Biological Journal of the Linnean Society, 2020, **129**, 334–346. With 4 figures.

Limited impacts of chronic eye fluke infection on the reproductive success of a fish host

VERONIKA NEZHYBOVÁ[1,2], MARTIN REICHARD[1,*], CAROLINE METHLING[1] and MARKÉTA ONDRAČKOVÁ[1*]

[1]*Institute of Vertebrate Biology, Czech Academy of Sciences, Květná 8, 603 65 Brno, Czech Republic*
[2]*Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic*

Received 19 September 2019; revised 22 November 2019; accepted for publication 25 November 2019

Parasitic infections may affect the reproductive success of the host either directly, through behavioural modification, or indirectly, by altering their reproductive investment in response to infection. We determined the effects of infection with the eye fluke *Diplostomum pseudospathaceum* (Trematoda) on the reproductive traits of European bitterling (*Rhodeus amarus*, Cyprinidae), an intermediate fish host with a resource-based mating system. Male bitterling infected by *Diplostomum* exhibited a larger but less pronounced red eye spot (sexually selected signal) than control males, suggesting that infected males were less preferred by females. The frequency of female ovulation and number of offspring were comparable between the infected and the control group, although there was a 1–2 week delay in the peak of ovulation and offspring production in infected fish, which is known to coincide with higher juvenile mortality. Chronic eye fluke infection had minimal metabolic costs (measured as oxygen consumption) and, consistent with these results, reproductive activity did not differ between infected and control fish in an experimental test of intersexual selection. Overall, the impact of eye fluke infection on the reproduction of European bitterling was limited. We consider the potential effect of favourable conditions during experiments (abundant food, access to spawning substrate and lack of predators and co-infections) on experimental outcomes and recognize that the effects of chronic eye fluke infection in natural conditions might be more pronounced.

ADDITIONAL KEYWORDS: *Diplostomum* – European bitterling – nuptial coloration – parasite impact – reproduction – respirometry.

INTRODUCTION

Parasite infections commonly compromise host reproductive success (Poulin, 2007). Reproduction is an energetically demanding process that requires investment not only into gonadal tissue and gametes but also into secondary sexual traits, territorial defence and parental care (Read, 1990; Wootton & Smith, 2015). Direct negative effects of parasites are often associated with compromised gonadogenesis and fecundity (e.g. Heins & Baker, 2003; Carter *et al.*, 2005). The energetic demands that parasites impose on their host can delay gametogenesis (Heins & Brown-Peterson, 2010) or inhibit gonadal development through disruption of endocrine system that governs reproductive functions (Arme, 1997; Geraudie *et al.*, 2010) or reduce egg size and quality (Heins & Baker, 2003).

Indirect effects of parasites on host reproduction include decreased expression of secondary sexual characteristics and reduction in courtship vigour (MacNab *et al.*, 2009). The Hamilton–Zuk hypothesis (Hamilton & Zuk, 1982) directly links parasite infections with sexual selection theory. Here, the prediction is that parasitized individuals (and males in particular) cannot develop costly sexual ornaments, implying that they represent phenotypic expression of 'good' genes. By choosing partners with costly sexual ornaments, females provide their offspring with superior immunity (Milinski & Bakker, 1990). In addition, in the case that parasites are transmitted directly, females can avoid males with high parasite loads and thereby minimize infection risk (Clayton, 1991; Houde & Torio, 1992).

*Corresponding author. E-mail: audrey@sci.muni.cz

It has been shown that parasite and host immune systems play an important role in individual-level trade-offs between reproduction and survival (Sheldon & Verhulst, 1996), manifested as plasticity in the reproductive investment of hosts (Agnew *et al.*, 2000). Acute non-persistent infections are expected to lead to downregulation of current reproduction to increase the chance of recovery, survival and future reproduction, whereas hosts with chronic persistent infections are predicted to increase current reproductive efforts because future reproductive opportunities might be limited (Schwanz, 2008).

Here, we investigate the mechanisms by which a chronic parasite infection may affect host reproductive success by testing multiple phenotypic (morphological and behavioural) traits related to reproduction. Our study system includes a widespread eye fluke parasite *Diplostomum pseudospathaceum* Niewiadomska, 1984 (Trematoda, Diplostomidae) and its common second intermediate host, the European bitterling, *Rhodeus amarus* (Bloch, 1782) (Dávidová *et al.* 2008). Natural infection intensity ranges from one to 28, with prevalence reaching up to 93% in particular populations (Dávidová *et al.* 2008). *Diplostomum* trematodes are common parasites of a wide range of freshwater fishes, with potential for chronic infections (living ≤ 4 years; Shigin, 1964) and compromising host survival (Michálková & Ondračková, 2014). Metacercariae (larval stage infecting fish eye lenses) of *D. pseudospathaceum* may cause serious pathologies (Shariff *et al.*, 1980) and considerable behavioural changes by reducing anti-predator behaviour, leading to an increased transmission to their definitive host (piscivorous birds). Accumulation of metacercarie in the eye lens of the host may compromise vision and impair the feeding efficiency of the fish (Owen *et al.*, 1993; Voutilainen *et al.*, 2008), leading to retarded growth (Voutilainen *et al.*, 2008) and increased susceptibility to predation (Seppälä *et al.*, 2004). However, its impact on fish host reproduction is not known.

The European bitterling (*R. amarus*) is a small, short-lived cyprinid fish with a resource-based mating system. During the breeding season, males develop red carotenoid-based coloration and defend territories around live unionid mussels that females use for oviposition. Males actively court females and lead them to mussels in their territories. Both intersexual (female choice) and intrasexual (male–male competition for resources and sperm competition) components of sexual selection are important for male reproductive success (Candolin & Reynolds, 2001; Reichard *et al.*, 2005). Females typically inspect several males and the quality of their oviposition sites before making a decision to spawn (Smith *et al.*, 2002). Spawning includes oviposition of one to six eggs into the mussel gill cavity, and male release sperm over the inhalant siphon of the mussel. The embryos develop inside the mussel and emerge after 4–5 weeks (Smith *et al.*, 2004). Females spawn in several seasonal bouts lasting 1–2 days and consisting of several clutches (Smith *et al.*, 2004), with a seasonal fecundity of 50–250 eggs. The extent of red pigment in the eye of male bitterling appears to play a particular role in female mate choice (Candolin & Reynolds, 2001; Reichard *et al.*, 2005) and appears to function as a signal of male quality as a mate (Reichard *et al.* 2008; Smith *et al.*, 2014).

In this study, we experimentally separated possible costs of infection by *D. pseudospathaceum* on bitterling reproduction. We investigated the energy allocation to gonadal development and somatic condition before and after the spawning period and quantified the energetic costs of chronic infection with an eye cataract by measuring host oxygen consumption during the breeding season. We also studied the effects of eye flukes on male and female reproductive behaviour related to sexual selection. Parasites are known to inhibit the expression of carotenoid pigmentation, possessing important immunological functions (Milinski & Baker, 1990; Houde & Torio, 1992). We hypothesized that *D. pseudospathaceum* infection would compromise the ability of males to develop a carotenoid-based red spot in the eye that serves as a signal of male quality during female mate choice (Reichard *et al.*, 2005).

Chronic infection with *Diplostomum* metacercariae is known to increase the metabolic rate in Arctic charr, *Salvelinus alpinus* L., 1758 (Voutilainen *et al.*, 2008). Consequently, we predicted increased energetic costs in infected bitterling, especially during the reproductive period when somatic resources are directed to the gonads. Although chronic infections may lead to fecundity compensation via increased current reproductive effort (Forbes, 1993), energy reduction as a direct cost of parasite infection results in reduced current reproduction (Schwanz, 2008). Here, two possible scenarios are predicted. First, increased energetic requirements lead to decreased reproductive investment, activity and output of infected fish. Second, little or no effect of the parasite on the host metabolism leads to increased reproductive effort. Finally, we predicted a compromise in the capacity of females to choose a high-quality mate owing to impairment of their vision.

MATERIAL AND METHODS

Collection and maintenance of experimental animals

All fish used in the experiments were captive-reared offspring of adult *R. amarus* collected in the River Kyjovka (48°46′45″N, 17°01′00″E) in the south-eastern

Czech Republic (Danube basin). Wild-collected adult fish were housed in outdoor fibreglass tubs (1.4 m × 1.4 m × 0.9 m) located in the garden of the Institute of Vertebrate Biology (IVB), Brno, Czech Republic and allowed to spawn into host mussels (*Unio tumidus* L., 1758) positioned in sand-filled flowerpots. The tubs were equipped with artificial vegetation and a gravel substrate. Parental fish foraged on algae that established in the tubs and were additionally fed daily with frozen chironomid larvae and occasionally with flake food (Nutron Tropical fish food). Juvenile bitterling that emerged from mussels were captured immediately after departing the host mussels and transferred to separate tubs. In November, juvenile fish were transferred for overwintering in semi-natural conditions to a large outdoor pool (12.4 m × 6.0 m × 1.5 m). Fish were placed into cages (cylinder shaped, 0.50 m deep, 0.45 m diameter, 1 mm mesh) with a maximal density of 20 fish and housed in the pool until the beginning of April. The pool was equipped with a layer of sediment, and fish were able to forage naturally on algae, detritus and invertebrates. In spring (beginning of April), fish were transferred back to the tubs.

Experimental mussels were collected from Týnecké oxbow lake (*U. tumidus*) and the River Kyjovka (*Anodonta anatina* L., 1758) before the start of the bitterling reproductive season, ensuring the absence of bitterling eggs in their gills. Mussels were placed in outdoor tubs, where they were able to feed on sediment and algae. After completion of the experiments and after all the experimental bitterling had departed from their gills, mussels were released at their original locations.

The gastropod mollusc *Lymnaea stagnalis* L., 1758, the first intermediate host of *D. pseudospathaceum*, originated from Vlkovský Pond (49°08′56″N, 14°43′51″E) and Bohdaneč Pond (50°05′17″N, 15°40′24″E) in the River Elbe basin, Czech Republic. Determination of infection state and identification of parasite species were completed in the Laboratory of Helminthology of the Institute of Parasitology of the ASCR in České Budějovice. Specimens of *L. stagnalis* were placed individually in transparent plastic containers 50 mm in diameter containing aged tap water. Shedding of cercariae was stimulated by exposure to daylight for 5 h. Released cercariae were observed under a light microscope and identified to species using morphological (Faltýnková *et al.*, 2007) and molecular methods (Georgieva *et al.*, 2013).

Infection procedure and fish dissection

Before infection, snails were placed in 1 L beakers with aged tap water to produce cercariae at room temperature (22–25 °C). After 2 h, the cercarial density was estimated under a binocular microscope by subsampling the cercarial suspension. Experimental fish were randomly divided into two groups (control and infected), individually placed in a container with 150 mL of water and exposed either to the required number of cercariae of *D. pseudospathaceum* (treatment group) or to aged tap water (control group). The handling procedure was identical for both groups. The cercariae used for infection were not older than 6 h to mitigate decreased infectivity with time (Karvonen *et al.*, 2003). Experimental infections were carried out in August, using juveniles released in June and July. Experiments were conducted during the year after infection, thus, fish were infected for several months. Fish used in Experiment 1 were infected with 200 cercariae per fish for 1.5 h, and 2 weeks later re-infected (120 cercariae for 2 h) owing to the low intensity of infection. Fish used in Experiment 2 were exposed to 300 cercariae per fish for 1 h, and fish used in Experiment 3 were infected with 180 cercariae for 1 h, with later re-infection with 240 cercariae for 2 h. The infection dose in particular treatments reflected the density of cercariae released and the size of individual fish in different experiments to ensure successful infection (Michálková & Ondračková, 2014). Note that all individual fish from the same experiment were exposed to the same number of cercariae.

At the end of the experiments, infection by *D. pseudospathaceum* in the eye lenses was quantified. Fish were anaesthetized with clove oil and killed by severing the spinal cord at the base of the skull. Fish were examined under a binocular microscope for the presence of all metazoan parasites using a standard protocol (Ergens & Lom, 1970). Except for one subordinate male (Experiment 1), all treated (infected) fish in all experiments were successfully parasitized by *Diplostomum* metacercariae, and no other parasites were found. All control fish were free from parasites. Dissection of fish from Experiment 1 was performed ~2 months after the end of the experiment. During this 2 month period, three infected males and one female and two control males died, resulting in dissection of 76 infected and 77 control fish. In Experiment 2, five infected females, four infected males, three control females and one control male did not survive until the end of the experiment and were excluded from further analyses.

Experimental design

Experiment 1: bitterling reproductive traits

Before the onset of the spawning season (April 2013), 160 experimental (1-year-old) fish were divided into 40 groups (20 infected and 20 control fish). Each replicate contained a dominant (large) and subordinate (smaller) male and two females. The males were

uniquely marked with a coloured visible implant elastomer (VIE; Northwest Marine Technology) close to the dorsal fin base. Fish were housed in tubs in the garden of the IVB. Each tub was divided into four identical sectors with a 1 mm mesh barrier. A single *U. tumidus* and single *A. anatina* mussel were added as a spawning substrate for each group of fish. Both mussel species are used by *R. amarus* for spawning and are abundant in the population from which the fish originated. Three variables were measured in control and infected bitterling from each experimental group: (1) the size and colour intensity of the red eye spot of the dominant male; (2) the number of females in spawning condition; and (3) the number of offspring released from mussels.

Eye spot coloration in dominant males: All males were photographed every second week over the entire spawning season (end of April to start of August) to determine the size and colour intensity of the red eye spot. Red nuptial coloration on the body and fins was not measured because, unlike eye pigmentation, it is under neuronal control and changes rapidly when a fish is captured. Eye spot coloration was quantified following the methods of Barber *et al.* (2000), Smith *et al.* (2002) and Reichard *et al.* (2005). Each male was gently captured and immediately released into a small photo-aquarium. No anaesthetics were used to prevent any alteration of colour pattern and intensity in response to anaesthesia. Each male was gently held in position against the front of the aquarium using a soft sponge and photographed using a Canon EOS Digital Rebel XTi camera (Canon Inc., Tokyo, Japan) sitting on a fixed tripod to ensure a constant distance from each fish. A ruler and colour standard were included in the frame. Light conditions were standardized by siting the photo-aquarium in a dark case and using flash illumination during image capture.

The number of females in spawning condition: Between mid-April and the beginning of August, the number of females with an extended ovipositor, which is an unambiguous indication of active reproductive status (i.e. presence of ovulated eggs), was recorded twice each week. Each female was gently captured with a dip net and immediately released into a plastic container, where the condition of her ovipositor was checked.

The number of offspring: Throughout the entire breeding season, juvenile bitterling released from host mussels were regularly captured using a fine-mesh dip net, counted and transferred to separate tubs, where they were reared until November.

A subsample of infected (N = 26) and control (N = 30) fish, individually marked by coloured visible implant elastomers, was measured, weighed and held in aquaria between the experimental infection and the onset of spawning. In April, the fish were dissected and measured for evaluation of parasite infection, condition, growth rate and gonad size before the spawning season. Likewise, at the end of Experiment 1, in October 2013, all experimental fish were dissected (for details, see above), their size [standard length (SL)], eviscerated mass (WE), gonad mass and liver mass were measured and the number of *D. pseudospathaceum* metacercariae in the eye lens was counted. The following indices were calculated for each individual fish: condition factor, $CF = WE \times 10^5 \times SL^{-3}$; hepatosomatic index (measure of energy storage), $HSI = W(\text{liver}) \times 10^2 \times WE^{-1}$; and gonadosomatic index (measure of physiological investment in reproduction), $GSI = W(\text{gonads}) \times 10^2 \times WE^{-1}$, using WE and weight of organs (W; in grams) and SL (in millimetres) (Schreck & Moyle, 1990). Growth rate was calculated as the difference in SL between the beginning and end of the experiment.

The size of the eye spot was analysed in IMAGEJ v.1.43n software (Rasband, 2011). The number of pixels of the eye, pupil and the red spot were measured. The size of the red spot was calculated as the proportion of the spot in the eye area minus pupil area. The colour intensity of the eye spot was analysed in Lucia G (v.5.0). In all eye spots, seven points were randomly selected (five in the central area and two in the upper part of the eye spot) and the red (R), green (G) and blue (B) values determined. The RGB values were also measured for four points from the red area of the colour standard that was included in every image. For all points, redness (Red) was expressed as the proportion of the brightness of R as a function of the sum of R, G and B values. The mean redness and variance were calculated from the seven points (or four for colour standards) and the standardized redness (SRed) was estimated for all males as: SRed = mean Red (spot)/mean Red (control). Repeated-measures ANOVA was used to test the effect of infection on the eye spot size and colour intensity. Spearman correlation was used to test the relationship between the intensity of infection and body indices (SL, weight, gonad weight, liver weight, GSI, HSI and growth rate).

Experiment 2: metabolic rate

A total of 64 1-year-old experimental fish (32 control and 32 infected fish; offspring of parental fish collected in the River Kyjovka in 2015) were marked with a unique combination of two coloured visible implant elastomers at the base of the dorsal fin before the start of the bitterling spawning season (April 2016). All fish were photographed and placed in groups of four females and four males in separate tubs in the garden of the IVB. The tubs were equipped with artificial plants and

one *U. tumidus* mussel as a spawning substrate. The fish were housed outdoors until September, ensuring natural light and temperature conditions. Fish were fed daily with frozen chironomid larvae and foraged on algae and invertebrates that established in the tubs, supplemented by commercial dry fish food. At the end of the experiment, fish were dissected (for details, see above). Their size (SL) and weight were measured, the number of *Diplostomum* metacercariae was counted and the number of developed eye cataracts in the lenses recorded. Cataract is considered to be extensive damage of the eye lens tissue around the location of metacercariae. All cataracts covered < 50% of the lens area.

The metabolic rate of infected and control fish was quantified by computerized intermittent flow-through respirometry (Steffensen, 1989), using a static respirometry system (Loligo Systems, Tjele, Denmark). Only fish in reproductive condition (males with intense nuptial coloration and females with an extended ovipositor) were measured during the breeding season (23 May to 16 June). Four respirometers (55.0 mL) were submerged in an outer holding tank of 50 L, supplied with dechlorinated, aerated tap water from a 50 L reservoir and passed under an ultraviolet lamp to minimize microbial respiration. Respirometers were darkened; therefore, the fish could not see each other. The temperature was maintained at 20.7 ± 1.3 °C. Experiments began in the morning, by subjecting fish to a 3 min chasing + 30 s air exposure protocol to elicit maximal metabolic rate (MMR; Clark *et al.*, 2013; Methling *et al.*, 2018), and they were immediately placed in the respirometry chamber. Chamber oxygen partial pressure (pO_2) was measured with an OXY-4 mini (PreSens GmbH, Germany) fibre-optic O_2 transmitter and recorded with the AutoResp4 software. Instantaneous mass-specific oxygen consumption rates (MO_2) were derived from a decrease in chamber pO_2 during a 5 min measurement period $[d(pO_2)/dt]$ according to: $MO_2 = V[d(pO_2)/dt]\alpha M - 1$, where V is the volume of the chamber, α is the specific oxygen solubility and M is fish wet weight. Chambers were flushed for 4 min with water from the holding tank, followed by a closed 1 min waiting period to reach steady state, before the next measurement period began. Measurements of MO_2 were recorded during the following 24 h. Fish were not fed 24 h before measurements.

Only MO_2 measurements where the regression coefficient of the slope $d(pO_2)/dt$ was > 0.96 were used for further analysis (Chabot *et al.*, 2016). If this resulted in < 30 MO_2 measurements, the individual was excluded from the data set. Standard metabolic rate (SMR) was estimated as the 20th percentile of MO_2 measurements (Chabot *et al.*, 2016). The MMR was determined as the highest of the first three MO_2 measurements after the chasing protocol. For some individuals, the MO_2 during spontaneous activity exceeded those measurements, and these values were then taken as MMR. Differences in SMR and MMR between infected and non-infected fish were analysed using linear mixed models, with infection status as a two-level fixed effect (infected or control) and experiment number (corresponding to the day on which measurements were made) as a random effect to account for between-experiment variation. To correct for size effects, ln(wet mass) was added as a covariate, assuming a linear relationship with ln-transformed SMR/MMR. Temperature was initially included as covariate, because this varied between experimental days (17.5–22.5 °C). For infected fish, the effect of infection load on SMR and MMR was analysed using linear models (LMs), with the total number of *D. pseudospathaceum* metacercariae (both eyes pooled) as an independent variable and sex and temperature initially added as covariates. The SMR and MMR were size standardized as the residuals from the ln(wet mass) minus ln(metabolic rate) regression analysis. For a subset of infected fish (N = 11) that had developed cataracts, the difference in SMR and MMR between non-infected and infected fish (with cataract) was analysed using LMs, with presence/absence as a two-level fixed effect and sex, temperature and wet mass as covariates. Model selection (stepwise removal of covariates) was based on the Akaike information criterion, and assumptions regarding homoscedasticity and normality of residuals were examined by visual inspection of residual plots and Q–Q plots of final models. Results were visualized in the package *visreg* (Breheny & Burchett, 2017). The intensity of *Diplostomum* infection between males and females was compared using a t-test for unequal variance.

Experiment 3: mate choice

The effect of *Diplostomum* infection on sexual selection was studied using offspring of fish collected in the River Kyjovka and infected in the previous year (for details, see above). Fish in reproductive condition (females with an extended ovipositor) were placed in aquaria measuring 750 mm × 400 mm × 400 mm. Experimental aquaria were isolated using opaque barriers to prevent interactions between fish from adjacent aquaria; they contained a layer of sand and were continuously aerated. An internal filter was used to maintain water quality when experimental fish were not present in aquaria, but disconnected when experiments were taking place. Artificial plants were placed in a central section to separate males visually from interference and divided the aquarium into two sections. The artificial vegetation also served as a refuge for females. Experimental

mussels (*U. tumidus*) were randomly selected from a stock of similar-sized individuals (mean ± SE shell size, 77 ± 5.4 mm). Aquaria were maintained under a natural light cycle (14 h light–10 h dark), and water temperature matched natural variations (17–20 °C). Experimental observations were conducted at the peak of the breeding season, between 10 and 26 May. Three complementary experimental set-ups were used to separate the effects of visual and olfactory cues and the role of spawning substrate (Fig. 1).

In the first set-up, only visual contact between the partners was allowed. Aquaria contained two transparent glass boxes (280 mm × 105 mm × 105 mm), in which experimental males were housed. The boxes were positioned in the left and right rear corners of each aquarium. A sand-filled flowerpot with a single *U. tumidus* mussel was placed next to each glass box containing an experimental male (Fig. 1A).

The second set-up allowed both visual and olfactory contact between partners. The glass boxes with males (200 mm × 100 mm × 100 mm) were perforated with three 50 mm × 50 mm openings covered with a net with a mesh size of 1 mm. The openings were situated on two adjacent walls of the box. One opening faced the mussel and two openings faced the central part of the aquarium. Artificial plants separated the males visually (Fig. 1B).

The third set-up allowed visual and olfactory contact between partners, but enabled the males (rather than the female) to have access to mussels. The two males were separated by a mesh divider (mesh size 1 mm) in a wooden frame. A mussel was placed in each section with a male and was inaccessible to the female (Fig. 1C).

Before experimental observations, fish were gently collected from outdoor tanks using a hand net and immediately transported to the aquarium. Two randomly selected males (one infected and one control) were introduced to their respective compartments and allowed to settle for 2–10 min. Upon resumption of normal swimming behaviour, a receptive female with an extended ovipositor was gently released into the aquarium. Behavioural recording started when the female left the artificial vegetation and exhibited an interest in the mussel. After completion of observations, the males were swapped between the left and right position in the aquarium, and a second observation period started after the fish had settled. Every 20 s, within a 20 min observation period, the following behaviours were recorded: female interaction (female strikes her head against the wall of the container; female swims in parallel with the male); female inspection (female inspects the exhalant siphon of the mussel; female skims over the siphon without inserting her ovipositor); and male interaction (male quivers his body and exposes his lateral side or swims in parallel with the female; male strikes his head against the wall of the container). When mussels were accessible to males (set-up C), male inspection (male inspects the mussel; male releases sperm) was recorded instead of female inspection. A maximal score of 120 was possible for each behaviour. The overall behaviour of every male and female was counted as the sum of all responses.

After each trial, experimental fish were captured, measured for SL and released back into their tub. A total of 17 replicates (each with two observations of 10 min) with control females and 21 replicates with infected females were completed in set-up A. Sixteen replicates with control and 16 with infected females were completed in set-ups B and C. The males used in the same replicate differed by < 2 mm of their body length. All fish were used only once in each set-up. At the end of the experiment, the fish were dissected (for details, see above); their size and weight were measured and the number of *Diplostomum* metacercariae was counted.

Reproductive activity and preference for an infected/control partner were tested using generalized linear models, with partner activity used as a covariate. All statistical analyses were carried out in R v.3.3.3 (Core Development Team, 2017).

Ethical note

The research was undertaken in line with the ethical requirements of the Czech Republic, and was approved by an appropriate ethics committee. The sampling, transportation, maintenance and care of experimental fish, the method of fish killing and the experimental procedures complied with legal requirements in the Czech Republic (§ 7 law no. 114/1992 about the protection of nature and landscape and § 6, 7, 9 and 10 regulation No. 419/2012 about the care, breeding and using experimental animals). Researchers involved in this study (V.N., M.O. and M.R.) are certified to work with experimental animals according to Czech legal requirements (§15, law no. 246/1992 on animal welfare).

RESULTS

Bitterling reproductive traits (Experiment 1)

Uninfected males possessed a higher intensity of red eye colour (repeated-measures ANOVA, $F_{7,32} = 3.2$, $P = 0.011$) but of a smaller area ($F_{7,32} = 4.6$, $P = 0.001$) compared with infected males. Red intensity and eye spot size increased seasonally, with red intensity peaking in mid-July (Fig. 2A) and spot size between mid-June and the beginning of July (Fig. 2B). Neither the extent nor the intensity of the red spot was correlated

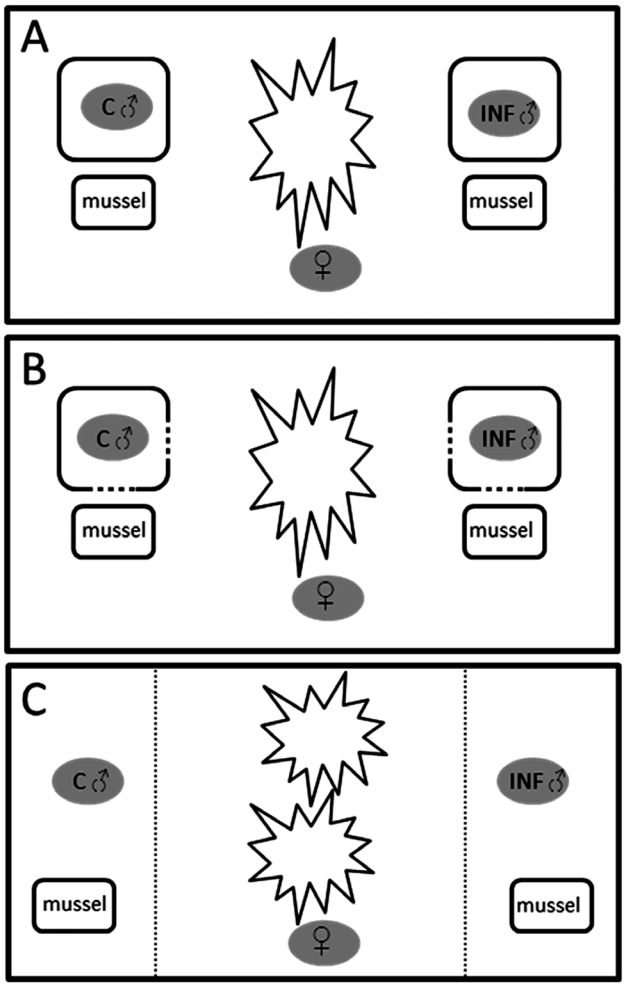

Figure 1. Diagrams of the experimental set-up. A, only visual contact among fish was allowed. B, both visual and olfactory contact among fish was allowed, and the spawning substrate (mussel) was accessible to the female. C, both visual and olfactory contact among fish was allowed, and the mussel was accessible to males. Abbreviations: C♂, control male; INF♂, infected male; and ♀, female.

with fish size or the intensity of *D. pseudospathaceum* infection (all $P > 0.05$).

The overall number of reproductively active females was the same in infected and control fish (*t*-test, $t_{19} = 0.08$, $P = 0.940$). However, spawning activity was significantly higher in control females during the main breeding season (May to mid-June; Student's paired *t*-test, $t_{13} = 3.10$, $P = 0.008$) and exhibited a peak ~2 weeks earlier (May/June) compared with infected fish (June/July) (Fig. 3). This pattern translated into an extended period of offspring production in infected fish (Fig. 4), but with no overall difference in the number of offspring produced over the entire breeding season ($t_{19} = 1.06$, $P = 0.300$).

Before the spawning period, growth increment, CF, HSI and GSI were similar for both infected and control fish. Infected fish exhibited increased variance in GSI (Bartlett test; males, $\chi^2_1 = 4.12$, $P = 0.027$; females, $\chi^2_1 = 4.12$, $P = 0.042$). After the spawning period, infected males had larger gonads and higher condition (GSI, $F_{1,71} = 5.61$, $P = 0.021$; CF, $F_{1,71} = 5.68$, $P = 0.020$), whereas control females exhibited a higher hepatosomatic index (HSI, $F_{1,75} = 13.77$, $P < 0.001$). There was no correlation between the intensity of infection and the initial and final body size, CF, GSI and HSI in males and females (all $P > 0.05$). The intensity of infection was 11.4 ± 6.6 (mean ± SD) in males and 10.5 ± 6.3 in females.

Bitterling metabolism (Experiment 2)

Diplostomum pseudospathaceum infection had no effect on SMR and MMR (Table 1). The intensity of infection assessed for surviving fish was comparable

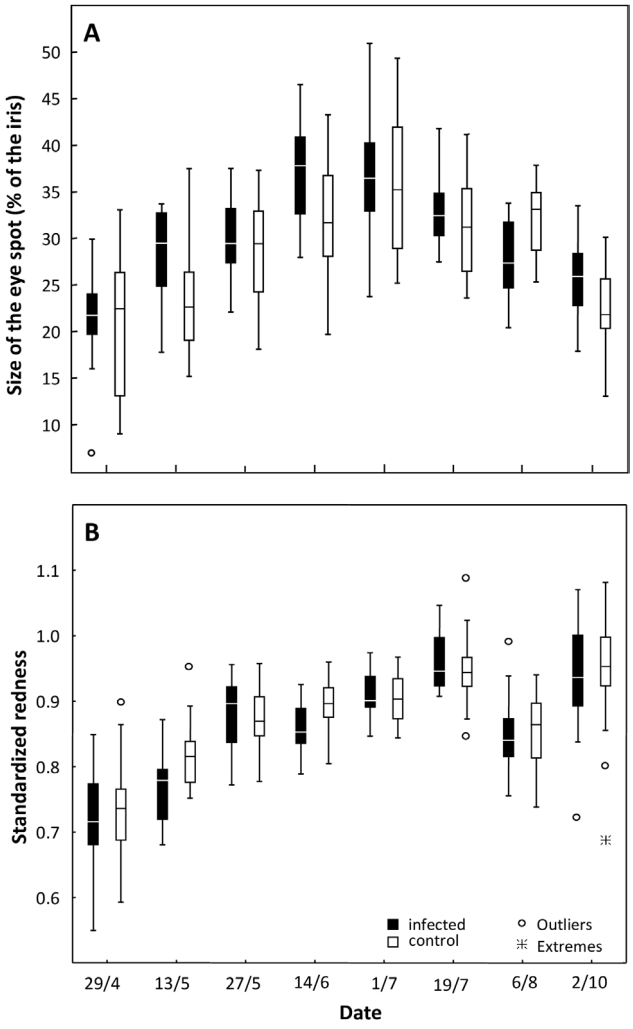

Figure 2. Seasonal variation in size of the red eye spot (A) and the colour intensity of the eye spot (B) of infected (black bars) and control males (white bars) during the bitterling breeding season. Box-plots represent the mean ± SE, and whiskers the 95% confidence intervals.

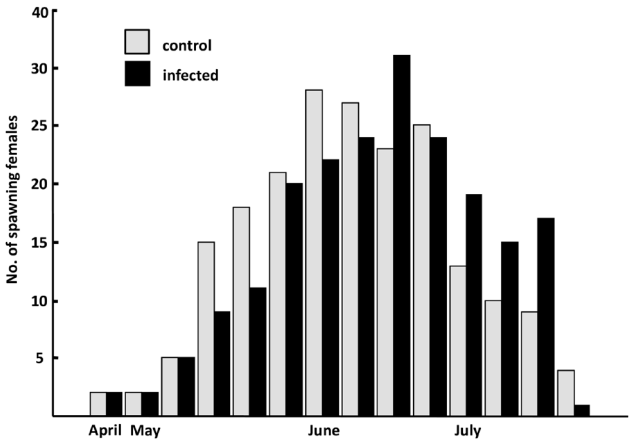

Figure 3. The number of bitterling females with extended ovipositor. Black bars, infected fish; grey bars, control fish.

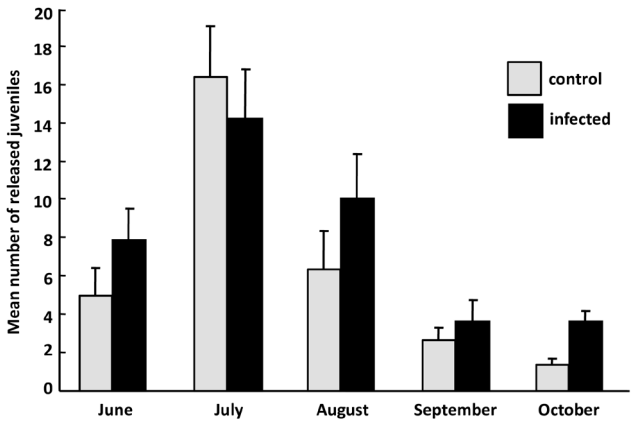

Figure 4. The mean number of offspring produced during the breeding season. Black bars, infected fish; grey bars, control fish.

Table 1. Summaries of the final linear mixed models with infection of *Diplostomum pseudospathaceum* metacercariae as the predictor of standard (SMR; N = 61 fish) or maximal metabolic rate (MMR; N = 62 fish), showing parameter estimates, standard error, t- and P-values for fixed effects and variance explained by random effects

		Ln(SMR)	Ln(MMR)
Intercept	Estimate (SE)	5.923 (0.062)	7.408 (0.409)
	t (d.f.)	95.749 (56.7)	18.127 (22.1)
	P-value	< 0.001	< 0.001
Fixed effects			
Infected (yes)	Estimate (SE)	0.018 (0.038)	0.007 (0.029)
	t (d.f.)	0.482 (56.4)	0.233 (46.9)
	P-value	0.630	0.816
Wet mass	Estimate (SE)	0.719 (0.092)	0.817 (0.077)
(ln-transformed)	t (d.f.)	7.792 (50.9)	10.609 (54.0)
	P-value	< 0.001	< 0.001
Sex (male)	Estimate (SE)	−0.182 (0.045)	−0.108 (0.053)
	t (d.f.)	4.016 (29.8)	−2.037 (26.0)
	P-value	< 0.001	0.042
Temperature	Estimate (SE)	–	−0.035 (0.019)
	t (d.f.)	–	−1.809 (22.8)
	P-value	–	0.071

Bold text indicates significant predictors of metabolic rates, and '–' denotes covariates dropped during model selection.

between males (mean ± SD = 10.3 ± 6.1) and females (9.9 ± 4.2) (t_{22} = 0.38, P = 0.71). Infection load did not explain any of the variation in SMR or MMR between infected and non-infected fish. Seven males and four females developed cataracts in their eyes. There was no difference in SMR (LM, t_{36} = 0.66, P = 0.52) or MMR (LM, t_{39} = 0.97, P = 0.34) between fish that developed cataracts and non-infected fish. The intensity of infection differed between the fish with cataract (intensity of infection range 8–25, mean = 13.5) and without cataract (range 2–16, mean = 7.7) (Mann–Whitney U-test, Z = 2.5, P = 0.012).

MATE CHOICE (EXPERIMENT 3)

There was no difference in reproductive behaviour between infected and control fish when only visual contact between partner fish was allowed (Table 2). However, both sexes tended to interact more intensively with a partner of the same health status (Table 2), i.e. infected females showed a weak tendency to respond to infected males ($F_{1,39}$ = 4.6, P = 0.039), control females to control males ($F_{1,31}$ = 3.1, P = 0.087) and control males to control females ($F_{1,35}$ = 4.6, P = 0.038).

When both visual and olfactory contact was permitted, there was no difference in reproductive behaviour between infected and control males and

Table 2. Results of general linear models showing the effects of female and male health status on female/male reproductive response to the partner in three experimental set-ups: set-up A, in which visual contact between the partners was allowed; set-up B, in which both visual and olfactory contact between partners was allowed and access to a mussel was permitted for the female; and set-up C, in which visual and olfactory contact between partners was allowed, with male access to a mussel

	Set-up A (N = 38)		Set-up B (N = 32)		Set-up C (N = 32)	
	F	P-value	F	P-value	F	P-value
Female response						
Male activity (covariate)	53.5	< 0.001	71.4	< 0.001	95.9	< 0.001
Health status of female	0.1	0.818	1.9	0.173	1.0	0.314
Health status of male	0.3	0.615	2.7	0.106	< 0.1	0.896
Male × female health status	7.6	0.008	2.0	0.166	0.2	0.682
Male response						
Female activity (covariate)	68.7	< 0.001	51.5	< 0.001	130.6	< 0.001
Health status of male	0.9	0.345	0.4	0.539	0.1	0.807
Health status of female	< 0.01	0.952	3.4	0.070	2.2	0.146
Male × female health status	7.2	0.009	< 0.01	0.985	0.1	0.786

females. Females followed the same trend in response to the infected/control male, irrespective of their health status (Table 2). All experimentally infected fish contained metacercariae of *D. pseudospathaceum* in their eye lenses. The mean intensity of infection was 4.9 (range 1–18) and was comparable among all groups.

DISCUSSION

Carotenoid-based coloration in males can function as an important trait for female mate choice (Andersson, 1994), including mate choice in European bitterling (Reichard *et al.*, 2005; Smith *et al.*, 2014). Our results showed that infection by metacercariae of the trematode *D. pseudospathaceum* was associated with a significant difference in the expression of this trait. *Diplostomum*-infected males exhibited larger, but less intensive red eye pigmentation than control males, and the difference was particularly pronounced at the beginning of the breeding season. The intensity of eye colour and male dominance (including body size and activity) are reliable predictors of male reproductive success in this species (Reichard *et al.*, 2005).

Carotenoid-based coloration has been found to indicate several aspects of male quality, including immunocompetence (McGraw & Ardia, 2003) and resistance to parasites (Barber *et al.*, 2001), and functions as a cue for female choice across a range of taxa (Andersson, 1994; Olson & Owens, 1998). Parasites inhibit expression of carotenoid-based ornamental displays and, consequently, can affect female mate choice in several fish species (Milinski & Baker, 1990; Houde & Torio, 1992). Bitterling are probably able to obtain an adequate amount of carotenoids from their diet, which includes green and red algae, potentially making the expression of carotenoid-based pigments less costly than in other species (Olson & Owens, 1998). Given that the fish were additionally fed with flake food containing carotenoids, it is unlikely that the difference in coloration between infected and control males was caused by an inability of infected males to acquire carotenoids through their diet. Nonetheless, carotenoids are a finite resource and are needed both for immune function and for ornamental display (Hill, 1999). It is possible that infected males were less able to direct carotenoids away from vital immunological functions and produce intense eye coloration during the first part of the breeding season.

A delay of 1–2 weeks in the peak of reproductive activity and offspring departure from host mussels was observed in experimental populations of fish infected with *D. pseudospathaceum*. Nonetheless, the overall number of females in a reproductive state, summed over the breeding season, and the total number of offspring produced were comparable between infected and control populations. This finding is in agreement with an assumption of delayed, impaired or reversed gonadogenesis in parasite-infected animals, especially those with increased energetic demands (Heins *et al.*, 1999; Heins & Baker, 2003). Nevertheless, chronic infection by *D. pseudospathaceum* had no significant effect on the SMR or MMR of European bitterling and, notably, there was no increase in SMR or MMR even in fish with cataracts in their eyes. This result contrasted with increased oxygen consumption seen in *Salvelinus alpinus* that were chronically infected with

the closely related parasite, *Diplostomum spathaceum* (Voutilainen *et al.*, 2008). In addition, prespawning examination of bitterling condition status demonstrated no correlation between the intensity of infection and the difference between initial and final body size, somatic condition, hepatosomatic and gonadosomatic index, further indicating a relatively low energetic demand of chronic infection. However, the high individual variation in gonad size exhibited by infected fish might have reflected variation in energy allocation related to the intensity of infection and/or host immune response (Rohlenová & Šimková, 2010) and thereby contributed at the population level to delayed reproductive activity compared with control fish. Thus, fish exhibiting smaller gonad size before the onset of reproduction might have included individuals that maximized their gonadal development during spring and, as a consequence, postponed reproductive development until later in the season. Significantly lower energy reserves, measured as HSI, at the end of breeding season might indicate increased investment in reproduction at the expense of lipid reserve storage, particularly in infected females. In that case, the elevated investment in reproduction might have implications for subsequent female survival and fecundity (Wootton & Smith, 2015).

The reproductive activity of infected and control fish reflected the lack of difference in metabolic rate and condition status between the groups. Instead, reproductive activity significantly reflected the activity of the partner, irrespective of the health status or degree of visual and/or olfactory contact. Likewise, a tendency to respond to mating partners of the same health status was demonstrated when determining female preference for infected/control males without olfactory contact. The intensity of male courtship is reinforced by a positive female response to that courtship (Reichard *et al.*, 2005) and, despite the potential negative impacts of *Diplostomum* infection on host vision (Owen *et al.*, 1993), reproductive activity in infected bitterling appeared to be unaffected.

Our results showed that the *Diplostomum* metacercaria was not an energetically demanding parasite during chronic infection of bitterling fish. The significant energetic costs of *Diplostomum* infection associated with immune function are expected during the acute infection, in the first hours after exposure (Laitinen *et al.*, 1996; Voutilainen *et al.*, 2008). However, according to our results, even several months postinfection of bitterling fish, *Diplostomum* can cause a short delay of host reproduction, with potential population-level consequences. Uninfected males, with more intense sexually selected signals, are predicted to have an advantage in attracting females, especially at the beginning of the breeding season (Kokko & Mappes, 2005; Borg *et al.*, 2006). Consequently, uninfected males might sire most offspring in the first part of breeding season, which has several advantages. First, bitterling embryo mortality is much lower at the beginning of the breeding season owing to density-dependent effects (Smith *et al.*, 2000). Second, later-born offspring suffer reduced growth and may fail to reach the threshold size and energetic competence for overwinter survival (Francová & Ondračková, 2013; Michálková & Ondračková, 2014). Small-sized fish have a lower energy reserve capacity and a higher basal metabolism than large-sized fish, making them more vulnerable to winter starvation (Schultz & Conover, 1999; Post & Parkinson, 2001). Indeed, later-hatched naturally infected European bitterling demonstrated a deterioration in body condition, whereas this trend was not seen in early-hatched fish, despite their high parasite infection rate (Francová & Ondračková, 2013). Finally, smaller individuals are also more susceptible to predation (Wootton, 1990).

To summarize, by performing a series of three independent experimental trials, we estimated the effects of infection by the eye fluke trematode *D. pseudospathaceum* on reproduction of its fish host. Despite high infection doses of cercariae during infections, the number of metacercariae in fish eye lenses was relatively low, corresponding to parasite loads in nature (Dávidová *et al.*, 2011). The intensity of cataracts in eye lenses of infected fish was related to infection intensity and, in most cases, the cataract was partial, covering a maximum of 50% of the lens area. The vision of the fish was therefore affected only partially, suggesting more pronounced effects in the case of higher infection intensities with more pronounced cataract. Overall, the impact of infection was lower than expected. Contrary to predictions, no effect on host metabolism was detected. Infection had only a limited effect on the sexually selected trait of eye redness. Nevertheless, a delay in reproductive activity of infected females and, consequently, later offspring emergence, could potentially result in decreased offspring condition, overwinter survival and risk of predation. This phenological shift has the potential to affect fish population dynamics in parasite-affected environments. We highlight that in our study the experimental fish were held in benign conditions, with adequate food and breeding resources and with an absence of predators and non-experimental infections, potentially limiting the negative effects of parasite infection in comparison to more natural conditions (Candolin & Voigt, 2001).

ACKNOWLEDGEMENTS

The authors would like to thank to Luděk Šlapanský for help with the care of experimental fish and Carl Smith for correction of the English. We also thank two anonymous reviewers for their helpful comments.

This work was supported by the European Centre of IchthyoParasitology (ECIP); Centre of Excellence program of the Czech Science Foundation, project no. P505/12/G112. The authors declare no conflict of interest.

REFERENCES

Agnew P, **Koella JC**, **Michalakis Y. 2000.** Host life history responses to parasitism. *Microbes and Infection* **2:** 891–896.

Arme C. 1997. *Ligula intestinalis*: interactions with the pituitary-gonadal axis of its fish host. *Journal of Helminthology* **71:** 83–84.

Barber I, **Arnott SA**, **Braithwaite VA**, **Andrew J**, **Huntingford FA. 2001.** Indirect fitness consequences of mate choice in sticklebacks: offspring of brighter males grow slowly but resist parasitic infections. *Proceedings of the Royal Society B: Biological Sciences* **268:** 71–76.

Barber I, **Arnott SA**, **Braithwaite VA**, **Andrew J**, **Mullen W**, **Huntingford FA. 2000.** Carotenoid-based sexual coloration and body condition in nesting male sticklebacks. *Journal of Fish Biology* **57:** 777–790.

Borg AA, **Forsgren E**, **Amundsen T. 2006.** Seasonal change in female choice for male size in the two-spotted goby. *Animal Behaviour* **72:** 763–771.

Breheny P, **Burchett W. 2017.** Visualization of regression models using *visreg*. *The R Journal* **9:** 56–71.

Candolin U, **Reynolds JD. 2001.** Sexual signaling in the European bitterling: females learn the truth by direct inspection of the resource. *Behavioral Ecology* **12:** 407–411.

Candolin U, **Voigt HR. 2001.** No effect of a parasite on reproduction in stickleback males: a laboratory artefact? *Parasitology* **122:** 457–464.

Carter V, **Pierce R**, **Dufour S**, **Arme C**, **Hoole D. 2005.** The tapeworm *Ligula intestinalis* (Cestoda: Pseudophyllidea) inhibits LH expression and puberty in its teleost host, *Rutilus rutilus*. *Reproduction* **130:** 939–945.

Chabot D, **Steffensen JF**, **Farrell AP. 2016.** The determination of standard metabolic rate in fishes. *Journal of Fish Biology* **88:** 81–121.

Clark TD, **Sandblom E**, **Jutfelt F. 2013.** Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. *The Journal of Experimental Biology* **216:** 2771–2782.

Clayton DH. 1991. The influence of parasites on host sexual selection. *Parasitology Today* **7:** 329–334.

Dávidová M, **Blažek R**, **Trichkova T**, **Koutrakis E**, **Gaygusuz Ö**, **Ercan E**, **Ondračková M. 2011.** The role of the European bitterling (*Rhodeus amarus*, Cyprinidae) in parasite accumulation and transmission in riverine ecosystems. *Aquatic Ecology* **45:** 377–387.

Dávidová M, **Ondračková M**, **Jurajda P**, **Gelnar M. 2008.** Parasite assemblages of European bitterling (*Rhodeus amarus*), composition and effects of habitat type and host body size. *Parasitology Research* **102:** 1001–1011.

Ergens R, **Lom J. 1970.** *Causative agents of fish diseases*. Prague: Academia.

Faltýnková A, **Našincová V**, **Kablásková L. 2007.** Larval trematodes (Digenea) of the great pond snail, *Lymnaea stagnalis* (L.), (Gastropoda, Pulmonata) in Central Europe: a survey of species and key to their identification. *Parasite* **14:** 39–51.

Forbes MRL. 1993. Parasitism and host reproductive effort. *Oikos* **67:** 444–450.

Francová K, **Ondračková M. 2013.** Overwinter body condition, mortality and parasite infection in two size classes of 0+ year juvenile European bitterling *Rhodeus amarus*. *Journal of Fish Biology* **82:** 555–568.

Georgieva S, **Soldánová M**, **Pérez-del-Olmo A**, **Dangel DR**, **Sitko J**, **Sures B**, **Kostadinova A. 2013.** Molecular prospecting for European *Diplostomum* (Digenea: Diplostomidae) reveals cryptic diversity. *International Journal for Parasitology* **43:** 57–72.

Geraudie P, **Boulange-Lecomte C**, **Gerbron M**, **Hinfray N**, **Brion F**, **Minier C. 2010.** Endocrine effects of the tapeworm *Ligula intestinalis* in its teleost host, the roach (*Rutilus rutilus*). *Parasitology* **137:** 697–704.

Hamilton WD, **Zuk M. 1982.** Heritable true fitness and bright birds: a role for parasites? *Science* **218:** 384–387.

Heins DC, **Baker JA. 2003.** Reduction of egg size in natural populations of threespine stickleback infected with a cestode macroparasite. *Journal of Parasitology* **89:** 1–6.

Heins DC, **Brown-Peterson NJ. 2010.** Influence of the pseudophyllidean cestode *Schistocephalus solidus* on oocyte development in the threespine stickleback *Gasterosteus aculeatus*. *Parasitology* **137:** 1151–1158.

Heins DC, **Singer SS**, **Baker JA. 1999.** Virulence of the cestode *Schistocephalus solidus* and reproduction in infected threespine stickleback, *Gasterosteus aculeatus*. *Canadian Journal of Zoology* **77:** 1967–1974.

Hill GE. 1999. Is there an immunological cost to carotenoid-based ornamental coloration? *The American Naturalist* **154:** 589–595.

Houde AE, **Torio AJ. 1992.** Effect of parasitic infection on male color pattern and female choice in guppies. *Behavioral Ecology* **3:** 346–351.

Karvonen A, **Paukku S**, **Valtonen ET**, **Hudson PJ. 2003.** Transmission, infectivity and survival of *Diplostomum spathaceum* cercariae. *Parasitology* **127:** 217–224.

Kokko H, **Mappes J. 2005.** Sexual selection when fertilization is not guaranteed. *Evolution* **59:** 1876–1885.

Laitinen M, **Siddall R**, **Valtonen ET. 1996.** Bioelectronic monitoring of parasite-induced stress in brown trout and roach. *Journal of Fish Biology* **48:** 228–241.

MacNab V, **Katsiadaki I**, **Barber I. 2009.** Reproductive potential of *Schistocephalus solidus* infected male three-spined stickleback *Gasterosteus aculeatus* from two UK populations. *Journal of Fish Biology* **75:** 2095–2107.

McGraw KJ, **Ardia DR. 2003.** Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. *The American Naturalist* **162:** 704–712.

Methling C, **Douda K**, **Liu H**, **Rouchet R**, **Bartáková V**, **Yu D**, **Smith C**, **Reichard M. 2018.** Energetic costs in the relationship between bitterling and mussels in East Asia. *Biological Journal of the Linnean Society* **125:** 750–759.

Michálková V, **Ondračková M. 2014.** Experimental evidence for parasite-induced over-winter mortality in juvenile *Rhodeus amarus*. *Journal of Fish Biology* **84:** 1377–1388.

Milinski M, **Bakker TCM. 1990.** Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. *Nature* **344:** 330–333.

Olson VA, **Owens IP. 1998.** Costly sexual signals: are carotenoids rare, risky or required? *Trends in Ecology & Evolution* **13:** 510–514.

Owen SF, **Barber I**, **Hart PJB. 1993.** Low level infection by eye fluke, *Diplostomum* spp., affects the vision of three-spined sticklebacks, *Gasterosteus aculeatus*. *Journal of Fish Biology* **42:** 803–806.

Post JR, **Parkinson EA. 2001.** Energy allocation strategy in young fish: allometry and survival. *Ecology* **82:** 1040–1051.

Poulin R. 2007. *Evolutionary ecology of parasites*. Princeton: Princeton University Press.

R Core Team. **2017.** *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing. ISBN 3-900051-07-0. Available at: http://www.Rproject.org/

Rasband WS. 2011. *ImageJ*. Bethesda: US National Institutes of Health. Available at: http://imagej. nih. gov/ij/

Read AF. 1990. Parasites and the evolution of host sexual behaviour. In: Barnard CJ, Behnke JM, eds. *Parasitism and host behaviour*. London: Taylor and Francis, 95–116.

Reichard M, **Bryja J**, **Ondračková M**, **Dávidová M**, **Kaniewska P**, **Smith C. 2005.** Sexual selection for male dominance reduces opportunities for female mate choice in the European bitterling (*Rhodeus sericeus*). *Molecular Ecology* **14:** 1533–1542.

Reichard M, **Smith C**, **Bryja P. 2008.** Seasonal change in the opportunity for sexual selection. *Molecular Ecology* **17:** 642–651.

Rohlenová K, **Šimková A. 2010.** Are the immunocompetence and the presence of metazoan parasites in cyprinid fish affected by reproductive efforts of cyprinid fish? *Journal of Biomedicine and Biotechnology* **2010:** 418382.

Schreck CB, **Moyle PB. 1990.** *Methods for fish biology*. Bethesda: American Fisheries Society.

Schultz ET, **Conover DO. 1999.** The allometry of energy reserve depletion: test of a mechanism for size-dependent winter mortality. *Oecologia* **119:** 474–483.

Schwanz LE. 2008. Chronic parasitic infection alters reproductive output in deer mice. *Behavioural Ecology and Sociobiology* **62:** 1351–1358.

Seppälä O, **Karvonen A**, **Valtonen ET. 2004.** Parasite-induced change in host behaviour and susceptibility to predation in an eye fluke–fish interaction. *Animal Behaviour* **68:** 257–263.

Shariff M, **Richards RH**, **Sommerville C. 1980.** The histopathology of acute and chronic infections of rainbow trout *Salmo gairdneri* Richardson with eye flukes, *Diplostomum* spp. *Journal of Fish Diseases* **3:** 455–465.

Sheldon BC, **Verhulst S. 1996.** Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends in Ecology & Evolution* **11:** 317–321.

Shigin AA. 1964. The life-span of *Diplostomum spathaceum* in the intermediate host. *Trudy Gel'mintologicheskoi Laboratorii. Akademiya Nauk SSSR* **14:** 262–272. [In Russian.]

Smith C, **Douglas A**, **Jurajda P. 2002.** Sexual conflict, sexual selection and sperm competition in the spawning decisions of bitterling, *Rhodeus sericeus*. *Behavioral Ecology and Sociobiology* **51:** 433–439.

Smith C, **Phillips A**, **Polačik M**, **Reichard M. 2014.** Male coloration signals direct benefits in the European bitterling (*Rhodeus amarus*). *Environmental Biology of Fishes* **97:** 335–341.

Smith C, **Reichard M**, **Jurajda P**, **Przybylski M. 2004.** The reproductive ecology of the European bitterling (*Rhodeus sericeus*). *Journal of Zoology* **262:** 107–124.

Smith C, **Reynolds JD**, **Sutherland WJ**, **Jurajda P. 2000.** Adaptive host choice and avoidance of superparasitism in the spawning decisions of bitterling (*Rhodeus sericeus*). *Behavioral Ecology and Sociobiology* **48:** 29–35.

Steffensen JF. 1989. Some errors in respirometry of aquatic breathers: how to avoid and correct for them. *Fish Physiology and Biochemistry* **6:** 49–59.

Voutilainen A, **Figueiredo K**, **Huuskonen H. 2008.** Effects of the eye fluke *Diplostomum spathaceum* on the energetics and feeding of Arctic charr *Salvelinus alpinus*. *Journal of Fish Biology* **73:** 2228–2237.

Wootton RJ. 1990. *Ecology of teleost fishes*. London: Chapman & Hall.

Wootton RJ, **Smith C. 2015.** *Reproductive biology of teleost fishes*. Oxford: John Wiley & Sons.