodelphys clavata* (JX986908, OQ621744, KR271444) in a well-supported clade of the phylogenetic tree obtained from the ML analysis based on Alignment 1 (*cox*1; 365 nt) (Fig. 1).

The intraspecific variation between our sequences and those deposited in the GenBank demonstrated low divergence, ranging between 0% and 1.1% (0–4 nt). The interspecific variation between our sequences of *T. clavata* (E1–E19) and other species of *Tylodelphys* ranged between 9.9% and 15.8% (36–57 nt).

The phylogenetic tree obtained from the ML analysis based on Alignment 2 (ITS1-5.8S-ITS2; 817 nt) is presented in Fig. 2A. The novel sequence of *Tylodelphys* (E3) clustered with sequence of *T. clavata* (MW001147, MW135143, JQ665459, MW135176, MW001144) in a well-supported clade (Fig. 2A). The intraspecific variation between our sequence of *T. clavata* (E3) and those deposited in the GenBank demonstrated a low level of divergence, ranging between 0% and 0.2% (0–2 nt). The interspecific variation between our sequences (E3) and other species of *Tylodelphys* ranged between 1.0% and 7.2% (8–59 nt).

The phylogenetic tree obtained from the ML analysis based on Alignment 3 (28S; 1,144 nt) is presented in Fig. 2B. The novel sequence of *Tylodelphys* (E3) clustered with a sequence of *Tylodelphys* sp. (OQ672262) collected from *R. auricularia* in Poland (Kanarek et al. 2024). The genetic divergence between both sequences was null. Our results suggest that our isolate (E3) and *Tylodelphys* sp. (OQ672262) are conspecific with *T. clavata* (Fig. 2B). The interspecific variation between our sequence (E3) and other species of *Tylodelphys* ranged between 1.5% and 3.1% (17–36 nt). The identification of our isolates as *T. clavata* was confirmed in the analysis of the *cox*1 and ITS1-5.8S-ITS2.

Fourteen novel sequences were generated for 13 isolates of *Sanguinicola* sp. The phylogenetic relationships of the *Sanguinicola* sp. found in the present study with membership of the Sanguinicolidae were assessed based on the partial 28S rDNA sequences. A sequence of *cox*1 was generated for one isolate (E31) and was deposited in the Genbank to contribute to further studies. The phylogenetic tree obtained from the ML analysis based on Alignment 4 (28S rDNA; 1,128 nt) is presented in Fig. 3. Our specimens of *Sanguinicola* sp. clustered with a sequence of *Sanguinicola* cf. *inermis* Plehn, 1905 (AY222180) collected from *Lymnaea stagnalis* (Linnaeus) in Poland (Kanarek et al. 2024), with strong support (Fig. 3). The sequence divergence between our isolates was null and the divergence between our sequences and *S.* cf. *inermis* was 2.3% (26 nt). The interspecific genetic variation in comparison between our species and other members of the Sanguinicolidae was higher than 6.1% (68 nt).

Photomicrographs of live cercariae *T. clavata* and *Sanguinicola* sp. used in this study are presented in Fig. 4. GenBank accession numbers of newly generated sequences are presented in Supplementary Table S3.

Eight novel sequences of the internal transcribed spacer 2 (ITS2) were generated in the present study for snails to confirm the morphological evaluation. The following species were molecularly identified: *Ampullaceana balthica* from Lake Medard (n = 4) and *R. auricularia* from Lake Otakar (n = 4) (Supplementary Table S4). Molecular results confirmed our preliminary identification based on morphology.

Prevalence and measurements of experimental species

During the summer and autumn seasons 2021, a total of 819 *A. balthica* snails were collected from Lake Medard (Table 1). Of these, 13% snails were infected with trematodes across seasons, with *T. clavata* being the dominant species in both seasons. The overall prevalence of trematode infections was lower in July than in September, but

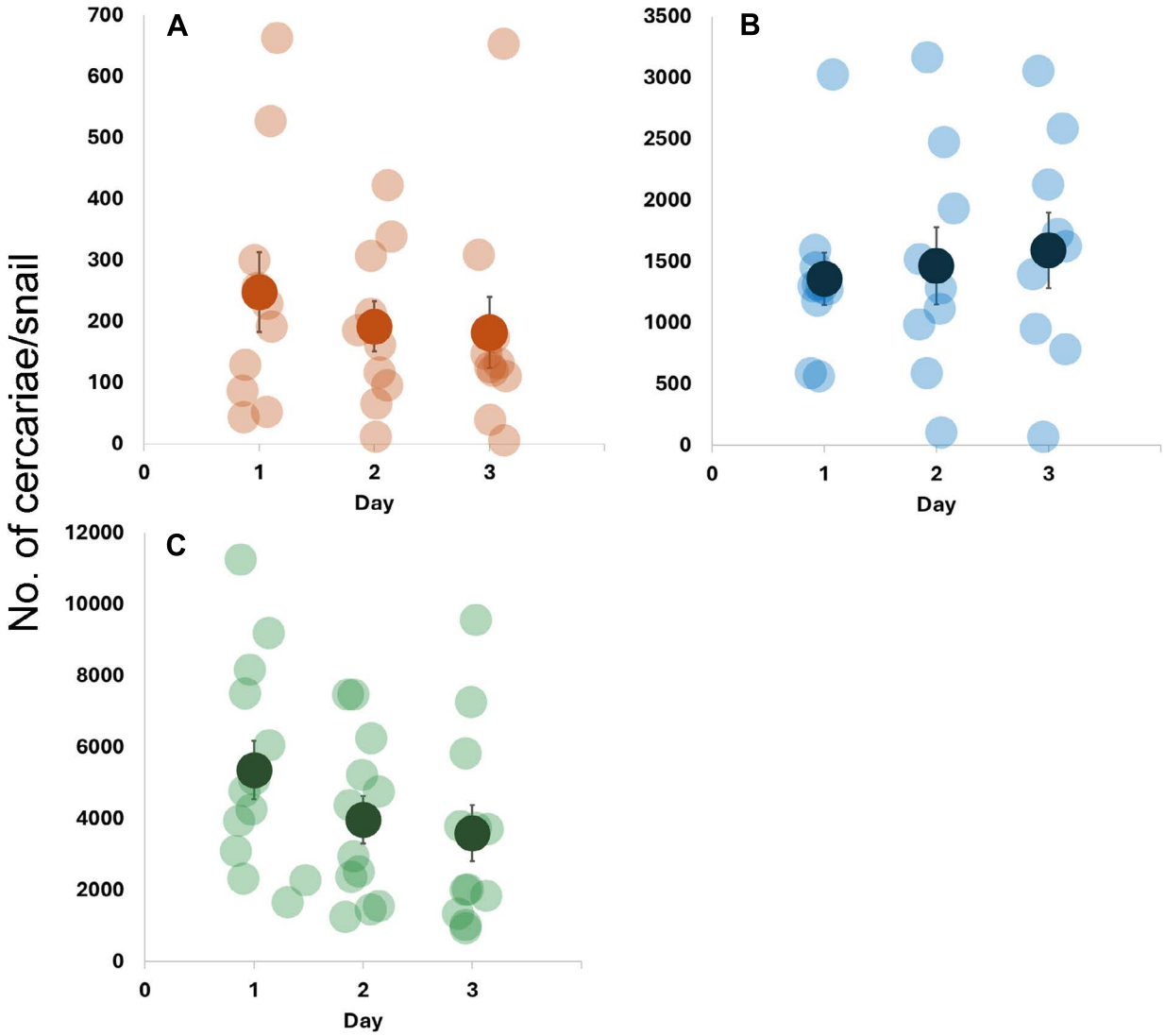

Fig. 5. Daily output rates of cercariae of **A** – *Tylodelphys clavata* (von Nordmann, 1832) ex *Ampullaceana balthica* (Linnaeus) in August 2021 (orange), **B** – *T. clavata* (ex *A. balthica*) in September 2021 (blue) and **C** – *Sanguinicola* sp. ex *Radix auricularia* (Linnaeus) in August 2021 and July 2022 considered as August (green). The transparent points represent the number of cercariae emerged from individual snails; full points represent the overall daily mean with bars indicating the standard error (± SE). The data are shown separately for each experimental day. Note different Y-axes.

Table 3. Results of repeated measures ANOVA evaluating the effect of time (daily intervals) and day (experimental days) on the emergence of cercariae from naturally infected snail hosts with *Tylodelphys clavata* (von Nordmann, 1832) and *Sanguinicola* sp. Statistically significant results ($P < 0.05$) are indicated in bold.

Trematode	Factor tested	Season	df[b]	MS[c]	F[d]	*P*[e]
T. clavata		**August** (n=10)[a]				
	Time		3	10.02	10.31	**<0.001**
	Day		2	6.21	6.68	**<0.01**
	Time × Day		6	2.08	3.44	**<0.01**
		September (n=9)[a]				
	Time		3	11.04	8.15	**<0.001**
	Day		2	0.21	0.28	0.756
	Time × Day		6	0.65	1.34	0.258
***Sanguinicola* sp.**		**August/July**[f] (n=12)[a]				
	Time		3	28.84	12.94	**<0.001**
	Day		2	18.45	13.47	**<0.001**
	Time × Day		6	9.96	5.28	**<0.001**

[a]Number of infected snails used in analyses; [b]Degrees of freedom; [c]Means of squares; [d]Test criterion value; [e]Probability value; [f]Pooled data from August 2021 and July 2022 considered as August.

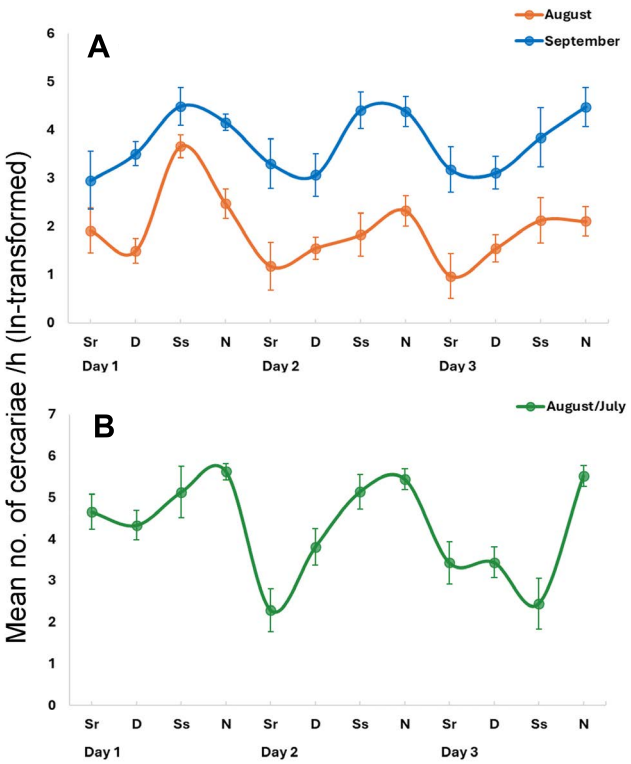

Fig. 6. Patterns in daily cercarial emergence of experimental trematodes from their snail hosts (converted to 1 h) for **A** – *Tylodelphys clavata* (von Nordmann, 1832) ex *Ampullaceana balthica* (Linnaeus) in August 2021 (orange line) and September 2021 (blue line), and **B** – *Sanguinicola* sp. ex *Radix auricularia* (Linnaeus) in August 2021 and July 2022 considered as August (green line). Points represent mean numbers of emerged cercariae for each daily interval (Sr – sunrise; D – day; Ss – sunset; N – night) pooled across all snail replicates and displayed for three experimental days; bars represent ± SE (standard error).

not statistically different (χ^2 = 1.25, P = 0.26), while the prevalence of *T. clavata* was slightly higher in July than in September, but also not significant (χ^2 = 3.23, P = 0.07) (Table 1). Post-experimental dissection of snails revealed a higher infection intensity of *T. clavata* in September compared to July (Table 1, Supplementary Table S2).

In Lake Otakar, 117 *R. auricularia* snails were sampled in the summer season 2021 and 2022 (Table 1). Of these, 21% harboured trematode infections, with most infections caused by *Sanguinicola* sp. (17%). Interestingly, our two-year seasonal sampling (May, July, September and October; data not shown) showed the exclusive presence of *Sanguinicola* sp. in the summer months, especially in July, with a higher prevalence in 2022 (Table 1). Snail dissection of the experimental *R. auricularia* specimens showed high infection intensity (Table 1), with almost half of the snails having the hepatopancreas completely occupied by *Sanguinicola* sp. sporocysts (six out of 13 snails with 100% occupancy; Supplementary Table S2), indicating a maximum potential for daily cercarial production.

All snails infected with the experimental species exhibited patent infections. Of the six snails infected with *Sanguinicola* sp. sampled in July 2021, three snails died during sample processing, i.e., before the start of acclimatisation. In total, 31 experimental snails survived the three-day experiments (19 infected with *T. clavata* and 12 infected with *Sanguinicola* sp.) (Table 1).

The mean total body length (sum of body, tail stem and furca) ± SD of *T. clavata* cercariae (n = 6) used in the experiment was 652 ± 34 µm, ranging from 613 to 699 µm, while the mean total body length ± SD of *Sanguinicola* sp. cercariae (n = 5) was 372 ± 19 µm, ranging from 348 to 400 µm (Fig. 4).

Cercarial output rates

Cercarial output rates varied among experimental snails, seasons and trematode species (Supplementary Table S2, Supplementary Fig. S1). For *T. clavata*, the mean daily cercarial emergence rate in August was 207 cercariae / snail / day, ranging from 6 to 663 (Fig. 5A), while the daily output rate in September was significantly higher, with a mean daily production of 1,469 cercariae / snail / day, ranging from 72 to 3,171 (Fig. 5B). In both experimental seasons, the pooled mean daily emergence rate was 805 cercariae / snail / day. The significant difference in output rates between seasons was confirmed by the result of GLM-ANOVA performed for dataset 1, which showed the significant effect of season ($F_{1,34}$ = 7.90, P < 0.05; *post-hoc* Tukey's HSD test with P < 0.001) as well as the pronounced effect of snail length ($F_{1,34}$ = 24.0, P < 0.001).

In contrast, mean emergence rates of *Sanguinicola* sp. in August were 4,205 cercariae / snail / day, ranging from 933 to 11,265 (Fig. 5C), indicating significantly higher daily cercarial production than *T. clavata*, regardless of the season. This was confirmed by the GLM-ANOVA results for dataset 2, which revealed a significant effect of trematode species ($F_{1,44}$ = 27.29, P < 0.001; *post-hoc* Tukey's HSD test with P < 0.001) and snail length ($F_{1,44}$ = 108.93, P < 0.001). Similarly, the GLM-ANOVA for dataset 3 showed a significant effect of trematode species ($F_{1,42}$ = 11.94, P < 0.01; *post-hoc* Tukey's HSD test with P < 0.001).

Patterns of cercarial emergence

The raw numbers of cercariae emerged during different daily intervals indicated a nocturnal emergence pattern (only the night interval) in both trematode species (Table 2). After conversion to 1 h to ensure comparability between intervals, the highest numbers of emerged cercariae appeared to be during the two daily intervals, sunset and night, as indicated by the majority of emergence patterns from individual snail hosts (Supplementary Fig. S1A–C). The results of the separate RMAs showed a significant effect of time (daily intervals), experimental day and their interaction on the emergence of cercariae of *T. clavata* in August and *Sanguinicola* sp. in August (Table 3).

In experiments with *T. clavata* in September, only a significant effect of time was detected (Table 3). The cercarial emergence was circadian in both trematodes and consistent across all three experimental days (Fig. 6). *Post-hoc* tests (Tukey's HSD) revealed the highest emergence of cercariae *T. clavata* at sunset and night in both seasons (Supplementary Table S5, Fig. 6A–B), while a nocturnal peak was observed for *Sanguinicola* sp. (Supplementary Table S5, Fig. 6C).

The results of the combined seasonal RMA for *T. clavata* revealed a significant effect of season ($F_{1, 17} = 19.67$, $P < 0.001$), time ($F_{3, 51} = 18.02$, $P < 0.001$), experimental day ($F_{2, 34} = 4.29$, $P < 0.05$) and interactions time and day ($F_{6, 102} = 2.41$, $P < 0.05$) and time, day and season ($F_{6, 102} = 2.45$, $P < 0.05$) on the number of cercariae emerged at specific daily intervals. However and most importantly, the interaction between time and season was not significant ($F_{3, 51} = 0.31$, $P = 0.82$), indicating consistency in emergence patterns of *T. clavata* cercariae in both seasons. The *post-hoc* HSD tests for the combined seasonal RMA for *T. clavata* matched the results of the separate tests, thus confirming the peaks of emergence at sunset and night (Supplementary Table S5).

Effects of environmental factors on the emergence

Pearson's correlation analysis revealed no relationship between water temperature and the number of cercariae emerged at specific daily intervals in either experimental species (Supplementary Table S6), indicating stable conditions in the experimental setup. A negative correlation between light intensity and cercarial emergence was observed only in the pooled data for *T. clavata* in both experimental seasons, while no significant correlation with light intensity was observed for *Sanguinicola* sp. (Supplementary Table S6).

Experimental snails infected with *T. clavata* were significantly larger and showed a higher infection intensity in September (Table 1; see also Supplementary Table S2 and results for snail size by one-way ANOVA above; results for infection intensity: one-way ANOVA, $F_{1,17} = 9.56$, $P < 0.01$). However, no significant correlation was found between cercarial emergence rates and snail length within each season (Supplementary Table S6), as a result of selecting similar-sized experimental snails. In contrast, a positive effect of snail size on the cercarial emergence was observed for *Sanguinicola* sp. (Supplementary Table S6), as it was not possible to select snails of similar size due to the small number of infected snails with patent infections (Table 1; see the range of snail sizes in Supplementary Table S2).

There was no significant correlation between the infection intensity and the number of cercariae of *Sanguinicola* sp. and *T. clavata* in August (Supplementary Table S6). For *T. clavata* in September, however, a positive correlation was only detected on the second and third day of the experiment (Supplementary Table S6). This was most likely related to two snail individuals whose hepatopancreas was 100% occupied with sporocysts and which simultaneously showed a higher emergence rate on these days than on the first day (see Fig. 5B for snail individuals with an emergence rate between 2,000 and 2,500 cercariae).

DISCUSSION

The present study provides the first detailed data on the daily emergence rates and patterns of cercariae of *Tylodelphys clavata* and *Sanguinicola* sp. from small lymnaeid snail hosts *Ampullaceana balthica* and *Radix auricularia* under natural photoperiod and controlled temperature conditions, and on the effects of selected environmental factors influencing emergence. Our study also extends the limited knowledge on the distribution and prevalence of both trematodes in European freshwater snails and provides their molecular characterisations, including the first genetic data for cercariae of *Sanguinicola* sp. from the Czech Republic.

Molecular identification of our experimental trematode species using *cox*1 and ITS1-5.8S-ITS2 sequences confirmed that the diplostomid cercariae in our emergence experiments belong to *T. clavata*. Based on the 28S alignment, our isolate clustered with the sequence OQ672262, which was identified as *Tylodelphys* sp. ex *R. auricularia* in GenBank (Kanarek et al. 2024) but was incorrectly labeled as *Tylodelphys excavata* (Rudolphi, 1803) ex *Planorbarius corneus* (Linnaeus) in the authors' publication (see Table 2 in Kanarek et al. 2024). Based on the confirmation of *cox*1 and ITS1-5.8S-ITS2, our isolate is the first validated 28S sequence of *T. clavata* in GenBank.

The second experimental cercariae morphologically resembled those of *Sanguinicola inermis* Plehn, 1905 (Našincová 1992, Faltýnková et al. 2007), but the molecular identification was less straightforward. The novel 28S sequences generated in the present study clustered with *Sanguinicola* cf. *inermis* (AY222180) (Olson et al. 2003). However, we consider the divergence of 2.3% (26 nt) between the sequences too high to classify them as intraspecific for a conservative gene such as 28S. This suggests that our sequences and that of *Sanguinicola* cf. *inermis* (AY222180) probably represent separate species, preventing confirmation of our preliminary morphological identification.

Nevertheless, we consider it possible that the cercariae used in the present study still represent *S. inermis*. First, the only available sequence of *S. inermis* in GenBank is labeled "cf." (i.e., "confer"), indicating considerable uncertainty in its identification by the authors (Olson et al. 2003). Secondly, this sequence of *Sanguinicola* cf. *inermis* (AY222180; Olson et al. 2003) originates from the snail host *Lymnaea stagnalis* which is considered non-susceptible to infection by this trematode (Kirk and Lewis 1992, Zhokhov et al. 2021), whereas *R. auricularia*, the host used in our study, is a susceptible host (Kirk and Lewis 1992).

Finally, the fish population in Lake Otakar, where snails *Sanguinicola*-infected were sampled, consists mainly of common carp (*Cyprinus carpio*), which is the most common definitive host of *S. inermis* (see Zhokhov et al. 2021). However, due to the lack of conclusive confirmation, we treated the trematode in our study as *Sanguinicola* sp. Future research should focus on the recovery of adult sanguinicolids from common carp in Lake Otakar to apply the 'best practice' approach and confirm or refute our suspicion (Blasco-Costa et al. 2016).

The output rates (daily production) and emergence patterns (daily rthythms) of cercariae showed remarkable differences between trematode species, including seasonal differences. This is consistent with our first hypothesis of interspecific variation in these basic features of cercarial emergence from snail hosts in relation to the parasites' life-history traits and life cycle. In particular, cercarial

output rates of *Sanguinicola* sp. were considerably higher, ranging from 3 to 20 times that of *T. clavata*.

This discrepancy may be due to size differences, with cercariae of *T. clavata* being much larger (~650 µm) than those of *Sanguinicola* sp. (~370 µm), similar to the previously and repeatedly observed negative correlation between output rates and size of cercariae in different snail-trematode systems (Loker 1983, Thieltges et al. 2008b, Preston et al. 2013, Prokofiev et al. 2016, Khosravi et al. 2023). This could simply be because smaller-bodied species with smaller volumes provide more space in the rediae/sporocysts for asexual reproduction.

In addition, cercarial output rates of *T. clavata* were 7 times higher in the colder (20° C, September) compared to the warmer season (22° C, August). This was surprising and contradicts the hypothesis of higher cercarial production at higher temperatures (here August) based on literature data (e.g., Żbikowska 2004, Poulin 2006, Morley et al. 2010, Morley and Lewis 2013), although there are exceptions (e.g., Thieltges and Rick 2006).

One possible cause could be snail size (Loker 1983, Thieltges et al. 2008b, Morley et al. 2010, Vyhlídalová and Soldánová 2020) and the proportion/intensity of trematode larvae occupying the snail's hepatopancreas (Massoud 1974, Le Clec'h et al. 2019, Vyhlídalová and Soldánová 2020), both of which have been shown to correlate positively with cercarial production. However, our data show that the effects of these biotic factors were species- and season-specific. Higher cercarial production of *Sanguinicola* sp. was observed from larger snails in summer (August), but with similar infection intensities, which can be attributed to the inability to select experimental snails of similar size due to the small sample size.

Snails infected with *T. clavata* in autumn (September) were larger and more heavily parasitised than in summer (August), suggesting that natural seasonal changes in snail size and infection intensity outweighed the effects of temperature on cercarial emergence. This is probably due to the annual turnover of size cohorts in the snail population and the associated changes in trematode recruitment and development. Small lymnaeid snails reproduce in spring and new cohorts grow continuously towards the autumn (Vinarski and Aksenova 2023), while they rapidly accumulate trematode infections (Soldánová and Kostadinova 2011), which is consistent with our observations in both seasons.

The lack of correlation between *T. clavata* output rates and snail size in each season and the only minor effect of infection intensity was due to the deliberate selection of similarly sized experimental snails, as well as the interplay between the natural seasonal development of snails and the recruitment of new trematode infections into the snail population.

Although some research has addressed the cercarial emergence of fish pathogens within the family Diplostomidae from small lymnaeids (Brassard et al. 1982, Karvonen et al. 2006, Vyhlídalová and Soldánová 2020), no study to date has addressed the cercarial emergence of the diplostomid *T. clavata* from snails, despite its importance for fish health and economic losses in aquaculture (Chappell et al. 1994, Stumbo and Poulin 2016, Heneberg and Sitko 2021, Unger et al. 2022). In particular, cercarial output rates of congeneric trematodes of the genus *Diplostomum* von Nordmann, 1832 can be compared, as both species exhibit similar transmission pathways by sharing species of small lymnaeid snails *Ampullaceana* and *Radix* as first intermediate hosts and some fish species as second intermediate hosts in their life cycles (Kozicka and Niewiadomska 1960, Burrough 1978, Kennedy 1981, Faltýnková et al. 2016, Kudlai et al. 2017).

However, the mean daily number of *T. clavata* cercariae in our study was much lower than that of *Diplostomum* spp. cercariae from small lymnaeids reported in previous European studies, depending on seasonal conditions (August and September in this study: 207 and 1,469 cercariae / snail / day *vs* May, July and September: 2,304–17,044 cercariae / snail / day in Karvonen et al. 2006 and Vyhlídalová and Soldánová 2020).

This large discrepancy may be due to the smaller snails and the seasonal timing of the experiments in our study. In particular, the emergence of *T. clavata* cercariae could not be investigated in May, as there were no infected snails. It is likely that cercarial emergence is much higher in spring and is related to the reproductive cycle of the upstream hosts. European perch, the most common fish host for *T. clavata*, spawns in spring, leading to increased activity and density of fish in the littoral habitat (Thorpe et al. 1977) and possibly to increased parasite transmission dynamics in the form of increased cercarial output rates, as has been observed in fish-infecting *Diplostomum* spp. (Vyhlídalová and Soldánová 2020). Future studies should therefore investigate the cercarial emergence during peak production seasons to validate this hypothesis.

In the absence of data on the daily output of cercariae of *Sanguinicola* sp. the comparison proved to be difficult. Cercarial output rates of related blood flukes from the family Schistosomatidae, especially bird schistosomes (Soldánová et al. 2016, Soldánová et al. 2022a), also make a comparison unreliable, as these cercariae are many times larger (810–1,368 µm) (Podhorský et al. 2009, Jouet et al. 2010, Soldánová et al. 2022b) and have a completely different life cycle (Soldánová et al. 2013). The lack of emergence data may stem from the generally low prevalence of *Sanguinicola* species in European snails (Zhokhov et al. 2021), which makes it challenging to obtain enough infected snail replicates for emergence experiments.

For the same reason, the possible seasonal differences in the cercarial emergence of this trematode could not be investigated. The strictly seasonal occurrence of *Sanguinicola*-infected snails with a high prevalence exclusively in summer coincides with the biology of carp in spring and summer when fish aggregate for spawning in the shallow littoral habitats (Chizinski et al. 2016) and are more exposed to infection by *Sanguinicola* sp. cercariae.

Circadian rhythms are the result of an autonomous timekeeping system called the circadian clock; a biological mechanism regulated by environmental stimuli (Patke et al. 2020). This clock is genetically controlled in both free-living (Young and Kay 2001) and parasitic organisms

(Théron 2015). Many environmental factors can influence the emergence of cercariae (see *Introduction*), but the most important are temperature and light intensity (e.g., Smyth and Halton 1983, Morley 2012, Théron 2015). However, while temperature has a greater influence on the daily production of cercariae (Poulin 2006), the periodicity/rhythm in cercarial emergence is generally more controlled by light, although there are exceptions (Prokofiev et al. 2016, 2023). In the present study, temperature remained intentionally constant, without diurnal fluctuations that could lead to an increase in the cercarial production at specific daily intervals. This suggests that the primary factor in maintaining daily rhythms in cercarial emergence was light intensity, confirming previous findings.

Cercariae of both trematode species emerged in the highest numbers during daily periods when light intensity decreased or was absent, albeit with slight differences. Cercariae of *T. clavata* showed peak emergence at sunset and night, and this pattern was consistent in both experimental seasons (August and September). Cercariae of *Sanguinicola* sp., investigated during a single (summer) season in two consecutive years, emerged in the highest numbers at night. These observations support our hypothesis of interspecific variation and that the cercariae of *T. clavata* exhibit adaptability in emergence rhythms in response to changing seasonal photo- and thermoperiod conditions.

Nocturnal emergence has also been observed in other fish-infecting trematodes (Lewis et al. 1989, Faltýnková et al. 2009, Vyhlídalová and Soldánová 2020), even with seasonally independent emergence rhythms (Soldánová et al. 2016, 2022a, Vyhlídalová and Soldánová 2020). Therefore, similar independence of cercarial emergence from the seasonally varying photo- and thermoperiod in *Sanguinicola* sp. cercariae can also be expected in seasons other than August. Hower, further studies are needed to explore the overall consistent pattern of seasonally independent rhythms as well as the single effect of seasonal photoperiod in terms of shifted rhythms.

In contrast to the lack of emergence data for *T. clavata*, several studies have addressed the emergence patterns of *Sanguinicola* spp. cercariae. It has been found that the cercariae of *Sanguinicola* sp. from *Ancylus fluviatilis* Müller (Martin and Vazquez 1984) and *S. inermis* from *Peregriana peregra* (Kirk and Lewis 1993) exhibit diurnal circadian emergence with a peak between 16:00 and 20:00, or in 'late afternoon' (Sommerville and Iqbal 1991). These results disagree with our findings on the emergence of *Sanguinicola* sp. from *R. auricularia* snails, which showed a clear nocturnal peak, possibly due to different species of *Sanguinicola* and cercarial preference for different definitive fish hosts. In addition, the number of snail individuals was not reported, limiting direct comparability with our data. Similar variability in emergence rhythms at the trematode species level has been documented in some snail-trematode systems (Théron 1989, Théron et al. 1997, Prokofiev et al. 2016, Vyhlídalová and Soldánová 2020), including schistosomes (e.g., Lu et al. 2009, Mouahid et al. 2012, Soldánová et al. 2016, 2022a), suggesting that circadian rhythms in the cercarial emergence may represent a widespread adaptive strategy in similar blood-dwelling trematodes. Future studies should consider employing molecular methods for confirmation of the model species to ensure comparability among studies.

Our results demonstrate the undeniable effect of light on emergence rhythms, but the negative correlation between light intensity and the number of emerged cercariae was only demonstrated for *T. clavata*, suggesting that other mechanisms must be involved in this process. Trematodes have evolved the synchronous emergence of cercariae from molluscan hosts with the activity and occurrence of the next host in the life cycle to increase contact and chances of successful transmission (Combes et al. 1994, 2002). Our findings of species-specific circadian rhythms in cercarial emergence of *T. clavata* and *Sanguinicola* sp. also suggest a temporal timing in cercarial emergence with the occurrence of their upstream hosts, as has been documented for many other trematodes in both marine and freshwater ecosystems (Shostak and Esch 1990, McCarthy et al. 2002, Karvonen et al. 2004, 2006, Faltýnková et al. 2009, Théron 2015, Prokofiev et al. 2016, Soldánová et al. 2016, 2022a, Vyhlídalová and Soldánová 2020).

Cercariae of *T. clavata*, emerging mainly at sunset and night, infect fish as a second intermediate host (Kozicka and Niewiadomska 1960). Snails infected with *T. clavata* were collected in Lake Medard, where nine of 12 fish species are suitable hosts (Supplementary Table S1; Burrough 1978, Pojmanska et al. 1980, Holland and Kennedy 1997, Morozińska-Gogol 2009), with the European perch (*Perca fluviatilis*) being the most abundant in the lake (Peterka et al. 2022) and generally the most frequently infected with *T. clavata* metacercariae at high densities (Kozicka and Niewiadomska 1960, Dzika et al. 2008, Vivas Muñoz et al. 2017). Adult perch exhibit specific seasonal horizontal migrations in lakes. In summer and early autumn (as our experimental period), adult fish reside in pelagic habitats during the daylight and migrate to the shallower littoral zone at sunset, where they remain throughout the night (Jacobsen et al. 2015, Nakayama et al. 2018). Similarly, juvenile perch migrate to shallower surface areas after sunset to avoid predation (Sajdlová et al. 2018).

The common roach *Rutilus rutilus* (Linnaeus) is the second most abundant in Lake Medard (Peterka et al. 2022) and also serves as intermediate host for *T. clavata* (Gibson et al. 2005). It can also acquire infections, albeit opportunistically, due to distinct daily activity patterns with foraging at sunrise and sunset (Baruš and Oliva 1995, Kottelat and Freyhof 2007). However, it is likely that roaches become less frequently infected only during their activity at sunset, which is supported by the lower prevalence and infection intensity of metacercariae compared to those in perch (Kadlec et al. 2003, Valtonen et al. 2003, Dzika et al. 2008). This suggests a possible niche preference and temporal partitioning of host infection by *T. clavata*, with perch being the primary and potentially most suitable intermediate host. Similar emergence patterns, peaking in the early evening and at night, have been observed in strigeid cercariae infecting mainly European perch (Faltýnková et al. 2009), providing further evidence

that the emergence of *T. clavata* cercariae is synchronised with the same fish host.

The nocturnal emergence of *Sanguinicola* sp. can also be associated with the dominant fish in Lake Otakar, the common carp (*C. carpio*) (90% of the fish fauna; Czech Anglers Union, pers. comm.), which is also the most important definitive host for *Sanguinicola* species (Kirk and Lewis 1993, Zhokhov et al. 2021). Carp foraging activity is light-dependent, with food-searching in the littoral zone increasing shortly after sunset and peaking at night (Bajer et al. 2010, Žák 2021), which is consistent with the nocturnal emergence of *Sanguinicola* sp. cercariae observed in this study. The occurrence of lymnaeid snails in shallow littoral waters up to a maximum depth of five metres (Fretter and Peake 1975, Stift et al. 2004, Zhuykova 2020) combined with the restricted mobility of cercariae due to their small size and energy limitations (Ginetsinskaya 1960), implies that the emergence patterns of *T. clavata* and *Sanguinicola* sp. cercariae are finely tuned to synchronise with the presence of their preferred fish hosts and are thus closely adapted to the "host-time and space".

In addition, nocturnal emergence may be beneficial to avoid visual predators, as predation on cercariae decreases significantly in low-light conditions (Orlofske et al. 2015). This precise timing of the cercarial emergence, which significantly influences the likelihood of encountering and infecting subsequent hosts, is particularly important for fish-infecting trematodes, which are the fastest moving, so their energy glycogen reserves must be effectively depleted (Morley 2020). This indicates a high risk of infection for fish hosts, maintaining the spread, circulation and persistence of parasites in the ecosystem.

In conclusion, we have presented the first comprehensive dataset on the emergence of two furcocercous cercariae of *T. clavata* and *Sanguinicola* sp. from their small lymnaeid snails, revealing interspecific variations due to specific parasite life cycles and life history traits, and demonstrating the adaptability of *T. clavata* to changing seasonal conditions in terms of output rates and the uniformity of emergence rhythms, as well as the importance of biotic factors for cercarial emergence, particularly the biological and ecological aspects of next fish hosts.

In addition, determining the exact timing in the cercarial emergence provides valuable insight into the extent of parasite adaptations to maximise transmission and complete the life cycle. This is highly relevant for understanding the ecological and epidemiological aspects of specific adaptation strategies in these species and the implications for infection risk in fish.

Furthermore, further seasonal research involving snails and molecularly characterised conspecific trematodes inhabiting different geographical areas would be of great benefit to obtain a more comprehensive picture of cercarial emergence patterns in members of the genera *Tylodelphys* and *Sanguinicola* and to investigate the intensity of metacercariae infections in fish, thus underpinning and confirming the implications of our results.

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