



Parasite infectious stages provide essential fatty acids and lipid-rich resources to freshwater consumers

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Received: 18 February 2019 / Accepted: 2 December 2019 / Published online: 13 December 2019
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Abstract

Free-living parasite infectious stages, such as motile cercariae of trematodes (flatworms), can constitute substantial biomass within aquatic ecosystems and are frequently eaten by various consumers, potentially serving as an important source of nutrients and energy. However, quantitative data on their nutritional value (e.g., essential fatty acids [EFA]) are largely lacking. As EFA are leading indicators of nutritional quality and underpin aquatic ecosystem productivity, we performed fatty acid (FA) analysis on an aggregate of ~30,000 cercariae of the freshwater trematode, *Ribeiroia ondatrae*. Individual cercariae contained 15 ng of total FA, and considerable quantities of EFA, including eicosapentaenoic (EPA, at 0.79 ng cercaria⁻¹) and docosahexaenoic (DHA, at 0.01 ng cercaria⁻¹) acids. We estimated annual EFA production by *R. ondatrae* cercariae for a series of ponds in California to be 40.4–337.0 $\mu\text{g m}^{-2} \text{yr}^{-1}$ for EPA and 0.7–6.2 $\mu\text{g m}^{-2} \text{yr}^{-1}$ for DHA. To investigate viability of cercariae as prey, we also compared growth and FA profiles of dragonfly larvae (naiads of *Leucorrhinia intacta*) fed equivalent masses of either *R. ondatrae* or zooplankton (*Daphnia* spp.) for 5 weeks. Naiads raised on the two diets grew equally well, with no significant differences found in their EFA profiles. While zooplankton are widely recognized as a vital source of energy, and an important conduit for the movement of EFA between algae and higher trophic levels, we suggest a similar role for trematode cercariae by ‘unlocking’ EFA from the benthic environment, highlighting their potential importance as a nutrient source that supports animal health.

Keywords Cercariae · Essential fatty acids · Nutrients · Trematode · Lipids

Introduction

More than 20 years ago, Marcogliese and Cone (1997) suggested that the explicit incorporation of parasites into aquatic food web models would facilitate a more accurate assessment of food web dynamics, including quantification of nutrient flows, energy transfer, and the strength of discrete trophic interactions. Subsequent empirical research has

made it apparent that parasites, including free-living stages (cercariae) of trematodes released by molluscan intermediate hosts, represent substantial biomass in aquatic ecosystems (Kuris et al. 2008; Kaplan et al. 2009; Dunne et al. 2013; Preston et al. 2013). For example, cercariae biomass in marine and freshwater environments can exceed that of predators, such as birds and aquatic insects (Kuris et al. 2008; Preston et al. 2013). Soldánová et al. (2016) estimated that 4.65 tons of cercariae were emitted annually into a freshwater pond, which is roughly equivalent to the mass of an Asian elephant. Because a large portion of cercariae likely fail to find, and infect, a suitable second intermediate host within their brief lifespan (~24 h), much of their annual production is available to be consumed by predators or accumulate as detritus (Orlofske et al. 2015).

Given their overlap in size with many zooplankton (Morley 2012), cercariae serve as prey for various aquatic invertebrates and vertebrates, such as larval insects, oligochaetes, copepods, bivalves, and fishes (e.g., Schotthoefer et al. 2007; Kaplan et al. 2009; Orlofske et al. 2012, 2015; Catania et al.

Communicated by Lisa Belden.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00442-019-04572-0>) contains supplementary material, which is available to authorized users.

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2016; Vielma et al. 2018). Because predation on parasite infectious stages could limit or reduce infection for subsequent hosts, cercariae consumption has been primarily considered in the context of reduced transmission (e.g., Orlofske et al. 2012; Thieltges et al. 2013; Welsh et al. 2017). However, there are likely additional consequences for aquatic ecosystems, including influences on food web topography, nutrient dynamics, and energetics (Thieltges et al. 2008; Johnson et al. 2010). Free-living infectious stages such as cercariae could thus represent an important pathway through which parasites exert additional effects on food webs, particularly if their consumption provides a major, and heretofore unappreciated, source of energy and nutrients (Thieltges et al. 2008; Preston et al. 2013).

Lipids represent the primary energy currency in aquatic ecosystems and are essential to animal health and survival (Müller-Navarra 1995; Arts and Wainman 1999; Arts et al. 2009). Bioactive lipids, such as long-chain polyunsaturated fatty acids (LC-PUFA), are considered essential fatty acids (EFA) because they act as essential signalling molecules and play fundamental roles in reproduction, cell membrane function, and neural and ocular development (Stillwell and Wassall 2003; Hoffman et al. 2009). However, many animals have only a limited ability to convert shorter-chain PUFA like alpha-linolenic acid (ALA; 18:3n-3) and linoleic acid (LNA; 18:2n-6) to LC-PUFA, including eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3), and arachidonic acid (ARA; 20:4n-6). Sources of pre-formed LC-PUFA are, therefore, especially pivotal. LC-PUFA are largely synthesized by aquatic primary producers such as algae (e.g., diatoms) and other protists (Arts et al. 2009) and retained as they are transferred up trophic levels (Kainz et al. 2004); however, LC-PUFA availability to consumers also depends on the composition of the zooplankton communities that facilitate transfer of these essential biomolecules across the plant–animal interface (Ravet et al. 2010; Burns et al. 2011).

Aquatic snails serve as the first intermediate host for many trematodes, wherein asexual reproduction occurs. Cercariae develop within sporocysts or rediae infecting the snail's digestive gland-gonad complex (Fried et al. 1993). Sporocysts absorb host-derived nutrients while rediae consume host tissue with a mouthpart (Moore and Halton 1975). Because freshwater snails often consume large quantities of algal derived EFA (Pinowska 2002; Lombardo and Cooke 2002), trematodes feeding on snail hosts may ingest such EFA and invest them into developing cercariae (Oudejans and Van der Horst 1974). Consequently, although free-living trematode cercariae are short lived and do not feed, their EFA profiles are likely similar to that of secondary consumers, such as zooplankton feeding on phytoplankton.

While previous investigations have shown that cercariae can be rich in neutral lipids and glycogen (e.g., Fried et al.

1998; Marsit et al. 2000a, b), little is known about their full FA profile or the availability of vital EFAs. The coupling of such information with annual cercariae production estimates for specific trematode species would help reveal their EFA contributions within aquatic ecosystems. A key priority is thus to determine the degree to which free-living parasite stages accumulate and transfer EFA, similar to the widely recognized roles in zooplankton (e.g., Kainz et al. 2004). In addition, as aquatic ecosystems are the primary source of pre-formed n-3 LC-PUFA, it is essential to understand the various pathways and sources for the transfer of these bioactive molecules to adjacent terrestrial areas. For example, the emergence of aerial or terrestrial adult insects from aquatic larval stages is one such known pathway for EFA transfer (e.g., Martin-Creuzburg et al. 2017). Larval odonates such as dragonflies are generalist freshwater predators that consume various prey, including zooplankton (Pritchard 1964) and trematode cercariae; larval *Leucorrhinia intacta* feed on cercariae even when *Daphnia pulex* is offered as an alternative prey item (Catania et al. 2016). These observations highlight the potential for larval dragonflies and other emergent aquatic insects that consume trematode cercariae to function as trophic link for the transfer of n-3 LC-PUFA from aquatic to terrestrial ecosystems, although whether cercariae represent a nutritionally comparable food source for secondary consumers relative to zooplankton remains conjectural.

Here, we tested the hypothesis that cercariae contain biologically relevant amounts of EFA and contribute substantial quantities of these vital nutrients to a key consumer of parasites within freshwater ecosystems. EFA are synthesized in high quantities by algae, and cercariae could thus acquire these while developing within their herbivorous gastropod intermediate host. Therefore, we were interested in quantifying EFA contents of *Ribeiroia ondatrae* cercariae obtained from field-collected snails within a series of freshwater ponds. To compare the influence of parasites on the diet of a common secondary consumer, we experimentally evaluated growth, total lipid content, and FA composition of *L. intacta* naiads fed diets consisting of equivalent masses of either trematode cercariae (*R. ondatrae*) or freshwater zooplankton (*Daphnia* spp.). Owing to their similarities in trophic level, size, and potential lipid composition, we predicted that dragonfly naiads fed *R. ondatrae* cercariae would show similar growth, as well as EFA profiles, compared to those fed *Daphnia* spp., thus confirming that cercariae represent a previously undescribed but substantial source of LC-PUFA that may be transferred to consumers in aquatic and, eventually, terrestrial ecosystems.

Materials and methods

Acquisition of predators and prey

Aquatic snails (*Helisoma trivolvis*) were collected in July–2017 from two ponds in Pleasanton Ridge Regional Park in California, USA (37.6154094° N, –121.8823708° W) and transported to Ryerson University for screening of infection by the trematode *R. ondatrae* using established procedures. This trematode species was chosen because their cercariae are known to be consumed by aquatic insects, including larval odonates (Schotthoefer et al. 2007; Orlofske et al. 2012), and because estimates of yearly cercariae biomass production for California ponds in the same park were also available (Preston et al. 2013). Zooplankton (*Daphnia* spp.) and a common freshwater predator, naiads of the dragonfly species *L. intacta* (whose distribution extends throughout much of North America; Paulson 2017), were collected using dipnets from the Koffler Science Reserve (KSR) in King City, Canada (44.0296° N, 79.5316° W) and transported to Ryerson University.

Leucorrhinia intacta naiads mature over a 10-month period before emerging onto the terrestrial landscape as adults (Wissinger 1989). *Daphnia* prey were a mix of two species (*D. pulex* and *D. magna*), but predominantly contained *D. pulex*, and are hereafter referred to as “*Daphnia*”. Dragonfly naiads and *Daphnia* were maintained in 13.2 L containers in dechlorinated water on a 13:11 h light/dark cycle to mimic summer daylength hours. Importantly, *R. ondatrae*-infected snails and *Daphnia* were fed using the same green algae culture (*Chlamydomonas* sp.; Merlan Scientific). This ensured that the EFA produced by *Chlamydomonas* was available to both prey items; assuming that *Daphnia* acquired EFA through direct consumption, and cercariae did so while developing within algae-consuming host snails.

Cercariae and *Daphnia* fatty acid analysis

Cercariae for FA analysis were obtained from snails collected from Pleasanton Ridge, and *Daphnia* for FA comparison were collected from KSR. A minimum of 15 mg dry weight (DW) of tissue was desirable in order to be able to accurately quantify cercariae FA composition. Therefore, based on the known DW of individual *R. ondatrae* cercariae (Lambden and Johnson 2013), it was estimated that ~30,000 cercariae were needed. This agrees with a previous study that used ~60,000 *S. mansoni* cercariae for FA analysis (Smith et al. 1966) which are ~255 µm in length (Pereira et al. 2013); *R. ondatrae*, at ~800 µm, are just over double this size (Szuroczki and Richardson

2009). Cercariae were collected for ~3 weeks using the procedure described below for the experimental feeding component and then transferred into microcentrifuge vials in known quantities and frozen at –85°C. Three aliquots of 40 *Daphnia* were frozen at –85°C prior to FA analysis.

Prior to analysis, *R. ondatrae* cercariae were thawed at 23 °C for 20 min and collected on a 47 mm-diameter, 5.0 µm pore size silver metal membrane filter (General Electric, #X12CP04725) inside a sealed filtration apparatus attached to a pressure-controlled vacuum pump (1VAF-10-M100X, GAST). The cercariae were filtered at 3.0 Barr to ensure that they would not burst. The filter was very gently scraped with a pliable solvent-cleaned soft silicone spatula during and after filtration to ensure that cercariae were not forced into the filter during filtration. The aggregate cercariae sample (wet weight = 19.84 mg) was weighed using a microbalance (Mettler Toledo #XP6) with 1 µg precision. The three aliquots containing 40 *Daphnia* were freeze-dried and the % moisture of their tissue was calculated. Each aliquot was ground into a fine powder in liquid nitrogen using a mortar and pestle and the sample weighed using the same microbalance.

Samples were homogenized using a Polytron PCU-2-110 homogenizer (Brinkmann Instruments, Rexdale, ON, Canada) and the lipids were extracted with 2 mL of a 2:1 mixture of chloroform: methanol (volume/volume %) following Folch et al. (1957). Chloroform-extracted water was then added to bring the ratio of chloroform:methanol:water to 8:4:3. A known amount of tricosanoic acid standard (23:0 methyl ester, Nu-Chek Prep. Inc, N-23-M) was added to the sample. This internal standard was selected based on previous lipid profiles of freshwater zooplankton (Boissonnot et al. 2016), which generally lack 23:0 and are comparable to cercariae in terms of their food sources and environment. After the addition of 0.8 mL of 0.88% aqueous KCl (weight/volume %), the sample was homogenized using a vortex and centrifuged at 2000 rpm for 5 min to separate the phases. The bottom organic layer containing chloroform and lipids was recovered using a double-pipetting technique (Parrish 1999). More chloroform was added, and the sample was re-extracted (a total of 3X). The pooled extracts were placed in a solvent-washed glass round bottom test tube and evaporated to dryness under a continuous N₂ stream. The sample was reconstituted by adding 2 mL of hexanes and ensuring that the sides of the tube were washed down during the addition of the hexanes. Gravimetric analysis was done by first recording the weight of two empty tin capsules (D1029, Isomass Scientific Inc., Calgary CA) and placing 100 µL aliquots of hexanes into each tin capsule. The solvents were then evaporated at room temperature and the tin cups were re-weighed to provide an estimate of total lipid.

Fatty acid methyl esters (FAME) were produced with the acid-catalyzed transesterification method, using 2 mL of

H₂SO₄ in methanol (1%, v/v) (Christie 1989). The samples were sealed in the tube with N₂ gas and heated at 90 °C for 90 min. After cooling for 5 min, 1.5 mL of Milli-Q water and 4 mL of hexanes were added to stop the reaction. The solution was vortexed and centrifuged at 2000 RPM for 3 min at 4°C. The top layer (hexanes) was decanted with a short Pasteur pipette, and 4 mL of hexanes was added to the bottom layer (water); this process was repeated 3X. The final sample was evaporated to dryness under an N₂ stream, reconstituted with 100 µL of hexanes, and transferred via Pasteur pipette into a gas chromatography (GC) vial with a straight 400 µL insert. This process was repeated, adding another 100 µL of hexanes to the vial. The vial was then evaporated to dryness and 100 µL of hexanes added as the final sample volume. The sample was stored at –85 °C until analysis.

The sample was analyzed using a GC (Shimadzu GC-2010 plus) equipped with an FID detector. Helium was used as the carrier gas. The operating parameters were: splitless injection, initial column temperature of 60 °C, temperature ramp to 250 °C over 37 min, and then holding at 250 °C for 23 min. Fatty acid methyl esters (FAME) were identified based on their retention times. The FAME were quantified by comparing their responses (peak areas) to a standard concentration curve prepared using a standard FAME mix (GLC 68E, Nu-Check Prep, Inc.) and standard QA/QC was performed in relation to the internal standard.

Dragonfly diet and growth experiment

The feeding experiment consisted of two different diet treatments whereby *L. intacta* naiads received equivalent masses of either *R. ondatrae* cercariae or *Daphnia*. Equivalent mean DW of individual *D. pulex* (0.75–1 mm carapace length) and *R. ondatrae* cercariae were estimated to be 5.2 and 0.880 µg individual^{–1}, respectively (Dumont et al. 1975; Lambden and Johnson 2013). The two food treatments thus consisted of either 126 live *R. ondatrae* cercariae (~110.88 µg DW) or 21 live *Daphnia* (~109.2 µg DW) fed to each dragonfly naiad 2X week^{–1}, 2–3 days apart. Naiads were provided with 42 *Daphnia* week^{–1} to ensure they were receiving adequate amounts of prey as *L. intacta* of the size used here (see below) typically consume ~5–7 *Daphnia* day^{–1} (McCauley 2008), or 35–49 per week; however, unconsumed prey items were not explicitly measured. To make sure that *Daphnia* were of the appropriate size, these were sieved through two different mesh sizes to retain those sized 0.75–1 mm.

The two diet treatments consisted of 21 replicates for *R. ondatrae* and 18 for *Daphnia* ($N=39$ total owing to *L. intacta* death within first 2 days), represented by 1 L plastic containers that each held the following: 1 naiad of *L. intacta*, a single 4-cm piece of floating plastic vegetation (Green Cabomba, Big Al's Aquarium Supplies), and ~800 mL of dechlorinated water. The placement of the 39 containers

was randomized among shelves on two separate side-by-side racks. Evaporated water was replaced weekly. *Ribeiroia ondatrae* cercariae and *Daphnia* of the required size range were collected and counted the morning of each feeding day. *Ribeiroia ondatrae* cercariae are nocturnal and emerge at night; infected snails were thus individually placed in water-filled 50 mL centrifuge vials and left overnight (Hannon et al. 2017). Emerged cercariae were collected early the next morning from ~50 snails and pooled together to account for intraspecific variation before allocation to individual naiad containers.

Digital calipers were used to measure naiads, and only those 4–6 mm in head width (a standard measure of larval dragonfly size) were kept (e.g., Catania et al. 2016). This range was chosen as *L. intacta* of this size have been previously found to prey on cercariae that were ~300 µm in total length (body + tail), as well as *Daphnia* sized ~750–1500 µm (Catania et al. 2016). In comparison, *R. ondatrae* cercariae are ~800 µm long (Szuroczki and Richardson 2009). Because larval dragonflies typically grow 3–4X their original size before metamorphosis, using relatively small individuals allows the best chance to observe the effect of diet on their growth prior to emergence (Marczak et al. 2006). The starting head width of each dragonfly naiad was recorded before commencing the feeding experiment in August 2017. Due to the small number of *L. intacta* naiads available at the beginning of the experiment, two separate feeding trials were conducted. A first trial ($N=23$) began in week 1, and a second trial ($N=16$) was added in week 3. However, naiads in both trials had an identical feeding regimen and experimental set-up, and the experiment ultimately ran for 5 weeks in each trial. After taking final head width measurements at the end of the feeding experiment, the growth of each naiad was calculated using the following equation: % growth = [(final head width – start head width) / start head width] × 100. Individual dragonfly naiads were weighed, placed into labeled 2.5 mL cryogenic vials, and frozen at –85°C for lipid analysis as described above for *Daphnia*.

Prior to analyses, response variables were plotted to evaluate normality and homogeneity of variance. To assess effects of diet on dragonfly growth, as well as on their MUFA (monounsaturated FA), PUFA (polyunsaturated FA), and SFA (saturated FA) content, respectively, a series of separate general linear models (GLM) was used with trial as a categorical fixed effect. MUFA, PUFA, and SFA were separately considered in terms of their % composition of total FA, as well as their absolute values (e.g., µg of MUFA mg^{–1} DW). Dragonfly growth was arcsine (sqrt)-transformed prior to use, as was the % composition of the various FA, respectively, but their absolute values were log₁₀-transformed. To test whether diet had an effect on the FA in the naiads specifically considered as essential (i.e. EFA), a multivariate GLM

with diet as a fixed categorical effect (cercariae or *Daphnia*) was used, also with trial as a fixed categorical effect. As above, the proportional values for ALA, LNA, EPA, ARA, and DHA (i.e. those considered as EFA) were first analyzed as the dependent variables, followed by a separate analysis that considered the absolute values of these EFA (μg of each FA mg^{-1} DW). Significant multivariate results were followed by univariate tests to identify the source of influential findings. Analyses were performed using IBM SPSS (version 25.0).

Estimation of cercariae EFA contributions within natural ecosystems

The annual production and total mg of five EFA (DW) by *R. ondatrae* cercariae in three California ponds were estimated using data from Preston et al. (2013), who reported annual *R. ondatrae* cercariae biomass production (DW) in each pond in $\text{mg m}^{-2} \text{yr}^{-1}$ (see Table 1). These production estimates by Preston et al. (2013) were calculated based on the seasonal period during which cercariae were found to emerge from snails (~90 days), and assumed relatively constant cercariae production during this period based on patterns in snail density, infection prevalence, and cercariae release from a related study (Paull and Johnson 2018). The subsequent calculations for pond-level EFA contributions by cercariae are summarized in Table 1, but are based on the ng of EFA per μg DW of the aggregate sample (see Results and electronic supplementary material—ESM). This approach was preferable to utilizing estimates of per cercaria levels of each EFA owing to possible error propagation stemming from the range reported for individual *R. ondatrae* cercaria size (Lambden and Johnson 2013), i.e. the variance associated with values at fine scales can result in ecosystem-level estimates with a wide range after scaling up multiple orders of magnitude (e.g., Lehrter and Cebrian 2010). Related to this, variance in pond-level cercariae EFA contributions were calculated considering the variance in annual *R. ondatrae* biomass production in $\text{mg m}^{-2} \text{yr}^{-1}$ (Preston et al. 2013) to create 95% confidence intervals using an online error propagation tool (<https://www.eoas.ubc.ca/courses/eosc252/error-propagation-calculator-fj.htm>).

Results

Cercariae and *Daphnia* fatty acid composition

Total lipid extracted from the aggregate sample of ~30,000 (± 500) *R. ondatrae* cercariae (11 mg DW) was 8.64% of the DW, allowing for an estimation of ~0.032 μg of total lipid per individual cercaria. The aggregate sample contained 45 μg of total identified FA, which provides an estimated

15.0 ng of total identified FA cercaria⁻¹. As a whole, *R. ondatrae* cercariae contained high amounts (reported as ng μg^{-1} DW of the aggregate sample, hereafter shortened to “ng”) and proportions (% of total identified FA in the aggregate), respectively, of: 16:0 (palmitic acid) at 7.8 ng (19.0%); 18:0 (stearic acid) at 5.6 ng (13.6%); 18:1n-9 (oleic acid) at 2.5 ng (6.2%), and 20:3n-3 (eicosatrienoic acid) at 2.8 ng (6.8%) (see ESM). The aggregate cercariae sample also contained the following EFA: ALA (5.0 ng, 12.3%), LNA (1.9 ng, 4.6%), EPA (2.2 ng, 5.3%), DHA (0.04 ng, 0.1%), and ARA (0.7 ng, 1.7%). FA sums indicated that SFA had the highest proportion and mass fraction of FA (17.9 ng; 43.8%), followed closely by PUFA (13.9 ng; 34.0%), and MUFA (9.1 ng; 22.2%). Finally, the aggregate sample of *R. ondatrae* cercariae contained greater proportions of n-3 FA (10.0 ng; 24.5%) compared to n-6 FA (3.9 ng; 9.6%). Per cercaria FA values can be found in the ESM (see Online Resource 1).

Gravimetric analysis revealed the total lipid extracted from the aggregate sample of 40 *Daphnia* to be $18.3 \pm 2.5\%$ of the dry weight of the extracted tissue. The *Daphnia* samples contained an average of $91.8 \pm 7.9 \mu\text{g}$ of total identified FA, which provides the estimation of 2.3 μg of identified FA per individual *Daphnia*. The aggregate *Daphnia* samples contained high amounts (hereafter reported as mean $\pm$ S.D ng μg^{-1} DW of the aggregate samples) and proportions (mean $\pm$ SD % of total identified FA in the aggregates), respectively, of: 16:0 (24.2 ± 2.4 ng, $22.3 \pm 0.5\%$); 18:0 (14.7 ± 1.7 ng, $13.8 \pm 0.9\%$), and 18:1n-9 (9.8 ± 0.9 ng, $9.3 \pm 0.4\%$) (see ESM). The *Daphnia* samples also contained all five EFA: ALA (7.7 ± 1.2 ng, $7.1 \pm 0.02\%$); LNA (6.3 ± 0.9 ng, $5.9 \pm 0.1\%$); EPA (7.3 ± 1.1 ng, $6.8 \pm 0.2\%$); DHA (1.1 ± 0.3 ng, $1.0 \pm 0.2\%$); and ARA (7.9 ± 1.3 ng, $7.2 \pm 0.5\%$). FA sums indicated that SFA had the highest proportion and mass fraction of FA (46.1 ± 4.4 ng; $43.4 \pm 1.1\%$), followed closely by PUFA (38.3 ± 5.7 ng; $35.4 \pm 1.0\%$), and finally, MUFA (22.7 ± 2.7 ng; $21.2 \pm 0.4\%$).

Dragonfly diet and growth

Diet type (*R. ondatrae* cercariae or *Daphnia*) had no effect on % growth of dragonfly naiads ($F_{1,36} = 1.032$, $p = 0.316$), indicating that *L. intacta* achieved similar growth regardless of the prey upon which they fed (Fig. 1). The mean ($\pm$ SD) % growth of naiads fed *R. ondatrae* was 4.36 ± 0.03 , and that of those fed *Daphnia* was 3.44 ± 0.03 . Naiads fed either *R. ondatrae* or *Daphnia* showed no significant difference in their % MUFA ($F_{1,36} = 1.386$, $p = 0.247$), PUFA ($F_{1,36} = 0.002$, $p = 0.962$), or SFA ($F_{1,36} = 0.308$, $p = 0.583$; Table 2) content. Similarly, diet had no effect on absolute (μg of FA mg^{-1} DW) SFA ($F_{1,36} = 1.312$, $p = 0.260$), MUFA ($F_{1,36} = 0.641$, $p = 0.428$), and PUFA ($F_{1,36} = 1.051$, $p = 0.312$) composition. Diet also had no significant overall

Table 1 Estimates of annual essential fatty acid (EFA) contributions in dry weight (DW) by *Ribeiroia ondatrae* cercariae (cerc) in three California ponds

Pond	EFA	Ng of EFA mg ⁻¹ DW of cercariae ^a	Annual cerc bio- mass production (mg m ⁻² yr ⁻¹) ^b	95% CI ^b	Mean cerc EFA (ng m ⁻² yr ⁻¹) ^c	Upper cerc EFA (ng m ⁻² yr ⁻¹) ^c	Lower cerc EFA (ng m ⁻² yr ⁻¹) ^c	Pond area (m ²) ^b	Mean total cerc EFA (mg yr ⁻¹) ^d	Upper total cerc EFA (mg yr ⁻¹) ^d	Lower total cerc EFA (mg yr ⁻¹) ^d
Sheep	ALA	5020	156	86	783,120	1,214,840	351,400	453.95	355.5	551.5	159.5
	LNA	1860	156	86	290,160	450,120	130,200	453.95	131.7	204.3	59.1
	EPA	2160	156	86	336,960	522,720	151,200	453.95	153.0	237.3	68.6
	DHA	40	156	86	6240	9680	2800	453.95	2.8	4.4	1.3
	ARA	680	156	86	106,080	164,560	47,600	453.95	48.2	74.7	21.6
Total											
Quick	ALA	5020	88	33	441,760	607,420	276,100	2679.1	691.2	1072.2	310.1
	LNA	1860	88	33	163,680	225,060	102,300	2679.1	1183.5	1627.3	739.7
	EPA	2160	88	33	190,080	261,360	118,800	2679.1	438.5	603.0	274.1
	DHA	40	88	33	3520	4840	2200	2679.1	509.2	700.2	318.3
	ARA	680	88	33	59,840	82,280	37,400	2679.1	9.4	13.0	5.9
Total											
North	ALA	5020	19	8	95,380	135,540	55,220	134.5	12.8	18.2	7.4
	LNA	1860	19	8	35,340	50,220	20,460	134.5	4.8	6.8	2.8
	EPA	2160	19	8	41,040	58,320	23,760	134.5	5.5	7.8	3.2
	DHA	40	19	8	760	1080	440	134.5	0.1	0.1	0.1
	ARA	680	19	8	12,920	18,360	7480	134.5	1.7	2.5	1.0
Total											
Total											

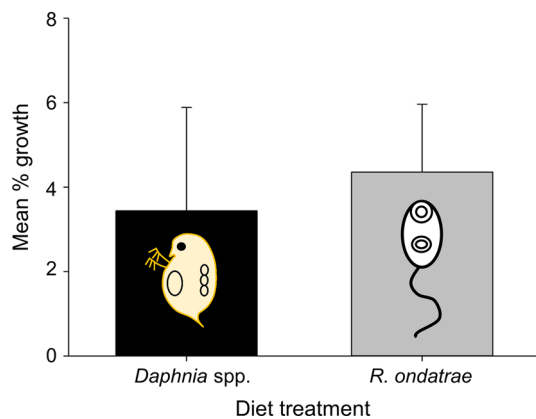


Fig. 1 Mean ($\pm$ SD) % growth (head width change) over 5 weeks for dragonfly (*Leucorrhinia intacta*) naiads ($N=39$) fed mass equivalents of two different prey (*Daphnia* spp. or *Ribeiroia ondatrae* cercariae). No significant difference was found between the two diet treatments

Table 2 Mean ($\pm$ SD) proportional (%) and absolute (μg of FA mg^{-1} DW) fatty acid (FA) and essential fatty acid (EFA) composition of *Leucorrhinia intacta* naiads ($N=39$) fed two different diet treatments

Fatty acid composition	Diet treatment	
	<i>Daphnia</i> spp.	<i>Ribeiroia ondatrae</i> cercariae
%		
SFA	21.3 $\pm$ 4.90	22.4 $\pm$ 3.44
MUFA	18.2 $\pm$ 2.07	18.1 $\pm$ 3.06
PUFA	60.5 $\pm$ 6.19	59.5 $\pm$ 5.38
ALA	15.4 $\pm$ 2.91	14.5 $\pm$ 4.53
LNA	8.0 $\pm$ 1.17	7.2 $\pm$ 2.07
ARA	2.8 $\pm$ 0.445	2.7 $\pm$ 0.882
EPA	30.2 $\pm$ 4.24	30.5 $\pm$ 8.44
DHA	0.010 $\pm$ 0.019	0.004 $\pm$ 0.003
Absolute (μg of FA mg^{-1} DW)		
SFA	3.53 $\pm$ 2.07	5.34 $\pm$ 4.62
MUFA	2.98 $\pm$ 1.52	4.08 $\pm$ 3.20
PUFA	10.2 $\pm$ 6.35	14.4 $\pm$ 14.2
ALA	2.61 $\pm$ 1.65	3.56 $\pm$ 3.80
LNA	1.37 $\pm$ 0.902	1.80 $\pm$ 1.64
ARA	0.474 $\pm$ 0.291	0.762 $\pm$ 0.952
EPA	5.10 $\pm$ 3.30	7.16 $\pm$ 6.76
DHA	0.001 $\pm$ 0.002	0.001 $\pm$ 0.002

Fatty acids grouped by type as MUFA (monounsaturated FA), PUFA (polyunsaturated FA), and SFA (saturated FA); ALA, LNA, ARA, EPA, and DHA represent select EFA

effect on % EFA content (combined ALA, LNA, EPA, DHA and ARA) of dragonfly naiads (Wilk's $\lambda=0.907$, $F_{5,32}=0.656$, $p=0.659$) (Table 2; Fig. 2a). The follow-up univariate tests also revealed no effect of diet on proportional

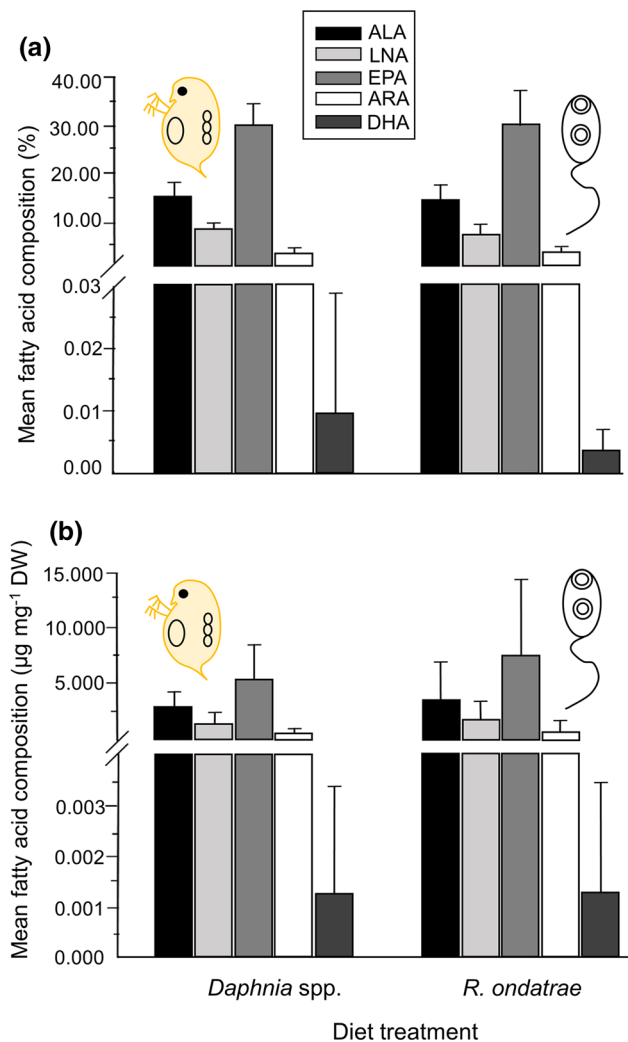


Fig. 2 Mean ($\pm$ SD) proportional (a) and absolute (b) essential fatty acid (ALA, LNA, EPA, ARA, and DHA) composition of *Leucorrhinia intacta* naiads ($N=39$) fed mass equivalents of two different prey (*Daphnia* spp. or *Ribeiroia ondatrae* cercariae)

composition for any of the five EFA when considered separately (all $p>0.253$). Diet also had no overall effect on absolute composition (ng of FA μg^{-1} DW) of EFA (Wilk's $\lambda=0.918$, $F_{5,32}=0.572$, $p=0.721$; Fig. 2b), with follow-up univariate tests also revealing no effects on ALA, LNA, EPA, DHA, or ARA (all $p>0.306$).

Cercariae fatty contributions within select California ponds

The annual (DW in $\mu\text{g m}^{-2} \text{yr}^{-1}$) production of EFA from *R. ondatrae* cercariae within Sheep, Quick, and North ponds, respectively, ranged from 93.9 to 783.1 for ALA, 34.8 to 290.2 for LNA, 40.4 to 337.0 for EPA, 0.7 to 6.2 for DHA, and 12.7 to 106.1 for ARA (see Table 1). Based on the size of each pond, this represents annual contributions as high as 1183.5 and 509.2 mg DW yr^{-1} for ALA and EPA,

respectively, in Quick pond (the largest). Estimates of cercariae EFA contributions at the pond level that incorporated error propagation based on variation in annual *R. ondatrae* cercariae biomass production at a small scale ($\text{mg m}^{-2} \text{yr}^{-1}$) resulted in a considerable range (e.g., upper and lower values of 551.5 and 159.5, respectively, for annual ALA in mg DW for Sheep pond; Table 1). Although these extrapolations serve as rough approximations of the EFA contributed by *R. ondatrae* cercariae in aquatic ecosystems, they represent the first attempts at such quantification.

Reducing or increasing annual cercariae productivity estimates by 25% gave a range of 84.3–978.9 (DW in $\mu\text{g m}^{-2} \text{yr}^{-1}$) for *R. ondatrae* cercariae ALA contributions among the three ponds, and a range of 36.3–421.2 (DW in $\mu\text{g m}^{-2} \text{yr}^{-1}$) for EPA (see Online Resource 3 of ESM). Reducing or increasing cercariae EFA content by 10% gave a range of 84.5–861.1 (DW in $\mu\text{g m}^{-2} \text{yr}^{-1}$) for *R. ondatrae* cercariae ALA contributions among the three ponds, and a range of 36.3–302.6 (DW in $\mu\text{g m}^{-2} \text{yr}^{-1}$) for EPA.

Discussion

Our findings highlight the lipid-rich nature of *R. ondatrae* cercariae, illustrating their potential to contribute significantly to the flow of EFA within aquatic ecosystems, as well as to provide these critical biomolecules to surrounding terrestrial ecosystems by way of emerging insects. By using a controlled laboratory study, we found that these nutrient-rich cercariae can support the growth of a top freshwater predator (dragonfly naiads) equally as well as if they had subsisted solely on a common zooplankter (*Daphnia*), which are considered to be important prey resources in many aquatic food webs. Finally, through an extrapolation of these data to determine annual ecosystem production values, we estimated annual EFA production (DW in $\mu\text{g m}^{-2} \text{yr}^{-1}$) by *R. ondatrae* cercariae for some representative freshwater ponds to be as high as 326.2 for ALA, 120.5 for LNA, 140.2 for EPA, 2.3 for DHA, and 44.3 for ARA. Bearing in mind that this trematode species is only one of many that are known to occur in these habitats (e.g., Preston et al. 2013), overall EFA contributions by cercariae are likely higher when considering their combined annual biomass production. As our calculations assume relatively constant cercariae production over a maximum of 90 days, more fine-scale temporal data will be helpful in refining these EFA contribution estimates, along with additional trematode- and site-specific information for extrapolation to other systems.

Our finding that each individual *R. ondatrae* cercaria contains 31.7 ng of total lipid compares favorably to a previous estimate of 13.3 ng individual⁻¹ for *S. mansoni* cercariae that are about half the size (Smith et al. 1966). Total FA levels in cercariae have been previously reported for three

other species of freshwater trematode: *Z. lunata* (160 ng cercaria⁻¹), *S. mansoni* (4.6 ng cercaria⁻¹), and *Echinostoma caproni* with 0.53 ng cercaria⁻¹ (Marsit et al. 2000a, b; Schariter et al. 2002). We report a value of 15 ng of total FA cercaria⁻¹ for *R. ondatrae*. *Ribeiroia ondatrae* cercariae are larger (~800 μm long) than those of *S. mansoni* and *E. caproni* at ~255 μm and ~400 μm , respectively (Szuroczi and Richardson 2009; Pereira et al. 2013); therefore, size could partly account for the higher levels of total lipid and FA in *R. ondatrae*, as well as in relatively large (~1200 μm) *Z. lunata* cercariae (Peoples and Fried 2008). However, it is possible that cercariae of different trematode species could vary in their FA on a per unit DW basis, and we, therefore, suggest that future investigations would be helpful in clarifying this point. The FA composition of cercariae from the marine trematode *H. elongata* has also been recently reported, with values for PUFA, EPA, and DHA similar to those found here (Fokina et al. 2018).

Individual *R. ondatrae* cercariae contained significant quantities of SFA (including palmitic [16:0] and stearic [18:0] acids), MUFA (including oleic acid [18:1n-9]), n-3 PUFA (including ALA, EPA, and DHA), and n-6 PUFA (including LNA and ARA). Smith et al. (1966) identified proportions of ARA (3.6%) and EPA (1.3%) FA within *S. mansoni* cercariae, whereas we identified ARA and EPA in proportions of 1.7 and 5.3%, respectively, for *R. ondatrae*. The precise function of each individual FA within cercariae is still speculative; however, it has been suggested that they generally play a role in buoyancy regulation, maintaining adequate energy reserves for movement, and in the management of metabolic waste products (Marsit et al. 2000a; Fried and Toledo 2009; Fokina et al. 2018).

Based on our experimental trials over 32 days, *L. intacta* naiads fed equivalent biomasses of *Daphnia* and *R. ondatrae* showed no differences in their relative growth. As importantly, their proportional and absolute FA compositions were similar for SFA, MUFA, and PUFA, as well as for EFA (i.e. ALA, LNA, EPA, DHA, and ARA). *Ribeiroia ondatrae* and *Daphnia* also contained similar absolute compositions of EFA and PUFA, which corroborates our findings in the dragonfly naiad consumers, and the potential of these two different prey items to contribute similarly to the growth of aquatic predators that consume them. Although cercariae and *Daphnia* differed in the absolute mass fractions of their individual FA, and in the amount of total lipid they contained (*Daphnia* had ~2X of each owing to their greater size), all five EFA were identified in both prey items, and these exhibited similar proportional compositions. These results indicate that *R. ondatrae* cercariae and *Daphnia* may serve as comparably nutritious prey items to aquatic invertebrates with respect to their lipid profiles. The similarities in growth and FA profiles of *L. intacta* given the two diets may also be explained by the relatively smaller size of cercariae,

their lack of a tough chitinous exoskeleton, and the absence of tail spines and/or neck teeth, which could potentially render cercariae easier to handle, consume, digest, and assimilate; however, future studies should examine such aspects, as well as consumer growth and FA profiles over longer periods of time as well.

As LC-PUFA are consumed and generally selectively retained by other aquatic organisms at higher trophic levels such as benthic invertebrates, molluscs, and fish (Kainz et al. 2004; Lands 2009; Taipale et al. 2013), they are key drivers of aquatic ecosystem productivity (Müller-Navarra 1995; Müller-Navarra et al. 2000), and it is vital to understand their dynamics at the base of aquatic food webs. For instance, zooplankton and other aquatic crustaceans only achieve optimal growth rates when consuming dietary LC-PUFA (Jones et al. 1979; Kanazawa et al. 1985), and there is a demonstrated strong correlation between EPA content of phytoplankton species and their quality as food for other aquatic predators (Müller-Navarra 1995). As such, the identification of a novel source of LC-PUFA in the form of freshwater cercariae (which had a similar FA composition to *Daphnia*) is important to understanding overall aquatic ecosystem productivity. That we found substantial quantities of EFA, including EPA and DHA, in cercariae indicates their ability to make these molecules available, pre-formed, to aquatic consumers. In addition, their potential to act as trophic upgraders via de novo synthesis of PUFA warrants investigation given that Kabeya et al. (2018) reported that various invertebrate taxa possess the genetic sequences for the necessary enzymes. This is critical because LC-PUFA like EPA and DHA provide physiologically vital functions to all aquatic animals (Kainz et al. 2004) and serve as key mediators and regulators of inflammation and immunity in vertebrates (Calder 2010).

Our findings suggest that cercariae make substantial quantities of EFA available to consumers in aquatic ecosystems based on their overall biomass, as well as their nutritional value as prey items. Importantly, cercariae can ‘unlock’ EFA from benthic environments and thus facilitate its transfer to pelagic regions. This is because cercariae develop and emerge from aquatic snails, contributing to the redistribution of EFA, and thereby strengthening benthic-pelagic coupling in these systems. These inputs could be critical as nutrient subsidies from the benthos act to stabilize population dynamics of pelagic carnivores (reviewed by Schindler and Scheuerell 2002). While it has been suggested that cercariae and zooplankton serve similar roles within aquatic ecosystems (Morley 2012), this has yet to be shown empirically. Although cercariae and zooplankton are consumed by a similar range of invertebrate and vertebrate predators (e.g., Schotthoefer et al. 2007; Thieltges et al. 2008; Kaplan et al. 2009; Orlofske et al. 2012, 2015; Welsh et al. 2017; Mironova et al. 2019), our findings demonstrate that

cercariae exhibit similar FA profiles to zooplankton and that they may, therefore, play a previously underappreciated role in transferring EFA from the base of aquatic food webs to higher trophic levels (Müller-Navarra 1995; Müller-Navarra et al. 2000). However, to further understand the potential roles of cercariae with respect to ecosystem productivity, it will be essential to carefully assess and compare the relative composition, consumption, and temporal occurrence of zooplankton and cercariae in natural systems.

When assessing the potential for cercariae to contribute to ecosystem productivity, the temporal variation in their peak abundance and that of zooplankton, such as *Daphnia*, should be taken into account. The emergence of cercariae in temperate areas generally shows a positive association with water temperature and is usually restricted to warmer summer months (Morley 2011). In contrast, freshwater zooplankton populations typically show a bloom in spring, before cercariae emergence peaks, and then decline over summer before exhibiting a resurgence in the fall (Burns 1992). As such, cercariae may serve as an important food source post-zooplankton spring bloom through to the early autumn months. Peaks of cercariae emergence vary among trematode species, and typically occur in mid-summer, but can occur anytime between late spring and early fall (e.g., Crews and Esch 1986; Schmidt and Fried 1997). This potential temporal disconnect needs further investigation, but may allow cercariae to buffer potential shortages in energy and nutrients for their predators by serving as an alternative source. Not only do cercariae represent substantial biomass in freshwater ecosystems (Preston et al. 2013; Rosenkranz et al. 2018), but also cercariae from as many as 18 different species of trematode can simultaneously occur in a single freshwater pond (Loy and Haas 2001), resulting in huge variation in their availability as prey. Detailed FA profiles of cercariae from more species, along with estimates of their annual biomass production, will be needed to better understand the broad nutritional contributions of this one free-living parasite infectious stage to aquatic ecosystems.

Our study, which represents the first attempt to quantify the contributions of cercariae to EFA production in aquatic ecosystems, estimated that *R. ondatrae* cercariae alone can contribute as much as 509.2 mg yr⁻¹ of EPA and 9.4 mg yr⁻¹ of DHA within a freshwater pond. Although our estimates are approximations, they nonetheless highlight the potential for cercariae to accumulate and transfer non-trivial quantities of EFA to higher trophic levels, identifying an important yet previously unquantified source of EFA. Moreover, emergent insects that feed on trematode cercariae, such as *L. intacta* larvae, help to transfer aquatically derived lipid subsidies from their parasitic prey onto the terrestrial landscape. Therefore, cercariae may support animal growth and development across both aquatic and terrestrial ecosystems, the latter of which often rely

on emergent insects as a transport vector of LC-PUFA (Gladyshev et al. 2009; Martin-Creuzburg et al. 2017). The transfer of aquatic nutrient subsidies can be vital to the survival of riparian predators such as lizards, birds, spiders, and bats (Kato et al. 2004; Burdon and Harding 2008; Fritz et al. 2017). From a nutritional perspective, insects (with aquatic larval stages) can represent a superior food source relative to strictly terrestrial insects as they provide consumers with essential lipid subsidies (Martin-Creuzburg et al. 2017), and cercariae may be contributing significantly to EPA and DHA stores of some emergent insects if they are consumed by aquatic larval stages. In the California ponds of focus here, Odonata are likely the most important emergent insect taxon in this context owing to their abundance (Preston et al. 2013; McDevitt-Galles and Johnson 2018), with consumption of cercariae by both dragonfly and damselfly naiads (Orlofske et al. 2012, 2015) that typically spend one summer or more in this stage (e.g., Wissinger 1988). However, future investigations should aim to determine whether cercariae are consumed by other emergent insect taxa, and to consider how the seasonality of emergent insects might both influence—and be influenced by—the availability of free-living cercariae. It will also be important to more broadly account for other ways in which cercariae EFA may be trophically transferred, especially given the variety of invertebrates and vertebrates reported as cercariae consumers to date (e.g., Schotthoefer et al. 2007; Kaplan et al. 2009; Orlofske et al. 2012, 2015; Catania et al. 2016; Vielma et al. 2018).

While contemporary research on the predation of parasite free-living infectious stages has largely focused on the consequences for transmission to the next host, as well as how their presence may affect prey selection (e.g., Orlofske et al. 2012, 2015; Thieltges et al. 2013; Catania et al. 2016), there is increasing recognition that they should be considered as prey in their own right (Vielma et al. 2018). Ours is the first quantitative demonstration that cercariae can be similar in FA composition and prey quality to zooplankton, and may, therefore, function as important nutrient- and energy-rich prey items bolstering benthic-pelagic nutrient cycling and supporting predator diets. In demonstrating that *R. ondatrae* cercariae are a significant conduit for the trophic transfer of EFA to a common freshwater predator, we provide novel evidence for a previously undescribed pathway supporting the health and productivity of aquatic ecosystems and also, through vital EFA subsidies, terrestrial ecosystems.

Acknowledgements The authors thank Travis McDevitt-Galles, Wynne Moss, Lucy Santos and Jamie Nguyen for their assistance, as well as the Blue Oak Ranch and Koffler Scientific Reserves. This work was supported by Discovery Grants from the Natural Sciences and Engineering Research Council of Canada (04537-2014 and 05566-2015, respectively) (MTA and JK), as well as the David and Lucile

Packard Foundation, the National Science Foundation (1149308, 1754171), and the National Institutes of Health (R10 GM109499) (PTJJ).

Author contribution statement KMM, JK and MTA conceived and designed the protocols and laboratory experiments. KMM performed the experiments and fatty acid analysis, as well as statistical analyses in consultation with JK, MTA, and PTJJ. All authors contributed to the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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