University of South Bohemia in České Budějovice

Faculty of Science

Department of Parasitology

Master thesis

Production and periodicity in the emergence of cercariae of *Diplostomum* spp. (Digenea) from snails *Radix lagotis* (Lymnaeidae)

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České Budějovice 2019

Vyhlídalová, T., 2019: Production and periodicity in the emergence of cercariae of *Diplostomum* spp. (Digenea) from snails *Radix lagotis* (Lymnaeidae). MSc. Thesis, in English. – 57 pp., Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic.

Annotation

The cercarial emergence patterns of three species of *Diplostomum* (*D.* '*mergi*', *D. spathaceum* and *D. pariventosum*) parasitising freshwater first intermediate host *Radix lagotis* were studied under various experimental conditions, i.e., field, laboratory and incubator, and seasons, i.e., spring, summer and autumn. This study provided novel data on the production and periodicity in cercarial emergence and revealed both interspecific and intraspecific variaons related to the species-specific adaptive nature of cercariae to facilitate transmission to second intermediate fish hosts.

Declaration

Prohlašuji, že svoji diplomovou práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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České Budějovice, 2019 Bc. Tereza Vyhlídalová

Acknowledgements

Many thanks belong to my supervisor Miroslava Soldánová for her professional guidance and patience during the experiments as well as compilation of this thesis. Moreover, I would like to thank Tomáš Scholz for his advices, proofreading and the opportunity to be part of his team. I higly appreciate the help and support of all members of the Laboratory of Helminthology, especially Martina Borovková, who helped me with collections of snails in the field, and Ana Born-Torrijos and Camila Pantoja for helping me with the molecular analysis. Futhermore, I would like to express my gratitude also to Daniel Bartoň, Lukáš Vejřík and other colleagues from the Hydrobiological Institute, Biological Centre, BC CAS for providing insight into fish biology and ecology. At last, I would like to thank my family and friends for their support during my studies.

Financial support

This study was supported by the Czech Science Foundation (Project No. 17-20936Y).

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1. Introduction and Aims

Parasites are ubiquitous organism as almost all major groups of animals have parasitic members (e.g., Poulin and Morand 2004). They have complex life cycles, involving different life stages infecting variety of host taxa. Transmission of parasites often relies on predator-prey interactions to complete the life cycle. Such a close host-parasite relationship at many trophic levels typically reflects host diversity and abundance, and hence the ecosystem-level dynamics and structure of food webs (e.g., Marcogliese 2003, Hechinger and Lafferty 2005, Fredensborg et al. 2006). Indeed, recent studies have demonstrated an important role in which parasites act as drivers of key processes in natural systems (see Sures et al. 2017 and references therein).

Trematodes (Digenea) are essential and integral components of aquatic ecosystems that play a crucial role in population and community structure with broader consequences for food web topology, stability and functioning (Lafferty et al. 2006, 2008, Hechinger et al. 2007, Kuris et al. 2008). Trematode-induced changes in host behaviour, feeding and foraging activity, physiology and morphology or reproduction and fecundity (e.g., Sorensen and Minchella 2001, Marcogliese 2004, Poulin 2010, Voutilainen 2010), leading to a regulation of ecological populations and communities, translate to modifications of trophic interactions strength and thus energy flow in aquatic ecosystems (Thieltges et al. 2008a, 2013, Sukhdeo 2010).

Trematodes are common and abundant in marine and freshwater habitats worldwide and their typically three-host life cycle requires molluscs as first intermediate hosts and wide range of invertebrates and vertebrates as second and definitive hosts (Combes et al. 2002, Galaktionov and Dobrovolskij 2003). Free-swimming larvae, cercariae, emerging from molluscs, have been found significant ecological elements with a strong potential to transfer substantial biomass within the whole ecosystem's food chains, as they are usually produced in tremendous numbers (Kuris et al. 2008, Thieltges et al. 2008a, Preston et al. 2013, Soldánová et al. 2016, Rosenkranz et al. 2018) and frequent subject to predation (e.g., Kaplan et al. 2009, Welsh et al. 2017, Vielma et al. 2019).

As non-feeding and short-lived (typically 24–72 hours), cercariae represent a functionally crucial stages, aiming at infecting as many hosts as possible (Esch et al. 2001, Combes et al. 2002, Morley 2012). Besides the strong dilution effect described for many different predator-prey systems (e.g., Thieltges et al. 2008b, Johnson et al. 2010, Johnson and Thieltges 2010, Goedknegt et al. 2012), other extreme environmental factors contribute to the reduced cercarial population, thereby preventing successful completion of the life cycle (e.g., Pietrock and Marcogliese 2003). This short transmission opportunity is balanced by the great

diversity of cercarial behaviours related to processes of host finding and recognition as well as emergence patterns from their molluscan hosts, resulting from long coevolutionary processes/interaction with molluscs and challenging environment (e.g., Combes et al. 1994, Haas 2003). Thus, trematode cercariae are well adapted to be more effective in their primary functional role to increase chances of their transmission to next hosts (Combes 2001, Esch et al. 2001, Poulin 2007, Reece et al. 2017).

There are many species-specific variations in patterns of cercarial emergence, of which the most common is that cercariae are released in high numbers with continuous production (Combes et al. 1994). In many mollusc-trematode systems the timing of cercarial output is synchronised with activity and/or behaviour of the potential next hosts to ensure their mutual overlap in space and time (Anderson et al. 1976, Combes et al. 1994, Combes et al. 2002, Théron 2015). A typical example involves schistosomes (Digenea, Schistosomatidae) that utilise birds as definitive hosts peaking in early morning hours (e.g., release of *Trichobilharzia szidati* Neuhaus, 1952 from the lymnaeid freshwater snail *Lymnaea stagnalis* (Linnaeus, 1758) – Soldánová et al. 2016) and human schistosomes, also infecting a range of mammalian definitive hosts, with three different circadian chronotypes within a single parasite species (*Schistosoma mansoni* Sambon, 1907 – Mouahid et al. 2012) or distinct interspecific diurnal (*S. haematobium* (Bilharz, 1852) infecting humans) and nocturnal (*S. rodhaini* Brumpt, 1931 infecting rodents) emergence patterns (e.g., Mintsa-Nguéma et al. 2014, Théron 2015).

A wide range of environmental factors affecting cercarial emergence is well-documented. It is regulated by exogenous abiotic conditions such as salinity, water pressure, tidal cycles or UV radiation (Mouritsen 2002, Koprivnikar and Poulin 2009a,b, Studer et al. 2012) as well as biotic factors such as the size of molluscan hosts (e.g., Poulin 2006, Morley et al. 2010) or history of trematode infection related to the number of miracidia infecting snails (e.g., Massoud 1974, Sluiters et al. 1980, Gustafson and Bolek 2015). The primary role is played by the effect of temperature as cercarial emergence generally increases with increasing temperature (e.g., Lyholt and Buchmann 1996, Fingerut et al. 2003, Poulin 2006), but trend of decreased emergence was also observed (Thieltgest and Rick 2006, Koprivnikar and Poulin 2009a,b). Photocycle and changes in light intensity has been also found important in controlling cercarial emergence and output rates (e.g., Umadevi et al. 1997, Kaewkes et al. 2012, Soldánová et al. 2016, Théron 2015).

The cercarial emergence from molluscan hosts is usually circadian (one peak during 24 h) and diurnal (maximum number of emerged cercariae during the daylight period) (Combes et

al. 1994). Although, the effect of thermo- and photoperiod on cercarial emergence has been largely studied in many systems, most information available originates from laboratory-controlled settings and only few studies provides data simulating natural conditions (mainly by conducting experiments near a window in the laboratory, e.g., Taskinen 1998, Morley et al. 2010, Soldánová et al. 2016). However, cercarial output measurements by keeping molluscs directly in the field under natural light and temperature conditions are generally scarce (Brassard et al. 1982, Fingerut et al. 2003, Preston et al. 2013, Prokofiev et al. 2015) and no study has been so far conducted in freshwater systems in temperate European regions. In addition, seasonal variation in cercarial output in these habitats is strongly temperature-dependent, peaking in warm summer months and decreasing towards winter period when temperature drops to a minimum threshold, ceasing the emergence (Galaktionov and Dobrovolskij 2003, Morley 2012).

Trematodes of the genus *Diplostomum* von Nordmann, 1832 (Diplostomidae) utilize a three-host life cycle involving freshwater gastropods of the family Lymnaeidae as first intermediate hosts, fish as second intermediate hosts and fish-eating birds as definitive hosts (Niewiadomska 1986, Chappell et al. 1994, Karvonen 2012). Adult trematodes inhabiting the intestine of the definitive host (Figure 1A) release eggs via the bird's faeces into the water (Figure 1B). Miracidia hatched from the eggs actively seek the first intermediate snail host (Figure 1C), where asexual reproduction through sporocysts takes place and furcocercariae (i.e., cercariae with a bifurcated tail) are produced (Figure 1D). After leaving the snail, cercariae actively penetrate the second intermediate host (Figure 1E), enter through the epithelium of gills or skin and migrate through the tissues to the eye where metacercariae develop (lens, vitreous humour or region of retina). Definitive hosts become infected after ingestion of fish harbouring metacercariae, which develop into adults, mate and produce eggs.

Metacercariae of *Diplostomum* spp. are important pathogens of fish causing eye cataracts leading to impaired vision and even blindness, making fish more vulnerable and prone to predation (Chappell et al. 1994, Seppälä et al. 2004, Karvonen 2012). Depending on the snail host, furcocercariae are released in tens of thousands every day (up to 58, 000 snail^{-1} day^{-1}) and the emergence is diurnal (i.e., higher numbers released during a daylight period) (Lyholt and Buchmann 1996, Karvonen et al. 2004a).

Even though species of *Diplostomum* were studied intensively, especially with respect to the infection capability of fish intermediate hosts and induced changes in their behaviour (e.g., Majoros 1999, Seppälä et al. 2004, 2007, Larsen et al. 2005, Gopko et al. 2015), little is

Figure 1. Life cycle of *Diplostomum* spp. (**A)** Adult trematodes inhabit the intestine of definitive host, the fish-eating bird. (**B)** Eggs are released into the water via the bird's faeces. **(C)** Miracidia hatch from the eggs and seek out the first intermediate snail host. **(D)** Emerged furcocercariae actively penetrate the second intermediate fish host. **(E)** Larvae migrate through the fish tissues to the eye where metacercariae develop. Drawings of hosts and trematode larval stages were taken from Karvonen (2012).

known about the daily output rates (production) and patterns of cercarial emergence (periodicity). At least some data exist for lymnaeid snails *Lymnaea stagnalis* – Lyholt and Buchmann (1996), Karvonen et al. (2004a, 2006a), *Lymnaea arctica* Lea, 1864 – Brassard et al. (1982) and *Myxas glutinosa* (O. F. Müller, 1774) – Karvonen et al. (2006a). However, only Brassard et al. (1982) followed emergence in the field under natural photocycle and thermal regime, whereas remaining studies conducted research in the laboratory or incubator with

standardised conditions. Moreover, in these studies cercarial output was monitored mainly every 24–48 h focusing on seasonal differences or diurnal *vs* nocturnal preference in daily emergence. Therefore, the poorly studied chronobiology and seasonality of cercarial emergence of *Diplostomum* spp. remain to be explored.

Due to serious gap in the current knowledge together with complete lack of data for lymnaeid snails of the genus *Radix* Montfort, 1810, the present study aims (i) to investigate emergence of cercariae of *Diplostomum* spp. from the first intermediate snail host *Radix lagotis* (Schrank, 1803) (Lymnaeidae) in a temperate region, (ii) to evaluate temporal variation and daily periodicity in cercarial emergence, and (iii) to assess the possible interspecific (between species) and intraspecific variation (between two populations of one species) in response to seasonal climatic conditions. We followed the cercarial production and periodicity in emergence of three *Diplostomum* species from naturally infected snails *Radix lagotis* in three types of experimental treatments and three seasons characterised by various thermal and light conditions to determine daily output rates, peaks during specific day-time intervals (i.e., sunrise, day, sunset and night) and seasonal variation in emergence of cercariae.

2. Materials and Methods

2. 1. Snail samples and study site

Snails of the genus *Radix* were collected from Most Lake in northern Bohemia, Czech Republic (50°32'13"N, 13°38'40"E) once a month in July and September 2017, and May 2018 (Figure 2A). Most Lake is an oligotrophic man-made water reservoir with a surface area of 309 ha, an elevation 199 m a.s.l, a maximum depth of 75 m, and a mean depth of 22 m (www.pku.cz/jezera/most/). It was created between 2008 and 2014 by flooding the former coal quarry of Most-Ležáky, serving for brown coal mining since 1970s to 1999. Nowadays, Most Lake is the largest hydric reclamation in the Czech Republic, supporting viable and species-rich fauna, including molluscs, fish and birds, important hosts for trematodes (Beran 2013, Peterka et al. 2013, Peterka 2018, Bažant 2015, 2017). Among six species of freshwater molluscs (five gastropods and one bivalve) belonging to four families recorded at the early stage of habitat succession, *Radix auricularia* (Linnaeus, 1758) was the only lymnaeid snail detected in the Most Lake (Beran 2013). The fish fauna consists of eight species from five families, namely Cyprinidae, roach *Rutilus rutilus* (Linnaeus, 1758), tench *Tinca tinca* (Linnaeus, 1758), rudd *Scardinius erythrophthalmus* (Linnaeus, 1758); Esocidae, pike *Esox lucius* Linnaeus, 1758; Percidae, perch *Perca fluviatilis* Linnaeus, 1758, ruffe *Gymnocephalus cernua* (Linnaeus, 1758); Salmonidae, maraena whitefish *Coregonus maraena* (Bloch, 1779); and Siluridae, catfish *Silurus glanis* Linnaeus, 1758 (Peterka et al. 2013, Peterka 2018). Due to the current status of mining area inaccessible to the public, Most Lake became one of the most important ornithological localities in the Czech Republic for around 52 species of waterfowls belonging to 10 orders, out of which more than half is particularly endangered (Bažant 2015, 2017). The importance lies in great opportunities for feeding, nesting and breeding, but especially in providing shelter for wintering and migratory waterfowls passing through Europe (Bažant 2015, 2017). The abundant fish stock attracts many fish-eating birds such as grebes, herons, gulls and cormorants (ca 35,500 fish individuals older than 0+, total weight of nine tones; Peterka 2018).

Altogether, 880 snails of different size cohorts were randomly sampled by hand from stones in shallow littoral zone in two localities (Table 1, Figures 2B, C). The delineation of species within the genus *Radix* based on shell or reproductive system characteristics is rather problematic due to overlapping morphological features (Huňová et al. 2012). Therefore, the foot tissue of six specimens (out of 16 snails that survived experiments, Table 1) was preserved

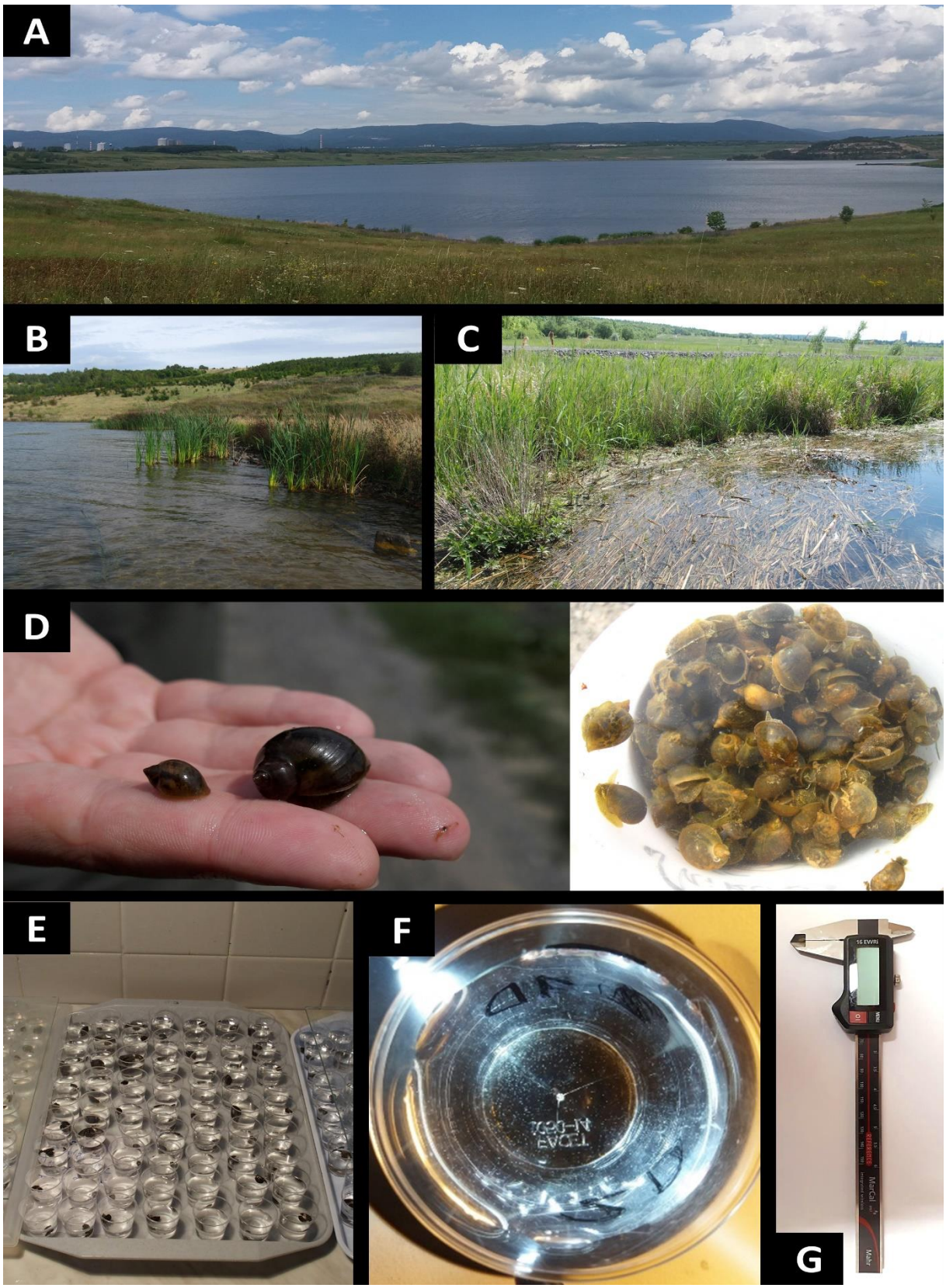

Figure 2. Sampling locality and snail processing. (A) Most Lake in northern Bohemia, Czech Republic. **(B, C)** Snails collected from two sampling sites. **(D)** Model lymnaeid snail *Radix lagotis*. **(E)** Snails individually placed into transparent plastic beakers. **(F)** Screening for patent trematode infections. **(G)** Measurements of snail shell length and width using an electronic digital calliper.

in molecular-grade ethanol for DNA isolation and sequencing. The subsequent molecular identification using sequence analysis of ITS-2 rDNA according to Huňová et al. (2012) assigned these isolates to *Radix lagotis* (Figure 2D).

After transportation to the laboratory, snails were individually sorted out into transparent 40-ml plastic beakers filled with lake water under a light source for 24 h (Figure 2E). Thereafter, snails were examined for patent trematode infections (cercarial release, Figure 2F) and measured for shell length and width with electronic digital calliper (Figure 2G). Prior experiments, all snails infected with *Diplostomum* spp. were isolated in aerated lake water and maintained in laboratory at ambient temperature and natural photoperiod (depending on the season) for at least 48 h for acclimatization. Meantime, snails were fed *ad libitum* with lettuce (*Lactuca sativa* Linnaeus, 1758.) and repeatedly checked for presence of double infections, those being excluded from experiments.

2. 2. Model trematode species and identification

Cercariae of three species *Diplostomum* found in snails *R. lagotis* in Most Lake, i.e., *Diplostomum* '*mergi*' species complex (hereinafter referred to as *D.* '*mergi*'; Figure 3A), *D. spathaceum* (Rudolphi, 1819) (Figure 3B) and *D. parviventosum* Dubois, 1932 (Figure 3C), were used in emergence experiments. Given the lack of previous studies, the pooling of species was based on the assumption of analogous cercarial emergence patterns and daily production due to specific traits characteristic at the genus level (morphotype, behaviour and transmission mode in which species of *Diplostomum* utilize similar vertebrate taxa, lymnaeid snails-fish-piscivorous birds). Wide range of hosts have been recorded for the three species of *Diplostomum* in question (e.g., Shigin 1986, Moravec 2001, Sitko et al. 2006, Georgieva et al. 2013), among which up to eight species of fish and 25 species of waterfowl serve as second intermediate and definitive hosts in Most Lake, depending on the species of *Diplostomum* (Table 2).

Live cercariae were identified to species level based on differential morphological features described by Selbach et al. (2015). The *D.* '*mergi*' species complex includes three genetically and morphologically distinct species-level lineages. However, Lineage 4 is unique in having three pairs of penetration gland-cells compared to those other two lineages with two pairs only. Additionally, Lineage 3 differs in a smaller ventral sucker, number and organisation of pre-oral spines and other characteristics, which make us confident we have used only Lineage 2 in our experiments. Detailed microphotographs of cercariae of all *Diplostomum* species were taken with an Olympus UC30 digital camera on Olympus BX51 light microscope (Figure 3).

Table 1. Number of examined snails *Radix lagotis,* prevalence of infection with larvae of *Diplostomum* spp.* and snail shell size (length and width).

Parameter/Season	**July**	**September**	**May**	**Total**
No. of examined snails	261	387	232	**880**
No. of infected snails (overall prevalence, %)	159 (60.9)	238 (61.5)	31 (13.4)	**428 (48.6)**
No. of infected snails with *Diplostomum* spp. (prevalence, %)*	40 (15.3)	21 (5.4)	9 (3.9)	**70 (8.0)**
No. of infected snails with *D.* '*mergi*' (prevalence, %)	25 (9.6)	6 (1.6)	1 (0.4)	**32 (3.6)**
No. of infected snails with *D. parviventosum* (prevalence, %)	4 (1.5)	2 (0.5)	1 (0.4)	**7 (0.8)**
No. of infected snails with *D. spathaceum* (prevalence, %)	11 (4.2)	13 (3.4)	7 (3.0)	**31 (3.5)**
No. of snails in experiments, initial/survived	12/5	12/7	8/4	**32/16**
Mean snail length ± SD (range, mm)	14.5 ± 4.6 (8.4–21.1)	17.4 ± 3.5 (13.6–22.1)	22.2 ± 2.7 (18.4–25.1)	**17.5 ± 5.0 (8.4–25.1)**
Mean snail width ± SD (range, mm)	9.5 ± 2.9 (5.9–14.0)	11.2 ± 2.1 (8.9–14.0)	14.0 ± 2.2 (11.8–17.5)	**11.2 ± 3.1 (5.9–17.5)**

**Diplostomum* '*mergi*' species complex (according to Georgieva et al. 2013 and Selbach et al. 2015), *D. parviventosum* and *D. spathaceum*

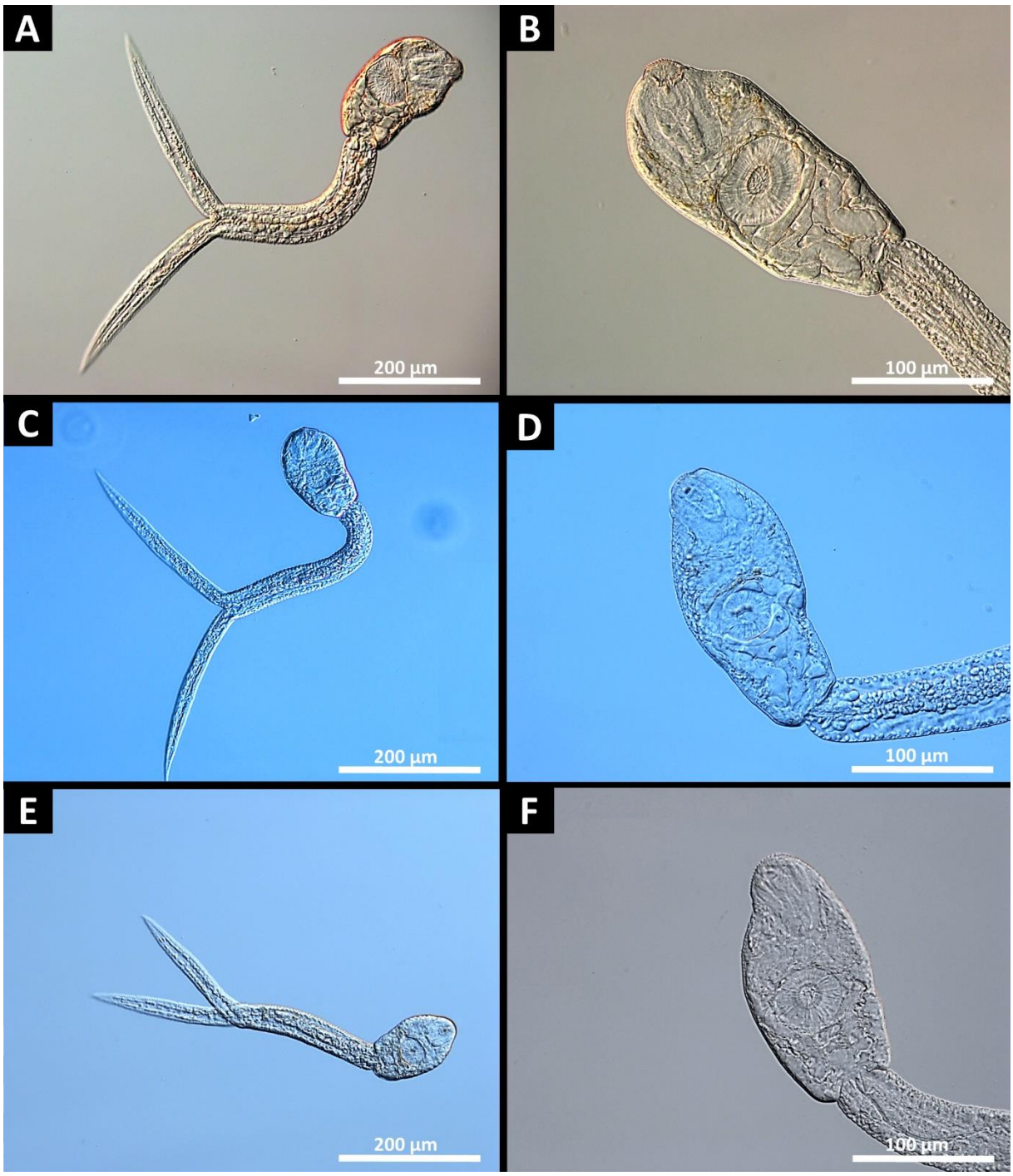

Figure 3. Photomicrographs of live furcocercariae of model *Diplostomum* species. (A, B) *Diplostomum* '*mergi*', **(C, D)** *D. spathaceum*, and **(E, F)** *D. parviventosum*. A, C, E – total view, ventrally; B, D, F – body with apical organ, ventral sucker and two pairs of penetration glands.

Table 2. Fish intermediate hosts and bird definitive hosts of *Diplostomum* spp. whose larvae were found in *Radix lagotis* in Most Lake, northern Bohemia, Czech Republic. Records from the Czech Republic are indicated by an asterisk.

Species	Second intermediate host	Definitive host
D. '*mergi*' Dubois, 1932	*Coregonus maraena*[a], *Gymnocephalus cernua*[a], *Perca fluviatilis*, *Rutilus rutilus*, *Scardinius erythrophthalmus*, *Silurus glanis*[a], *Tinca tinca*[a]	*Chroicocephalus ridibundus*, *Larus argentatus*, *L. cachinnans*[a], *L. canus*, *L. michahellis*[a], *Mergellus albellus*, *Mergus merganser**, *M. serrator*, *Podiceps auritus*[a], *P. cristatus*, *P. grisegena*, *P. nigricollis*[a], *Tachybaptus ruficollis*[a]
D. parviventosum Dubois, 1932	*Coregonus maraena*[a], *Esox lucius*, *Gymnocephalus cernua*[a], *Perca fluviatilis*, *Rutilus rutilus*, *Scardinius erythrophthalmus*, *Tinca tinca*[a]	*Chroicocephalus ridibundus*, *Larus argentatus*[a], *L. cachinnans*[a], *L. canus*[a], *L. michahellis*[a], *Melanitta fusca**, *M. nigra*[a], *Mergellus albellus*[a], *Mergus merganser**, *M. serrator*[a]
D. spathaceum (Rudolphi, 1819) Braun, 1893	*Coregonus maraena*[a], *Esox lucius**, *Gymnocephalus cernua**, *Perca fluviatilis**, *Rutilus rutilus**, *Scardinius erythrophthalmus**, *Silurus glanis**, *Tinca tinca**	*Ardea alba*[a], *A. cinerea**, *Aythya ferina*[a], *A. fuligula*[a], *A. marila*[a], *Botaurus stellaris*[a], *Chlidonias niger*[a], *Chroicocephalus ridibundus**, *Gavia arctica*[a], *G. stellata*, *Larus argentatus**, *L. cachinnans**, *L. canus**, *L. michahellis**, *Phalacrocorax carbo*, *Podiceps auritus*[a], *P. cristatus*, *P. grisegena*[a], *P. nigricollis*[a], *Tachybaptus ruficollis*[a]

Hosts are provided based on records in the Host-Parasite Database of the Natural History Museum, London (Gibson et al. 2005) and literature data (Shigin 1968, 1986, Niewiadomska 1984, 2003, Moravec 2001, Sitko et al. 2006, Georgieva et al. 2013, Peterka et al. 2013, Peterka 2018, Bažant 2015, 2017, Kudlai et al. 2017). Accidental non-fish-eating bird hosts are not considered. Records of bird families Podicipedidae (*D.* '*mergi*') and Laridae (*D.* '*mergi*' and *D. parviventosum*) may represent erroneous identifications.

[a]Possible hosts inferred from records available for congeneric hosts' life cycle.

Representative samples of each trematode species were fixed in 4% formaldehyde solution as well as in molecular grade ethanol as voucher samples and for future molecular studies.

2. 3. Experimental design

Using a factorial design, we examined the influence of photoperiod light/dark regime, water temperature and season on cercarial emergence of *Diplostomum* spp. from naturally infected *R. lagotis*. Daily output rates (overall cercarial production) as well as patterns in cercarial emergence (periodicity) were investigated in three months representing three seasons (July and September of 2017 and May 2018) under various experimental conditions in a series of three types of experiments (Figure 4). A "field experiment" was carried out to follow natural climatic conditions, i.e., photo- and thermoperiod regime, using a device designed to measure the output of cercariae *in situ* (Figures 4A, 5). Due to the strict restrictions to public access at Most Lake, field experiments took place in Vlkovský fishpond in southern Bohemia (49°08'56"N, 14°43'51"E). A metal rod was placed into the pond substrate (Figure 5A), to which a plastic holder with beakers containing filtered water and snails was attached (Figure 5B). After tight covering of experimental beakers with transparent plastic lid and clips (Figures 5C, D), holders were fastened to a metal construction and partially submerged in the pond near the shore (at a distance of ca 1 m). At the same time, a control was carried out by allotting four extra beakers containing water without snails, ensuring that no cercariae were present prior experiment initiation. Cercariae were not found in these control beakers during the course of the study, illustrating the effectiveness of the *in situ*-device. To avoid the potential effect of different chemical composition of water (oligotrophic lake *vs* eutrophic fishpond), water from the Most Lake was used in all experiments. Lake water was filtered using 12 μm-pore filter membrane (Whatman, Nuclepore Track-Etch Membrane) eliminating the random presence of trematode cercariae in collected water samples.

A "laboratory experiment" was performed simulating the field light and thermal conditions. Snails produced cercariae in experimental beakers that were placed into an aquarium in a room near opened window to ensure a normal range of environmental climatic factors (Figure 4B). The water temperature was maintained around the mean value that was measured in the field, using a heater (Xilong AT-700 25W) placed into the aquarium.

Lastly, an "incubator experiment" was run in simulations of field conditions but under controlled light (artificial illumination) and temperature with minimal possibilities for their fluctuation (Figure 4C). Before the start of each experiment, the lake water was pre-warmed to the required temperature.

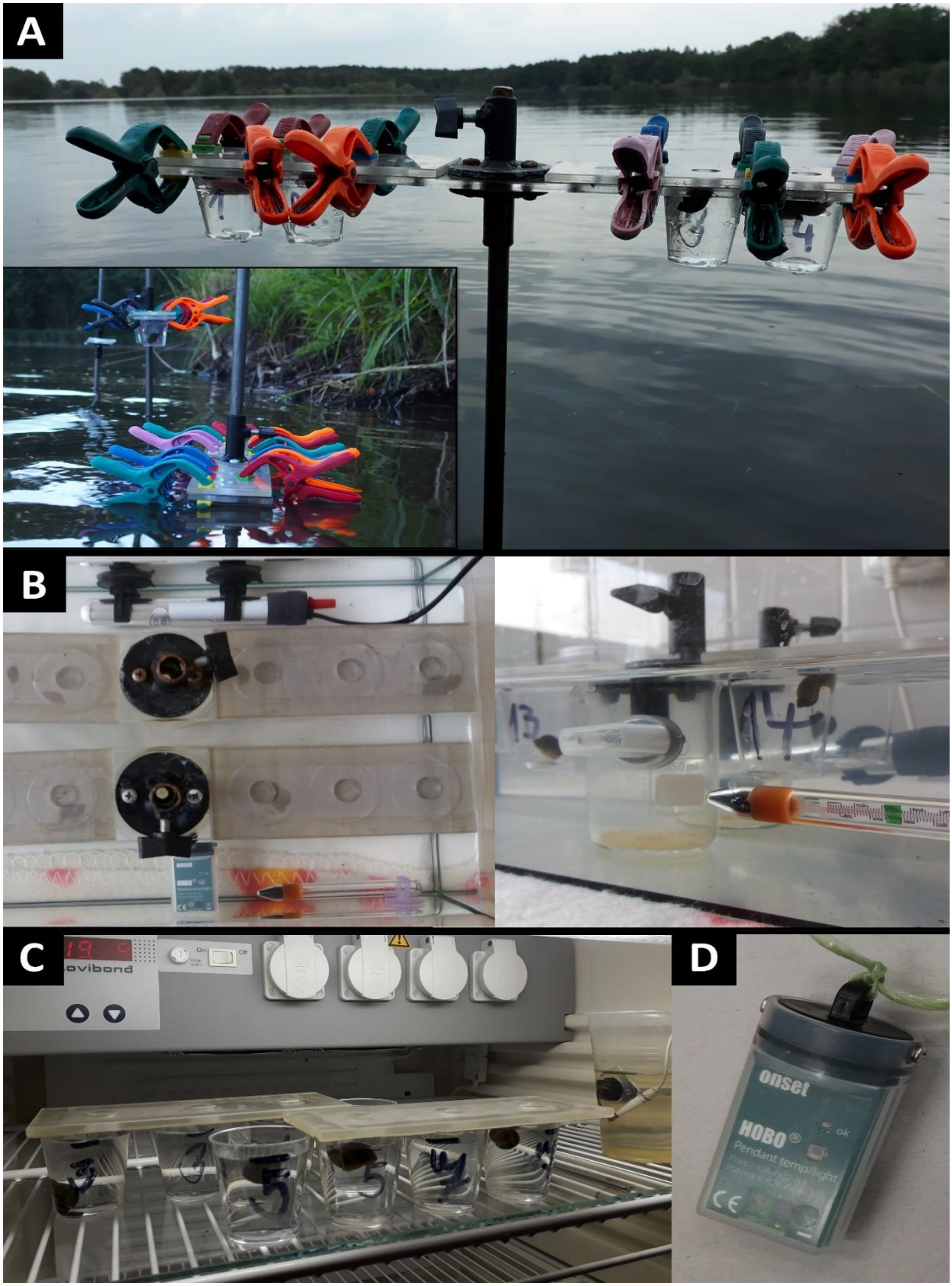

Figure 4. Methodology of experimental treatments under various thermal and light conditions. (A) Field experiment under natural climatic conditions. **(B)** Laboratory experiment simulating natural climatic conditions using the aquarium heated by a heater. **(C)** Incubator experiment under standardised conditions. **(D)** Data-loggers recording water and air temperature and illuminance/light intensity.

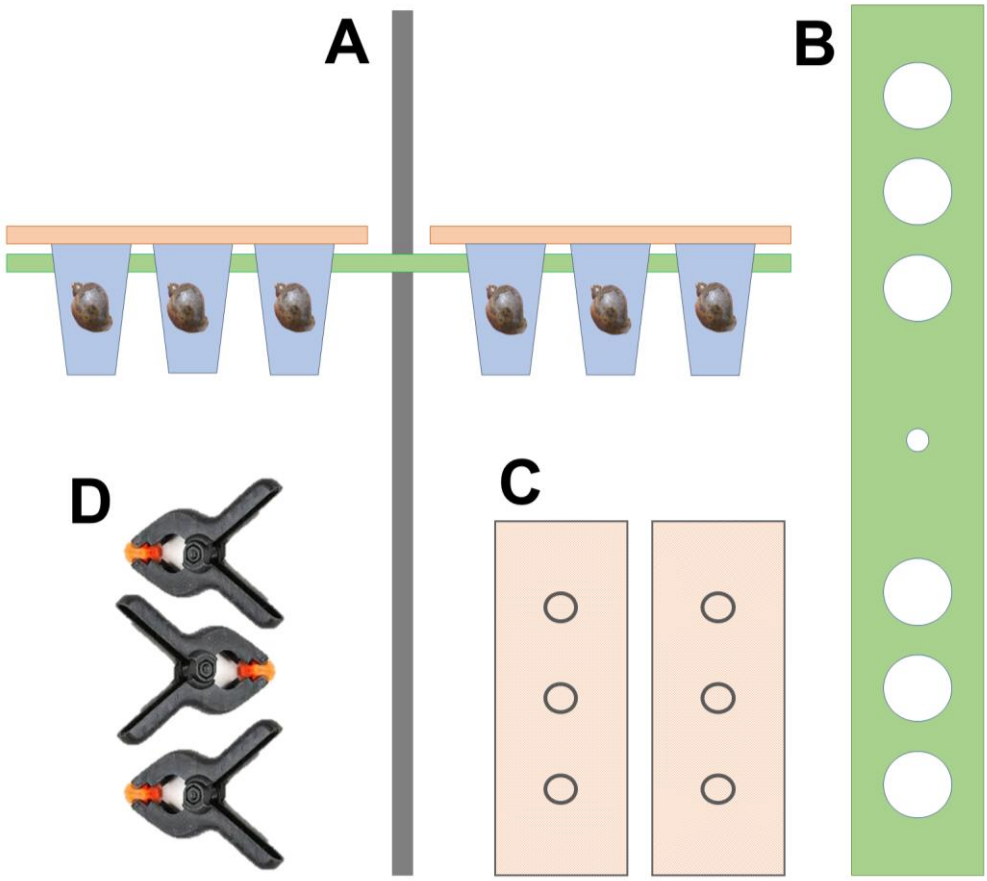

Figure 5. Device maintained *in situ* for emergence experiments in the field. (A) Metal rod placed in the pond substrate. **(B)** Plastic holder for 40-ml experimental beakers. **(C)** Plastic transparent lid with drilled holes and 0.5 µm net to cover plastic beakers containing snails **(D)** Plastic clips to hold transparent lids tightly.

Climatic parameters were monitored using data loggers (Onset HOBO UA-002-64 Pendant 64K) throughout the entire study (Figure 4D). The variation in temperature and illuminance/light intensity for the air and water under various experimental arrangements in the three seasons is presented in Table 3. Based on the seasonal field data, the water temperature in laboratory and incubator experiments were set up at ~22°C in July, ~16°C in September, and ~18°C in May (Table 3). The basic light-dark conditions were ~17:7 h, ~14:10 h and ~17:7 h of photocycle, respectively. Each experiment was performed over a 24 h period for three consecutive days with an interval of one free day in between when snails were allowed to acclimatize and supplied small amount of lettuce. In total, cercarial output was investigated in three successive experiments lasting for nine days in each season, resulting in nine experiments (Table 4).

Table 3. Summary of the mean (range) temperature and illuminance/light intensity for the air and water under various experimental conditions in three seasons.

Parameter/Season	**July**			**September**			**May**		
	Field	Laboratory	Incubator	Field	Laboratory	Incubator	Field	Laboratory	Incubator
Mean water temperature (range, °C)	22.0 (19.3–25.4)	23.0 (21.2–25.6)	21.0 (20.7–21.5)	15.9 (14.0–19.8)	15.1 (11.8–17.2)	16.1 (15.6–16.4)	18.5 (16.5–24.4)	20.5 (17.5–22.5)	17.2 (16.9–17.8)
Mean air temperature (range, °C)	17.2 (8.5–32.6)	24.1 (20.8–28.5)	22.4 (22.0–23.6)	14.8 (9.1–32.0)	14.5 (10.9–17.0)	16.7 (16.2–17.0)	15.4 (7.9–36.1)	21.6 (17.3–24.8)	18.2 (18.0–19.1)
Mean water illuminance water (range, lx)	76 (0–958)	241 (0–990)	382 (0–786)	6,153 (0–99,201)	587 (0–2,756)	274 (0–700)	7,649 (0–110,223)	926 (0–4,478)	647 (0–1,216)
Mean air illuminance (range, lx)	61 (0–969)	206 (0–969)	489 (0–904)	15,284 (0–187,379)	681 (0–3,100)	258 (0–678)	21,281 (0–198,401)	883 (0–4,133)	618 (0–1,152)

Table 4. Cercarial output of *Diplostomum* '*mergi*', *D. spathaceum* and *D. parviventosum* from naturally infected snails *Radix lagotis* under various experimental conditions in three seasons. The range, mean (±standard deviation, SD) and total number of emerged cercariae is given per snail pooled across three days.

Season/ Snail code	***Diplostomum* species**	**Field experiment (n = 12)**			**Laboratory experiment (n = 10)**			**Incubator experiment (n = 6)**		
JULY		**Range**	**Mean±SD**	**Total**	**Range**	**Mean±SD**	**Total**	**Range**	**Mean±SD**	**Total**
1J[a]	*D.* '*mergi*'	2,381–2,804	2,532±236	7,596	1,360–1,978	1,740±332	5,219	0–200	69±114	207
2J[c]	*D.* '*mergi*'	4,272–6,876	5,664±1,311	16,992	–	–	–	–	–	–
3J[a]	*D.* '*mergi*'	1,531–3,120	2,496±847	7,487	1,880–2,313	2,034±242	6,102	1,355-1,828	1,564±241	4,691
4J[c]	*D.* '*mergi*'	1,630–3,116	2,554±806	7,662	1,780–2,332	1,964±319	5,892	–	–	–
5J[b]	*D.* '*mergi*'	2,514–3,880	3,063±722	9,188	1,703–3,225	2,434±763	7,301	–	–	–
6J[a, b]	*D.* '*mergi*'	4,181–8,940	6,332±2,412	18,997	943–6,276	3,438±2,683	10,315	–	–	–
7J	*D.* '*mergi*'	4,176–11,978	8,117±3,902	24,350	9,417–15,440	11,513±3,404	34,538	4,459-8,104	6,324±1,824	18,971
8J[a]	*D.* '*mergi*'	3,940–4,572	4,151±364	12,454	6,433–9,518	7,748±1,592	23,245	3,464-5,176	4,595±979	13,784
9J[c]	*D.* '*mergi*'	1,610–7,924	4,254±3,280	12,763	–	–	–	–	–	–
10J[a]	*D.* '*mergi*'	670–1,624	1,291±538	3,873	1,508-8,748	6,115±4,003	18,344	2,460-4,508	3,525±1,026	10,576
11J[c]	*D.* '*mergi*'	–	–	–	–	–	–	–	–	–
12J[b]	*D.* '*mergi*'	1,449–1,700	1,572±126	4,716	–	–	–	–	–	–
SEPTEMBER		**Field experiment (n = 12)**			**Laboratory experiment (n = 7)**			**Incubator experiment (n = 7)**		
1S[c]	*D.* '*mergi*'	–	–	–	–	–	–	–	–	–
2S	*D. parviventosum*	894–2,708	1,957±946	5,870	1,459-2,792	1,971±718	5,913	2,380–3,564	2,984±592	8,952
3S[b]	*D.* '*mergi*'	–	–	–	–	–	–	–	–	–
4S[a, c]	*D.* '*mergi*'	–	–	–	–	–	–	–	–	–
5S[a]	*D. spathaceum*	878–1,697	1,352±424	4,055	2,227–2,476	2,358±125	7,075	2,804–3,688	3,275±445	9,824
6S[c]	*D. spathaceum*	–	–	–	–	–	–	–	–	–
7S[c]	*D. spathaceum*	–	–	–	–	–	–	–	–	–
8S	*D. spathaceum*	30–1,670	767±832	2,302	1,184–1,644	1,365±245	4,095	1,492–2,014	1,758±261	5,275
9S	*D. spathaceum*	659–6,114	3,044±2,791	9,133	3,647–5,679	4,922±1,111	14,767	6,028–6,934	6,365±496	19,095
10S	*D.* '*mergi*'	2,310–6,448	5,057±2,379	15,171	3,012–7,216	5,770±2,389	17,310	5,428–9,644	7,225±2,176	21,676
11S	*D. spathaceum*	5,027–10,186	7,157±2,695	21,470	7,988–13,205	10,567±2,609	31,702	10,744–13,572	12,243±1,422	36,728
12S	*D. spathaceum*	6,275–9,301	7,989±1,552	23,966	6,598–9,334	7,640±1,480	22,920	4,909–8,814	7,180±2,029	21,539

Table 4. Continued

Season/ Snail code	*Diplostomum* species	Field experiment (n = 8)			Laboratory experiment (n = 8)			Incubator experiment (n = 4)		
MAY		**Range**	**Mean±SD**	**Total**	**Range**	**Mean±SD**	**Total**	**Range**	**Mean±SD**	**Total**
1M[c]	*D. spathaceum*	15,312–21,414	18,004±3,114	54,012	–	–	–	–	–	–
2M[c]	*D. spathaceum*	19,968–22,884	21,566±1,478	64,698	–	–	–	–	–	–
3M	*D. spathaceum*	15,432–21,190	17,497±3,206	52,491	14,827–19,472	17,451±2,380	52,352	9,828–12,080	10,735±1,188	32,204
4M	*D. spathaceum*	7,652–44,263	23,210±18,914	69,631	28,622–33,408	31,812±2,763	95,437	12,548–24,240	18,881±5,907	56,644
5M	*D. spathaceum*	8,601–36,310	19,389±14,838	58,167	12,250–20,862	16,511±4,307	49,534	12,224–17,056	14,396±2,453	43,188
6M[b]	*D. spathaceum*	5,860–12,336	10,149±3,714	30,446	5,155–15,944	11,455±5,618	34,366	–	–	–
7M	*D. spathaceum*	13,062–20,546	15,960±4,017	47,880	13,453–18,765	15,620±2,788	46,860	8,444–12,736	10,060±2,334	30,180
8M[b]	*D. parviventosum*	12,971–17,409	15,717±2,399	47,150	11,538–23,994	18,024±6,244	54,072	–	–	–

[a]Snail with patent or prepatent double infection
[b]Snail died before the experiment
[c]Snail died during the experiment

In each season, cercarial emergence was monitored using different sets of snails naturally infected with *Diplostomum* spp., but the same snail individuals were involved in the three experiment treatments within one season. The selection of infected snails for emergence experiments was in particular affected by parasite's seasonal abundance, spontaneous death of snails or presence of double infections before experiments' initiation (Table 1). Hence, the initial number of 32 snails was entered into first field experiments. However, half snails had died during the course of study (Table 1), resulting in a different number of replicates in each experimental treatment and season (Table 4). *Diplostomum* '*mergi*' was the only trematode species in July experiments, but was also present only once in September. *Diplostomum parviventosum* was included into experiments in September and May (one case each), both seasons otherwise fully represented by *D. spathaceum* (Table 4). The main reason for combining trematode species was the basic assumption of no interspecific differences in cercarial emergence patterns and production rates.

2. 4. Data acquisition

To determine the daily production and periodicity in cercarial emergence, each snail was placed individually into 40-ml transparent plastic beaker and cercarial release was counted four times a day to follow natural light regime in specific day-time intervals, i.e., sunrise, day, sunset and night (Figure 6). Counts of cercariae in incubator experiments were conducted twice a day (light and dark period) due to the standardised light conditions when sunrise and sunset could not be simulated. At each counting point, snails were carefully transferred into clean beakers with filtered lake water and the spoon was washed up and wiped off thoroughly (Figure 7A). The water containing swimming cercariae of *Diplostomum* spp. (Figure 7B) was stirred to be homogenised prior taken subsamples from the cercarial suspension. Ten subsamples of one ml each were transferred into 6-well plates (Figure 7C) and the number of emerged cercariae was determined in each well under the stereomicroscope (Figure 7D). Before counting, few drops of 4% formaldehyde solution were added to subsamples to immobilise cercariae and thus facilitate counting. Numbers were recorded into a data sheet (Figure 7E). All experimental snails (Figure 7F) were dissected during (spontaneous death) and/or at the end of experiments to check the stage of infection in order to assess the extent to which the hepatopancreas was occupied with intramolluscan larval stages (sporocysts of *Diplostomum* and possibly other trematode taxa) (Figure 7D).

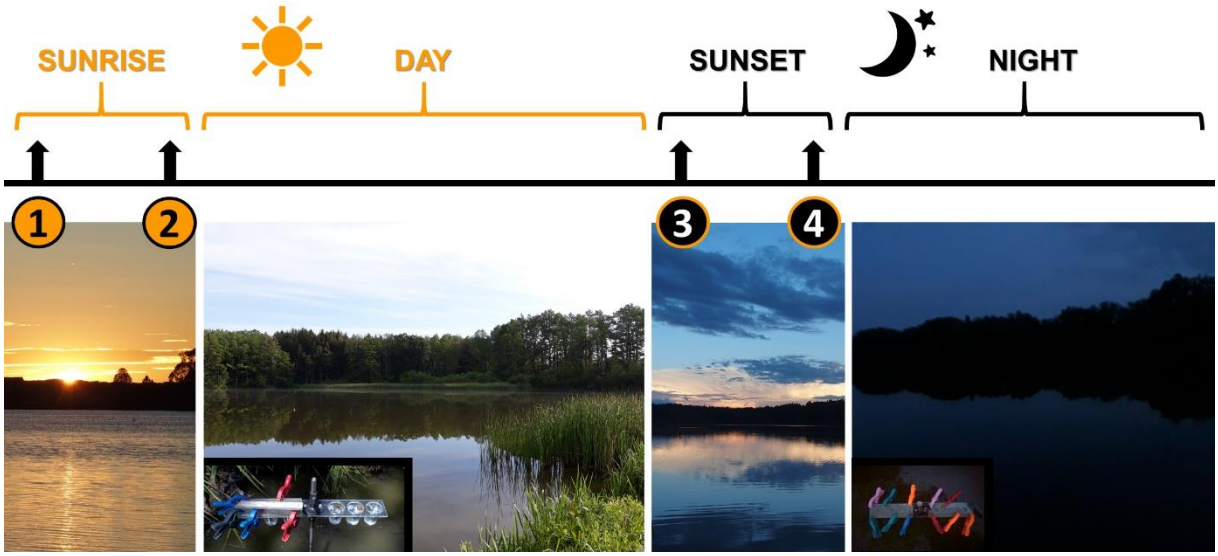

Figure 6. Experimental set-up. Cercarial counts carried out four times over 24 h period to monitor patterns in cercarial emergence during the main day-time intervals, i.e., sunrise, day, sunset and night, in field and laboratory experiments. Emergence of cercariae during two day-time intervals, i.e., light and dark periods, applies to incubator experiments (counting points one and four).

To estimate the total number of cercariae emerged from a single snail, a mean over 10 subsamples was calculated by summing cercariae counted in one ml and multiplied by the whole water volume (40 ml). The daily output rates of cercariae were calculated by pooling data across four day-time intervals, snail replicates and three experimental days and calculated on average, resulting in a mean number of cercariae produced snail^{-1} day^{-1} (hereinafter referred to as daily emergence rates). Circadian patterns/rhythms were assessed by estimating the total number of cercariae during each day-time interval separately and calculated on average, resulting in cercarial output specific for sunrise, day, sunset and night snail^{-1} day^{-1} (hereinafter referred to as time emergence rates). However, because of distinct duration of the specific day periods, the observed output of cercariae was recalculated to the shortest time interval within 24 h under assumption of constant cercarial emergence in all other day-time periods, allowing to determine clear peak(s) in cercarial emergence. Such conversion of raw data was done for each type of experiment in each season.

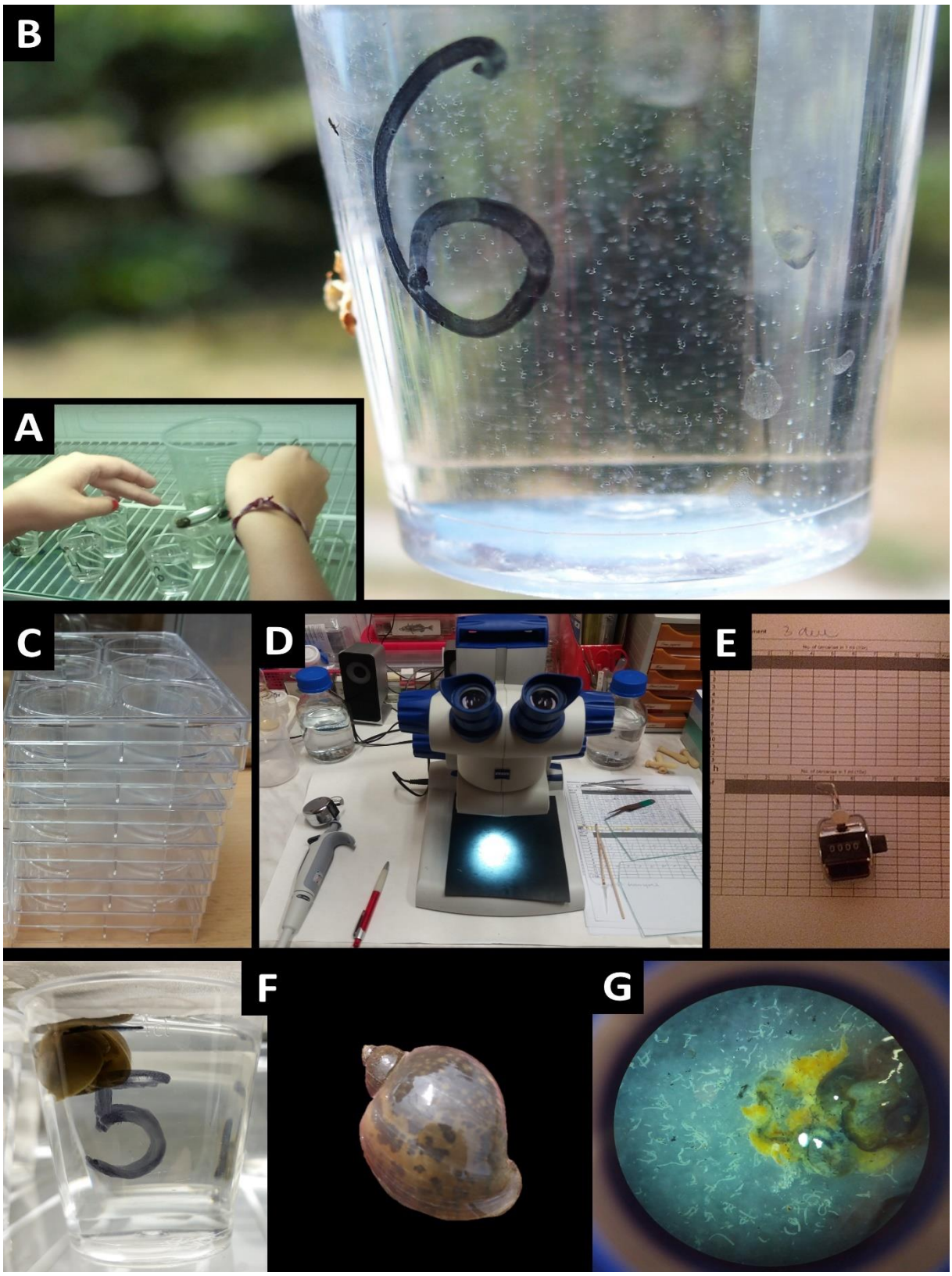

Figure 7. Methodology of assessing patterns in cercarial output and snail dissection. (A) Transferring snails to experimental beakers between each day-time interval. **(B)** Detail of emerged furcocercariae of *Diplostomum* spp. in the plastic beaker. **(C)** Six-well plates with water subsamples containing cercariae. **(D)** Counting under stereomicroscope. **(E)** Documentation of emerged cercariae using data sheet. **(F)** Dissection of *Radix lagotis* at the end of the incubator experiment or in case of spontaneous death of snails **(G)**.

2. 5. Data analyses

The following statistical analyses were performed on data sets excluding one surviving snail infected with *D.* '*mergi*' (September) and two with *D. parvinetosum* (September and May; Table 4). This reduced the bias due to potential differences in interspecific (between species) patterns in cercarial emergence and output rates. Hence, rhythms of these replicates are represented only graphically. During each sampling event, snails of different size cohorts were collected (Table 1). To account for the possible effect of snails' size on the number of emerged cercariae (e.g., Poulin 2006, Morley et al. 2010), univariate comparison by one-way ANOVA was performed first to test differences in snail length among seasons. Only measurements of snails that were used for field experiments were included into this analysis (Table 1). Second, the relationship between snail size and the total number of emerged cercariae pooled across three days was analysed separately for each experimental treatment within the corresponding season using a Spearman rank correlation. Furthermore, since double infections with other trematode species in some experimental snails were detected, Student's t-test was applied to examine differences in total numbers of emerged cercariae (pooled across three days) compared to singly-*Diplostomum*-infected snails. The test was performed on July data set from field and laboratory experiments due to a limited number of comparisons.

Emergence data were divided into two sets in order to assess: (i) seasonal variation in cercarial output rates (i.e., daily emergence rates) in relation to the three experimental treatments (field, laboratory and incubator) using observed number of emerged cercariae. We were specifically interested in the effect of season, day and type of experiment on the emergence rates of cercariae. The second data set was used to investigate: (ii) temporal variation in daily emergence patterns and cercarial output rates (i.e., time emergence rates) in relation to the specific day-time periods (sunrise, day, sunset and night) using recalculated data to the lowest time interval within 24 h. Here we analysed: (ii/A) within-seasonal differences by comparing time emergence intervals for each experiment separately; and (ii/B) between-seasonal differences by comparing data obtained from field and laboratory experiments only. Simultaneously, interspecific (between species) production and periodicity in emergence of *D.* '*mergi'* and *D. spathaceum* as well as intraspecific variation between two populations of *D. spathaceum* (September and May, Table 4) were examined in both data sets.

Snail size (mean shell length) differed among seasons (ANOVA; $F_{2, 20} = 7.14$, $p < 0.01$) with significantly larger snails in May ($p < 0.01$), whereas it was comparable in July and September ($p > 0.05$) (see Table 1 for mean snail length). Mean snail length also positively

correlated with the number of emerged cercariae in some experiments, indicating higher emergence rate from larger snails (Field-July, $r_s = 0.66$, $p < 0.05$; Laboratory-July, $r_s = 0.83$, $p < 0.05$; Laboratory-September, $r_s = 0.86$, $p < 0.05$; Incubator-September, $r_s = 0.89$, $p < 0.01$; Field-May, $r_s = 0.89$, $p < 0.01$). Therefore, a series of more complex general linear models (GLM-ANOVA) were performed to account for the effect of snail length, being treated as a covariate. Factors 'season', 'day', 'type of experiment' and 'time interval' were used as categorical variables with fixed effects in different combinations depending on the aim of analysis in statistical models ('i', 'ii/A' and 'ii/B'). Both observed and recalculated numbers of emerged cercariae were entered as dependent variable. In case of between-seasonal comparisons, factor 'snail identity' was entered as the random effect nested within season to account for different sets of snail replicates involved in each season. Prior to analyses, snail length was ln-transformed and numbers of emerged cercariae ln (x+1)-transformed in order to improve normality and homoscedascity. *Post-hoc* calculations were performed with Tukey's HSD-test where appropriate. All analyses were carried out using Statistica 7.0 software package (StatSoft Inc., Tulsa, Oklahoma, USA) with results considered as significant at $p < 0.05$.

3. Results

A total of 880 snails of *Radix lagotis* were examined for patent trematode infections (Table 1). The overall prevalence of infection was 48.6% across seasons, of which 8% consisted of *Diplostomum* spp. (Table 1). There was a seasonality in the infection with *D.* '*mergi*' with the highest prevalence in July, followed by a sharp decrease in September and low levels in May, whereas prevalence of *D. spathaceum* remained similar in summer, autumn and spring samples. Infections with *D. parviventosum* were rare in all seasons (Table 1).

Data on temperature and light intensity were highly variable depending on the season and experimental treatments performed under various thermal and light conditions (Table 3). In most cases, the lowest and highest values of measured parameters typically coincided with the coldest and warmest day-time periods, i.e., night and day. Nevertheless, the mean water temperature showed steady trend throughout each experimental treatment, providing similar conditions, and thus without strong influence on cercarial production among experiments. In contrast, mean illuminance of water and air fluctuated considerably, being higher during field experiments compared to two other experimental treatments (except for July).

The parasitological examination revealed that almost half of experimental snails with *Diplostomum* spp. had the whole hepatopancreas full of *Diplostomum* sporocysts (47% of 32 snails used in experiments), showing the maximum capacity for cercarial production per day. Infections in six and five snails occupied half and one quarter of hepatopancreas, respectively, and the remaining snails were found with less than one third of infections, mostly consisting of two trematode species within the snail individual. In total, seven experimental snails in July and September were with double infections (Table 4), of which one was patent (cercarial emergence of echinostome cercariae during the field experiment) and six prepatent (intramolluscan stages of echinostome rediae and *Opisthioglyphe ranae* (Froelich, 1791) detected in five and one dissected snails, respectively). No double infections were encountered in May. There were no significant differences in cercarial emergence from snails with double and single infection, although the overall productivity of *D.* '*mergi*' from double infections was somewhat lower in both field and laboratory experiments in July ($t = -0.62$, $p > 0.05$; $t = -0.11$, $p > 0.05$, respectively). Therefore, the doubly infected snails were not excluded from statistical analyses.

The mean daily emergence rate across three experiments in July was 3,962 cercariae snail^{-1} day^{-1} for *D.* '*mergi*' (reaching maximum of 15,440 cercariae); in September 5,199 cercariae snail^{-1} day^{-1} for *D. spathaceum* (reaching maximum of 13,572 cercariae); and 17,044 snail^{-1} day^{-1} in May (reaching maximum of 44,263 cercariae) (Table 5). The mean of cercariae emerged from a single snail infected with *D.* '*mergi*' in September was 6,017 snail^{-1} day^{-1}; and 8,130 snail^{-1} day^{-1} for *D. parviventosum* which emerged from the two snails in September and May experiments over three days. In total, the mean daily emergence pooled across trematode species and all nine experiments was 8,105 cercariae snail^{-1} day^{-1} (Table 5).

3. 1. Cercarial emergence patterns and seasonal variation

Data sets restricted to snails infected with *D.* '*mergi*' and *D. spathaceum* along without those that had died during or before experiments resulted in a reduced number in each experimental treatment entered into statistical analyses (see "n" for survived snails in Table 5). A comparative statistical assessment of observed output rates using GLM-ANOVA (model 'i') revealed pronounced effect of snail length as well as significant interaction between season and experimental treatment (Table 6, Figure 8). In addition, a significant effect of the experiment type was detected with lower cercarial emergence in incubator experiments compared to field and laboratory (Table 6). However, no differences were apparent in *post-hoc* test (Tukey's HSD; all $p > 0.05$). Daily cercarial emergence rates followed similar trend among days of experiments and no interactions with other factors were detected (Table 6). These results also demonstrate both intraspecific and interspecific seasonal variation in cercarial emergence. Mean daily output rates of cercariae of *D. spathaceum* exhibited significantly higher levels in all experimental treatments in May compared to September (all $p < 0.01$) with 3.5-fold increase (Table 5, Figure 8), except for comparable production under controlled conditions in incubator experiments ($p > 0.05$). Striking differences were also found for the conspecific *D.* '*mergi*' in July (all $p < 0.01$), as the average number of cercariae overall increased 2.9× in May (Table 5, Figure 8). When comparing experimental trials (field, laboratory and incubator) within each season, cercarial outputs distinctly differed only in July with notably lower levels under conditions in incubator (both $p < 0.01$; Table 5, Figure 8). Comparable emergence rates were observed between experiments in September as well as in May (all $p > 0.05$).

Table 5. Daily cercarial output of *Diplostomum* '*mergi*' (July) and *D. spathaceum* (September and May) from naturally infected *Radix lagotis* under various experimental conditions. Data are pooled across snail individuals. Number of survived snails in each experimental treatment is indicated by "n" in parentheses. Mean (± standard deviation, SD) represents number of emerged cercariae $snail^{-1}day^{-1}$.

Season	Type of Experiment	Day	Range	Mean±SD	Total
JULY	Field experiment (n = 11)	Day 1	670–8,940	4,299±2,604	47,294
		Day 2	1,449–8,196	3,754±2,074	41,289
		Day 3	1,531–11,978	3,409±3,051	37,495
	Total		**670–11,978**	**3,821±2,552**	**126,078**
	Laboratory experiment (n = 8)	Day 1	1,508–15,440	4,815±4,736	38,520
		Day 2	1,780–9,681	4,563±3,230	36,506
		Day 3	943–9,518	4,491±3,949	35,930
	Total		**943–15,440**	**4,623±3,843**	**110,956**
	Incubator experiment (n = 5)	Day 1	200–6,408	3,444±2,497	17,220
		Day 2	7–8,104	3,854±3,180	19,271
		Day 3	0–4,495	2,348±1,747	11,738
	Total		**0–8,104**	**3,215±2,445**	**48,229**
	Pooled data		**0–15,440**	**3,962±3,028**	**285,263**
SEPTEMBER	Field experiment (n = 5)	Day 1	30–9,301	3,545±4,045	17,727
		Day 2	1,670–10,186	5,611±3,865	28,056
		Day 3	603–6,275	3,029±2,524	15,144
	Total		**30–10,186**	**4,062±3,478**	**60,927**
	Laboratory experiment (n = 5)	Day 1	1,184–10,509	4,862±3,745	24,310
		Day 2	1,267–7,988	4,880±2,897	24,398
		Day 3	1,644–13,205	6,370±4,896	31,851
	Total		**1,184–13,205**	**5,371±3,713**	**80,559**
	Incubator experiment (n = 5)	Day 1	1,492–10,744	5,553±3,509	27,767
		Day 2	2,014–13,572	6,773±4,618	33,865
		Day 3	1,769–12,412	6,166±4,254	30,829
	Total		**1,492–13,572**	**6,164±3,879**	**92,461**
	Pooled data		**30–13,572**	**5,199±3,714**	**233,947**
MAY	Field experiment (n = 7)	Day 1	5,860–19,968	12,935±4,821	90,542
		Day 2	12,336–44,263	24,065±11,732	168,456
		Day 3	8,601–22,884	16,904±5,364	118,327
	Total		**5,860–44,263**	**17,968±8,898**	**377,325**
	Laboratory experiment (n = 5)	Day 1	12,250–33,408	19,149±8,468	95,743
		Day 2	13,453–28,622	18,742±6,189	93,708
		Day 3	5,155–33,407	17,820±10,224	89,098
	Total		**5,155–33,408**	**18,570±7,851**	**278,549**
	Incubator experiment (n = 4)	Day 1	8,444–12,548	10,761±1,964	43,044
		Day 2	12,080–19,856	15,432±3,684	61,728
		Day 3	9,000–24,240	14,361±6,906	57,444
	Total		**8,444–24,240**	**13,518±4,703**	**162,216**
	Pooled data		**5,155–44,263**	**17,044±7,844**	**818,090**
Total			**0–44,263**	**8,105±7,651**	**1,337,300**

Table 6. Results of general linear model (GLM) evaluating the effect of season, day and type of experiment on the cercarial emergence of *Diplostomum* '*mergi*' (July) and *D. spathaceum* (September and May) from naturally infected snails *Radix lagotis*, with snail length as a covariate. Snail identity was treated as random factor and nested within season. Observed numbers of emerged cercariae (dependent variable) were ln (x + 1)-transformed prior to the analysis. Statistically significant results are in bold. Abbreviations: df, decrees of freedom; MS, means of squares; F, test criterion value; P, level of significance.

Factor/interaction	Effect	df	MS	F	P value
Snail identity	*Random*	*20*	*1.36*	*2.04*	***< 0.05***
Snail size/length	Fixed	1	148.85	101.75	**< 0.001**
Season	Fixed	2	4.86	3.13	0.07
Day	Fixed	2	0.98	1.47	0.23
Type of experiment	Fixed	2	2.80	3.91	**< 0.05**
Season*Day	Fixed	4	0.91	1.36	0.25
Season*Type of experiment	Fixed	4	4.18	5.88	**< 0.001**
Day*Type of experiment	Fixed	4	0.62	0.93	0.45
Season*Day*Type of experiment	Fixed	8	0.43	0.64	0.74
Error		117	0.67		

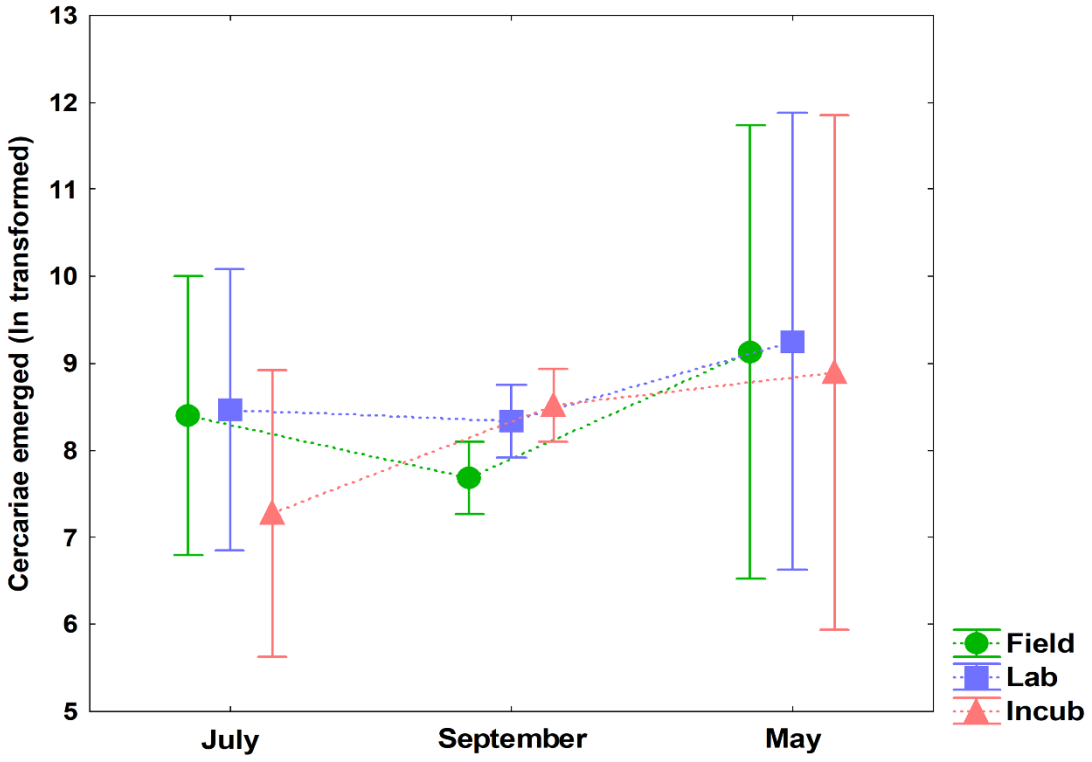

Figure 8. Seasonal variation in daily cercarial output rates (ln-transformed) of *Diplostomum 'mergi'* and *D. spathaceum* in relation to three experimental treatments with various conditions. Note that *D.* '*mergi*' corresponds to experiments in July and *D. spathaceum* in September and May. Factor snail length was entered as a covariate and snail identity as a random effect nested within season. Interactions (dashed lines) reflect significant differences in cercarial output rates between seasons. Symbols denote mean output of cercariae over three consecutive days and vertical bars 0.95 confidence interval.

Monitoring of cercarial emergence during the main four day-time intervals within 24 h showed large variation in cercarial output rates of *D.* '*mergi*' and *D. spathaceum* from individual snails, of which some maintained constantly higher production across experiments in each corresponding season. Also, that production patterns were circadian with clear single peak during the daylight throughout experimentation (Table 4, Figure 9). This is not surprising given it is the longest time interval. However, data recalculation/conversion to the shortest day-time period showed ultradian rhythms of both trematode species (Table 7, Figure 10). The natural light regime in each season is presented in Table 7.

Results of GLM-ANOVA models ('ii/A') used to assess periodicity in cercarial emergence in relation to each day-time interval (i.e., time emergence rates) indicated similar patterns between experiments within each season. In July, significant interaction between time interval and day, was detected in the field experiment, with emergence markedly higher in the first day of experiment (Field-July, $F_{6, 60} = 10.71$, $p < 0.001$), whereas only factor time was important under laboratory and incubator conditions (Laboratory-July, $F_{3, 83} = 26.07$, $p < 0.001$; Incubator-July, $F_{1, 23} = 4.99$, $p < 0.05$). In September, there was a pronounced effect of time interval on emergence rates in field and incubator experiments (Field-September, $F_{3, 47} = 11.13$, $p < 0.001$; Incubator-September; $F_{1, 23} = 62.63$, $p < 0.001$), and significant interactions between factors time and day, with lower emergence rates in the first day, for production in laboratory treatment (Laboratory-September, $F_{6, 47} = 2.86$, $p < 0.05$). In May, cercarial emergence in all experiments showed significant differences between time intervals and no effect of day of experiment (Field-May, $F_{3, 71} = 7.68$, $p < 0.001$; Laboratory-May, $F_{3, 47} = 3.32$, $p < 0.05$; Incubator-May; $F_{1, 17} = 12.02$, $p < 0.01$).

The series of pairwise comparison of the time emergence rates identified clear peaks during the sunrise and day for *D.* '*mergi*', depending on the experimental field and laboratory conditions (Figure 10A; Table 8; see also Table 7 for the mean of recalculated numbers). In contrast, more complex intraspecific variation in daily production patterns was revealed for *D. spathaceum*. In September, obvious peaks were observed during day and day and sunset (field and laboratory experiments, respectively; Table 8, Figure 10B). However, there was a lack of significant difference between day, sunset and night in both experimental treatments in May (Table 8, Figure 10C), despite the highest mean output rates during sunset (Field-May, 1,095 $snail^{-1}$ day^{-1} and Laboratory-May, 1,663 $snail^{-1}$ day^{-1}; Table 7). Models developed for incubator experiments confirmed generally diurnal output patterns/periodicity in emergence of both species of *Diplostomum* (Tables 7 and 8, Figure 10).

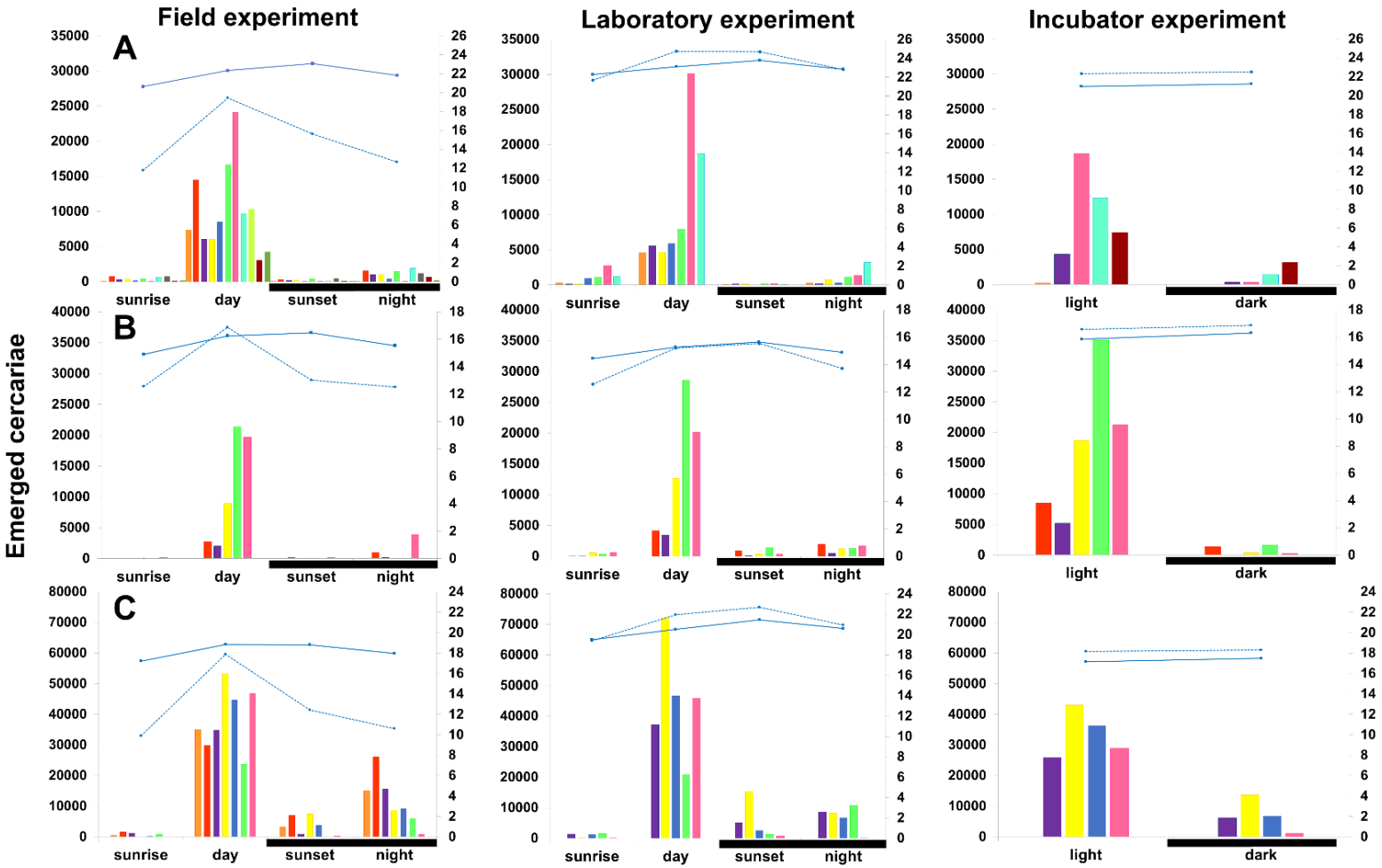

Figure 9. Emergence patterns and variability in cercarial output rates of *Diplostomum* spp. among individual snail replicates during the four day-time intervals in (A) July, **(B)** September and, **(C)** May. The observed number of emerged cercariae per snail, pooled across three consecutive days, is displayed for each experimental treatment under various conditions (field, laboratory, incubator) with plotted data for the mean air (dashed line) and water temperature (solid line; right y-axis). Black bars indicate the dark period between sunset and sunrise. Note the different y-axis.

Table 7. Daily cercarial output of *Diplostomum* '*mergi*' (July) and *D. spathaceum* (September and May) in experiments from naturally infected snails *Radix lagotis* during day-time intervals over 24 h (i.e., time emergence rates) under various experimental conditions. Emergence of cercariae during the two day-time intervals applies to the incubator experiments only. The total and mean number (in parentheses) of emerged cercariae is given for both, observed output (left part) and recalculated output to the shortest time interval (right part; indicated by "*").

Experiment-Season	**Day**	**Time interval/Observed emerged cercariae**				**Time interval/ Recalculated emerged cercariae**			
		Sunrise 4:30–5:15	Day 5:15–21:00	Sunset 21:00–21:45	Night 21:45–4:30	Sunrise 4:30–5:15*	Day 5:15–21:00	Sunset 21:00–21:45*	Night 21:45–4:30
Field-July	Day 1	1,910 (174)	36,644 (3,331)	1,860 (169)	6,880 (625)	1,910 (174)	1,745 (159)	1,860 (169)	764 (69)
	Day 2	1,294 (118)	38,336 (3,485)	116 (11)	1,543 (140)	1,294 (118)	1,826 (166)	116 (11)	171 (16)
	Day 3	559 (51)	35,640 (3,240)	105 (10)	1,191 (108)	559 (51)	1,697 (154)	105 (10)	132 (12)
	Total	**3,763 (114)**	**110,620 (3,352)**	**2,081 (63)**	**9,614 (291)**	**3,763 (114)**	**5,268 (160)**	**2,081 (63)**	**1,067 (32)**
Laboratory-July	Day 1	3,944 (493)	29,684 (3,711)	412 (52)	4,480 (560)	3,944 (493)	1,414 (177)	412 (52)	498 (62)
	Day 2	1,591 (199)	32,056 (4,007)	380 (48)	2,479 (310)	1,591 (199)	1,526 (191)	380 (48)	275 (34)
	Day 3	2,254 (282)	29,188 (3,649)	393 (49)	4,095 (512)	2,254 (282)	1,390 (174)	393 (49)	455 (57)
	Total	**7,789 (325)**	**90,928 (3,789)**	**1,185 (49)**	**11,054 (461)**	**7,789 (325)**	**4,330 (180)**	**1,185 (49)**	**1,228 (51)**
			Light 4:30–21:45		Dark 21:45–4:30*		Light 4:30–21:45		Dark 21:45–4:30*
Incubator-July	Day 1		14,916 (2,983)		2,304 (461)		5,837 (1,167)		2,304 (461)
	Day 2		17,496 (3,499)		1,775 (355)		6,846 (1,369)		1,775 (355)
	Day 3		10,488 (2,098)		1,250 (250)		4,104 (821)		1,250 (250)
	Total		**42,900 (2,860)**		**5,329 (355)**		**16,787 (1,119)**		**5,329 (355)**

Table 7. Continued

Experiment-Season	Day	Time interval/Observed emerged cercariae				Time interval/ Recalculated emerged cercariae			
		Sunrise 05:45–06:35	Day 06:35–19:15	Sunset 19:15–19:50	Night 19:50–05:45	Sunrise 05:45–06:35	Day 06:35–19:15	Sunset 19:15–19:50*	Night 19:50–05:45
Field-September	Day 1	179	13,542	156	3,820	126	624	156	225
		(36)	(2,708)	(31)	(764)	(25)	(125)	(31)	(45)
	Day 2	54	27,623	49	331	38	1,272	49	19
		(11)	(5,525)	(10)	(66)	(8)	(254)	(10)	(4)
	Day 3	50	13,717	428	948	35	632	428	56
		(10)	(2,743)	(86)	(190)	(7)	(126)	(86)	(11)
	Total	**283**	**54,882**	**633**	**5,099**	**199**	**2,528**	**633**	**300**
		(19)	**(3,659)**	**(42)**	**(340)**	**(13)**	**(168)**	**(42)**	**(20)**
Laboratory-September	Day 1	27	19,728	1,964	2,591	19	909	1,964	152
		(5)	(3,946)	(393)	(518)	(4)	(182)	(393)	(30)
	Day 2	374	21,868	743	1,413	262	1,007	743	83
		(75)	(4,374)	(149)	(283)	(52)	(201)	(149)	(17)
	Day 3	1,222	27,488	457	2,684	855	1,266	457	158
		(244)	(5,498)	(91)	(537)	(171)	(253)	(91)	(32)
	Total	**1,623**	**69,084**	**3,164**	**6,688**	**1,136**	**3,182**	**3,164**	**393**
		(108)	**(4,606)**	**(211)**	**(446)**	**(76)**	**(212)**	**(211)**	**(26)**
			Light 05:45–19:50		Dark 19:50–05:45		Light 05:45–19:50		Dark 19:50–05:45*
Incubator-September	Day 1		26,340		1,427		18,547		1,427
			(5,268)		(284)		(3,709)		(285)
	Day 2		33,120		745		23,321		745
			(6,624)		(149)		(4,664)		(149)
	Day 3		29,336		1,493		20,657		1,493
			(5,867)		(299)		(4,131)		(299)
	Total		**88,796**		**3,665**		**62,525**		**3,665**
			(5,920)		**(244)**		**(4,168)**		**(244)**

Table 7. Continued

Experiment-Season	Day	Time interval/Observed emerged cercariae				Time interval/ Recalculated emerged cercariae			
		Sunrise 04:20–05:05	Day 05:05–20:40	Sunset 20:40–21:25	Night 21:25–04:20	Sunrise 04:20–05:05*	Day 05:05–20:40	Sunset 20:40–21:25*	Night 21:25–04:20
Field-May	Day 1	2,852 (407)	45,684 (6,526)	4,252 (607)	37,754 (5,393)	2,852 (407)	2,199 (314)	4,252 (607)	4,094 (585)
	Day 2	598 (85)	133,010 (19,001)	12,952 (1,850)	21,896 (3,128)	598 (85)	6,402 (915)	12,952 (1,850)	2,374 (339)
	Day 3	930 (133)	89,615 (12,802)	5,799 (828)	21,983 (3,140)	930 (133)	4,313 (616)	5,799 (828)	2,384 (341)
	Total	**4,380 (209)**	**268,309 (12,777)**	**23,003 (1,095)**	**81,633 (3,887)**	**4,380 (209)**	**12,914 (615)**	**23,003 (1,095)**	**8,852 (422)**
Laboratory-May	Day 1	1,104 (221)	73,995 (14,799)	12,866 (2,573)	15,452 (3,090)	1,104 (221)	3,523 (705)	12,866 (2,573)	1,717 (343)
	Day 2	1,616 (323)	81,075 (16,215)	1,714 (343)	9,303 (1,861)	1,616 (323)	3,860 (772)	1,714 (343)	1,034 (207)
	Day 3	1,546 (309)	67,440 (13,488)	10,366 (2,073)	9,746 (1,949)	1,546 (309)	3,211 (642)	10,366 (2,073)	1,083 (217)
	Total	**4,266 (284)**	**222,510 (14,834)**	**24,946 (1,663)**	**34,501 (2,300)**	**4,266 (284)**	**10,594 (706)**	**24,946 (1,663)**	**3,834 (256)**
			Light 04:15–21:30		Dark 21:30–04:15		Light 04:15–21:30		Dark 21:30–04:15*
Incubator-May	Day 1		36,780 (9,195)		6,264 (1,566)		14,392 (3,598)		6,264 (1,566)
	Day 2		52,960 (13,240)		8,768 (2,192)		20,723 (5,181)		8,768 (2,192)
	Day 3		44,440 (11,110)		13,004 (3,251)		17,390 (4,347)		13,004 (3,251)
	Total		**134,180 (11,182)**		**28,036 (2,336)**		**52,505 (4,375)**		**28,036 (2,336)**

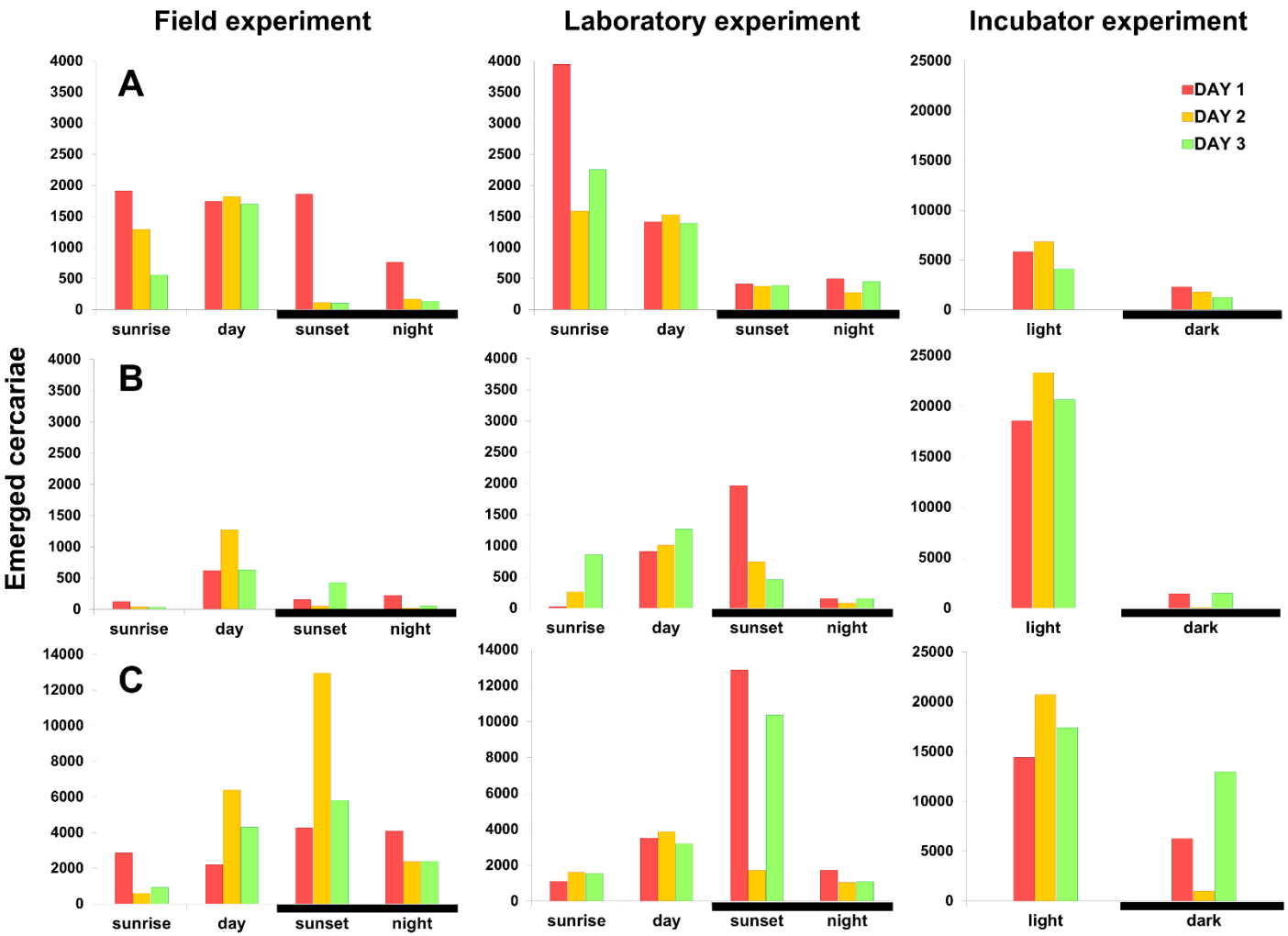

Figure 10. Periodicity in cercarial emergence during the four day-time intervals. (A) *Diplostomum* '*mergi*' in July, **(B)** *D. spathaceum* in September and **(C)** May. Daily output rates of cercariae, pooled across snail individuals, are recalculated to the shortest time interval within 24 h period and displayed for each experimental treatment with various conditions. Black bars indicate the dark period between sunset and sunrise. Note the different y-axis.

Table 8. Results from statistical pairwise comparison assessing the circadian periodicity in cercarial emergence of *Diplostomum* '*mergi*' (July) and *D. spathaceum* (September and May) from naturally infected snails *Radix lagotis*, using *post-hoc* Tukey's HSD tests in GLM models. Snail length was entered as a covariate. Emerged cercariae were recalculated to the shortest day-time interval within 24 h period and ln (x + 1)-transformed prior to the analysis. Statistically significant results are in bold, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; *ns*, not significant.

Experiment-Season	**Day-time intervals comparison**					
Field-July	Sunrise < Day **	Sunrise > Sunset **	Sunrise > Night **	Day > Sunset ***	Day > Night **	Sunset = Night *ns*
Laboratory-July	Sunrise = Day *ns*	Sunrise > Sunset ***	Sunrise > Night ***	Day > Sunset ***	Day > Night ***	Sunset = Night *ns*
Incubator-July	-	-	-	-	Day > Night *	-
Field-September	Sunrise < Day ***	Sunrise = Sunset *ns*	Sunrise = Night *ns*	Day > Sunset ***	Day > Night ***	Sunset = Night *ns*
Laboratory-September	Sunrise < Day ***	Sunrise < Sunset **	Sunrise = Night *ns*	Day = Sunset *ns*	Day > Night ***	Sunset > Night **
Incubator-September	-	-	-	-	Day > Night ***	-
Field-May	Sunrise < Day ***	Sunrise < Sunset **	Sunrise = Night *ns*	Day = Sunset *ns*	Day = Night *ns*	Sunset = Night *ns*
Laboratory-May	Sunrise < Day ***	Sunrise = Sunset *ns*	Sunrise = Night *ns*	Day = Sunset *ns*	Day = Night *ns*	Sunset = Night *ns*
Incubator-May	-	-	-	-	Day > Night **	-

Comparing the cercarial output of *D.* '*mergi*' and *D. parviventosum*, production was ultradian, but with contrasting diurnal and nocturnal patterns, respectively (Figure 11). While there was obvious preference for release of *D.* '*mergi*' during the sunrise and day (mean of 107 and 208 cercariae snail^{-1} day^{-1} in the field experiment; and 259 and 224 cercariae snail^{-1} day^{-1} in the laboratory experiment; Figure 11A), *D. parviventosum* favoured sunset and night (mean of 1,176 and 1,139 cercariae snail^{-1} day^{-1} in the field experiment; and 3,325 and 1,425 cercariae snail^{-1} day^{-1} in the laboratory experiment; Figure 11B). These results were confirmed following cercarial production of both species under controlled conditions in the incubator (Figures 11A, B). Although there was only one snail infected with *D.* '*mergi*', a similar trend was evident in comparison with results from above GLM-ANOVA models (ii/A) (see Figure 10A).

Results of GLM-ANOVA (model 'ii/B') used to assess between-seasonal differences in time emergence rates of cercariae showed a significant interaction between factors season, day and time interval in a model including field experiments, whereas cercarial emergence rates in laboratory conditions considerably differed between season and time interval only (Table 9, Figure 12). Maximum mean emergence rates were observed during day, sunset and night in May, but during sunrise in July, confirming interspecific variation in daily periodicity of cercarial emergence of *D. spathaceum* and *D.* '*mergi*' in general.

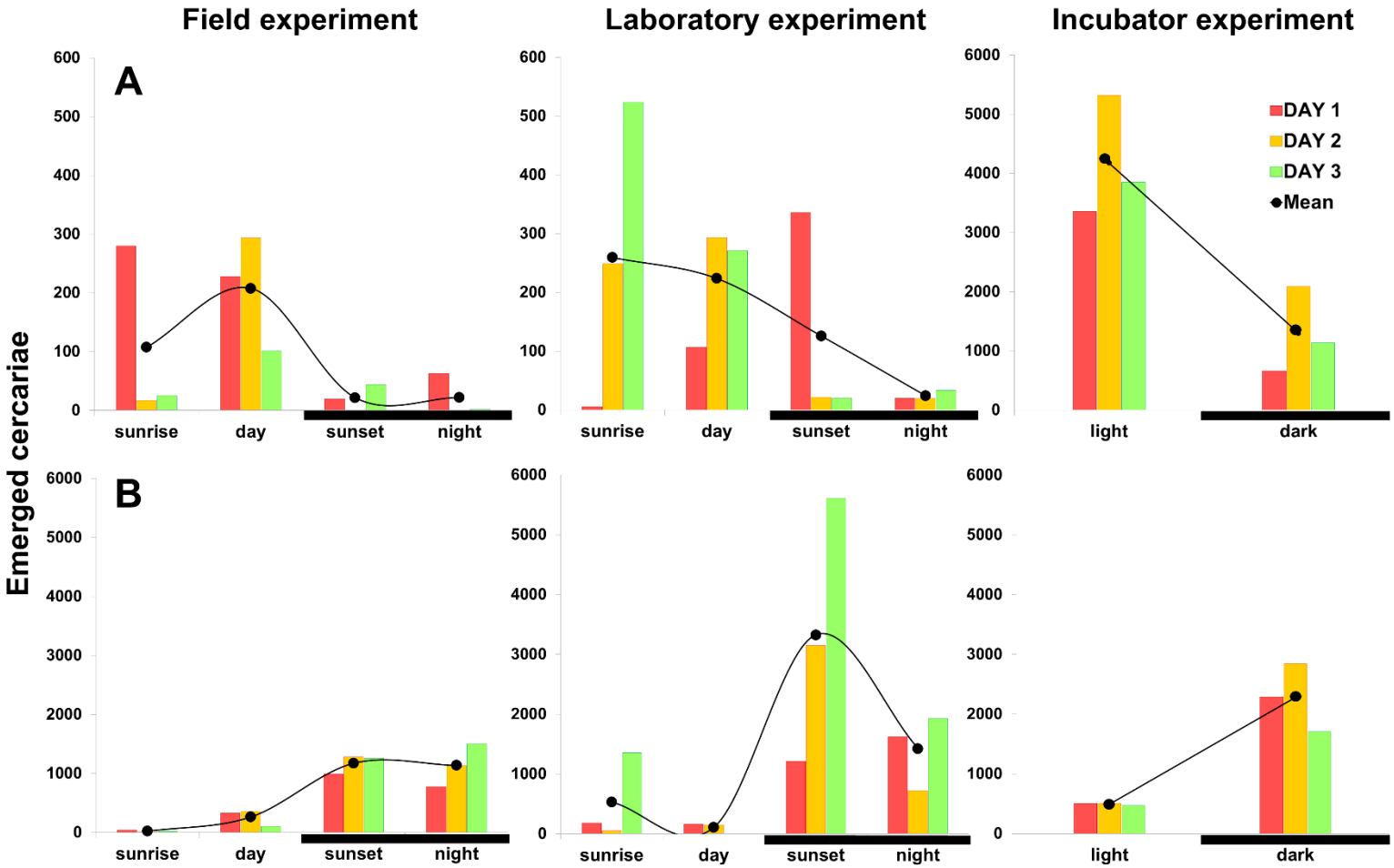

Figure 11. Comparison of periodic patterns in cercarial emergence during the four day-time intervals. (A) *Diplostomum* '*mergi*' and **(B)** *D. parviventosum*. Note that only one and two infected snails are included, respectively. Output rates of cercariae, pooled across snail individuals, are recalculated to the shortest time interval within 24 h period and displayed for each experimental treatment with various conditions. Black bars indicate the dark period between sunset and sunrise. Dots represent mean output of cercariae over three consecutive days. Note the different y-axis.

Table 9. Results of general linear model (GLM) evaluating the effect of season, day and day-time interval on the cercarial emergence of *Diplostomum* '*mergi*' (May) and *D. spathaceum* (September and May) from naturally infected snails *Radix lagotis*, with snail length as a covariate. Results are given separately for field experiments (left part) and laboratory experiments (right part). Snail identity was treated as random factor and nested within season. Recalculated numbers of emerged cercariae, were ln (x + 1)-transformed prior to the analysis. Statistically significant results are in bold. Abbreviations: df, decrees of freedom; MS, means of squares; F, test criterion value; P, level of significance.

	Field experiment					**Laboratory experiment**			
Factor/interaction	**Effect**	**df**	**MS**	**F**	**P value**	**df**	**MS**	**F**	**P value**
Snail identity	*Random*	*20*	*6.29*	*4.28*	***< 0.001***	*14*	*1.96*	*1.19*	*0.29*
Snail size/length	Fixed	1	1.96	1.33	0.25	1	101.64	51.35	**< 0.001**
Season	Fixed	2	71.75	17.21	**< 0.001**	2	14.45	7.17	**< 0.05**
Time interval	Fixed	3	58.82	39.33	**< 0.001**	3	23.35	13.98	**< 0.001**
Day	Fixed	2	7.14	4.85	**< 0.01**	2	0.84	0.51	0.60
Season*Time interval	Fixed	6	13.6	9.25	**< 0.001**	6	14.69	8.75	**< 0.001**
Season*Day	Fixed	4	12.23	8.32	**< 0.001**	4	3.04	1.84	0.12
Time interval*Day	Fixed	6	4.91	3.34	**< 0.01**	6	1.83	1.11	0.36
Season*Time interval*Day	Fixed	12	3.15	2.15	**< 0.05**	12	2.25	1.36	0.19
Error		219	1.47			165	1.65		

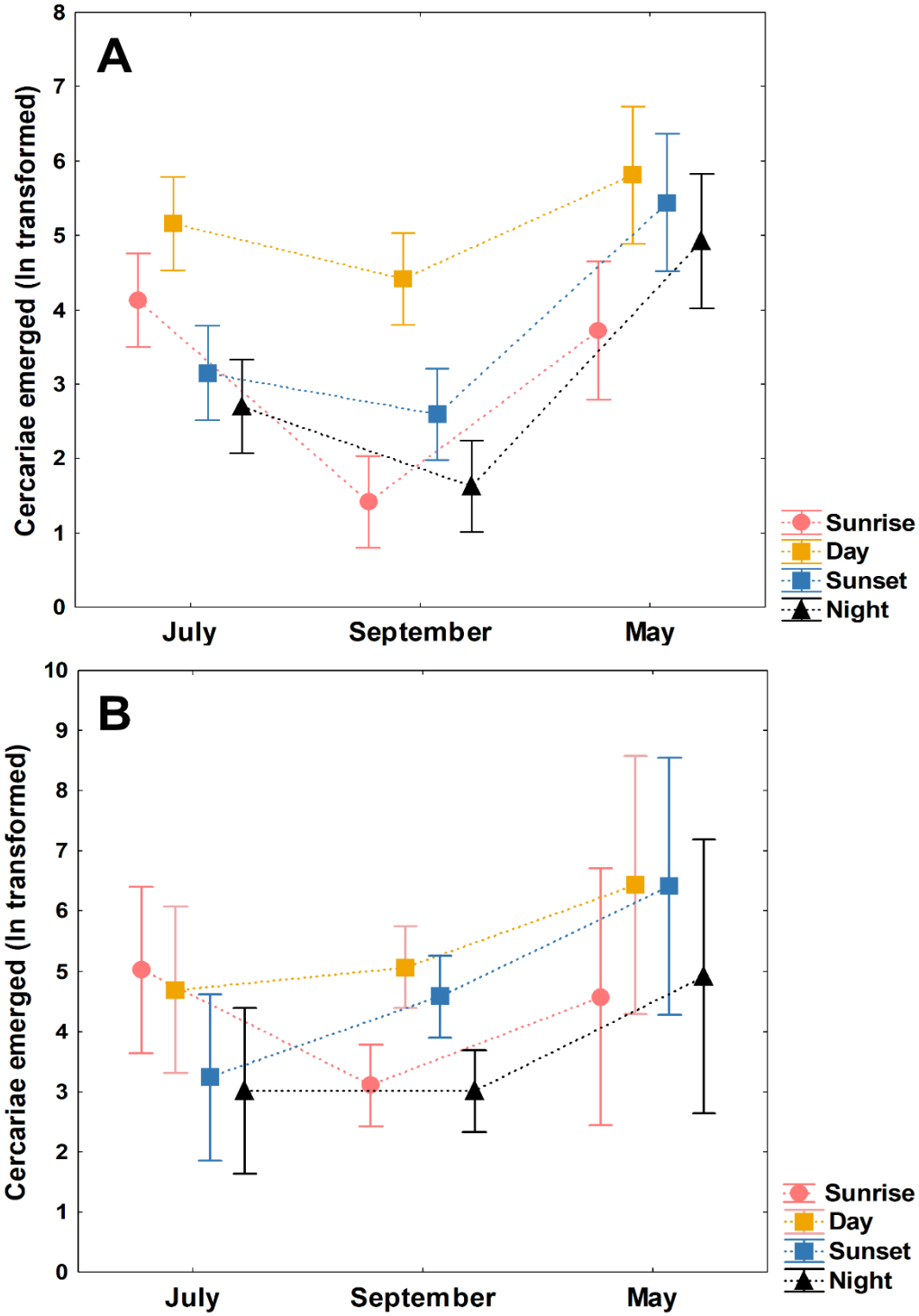

Figure 12. Seasonal variation in cercarial output rates (ln-transformed) of *Diplostomum* '*mergi*' and *D. spathaceum* in relation to the four day-time intervals and various experimental conditions. (A) Field experiments under natural climatic conditions, and **(B)** Laboratory experiments simulating natural climatic conditions. Note that *D.* '*mergi*' corresponds to experiments in July and *D. spathaceum* in September and May. Factor snail length was entered as a covariate and snail identity as a random effect nested within season. Interactions (dashed lines) reflect significant differences in cercarial output rates between seasons. Symbols denote mean of emerged cercariae over three consecutive days and vertical bars 0.95 confidence interval.

4. Discussion and Conclusions

This study presents the first detailed research on cercarial emergence patterns and daily output rates of three *Diplostomum* species, *D.* '*mergi*', *D. parvinevtosum* and *D. spathaceum* from the first intermediate lymnaeid snail *Radix lagotis*. Our study is unique in providing emergence data measured directly in the field conditions under natural photo- and thermoperiod. The only exception is Brassard et al. (1982), who investigated cercarial release of *D. spathaceum* from *Lymnaea arctica* by placing snails in the shallow pond zone. However, this concerned subarctic region in North America and no similar study has been conducted in freshwater temperate systems in Europe. Furthermore, we provide data on seasonality and the species-specific chronobiology patterns of cercarial emergence of *Diplostomum* species.

Our study also expands generally poor knowledge on the distribution of species of *Diplostomum* in lymnaeid snails of the genus *Radix* in Europe (Haarder et al. 2013, Selbach et al. 2015, Faltýnková et al. 2016) and the larvae trematode spectrum in *R. lagotis* so far limited to *Fasciola hepatica* Linnaeus, 1758, *Fascioloides magna* (Bassi, 1875) and *Trichobilharzia regenti* Horák, Kolářova & Dvořák, 1998 (Huňová et al. 2012, Skála et al. 2014). Trematodes of the family Diplostomidae are the most frequently recorded in lymnaeid snails in Europe (i.e., *Lymnaea stagnalis, Myxas glutinosa* and genera *Radix* and *Stagnicola* Jeffreys, 1830; Faltýnková et al. 2016), but infection records from *Radix* snails are ambiguous due to the controversial taxonomy and/or morphological misidentification of both snails (e.g., Bargues et al. 2001, Huňová et al. 2012., Lawton et al. 2015) and *Diplostomum* spp. (Georgieva et al. 2013, Horák et al. 2019). Virtually no data exist for the *R. lagotis-Diplostomum* spp. associations (Faltýnková et al. 2016). Beran (2013) found *R. auricularia* to be the only lymnaeid snail in the Most Lake, an indication of another erroneous identification based solely on shell morphology. The molecular analysis of our snail tissue samples revealed the compliance with *R. lagotis*, confirming its exclusive presence in Most Lake and occurrence in the Czech Republic (Huňová et al. 2012). The findings of *D.* '*mergi*' and *D. parviventosum* in Most Lake represent first records from the Czech Republic. However, since there is a high diversity of *Diplostomum* species in *R. auricularia* comprising six molecularly identified species/lineages (Selbach et al. 2015), the presence of other lineages within the *D.* '*mergi*' species complex in *R. lagotis* cannot be exclusively excluded.

The variation in seasonal occurrence of the three *Diplostomum* spp. in Most Lake with overall higher infection levels in summer corresponds to the temporal changes in prevalence of *Diplostomum* spp. in snail and fish hosts from temperate regions (Karvonen et al. 2006a,b,

Karvonen and Lindström 2018). The discrepancy for *D.* '*mergi*', showing apparent decreasing parasitism levels from July to September compared to the somewhat steady prevalence of *D. parviventosum* and *D. spathaceum* is associated with the abundance and distribution of bird definitive hosts. Most Lake is one of the most important wintering sites and migratory routes through Europe. Its varying seasonal occupancy by more than 50 species of waterfowls (Bažant 2015, 2017) deliver temporal load off by infectious stages into the ecosystem. In total, 13 (three families) and 10 species of birds (two families) may serve as definitive hosts for *D.* '*mergi*' and *D. parviventosum*, respectively. The generally low infection patterns of *D. parviventosum* may reflect reduced transmission opportunities due to the absence of anatids during warm months as well as the lack of grebes in the life cycle. In contrast, the combined effects of permanent presence of grebes, dominating gulls and life history traits of snails with two population turnovers during the one-year life span is likely responsible for the fluctuating seasonal infection levels of *D.* '*mergi*' (Islam et al. 2001, Yu and Wang 2013). Hence, the snail mortality reduces the parasitism as older and larger snails are replaced by new cohorts that have not yet become infected (e.g., Żbikowska et al. 2006, Brown et al. 2011). Contrary to recent findings on the highest prevalence of *Diplostomum* spp. in summer months (Karvonen et al. 2006a,b), the stable prevalence of *D. spathaceum* in Most Lake may be determined by the substantially wider range of definitive hosts (putative 21 waterfowl species of seven families). Congruent patterns of the lowest prevalence of all three *Diplostomum* species in spring could result from reduced survival of overwintering snails (Brassard 1982, Lyholt and Buchmann 1996, Karvonen et al. 2006b, Żbikowska et al. 2006). Nevertheless, above considerations shall be threated cautiously because some bird identities as hosts for *Diplostomum* spp. in the Palearctic region are uncertain and much of the published data may be based on erroneous identification. Overall, the heterogeneity in abundance of definitive hosts over time may have an important impact on the risk of parasitism and aggregation of infected individuals involved in the parasites' life cycle, taking into account the temporal selection pressure resulting from seasonal changes in cercarial emergence from snails.

Interestingly, despite the broad knowledge on the biology and ecology mostly related to pathology, physiology, behaviour and infectivity of *Diplostomum* spp., there are only few studies investigating daily output rates (production), rhythm patterns (periodicity) and seasonality in emergence of their cercariae (Karvonen et al. 2004a, 2006a, Lyholt and Buchmann 1996). All studies examined daily output rates of cercariae of *D. spathaceum* from *L. stagnalis*, either ranging between 7,279 and 37,418 cercariae snail^{-1} day^{-1} (Karvonen et al. 2004a), a mean of 11,966 (Karvonen et al. 2006a) or maximum production reaching 58,000

cercariae (Lyholt and Buchmann 1996). Furthermore, the mean daily cercarial emergence rate 4,471 of *D. gasterostei* Williams, 1966 was detected from the lymnaeid *M. glutinosa* (Karvonen et al. 2006a). In comparison to a former snail host, our results obtained by monitoring of cercarial release in three types of experiments over nine-day period showed mostly much lower production in dependence on the season; 3,962 *D.* '*mergi*' cercariae (July), 5,199 and 17,044 *D. spathaceum* cercariae snail^{-1} day^{-1} (September and May, respectively), whereas it was comparable with that from smaller snail *M. glutinosa* (except for output rates in May). In contrast, the maximum daily emergence of 44,263 cercariae snail^{-1} day^{-1} (*D. spathaceum* in May) almost reached that from *L. stagnalis* (58,000 – Lyholt and Buchmann 1996). This particular result indicates even higher production capacity from much smaller *R. lagotis*, as temperature usually triggers an increased cercarial emergence and the highest production appeared in May at 18°C, thereby 2°C lower than reported by Lyholt and Buchmann (1996). Apart from that, different size and number of experimental snails or temperature are probably responsible for differences in cercarial production (Lyholt and Buchmann 1996, Karvonen et al. 2004a, 2006a). However, it is difficult to draw a definite conclusion because of rather ambiguous data with respect to the methodology, results and identification of *D. spathaceum* (see the specificity of *D. pseudostahaceum* for *L. stagnalis* based on molecular evidence in Selbach et al. 2015).

Seven experimental snail individuals were infected with another trematode species (six with echinostomes and one with *Opisthioglyphe ranae*), but no significant differences were detected between snails with double and single infections. However, the overall cercarial emergence from doubly infected snails exhibited lower trend. Yet, the considerable suppression and resultant competitive elimination of the *Diplostomum* infection, as evidenced by a complete ceased emergence in a single snail individual with co-occurring patent echinostome infection in the July incubator experiment, leads to a conclusion that the interspecific competition takes place in our studied system (snail individual 1J). Collectively, this support previous findings on the echinostomes' dominance that are capable of active predation over subordinate sporocysts species (e.g., Lie 1967, Lim and Heyneman 1972, Kuris 1990, Kuris and Lafferty 1994), but see the capacity of *D. psedospathaceum* to prevent co-infections by rediae species due to priority of occupancy (Soldánová et al. 2012).

In our study, the comparative analysis revealed marked differences in total cercarial output rates (i.e., daily emergence rates) between seasons, but no changes among three experimental treatments. The cercarial emergence of *D. spathaceum* under controlled conditions is positively light- and temperature dependent (Lyholt and Buchman 1996,

Karvonen et al. 2004a), a common pattern leading to a substantial increase in cercarial production observed for other snail-trematode systems (e.g., Kaewkes et al. 2012, Born-Torrijos et al. 2014, Prokofiev et al. 2015). Despite a relatively high variation in temperature and illuminance/light intensity parameters, similar daily emergence rates among the field, laboratory and incubator experiments within each season demonstrate a successful simulation of natural light and temperature conditions, suggesting no substantial effect on the overall cercarial output. However, there was a considerably lower emergence in the July incubator experiment, but the mean water temperature was 1–2°C lower and the light intensity slightly higher by 141–306 lux than in preceding experiments, which proves that the effects of these two important abiotic factors was weak and can be eliminated. Although experiments differed in number of snails replicates potentially creating bias (11, 8 and 5 in field, laboratory and incubator, respectively), the impact of interspecific competition appears the most reasonable explanation given the link with the general tendency towards reduced cercarial emergence rates in four doubly infected snails out of five surviving in the incubator.

Seasonal variation in cercarial emergence of *D. spathaceum* from *L. arctica*, showing peaks in early August and to lesser extent in mid-September, have been attributed to temperature, overwintering parasite infections and snail population structure comprising two generations (Brassard 1982). Although some of these mechanisms may be involved in seasonal fluctuation in cercarial emergence in our study, data are virtually not comparable with Brassard's subarctic system due to parasites' adaptive responses to an extreme climate with prevailing low temperatures, long winter period and short transmission window (Bøhn and Amundsen 2004). In our study, the between-seasonal comparison revealed a rapid increase in cercarial emergence with maximum daily output rates in spring compared to congruent production between summer and autumn, which demonstrates both interspecific (between *D.* '*mergi*' in July and *D. spathaceum* in September and May) and intraspecific variation (between two populations of *D. spathaceum* in September and May). There are several possible scenarios explaining such conspicuous three times higher production of cercariae in May, namely the effect of 1) temperature and light intensity, 2) snail size, 3) infection dynamics in overwintering snails, 4) cyclic development of sporocysts' microhemipopulations within snails, and 5) synchronisation of cercarial emergence with fish second intermediate hosts.

(1) It is plausible that the marked difference between 22°C and 16°C water in summer and autumn, respectively, may be responsible for the lacking interspecific variation in daily emergence production, as the cercarial emergence of *Diplostomum* decreases with decreasing temperature (ca six-fold difference in a 10°C decline; Lyholt and Buchmann 1996). Because of

the opposite trend with output rates peaking in spring at 18°C, the role of water temperature in intraspecific differences is unlikely. Despite the highest mean water illuminance in spring, we consider a small effect of the light intensity as there was a high fluctuation among types of experiments ranging from 647–7,649 lux, but the production was generally congruent. This is supported by a similar varying pattern of light intensity among experiments in summer and autumn. (2) The increased emergence of cercariae could be affected by the snail size as larger snails provide more space and energetic resources for asexual multiplication and cercarial production (e.g., Graham 2003, Poulin 2006, Morley et al. 2010). In our case, significantly larger snails were collected in spring and there was also a positive relationship between snail length and the cercarial production. It is worth nothing that this multiple increase was not associated with snail size because its effect was accounted for as a precaution measure.

(3) The accumulation of trematode infections in snail hosts during winter months when cercarial release is ceased, but the asexual reproduction continues, can be an additional mechanism leading to the sudden burst in cercarial emergence in spring after water temperature exceeds a certain threshold (Galaktionov and Dobrovolskij 2003). Larvae of *Diplostomum* are capable to overwinter in snails (Brassard 1982, Żbikowska et al. 2006, Karvonen et al. 2006b, Soldánová et al. 2011). However, the development of sporocysts infrapopulations is not arrested, giving the advantage over other trematode species to sustain relatively high infection levels and effective transmission to next hosts (Soldánová et al. 2011). The water temperature of Vlkovský fishpond ranges between 10°C to 18°C from early to late April (pres. observation). In the context of field experiments reflecting real nature of host-parasite interaction, together with still relatively high cercarial emergence of *Diplostomum* at 10°C (maximum 10,000 per snail and day – Lyholt and Buchmann 1996), a significant progressive proliferation in cercarial release would appear likely even earlier than in May. (4) The cyclic development of sporocysts' microhemipopulations within snails may be responsible for the temporal fluctuations in cercarial emergence (Galaktionov and Dobrovolskij 2003). The process of periodical decrease and increase in the number of released cercariae is related to age composition of daughter sporocysts generations when the degenerating ones alternate with the sporocyst-producing cercariae (at this time, there is a maximum cercarial emergence from the snail). While this has been well documented for human schistosomes (Théron 1981) and suggested as one of the possible factors for the nine-fold increase in cercarial production rates of the bird schistosome *Trichobilharzia szidati* Neuhaus, 1952 (Soldánová et al. 2016), no such data are available for species of *Diplostomum*, except for a prepatent period of 4–10 weeks (Chapell 1994) or 50–60 days (Erasmus 1958). The period between our July and September experiments was 51 days

and it cannot therefore be ruled out that the peak could have been simply missed. Nonetheless, we rather do not speculate over the possible effect of oscillating development of sporocysts on the exceptionally high emergence rates in May due to obvious interplay of complex mechanisms between the host and parasite during winter.

(5) Finally, according to our opinion, the most realistic explanation appears to be the synchronisation of cercarial emergence of *D. spathaceum* with the activity and behaviour of its second intermediate fish hosts, making the emergence rhythms adaptive to enhance contact of short-lived cercariae with target hosts in space and time (Combes et al. 1994). It is plausible that high production in spring is in particular regulated by fish reproduction period due to their high abundance near-shore littoral zone (where infected snails also occur), thus creating ideal opportunities for facilitated transmission. This is in accordance with Soldánová et al. (2016), stating that maximal chances of cercariae of *T. szidati* for meeting the definitive bird host applies not only to a specific time of the day, but also during the reproduction period of ducks, because conditions are most favourable. With few exceptions, all eight fish species occurring in Most Lake serve as second intermediate hosts for *D.* '*mergi*', *D. parviventosum* and *D. spathaceum*, but roach, perch and rudd are the most common and dominant in terms of abundance and biomass (Peterka et al. 2013, Peterka 2018). The reproduction takes place once a year in spring, usually in May when these fish aggregate in shallow waters (Baruš and Oliva 1995, Kottelat and Freyhof 2007), thereby being more exposed to trematode infections. This is supported by observations on notably higher numbers of metacercariae acquired by fish in shallow habitats compared to deeper waters (Brassard 1982), proving that transmission of *Diplostomum* species is a function of spatial and temporal distribution as well as abundance of both cercariae and fish hosts. On the other hand, fish are able to detect the presence of parasite (Poulin et al. 1999) and exhibit avoidance behaviour in preventing infections in areas where high numbers of invasive cercariae of *Diplostomum* occur (Karvonen et al. 2004b). However, in our system, the effectiveness of avoidance performance in spring is less likely given the individual variability in fish response (Karvonen et al. 2004b), increased fish physiological susceptibility to trematode infections during spawning due to higher levels of sex steroids that lead to immunosuppression (Saha et al. 2002), rapid attachment of cercariae to fish skin (Haas et al. 2002), and massive cercarial production from *R. lagotis* snails. To support this, the roach, perch and rudd are much more active swimmers during their reproduction period (Baruš and Oliva 1995, Kottelat and Freyhof 2007, Vašek et al. 2009) and snail foraging activity is enhanced in the presence of fish as a result of *Diplostomum*-induced changes in snail behaviour (Voutilainen 2010). Furthermore, it has been documented that snails harbouring *Diplostomum*-

patent infections occur near the pond shoreline in April and May as a result of behavioural anapyrexia of the host which deliberately chooses microhabitats with lower temperature to prolong host survival and increase transmission success (Żbikowska 2011). All these aspects of the snail and fish life-history traits are particularly beneficial for passively-transmitted cercariae of *Diplostomum* which lack specific host-finding behaviour and respond to non-specific cues such as water current stimulating their attachment (Haas et al. 2002). Frequent water disruption emanating from fish movements along with high accumulation of hosts and infective stages thus create a short-term local hot-spots for highly effective host-parasite encounters.

This study also showed a large variability in cercarial emergence among snail individuals, and most importantly, contrasting inter- and intraspecific patterns in the periodicity of daily cercarial emergence from naturally infected snails *R. lagotis*. The history of infection may account for this disparate cercarial outputs, as the intensity of infection and consequently numbers of released cercariae strongly depend on the miracidial dose (Massoud 1974, Sluiters et al. 1980, Gustafson and Bolek 2015). This is evidenced by several highly-productive snails that have maintained great emergence rates throughout experiments in all seasons. Overall, the emergence of *Diplostomum* spp. was either circadian (one peak during 24 h) or ultradian (two or more peaks during 24 h), but with distinct species-specific daily emergence patterns, all depending on the type of experiment and season. Both *D.* '*mergi*' and *D. spathaceum* exhibited markedly diurnal patterns, whereas *D. parviventosum* exclusively nocturnal emergence. Despite using only two snails infected with *D. parviventosum*, the similar emergence pattern observed in two different seasons provide evidence for actual process in nature. Although the diurnal cercarial production of *D. spathaceum* matches with the finding of Karvonen et al. (2004a), these observations were surprising given our assumption of similar, if not identical, patterns of cercarial emergence due to traits that should be uniform at the genus level (morphotype, behaviour and transmission mode in which species of *Diplostomum* utilize similar host taxa). This expectation was in line with a conclusion by Prokofiev et al. (2015) that cercariae of conspecific trematodes, with the same type of host-searching behaviour and biology of hosts, have similar emergence rhythms. Yet, they also noticed differential emergence patterns between cercariae of closely related species due to the behavioural features of next hosts.

We observed increased "time cercarial emergence rates" during specific day-time periods (i.e., sunrise, day, sunset and night) for *D.* '*mergi*' peaking at sunrise and day, *D. spathaceum* during day and sunset, and *D. parviventosum* during sunset and night, but there

were there obvious peaks for *D. spathaceum* in May experiments. Karvonen et al. (2004a) detected significantly lower cercarial release during the night (counted every two hours) in July, but no differences in August, despite the rapid increase during last hour of darkness. However, the authors do not provide any further explanation for such emergence variation during the day and focus only on diurnal and nocturnal patterns.

While the interspecific variation may be associated with the feeding activity of fish, the distinct intraspecific differences observed between autumn and spring are likely to be again determined by their reproduction. In addition to elevated aggregation and activity, fish spawning continues throughout the day (Thorpe 1977, Wedekind 1996), explaining the prolonged duration of cercarial emergence until night hours, especially under extended 17:7 h photocycle in spring. Our data are congruent with one of the hypotheses suggested by Shostak and Esch (1990) that the periodicity in cercarial release increases chances to encounter a host, and this applies not only to mean daily emergence rates, but also cercarial emergence rhythms of the three species of *Diplostomum*. The three most dominant fish in Most Lake have a characteristic daily feeding activity, roach feeding after sunset, perch during sunrise and sunset and rudd preferring daylight (Baruš and Oliva 1995, Kottelat and Freyhof 2007). Hence, the species-specific daily rhythms of cercariae represent an adaptive evolutionary mechanism to maximise transmission success while avoiding competition for host resources. The fact that the infective potential of cercariae of *Diplostomum* is the highest within the first six hours after leaving the snail, and then decreases rapidly (Karvonen et al. 2003), supports the idea that parasites have diverged in their emergence strategies. The temporal overlap with patterns of periodic cercarial emergence would have important implications for the intensity of infection with metacercariae of *Diplostomum* in fish. For example, the nocturnal emergence of *D. parviventosum* would make the likelihood for infection highly probable in nocturnally-feeding roach, whereas considerably lower levels should be seen in other fish hosts. The significant lack of second peaks of emerged cercariae in the field compared to laboratory experiments in July and September may result from water transparency and associated light diffusion. Field experiments were performed in the eutrophic fishpond possessing turbid water, thereby reducing the light intensity. In contrast, in laboratory experiments snails produced cercariae in transparent beakers placed into an aquarium, thus facilitating the light access. Hence, we anticipate the presence of ultradian rhythms with two peaks in the nutrient-poor oligotrophic and far less turbid Most Lake (Jůza et al. 2019). This result highlights the importance of the abiotic habitat characteristics when performing experiments in the field, as brownification and

eutrophication of water bodies can play an important role in the periodicity of cercarial emergence.

In conclusion, the novel data on the production and periodicity in emergence of cercariae of three species of *Diplostomum* from naturally infected first intermediate snail host *R. lagotis* in a temperate freshwater ecosystem indicate the adaptive nature of cercariae leading to enhanced transmission to the second intermediate fish hosts. We found that a complex array of mechanisms can affect *Diplostomum* species-specific patterns in cercarial emergence, but their mutual interaction cannot be excluded. We conclude that the most significant regulation factor comprises behavioural characteristics of fish related to reproduction and feeding processes. Furthermore, our study expands current knowledge on the distribution of taxonomically problematic genus of *Diplostomum* and the lymnaeid *Radix lagotis* in Europe, as well as trematode spectrum in this snail host. The present study contributes to a better understanding of ecological and epidemiological aspects with respect to specific adaptive strategies compartmentalised among species of *Diplostomum* and consequences for infection risk in fish hosts. However, further experimental research is required to validate our findings. The outcomes described above are particularly important given the little attention paid to the seasonal variation, including both the prevalence of infection and emergence, rate of production and periodic release of cercariae of *Diplostomum*. It would be interesting to study temporal and spatial strategies in cercarial emergence resulting from parasite-induced manipulation of snail host behaviour, which can affect the local parasite abundance and transmission. This, in turn, may have significant consequences cascading throughout the ecosystem, as *Diplostomum* is able to regulate host populations via castration, mortality, predation risk or thermal preferences for certain habitats (e.g., Voutilainen 2010, Żbikowska 2011) and thus energy flow in food webs.

5. References

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