Oecologia
https://doi.org/10.1007/s00442-021-04944-5

ECOSYSTEM ECOLOGY – ORIGINAL RESEARCH

Parasites and their freshwater snail hosts maintain their nutritional value for essential fatty acids despite altered algal diets

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Received: 10 June 2020 / Accepted: 9 May 2021

Abstract

Despite their ubiquity and considerable biomass, the roles played by parasites in aquatic food webs are still not well understood, especially those of their free-living infectious stages. For instance, cercariae, the motile larvae of parasitic flukes (trematodes) may be a key source of nutrients and energy for consumers. As cercariae clonally reproduce within the digestive-gonadal gland complex of gastropod intermediate hosts that acquire nutritionally important polyunsaturated fatty acids (PUFA) mainly from their diets (e.g., by grazing on primary producers), cercariae could transfer snail-derived PUFA if consumed. Through fatty acid (FA) analysis, we explored whether a change in the diet of parasitized hosts altered the FA profiles of both snail-only and trematode-containing snail tissue, thereby affecting their nutritional values. Freshwater snails (*Stagnicola elodes*) infected with *Plagiorchis* sp. were fed three different diets (cyanobacteria, green algae, and diatoms) that differed in nutritional quality with respect to FA profiles. While diet influenced the overall FA composition of both snail-only tissue and snail tissue containing trematodes, levels of certain PUFA (mainly omega-3) were largely unaffected. Trematode-containing snail tissue also generally contained more PUFA relative to snail-only tissue. Notably, both tissue types had far higher levels of PUFA than found in their diets. Our results suggest that freshwater snail hosts, and possibly their associated trematode parasites, could be trophic upgraders of key PUFA despite anthropogenically induced changes in algal communities that may lead to overall diminished PUFA contents. As such, cercariae-mediated trophic transfers of PUFA may play important roles in aquatic food webs.

Keywords Trematodes · Gastropods · Algae · Essential fatty acids · Lipids

Introduction

Despite their ubiquitous presence, parasites (particularly their free-living infectious stages) have been largely neglected in discussions of food web dynamics (Marcogliese and Cone 1997; Lafferty et al. 2008). Trematodes (Phylum Platyhelminthes) have complex multi-host life cycles (Esch et al. 2002), including a larval stage situated within the digestive-gonadal gland complex (DGGC) of their molluscan first intermediate hosts (commonly aquatic snails). These larvae undergo asexual reproduction as parthenitae (sporocysts or rediae), either directly absorbing host nutrients (sporocysts) or consuming host tissue (rediae) (Kuris 1990; Esch et al. 2002). Cercariae are clonally produced within parthenitae, emerging as a motile stage into the environment to seek out their next host (Esch et al. 2002). Notably, cercariae biomass can exceed that of certain free-living taxa in many aquatic habitats (Kuris et al. 2008; Preston et al. 2013).

Due to their short lifespans and difficulty in locating a suitable second intermediate host, most cercariae are unsuccessful at transmission and have other fates (Morley 2012). It is estimated that ~ 50–80% of the total released cercariae biomass is consumed by non-host organisms, or dies and contributes to detritus (Johnson et al. 2010). Importantly, many cercariae fall within the same size range as other zooplankton (Morley 2012). This makes cercariae potentially attractive prey for aquatic predators such as copepods (Mironova et al. 2019), and insects (juveniles or adults) and fish (e.g., Orlofske et al. 2015). Trematode cercariae should, therefore, properly be considered as belonging to

Communicated by Jason Todd Hoverman.

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Published online: 18 May 2021

the zooplankton community (Morley 2012), and likely play key roles in transferring nutrients and energy throughout aquatic food webs. Thus, we argue that their nutritional and energetic properties should be examined further.

Lipids, including fatty acids (FA; especially saturated FA, or SFA) are key drivers for energy flow in aquatic food webs as they are the densest form of energy available to consumers, and play key roles in animal function, health, and survival (Parrish 2009). For instance, polyunsaturated FA (PUFA) are important constituents of biological cell membranes, contributing to their stability and function (Brett and Müller-Navarra 1997), and some are precursors of bioactive eicosanoid signalling molecules affecting hormonal and neural pathways (Twining et al. 2016). Notably, certain PUFA are considered essential because most animals are limited in their capacity to biosynthesize them (Twining et al. 2016). Essential fatty acids (EFA) are thus preferentially obtained from the diet as pre-formed molecules (Twining et al. 2016). EFA include the 18-carbon PUFA, such as α-linolenic acid (ALA, 18:3n-3) and linoleic acid (LNA, 18:2n-6), as well as long-chain PUFA (LC-PUFA; ≥ 20 carbons) for which ALA and LNA act as precursors, including eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), and arachidonic acid (ARA, 20:4n-6) (Das 2006).

Aquatic primary producers (e.g., algae and aquatic plants) are the main source of essential PUFA (Galloway and Winder 2015). These PUFA are then transferred to higher trophic levels through accumulation in the biomass of consumers (Gladyshev et al. 2009). Specifically, PUFA are selectively retained based on consumer physiological requirements and food preferences (Kainz et al. 2004; Gladyshev et al. 2009). As the FA composition of algae varies considerably by phylogeny (Ahlgren et al. 1992; Galloway and Winder 2015), consumers largely feeding on one taxonomic group of algae have a distinct FA profile following that of their diet, with different and characteristic levels of PUFA (e.g., Brett et al. 2006; Taipale et al. 2011). The composition of algal communities could therefore heavily influence food quality for multiple food-web members, especially in terms of the PUFA that can be retained by primary consumers and ultimately transferred to higher trophic levels.

Aquatic gastropods, which are commonly used by trematodes as first intermediate hosts, often feed on PUFA-rich periphyton (e.g., Dillon and Davis 1991; Lance et al. 2006), which should be reflected in snail FA composition. Snail FA profiles are somewhat dependent on dietary FA, as seen experimentally when fed non-algal diets (Fried et al. 1992). Importantly, trematodes are completely dependent on their molluscan hosts while parthenitae (Esch et al. 2002) and rely on host energy reserves (including lipids) to support their development and reproductive output (e.g., Arakelova et al. 2004). The FA composition of parthenitae thus likely resembles that of their host snails, and ultimately, host diet. Because cercariae are non-feeding (Esch et al. 2002), their FA profile should in turn reflect that of the sporocysts or rediae in which they developed (e.g., Furlong and Caulfield 1988). Critically, a recent study by McKee et al. (2020) determined that cercariae of the freshwater trematode *Ribeiroia ondatrae* have considerable amounts of omega-3 (n-3) and omega-6 (n-6) PUFA (including EPA and DHA), thereby representing a nutritional food source for consumers. Further, dragonfly larvae fed either *R. ondatrae* cercariae or *Daphnia* spp. had no significant differences in their FA profiles, including their PUFA content (McKee et al. 2020). As such, cercariae could serve as effective vectors for EFA from their intermediate hosts to consumers at higher trophic levels.

Although the FA composition of snail hosts and the larval trematodes developing within is almost certainly linked, this is likely influenced by many different factors, including host diet. Previous studies of host diet effects focused on the neutral lipids of infected snails given artificial diets (lettuce or fish food) and produced variable results (e.g., Beers et al. 1995). As such, no study has specifically examined the FA profiles of infected snails fed diets of different algae that range from nutritionally poor to rich (based on their FA content), as well as the consequent FA profiles of their trematodes. This is important to consider because common freshwater snails exhibit distinct feeding preferences, but will consume green algae, cyanobacteria, and diatoms (e.g., Dillon and Davis 1991; Lance et al. 2006; Rybak 2016).

Given this diversity of food sources, we may expect that different freshwater algae can affect the FA profiles of trematode-infected snails, especially because FA composition (and nutritional quality in terms of PUFA) varies considerably among algal taxa and cyanobacteria (Ahlgren et al. 1992; Galloway and Winder 2015). In other words, a clear contrast may be expected in the PUFA content of different purposely selected algal diets, with consequences for snail and trematode PUFA profiles. However, a number of invertebrates may have some capacity for de novo PUFA biosynthesis, including freshwater snails and select Platyhelminthes (Kabeya et al. 2018; Babaran et al. 2020). It is thus possible that trematode parthenitae maintain their PUFA profiles, and nutritional value, even when/if the diets of their snail hosts are deficient in this respect.

Here, we examined how freshwater primary producer diets ranging in FA composition affected the FA profiles of trematode-infected snails. Specifically, we selected a cyanobacteria meant to represent low EFA content, a green alga corresponding to moderate EFA content, and a diatom to represent high EFA content (Ahlgren et al. 1992; Galloway and Winder 2015). We tested the following hypotheses: (i) FA composition of snail-only tissue is significantly different (especially for EFA content) among algal diets; and (ii) sporocyst-containing snail tissue will have similar FA profiles to that of snail-only tissue, and thus also differ among diets.

Based on the trophic transfer pathway of essential PUFA described above, we predicted that infected snails on the cyanobacteria diet would have a low EFA content, those on the green algae diet would have an intermediate EFA content, and those on the diatom diet would have relatively high EFA content. As sporocysts depend on their snail hosts as a food source, the FA profiles of snail tissues (the DGGC) containing this larval stage (hereafter, trematode-containing tissue) should reflect those of snail-only tissue, as well as host diet. If diet-based differences in EFA composition were exclusively seen for the snail-only tissue, this would indicate that sporocyst (and cercariae) EFA are not affected by the diet of their host snails.

Materials and methods

Algal cultures

Starter algal cultures were ordered from the University of Waterloo's Canadian Phycological Culture Centre (CPCC). Freshwater microalgae found naturally as biofilm, and known to be rich in lipids, were preferentially selected based on CPCC recommendations and previous findings regarding the lipid content of cultured freshwater microalgae (e.g., Mata et al. 2010; Wahlen et al. 2011). In total, 3 CPCC cultures from three taxonomic groups (cyanobacteria, green algae, and diatom) were used. These were predicted to provide clear contrasts with regard to EFA content given the distinct FA profiles of different taxonomic groups (see Ahlgren et al. 1992; Galloway and Winder 2015). Specifically, we used CPCC 534 (*Synechocystis* sp.), CPCC 5 (*Tetradesmus obliquus,* formerly *Scenedesmus obliquus*), and CPCC 499 (*Nitzschia* sp.).

Techniques for algal culturing and maintenance were based on those provided by the CPCC (see Acreman 2013). All cultures were kept in sterile Erlenmeyer flasks containing sterile media that were chosen and made following standard CPCC protocols (Acreman 2013); BG-11 media was used for CPCC 534, BBM media was used for CPCC 5, and CHU-10 media was used for CPCC 499. All culture vessels were agitated manually (two times per day) rather than aerated to encourage growth. Cultures were kept under full-spectrum (448–630 nm) lights on a 14:10 light–dark cycle and at room temperature (averaging 23 °C during the culturing period).

All cultures were inoculated on a weekly basis to ensure that they remained in the exponential phase of growth, which was verified using optical density (OD) or absorbance (A) readings from a spectrophotometer (Spectronic 20 + model). The growth curves plotted the age of the culture (from the time of inoculation, in days) compared to the average OD readings at 685 nm ($n = 2$ flasks of algae per culture), and the OD was measured across the range of 600–950 nm. From this, a standard calibration curve was also constructed to determine the relationship between OD readings at 685 nm and the average algal cell density (no. of algal cells mL^{-1}), with the latter determined using a haemocytometer (Improved Neubauer Ultra Plane model).

After a week of growth, algae were harvested and stored at − 20 °C in 50 mL Falcon tubes. A few days before the start of the diet experiments, the harvested algae for each culture were thawed and transferred into 500 mL media bottles, with OD readings of each algal solution taken to confirm cell density. Algae-agar food cubes were then prepared for snails using a modified recipe from Imhof and Laforsch (2016). The recipe for 500 mL (500 food cubes) was as follows: 500 mL of algal solution, 51.2 g of agar powder, and 4.7 g of calcium carbonate powder (to maintain snail shells). The resulting paste was mixed and warmed on a hotplate at 30–50 °C, then poured into silicone trays with 1 cm^3 cells. These trays were stored at − 20 °C until use. The FA composition of each algal culture, and resulting food cubes, was later determined (see below).

Field collection and screening of snails

Freshwater snails (*Stagnicola elodes*) were collected from Brewer Park pond (45.3857° N, 75.6868° W) on two separate days in late July–early August 2019 and shipped to Ryerson University. Upon arrival, snails were transferred to aerated containers with dechlorinated tap water. Snails were screened for trematode infection using protocols adapted from Szuroczki and Richardson (2009). The majority of the collected snails were infected by a trematode within the family Plagiorchiidae. This infection was identified through external morphology of emerged cercariae (xiphidiocercariae of the armatae type), nocturnal emergence of cercariae, and use of *Stagnicola* spp. as a first intermediate host, using a standard guide (Schell 1985). The *S. elodes* infections here likely represented one genus (probably *Plagiorchis*) as the Plagiorchiidae are fairly specific with respect to snail host use (Blankespoor 1977; Schell 1985); however, genetic sequencing would be required to confirm this. Any snails with co-infections were excluded. We focused on snails parasitized by *Plagiorchis* sp. given their availability, thus "infected" refers to this specific genus from here on. Cercariae of *Plagiorchis* sp. are produced within sporocysts (Schell 1985). Infected snails were transferred into separate containers and fed a diet of raw organic spinach for 1 week before the diet experiment began.

Experimental design and protocols

The diet experiment used modified protocols from Beers et al. (1995). We fed infected snails food cubes from one of the three diets for 14 days. This duration was chosen because

Liess and Hillebrand (2006) found effects on the elemental composition of snails after 14 days of diet manipulation (N or P enrichment in periphyton), and distinct FA profiles were seen in snails fed two experimental diets for 7 days (Fried et al. 1992). We, therefore, assumed that FA profile changes could also be detected within infected snails after 14 days of exposure to algae-based diets ranging in FA content, especially because trematode-infected snails should continuously replenish their fat energy reserves during this period (Lafferty and Kuris 2009).

A total of 42 1-L glass jars were filled with dechlorinated tap water and randomly arranged on shelves of a single unit. These jars were left open but had mesh sheets to prevent snail escape. Fourteen jars (with two similarly-sized adult snails in each) were randomly assigned to one of three diet treatments: cyanobacteria, green algae, or diatom. Infected snails were transferred into these jars 1 week before the start of the diet experiment to acclimate while fed organic spinach. As pilot FA analysis had established the need to pool snail tissues to meet minimum sample weight requirements, we opted to house two snails/jar, which also ensured that food cubes were consumed fast enough to avoid water fouling.

Once the experiment began, snails were fed ad libitum with food cubes representing their specific diet (as described above), and then fed every 3–4 days to coincide with water changes. We estimated the no. of algal cells given to each snail per feeding period (no. of algal cells mL^{-1} or per food cube) based on the size of each food cube (~ 1 mL) and our measurements of algal density in the cultures. Each jar of snails was given one food cube on each feeding day. However, if the algal cell density of a specific culture (or diet) was substantially lower (as was seen in the diatom culture), then each jar with that diet was given two food cubes to ensure that all snails were receiving adequate amounts of algae. Snails were kept at ~ 24 °C on a 14:10 light–dark cycle and placed into clean jars after each water change. Jars were inspected daily, with dead snails immediately removed and kept frozen in labelled bags for later FA analysis. At the end of the feeding experiment, the remaining snails were also frozen for FA analysis.

Tissue sample preparation

To obtain tissue samples for FA analysis, snails from each diet were dissected to separate uninfected snail tissue from that containing parthenitae (Fried et al. 1998). Briefly, snails were placed in a Petri dish under a dissecting microscope and gently crushed to remove the shells. The snail body was then cut to obtain two specific portions: (i) the anterior-most portion represented only by head-foot tissue (i.e., without sporocysts); and (ii) the posterior portion containing the snail's DGGC with sporocysts (Esch et al. 2002). From here on, we consider the head-foot portion as "snail-only" tissue and the sporocyst-containing DGGC portion as "trematode-containing" tissue. Substantially degraded snails were not used for analysis ($n = 6$).

Although the nutritional value of cercariae is important, we focused on the sporocyst larval stage in which these develop for *Plagiorchis* sp. given that it is the parthenitae which derive nutrients directly from host snail tissue (Kuris 1990; Esch et al. 2002). Cercariae FA should thus strongly reflect that of the parthenitae in which they developed (e.g., Furlong and Caulfield 1988). Notably, when infected snails were given food labeled with the radioisotope ^{14}C, it was taken up by their parthenitae, and then seen in emerged cercariae within 4–5 days (Lewert and Para 1966). Transfer of snail FA to sporocysts was thus possible well within our 14-days feeding experiment. However, separating parthenitae from host tissue for FA analysis is difficult. Southgate (1970) was able to perform FA analysis on snail DGGC freed of rediae, whereas Fried et al. (1993a) could not; however, the latter found FA proportions from infected DGGC to be similar to those of separated rediae. Consequently, we did not attempt separate analyses of snail DGGC freed of sporocysts, or of sporocysts only.

After dissections, tissues were blotted dry before obtaining their wet weight, then transferred into microcentrifuge tubes. We first performed FA analysis (see below) for snails on the green algae diet. These tissues were pooled to meet minimum sample weight requirements for FA analysis. As such, each sample for snails fed the green algae cubes consisted of a single type of tissue (head-foot or DGGC) from ~ two different adult snails collected on successive days; however, while these pooled sample weights were sufficient for obtaining proportional (%) FA data, they were too low for absolute FA data. Consequently, we also pooled tissues for subsequent FA analysis of snails on the cyanobacteria and diatom diets, but each sample instead consisted of a single type of tissue from ~ 8 different adult snails. Although this reduced sample sizes, it better characterized the overall responses from the most FA-divergent diets (cyanobacteria vs. diatom) by generating absolute FA data for those samples.

This ultimately resulted in the following pooled sample sizes available for % FA data: $n = 24$ for head-foot tissue (four from cyanobacteria, four from diatom, and 16 from green algae diets, respectively); and, $n = 24$ for DGGC (four from cyanobacteria, four from diatom, and 16 from green algae diets, respectively). Pooled sample sizes available for absolute FA data were: $n = 8$ for head-foot tissue (four from the cyanobacteria, and four from the diatom diets, respectively); and, $n = 8$ for DGGC tissue (four from the cyanobacteria, and four from the diatom diets, respectively). All samples were frozen at − 80 °C prior to freeze drying, after which the dry weights were recorded. Prior to lipid

extraction, snail tissues were ground with a mortar and pestle (with additions of liquid nitrogen) to create a fine powder.

To analyze the FA profiles of the algae used to make the food cubes, algae cultured for at least 7 days (after inoculation) were transferred to 50 mL Falcon tubes and harvested by centrifugation (using modified protocols from Fuschino et al. 2011). Excess water from the samples was pipetted out, and the remaining algae pellets were transferred to microcentrifuge tubes. These samples were then frozen at − 80 °C prior to freeze drying, after which only the dry weights were recorded. Although agar was also a component of the food cubes fed to infected snails, we assumed that agar would contribute negligible FA compared to algae. To confirm this assumption, pure agar cubes were also prepared as samples for FA analysis.

Lipid extraction and FA analysis

Lipid extraction was conducted using a modified Folch et al. (1957) method. First, ~ 15–20 mg of dry tissue sample was transferred into a solvent-washed Kimax tube. The lipids in each sample were initially extracted using 4 mL of a 2:1 chloroform–methanol solution. Next, 800 µL of a 0.88% KCl solution (w v^{-1}) was added to each sample to remove any polar impurities and vortexed. A known amount of unmethylated internal standard (23:0) was also added to each sample tube (10–20 µL) and vortexed; this standard was used later to measure the methylation efficiency during the transesterification process. Each sample was then centrifuged at 2000 rpm and at 4 °C for 5 min to separate the solution into two layers: the aqueous KCl top layer and the chloroform–methanol extract bottom layer. A long Pasteur pipette was used to carefully recover and transfer the bottom layer of each sample (without disturbing the top layer) to a new Kimax tube. Each sample was re-extracted for a total of three times, with a 2 mL addition of an 86:14:1 chloroform–methanol-water solution after each centrifugation. The final extracts were then evaporated using an N_2 stream. When dry, 2 mL of hexanes was added to each sample tube. For the gravimetric analysis, two 100 µL aliquots of the total lipid extract were transferred to separate pre-weighed tin cups. The solvents were evaporated and re-weighed the next day, to provide a gravimetric estimate of total lipids.

A modified Christie (1989) method was then used to produce fatty acid methyl esters (FAME) from an acid-catalyzed transesterification reaction. First, 2 mL of a 1% H_2SO_4 in methanol solution (v v^{-1}) was added to each sample. Each sample tube was then sealed with N_2 gas and heated at 90 °C on a heating block for 90 min. After the samples cooled for 5 min, 1.5 mL of Milli-Q water and 4 mL of hexanes were added to each tube, effectively halting the reaction. The solutions were then vortexed and centrifuged at 2000 rpm and at 4 °C for 3 min, to create two distinct layers. The top layer of each sample was transferred to a new Kimax tube using a short Pasteur pipette. Each FAME sample was then re-extracted for a total of three times, with a 4 mL addition of hexanes after each centrifugation. The final FAME samples were evaporated using a N_2 stream and reconstituted with an appropriate amount of hexanes for the final volume (100–750 µL); this final volume was dependent on the organism and the estimates of total lipids in each sample (from the gravimetric analysis). A Pasteur pipette was then used to transfer each sample into labelled gas chromatograph (GC) vials. The final samples were stored at − 80 °C until analysis. FAME were analyzed using a Shimadzu GC-2010 plus that utilized a flame ionization detector and helium as the carrier gas. Depending on the estimates of total lipids in each sample, the samples were injected in split (i.e. animal tissues) or splitless (i.e. diets) mode. FAME were identified and quantified by comparing peak retention times and areas to a concentration curve of a standard FAME mix (GLC 68E, Nu-Check Prep Inc.).

Statistical analysis

FA composition of snail-only (head-foot) and trematode-containing (DGGC) tissues was obtained on a proportional (%) and absolute dry weight (µg mg^{-1} DW) basis. However, absolute data were only available for tissue samples from the cyanobacteria and diatom diets (see above). Thus, analyses on snail and parasite tissues focused mostly on proportional FA data. The FA composition (% and absolute) of food cubes from each diet were also obtained. Prior to statistical analysis, proportional FA data were square-root transformed while absolute FA data were $\log(x+1)$ transformed to assure data normality; homogeneity of variance for multivariate tests was confirmed with Q-Q plots, along with Levene's tests for homoscedasticity.

We first considered the FA composition of the food cubes. A 1-way permutational multivariate analysis of variance (PERMANOVA) was used to evaluate the effects of algae type (cyanobacteria, green algae, or diatom) on overall FA composition by considering all detected FA (37 were identified). The PERMANOVA was run upon Euclidean distance resemblance matrices and included an unrestricted permutation of data and a maximum of 9999 permutations. This was followed by a multivariate analysis of variance (MANOVA) to determine if algae type affected the total proportion of SFA, MUFA (monounsaturated FA), and PUFA present in the food cubes. Another MANOVA was conducted for the absolute DW sums for the three major FA groups. We also performed a 2-way ANOVA to examine the effect of sample type (food cube, snail-only tissue, or trematode-containing tissue) on % PUFA content across diets.

The FA composition of the tissue samples was then evaluated. We used a 2-way PERMANOVA to evaluate the main

and interactive effects of diet (cyanobacteria, green algae, or diatom) and tissue type (snail-only or trematode-containing) on sample FA composition for all 41 identified FA. After this, a separate 2-way MANOVA was performed for the FA within each of the three major groups (SFA, MUFA, and PUFA). Given that the snail tissues, not the food cubes, represented the food resources available to higher-level consumers (in our case, the parasites), we evaluated the three key FA groups in more detail, i.e. not just their total proportions or absolute sums. As such, the SFA group consisted of 9 FA, the MUFA group of 17 FA, and the PUFA group of 15 FA. Within the PUFA group, the EFA were also highlighted, including ALA (18:3n-3), LNA (18:2n-6), EPA (20:5n-3), DHA (22:6n-3), and ARA (20:4n-6).

As with the 2-way PERMANOVA, we considered the main effects of diet and tissue type (and their interaction) on % FA data for the MANOVAs, conducting post-hoc pairwise comparisons to identify how significant main effects influenced the individual FA in each group. We also conducted a 2-way MANOVA with the absolute data for the PUFA group to further analyze the composition of snail and parasite tissues from the most nutritionally divergent diets (cyanobacteria and diatom). The PERMANOVAs were done with PRIMER-E (Plymouth Routines in Multivariate Ecological Research; v.7.0.13) and PERMANOVA + add-on package, and the MANOVAs with IBM SPSS (Statistical Package for Social Sciences; version 25.0).

Results

FA and EFA characterization of host diets

The pure cultures had distinct FA profiles (see Online Resource 1 in electronic supplementary material—ESM), but we focus here on the food cubes as the composition of the latter did not fully reflect those of their respective algae, likely due to FA changes from heating while making the agar-algae mixture. Notably, the PUFA content of the food cubes was minimal relative to that in the corresponding pure culture (Online Resource 2). Within the food cubes, the proportion of PUFA (mean ± S.E.) was 1.2 (± 0.3) for the green algae diet, 1.3 (± 0.3) for the cyanobacteria diet, and 1.6 (± 0.2) for the diatom diet. In contrast, the raw cultures had PUFA content of 7.2% for green algae, 32.6% for cyanobacteria, and 5.9% for diatoms.

The PERMANOVAs indicated no overall effect of algae culture on food cube FA composition for both the % (*pseudo-F* = 1.4908, $p = 0.1148$) and absolute (*pseudo-F* = 1.8916, $p = 0.1197$) data as there were significant differences only between the cyanobacteria and diatom experimental diets (%, $t = 1.4747$, $p = 0.0454$; absolute, $t = 2.036$, $p = 0.0222$). The MANOVA for the total DW of SFA, MUFA, and PUFA also found no overall difference among the diets (Wilk's $\Lambda = 0.730$, $F_{6,50} = 1.418$, $p = 0.226$); however, diet had a significant effect on total % of each FA group (Wilk's $\Lambda = 0.567$, $F_{6,50} = 2.738$, $p = 0.022$), with % PUFA highest in the diatom food cubes. Importantly, there was a significant effect of sample type ($F_{2,69} = 858.94$, $p < 0.001$) on % PUFA because this was far lower in food cubes compared to either the snail-only ($p < 0.001$) or trematode-containing tissues ($p < 0.001$; see Fig. 1).

FA and EFA characterization of snail and parasite tissues

The following accounted for > 6% of the total FA in snail-only and trematode-containing tissues: 16:0, 18:0, 18:1n-9, 20:1, 22:4n-6, and ARA (Online Resources 3 and 4). Both tissues were generally characterized by high contents of ARA regardless of diet. The PERMANOVA indicated that the overall FA composition (%) of both tissues differed among diets (*pseudo-F* = 2.4636, $p = 0.0225$; Fig. 2). For instance, % PUFA content was highest for tissues from the green algae diet (mean 48.8 ± 0.2 S.E.), but tissues from the diatom diet had the highest SFA content (mean 23.7 ± 1.3 S.E.). FA composition also differed between tissue types (*pseudo-F* = 3.5181, $p = 0.0157$), but there was no interaction of diet and tissue type (*pseudo-F* = 1.15, $p = 0.30$). Focusing on % PUFA content, this was significantly higher in trematode-containing tissue (mean 47.4 ± 0.8 S.E.) vs. snail-only tissue (mean 46.5 ± 1.1 S.E.).

The MANOVAs indicated that diet influenced the FA composition (%) of snail-only (head-foot) and trematode-containing (DGGC) tissues within the SFA (Wilk's $\Lambda = 0.320$, $F_{18,68} = 2.906$, $p = 0.001$) and MUFA (Wilk's $\Lambda = 0.185$, $F_{34,52} = 2.027$, $p = 0.011$) categories, but not for PUFA (Wilk's $\Lambda = 0.323$, $F_{30,56} = 1.416$, $p = 0.129$; Online Resources 5–7). Tissue type influenced the FA (%) composition within the SFA (Wilk's $\Lambda = 0.373$, $F_{9,34} = 6.338$, $p < 0.001$), MUFA (Wilk's $\Lambda = 0.082$, $F_{17,26} = 17.015$, $p < 0.001$), and PUFA categories (Wilk's $\Lambda = 0.081$, $F_{15,28} = 21.071$, $p < 0.001$), with values generally higher in trematode-containing tissue, especially for PUFA. There was also a marginally insignificant interaction seen for the SFA (Wilk's $\Lambda = 0.466$, $F_{18,68} = 1.754$, $p = 0.051$).

Based on the between-subjects effects tests and pairwise comparisons within the MANOVAs, snails and their parasites were selectively retaining FA (within each FA group) as not all of these were affected by diet treatment. For example, in terms of the specific PUFA (%) composition of both snail-only and trematode-containing tissues, only docosapentaenoic acid (22:5n-3) differed among diets. In contrast, key EFA (represented by ALA, EPA, DHA) did not differ among diets for either tissue. For % PUFA, the difference between tissue types was greatest for 20:2n-6, 20:3n-6,

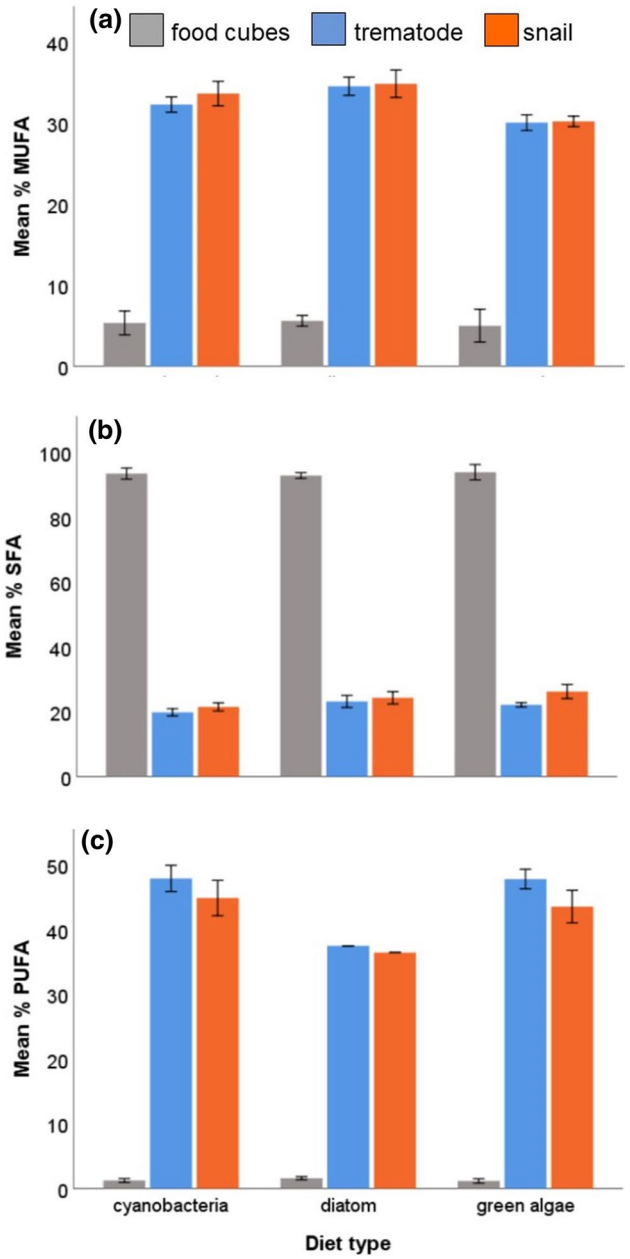

Fig. 1 Mean (±S.E.) proportion of total fatty acids (FA) in experimental food cubes ($n=30$), snail-only tissue, i.e. snail ($n=24$), and snail tissue containing trematode larvae, i.e. trematode ($n=24$), for each of the following three major FA groups: **a** monounsaturated FA (MUFA); **b** saturated FA (SFA); and, **c** polyunsaturated FA (PUFA)

20:3n-3, 22:2n-6, and 22:4n-6. Except for 22:4n-6, all of these were higher in trematode-containing tissue represented by the snail's DGGC (Table 1).

Considering absolute PUFA composition (μg mg^{-1} DW) for snails fed the two most nutritionally divergent diets (i.e., cyanobacteria and diatom), there was a marginally significant overall difference between tissue types (Wilk's $\Lambda=0.001$, $F_{1,12}=150.6$, $p=0.064$) as 20:3n-6, 20:3n-3, and 22:2n-6 were significantly higher in trematode-containing tissues (Table 1). The effect of diet was not significant (Wilk's $\Lambda=0.103$, $F_{1,12}=0.728$, $p=0.736$), nor was there a significant interaction (Wilk's $\Lambda=0.005$, $F_{1,12}=18.423$, $p=0.180$).

Discussion

The freshwater algae-based experimental diets used here varied in their overall FA composition, especially for PUFA, as did the overall FA profiles of host-only tissue from trematode-infected snails that fed on these diets, and the FA profiles of snail tissues containing the developing larval trematodes. As changes in FA profiles were seen for both tissues, this indicates that the primary producers upon which freshwater snails feed not only affect their FA composition, but also that of trematode parasites using them as hosts. Nonetheless, certain EFA (specifically, n-3 PUFA represented by ALA, EPA, and DHA) were remarkably consistent in their proportional abundance across diets for both the snail-only (head-foot) and trematode-containing (DGGC) tissues. Critically, PUFA content in both tissue types was far higher than that in their experiment diets and was greatest in trematode-containing tissues. As the latter contain parthenitae (sporocysts here) within which cercariae develop, this has implications for the potential contributions of these free-living parasite infectious stages to aquatic food webs, particularly in regard to cercariae-vectored transfer of EFA to higher trophic levels.

The FA composition of snail tissues was consistent with FA profiles characterized in field-collected freshwater snails (Fried et al. 1993b), as well as FA profiles for other freshwater molluscs (e.g., Dembitsky et al. 1992). Notably, we found that snail tissues were particularly enriched in ARA. This EFA is usually associated with benthic pathways in aquatic food webs (e.g., Feiner et al. 2018), and is generally present at higher amounts within freshwater vs. marine ecosystems (Parzanini et al. 2020). Many animals have limited capacities for ARA biosynthesis (see Twining et al. 2016). Moreover, pre-formed EFA (including ARA) are not always readily available to higher trophic level consumers given their patchy distribution across taxa, as well as the high rates of scavenging or biochemical alterations of these FA in their environment (e.g., by microbes; Galloway et al. 2013). That snails are rich in ARA is thus important considering its dietary value for many aquatic taxa. ARA-rich diets may improve growth, immunity, reproductive capacity, and survival for crustaceans and fish (reviewed by Glencross 2009). Furthermore, ARA plays an essential role in vertebrate brain development, including in fish (Stoknes et al. 2004), and ultimately to human learning and memory through fish consumption (e.g., Hadley et al. 2016).

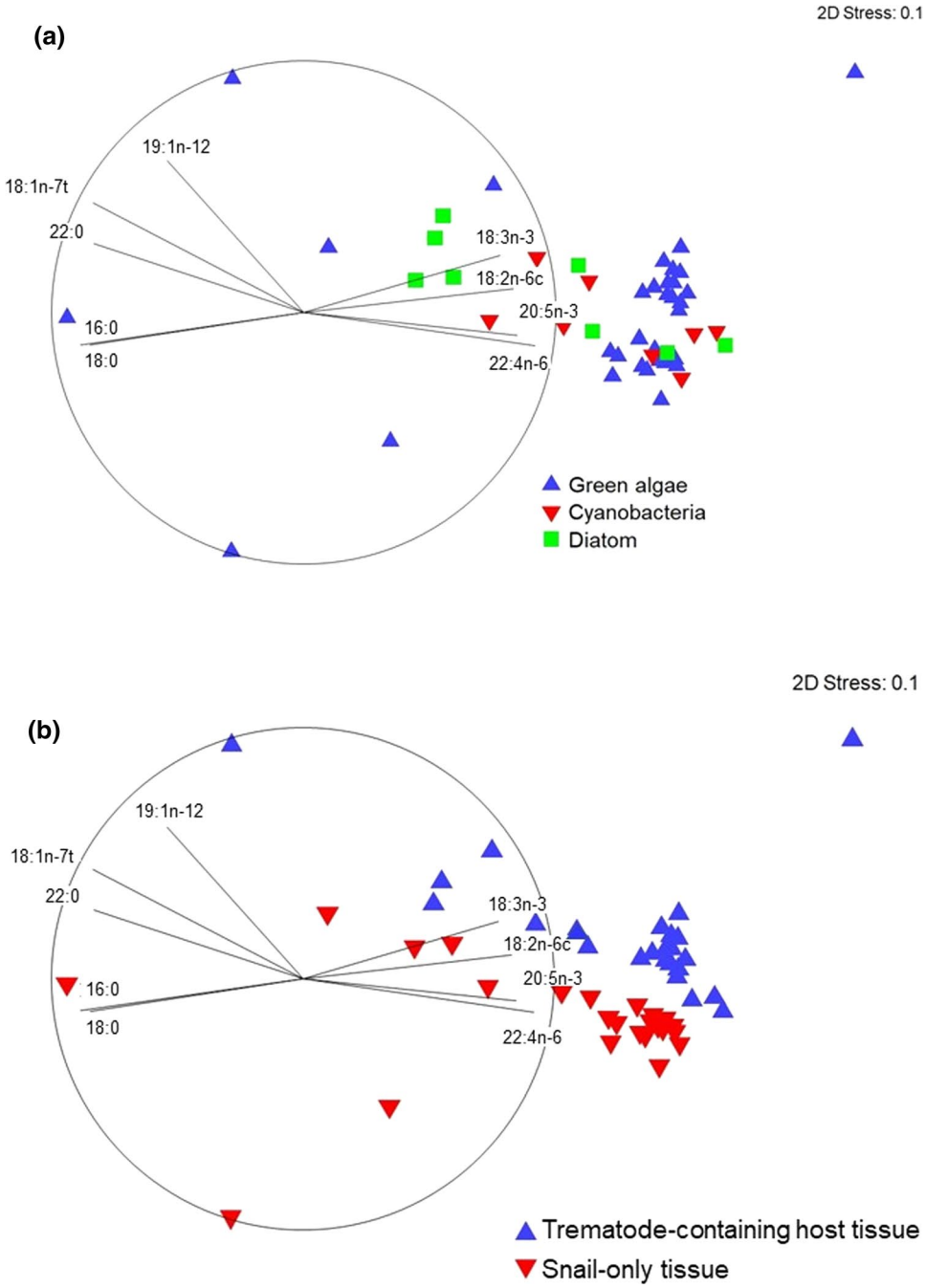

Fig. 2 Non-metric Multi-dimensional Scaling (NMDS) plots for proportional fatty acid (FA) content from tissue samples ($n = 48$) based on all 41 identified FA illustrating: **a** effects of experimental diet (diatom, cyanobacteria, and green algae); and, **b** tissue type (snail-only or trematode-containing)

Liess and Hillebrand (2006) observed significant changes in the C:P and N:P ratios of freshwater snails after 14 days of diet manipulation (N or P enrichment in periphyton), and we found compositional differences in the overall FA profiles of *S. elodes* host snails after 14 days of exposure to algal diets ranging in FA content. Our findings for *S. elodes* are also in keeping with FA changes seen in *Daphnia* spp. 6–14 days after algal diet manipulations (Brett et al. 2006; Taipale et al. 2011); however, diet did not affect all FA here. For instance, the proportions of some key EFA (ALA, EPA, and DHA) were consistent even though the food cubes were extremely deficient overall, indicating that snails were apparently able to adjust to food source quality to meet their physiological needs. Notably, *Daphnia* on different diets may selectively retain LC-PUFA such as EPA and ARA, and/or bioconvert them from their 18C-PUFA precursors (e.g. ALA, LNA) (Brett et al. 2006; Taipale et al. 2011).

To maintain LC-PUFA, snails here may have relied on stored FA reserves (Twining et al. 2016), or utilized compensatory biochemical pathways, including de novo EFA biosynthesis and/or bioconversion to counteract specific FA deficiencies. While the ability to convert short-chain PUFA

Table 1 Results for between-subjects effects tests and post-hoc pairwise comparisons (following significant main effects from 2-way MANOVAs), examining the influences of diet (cyanobacteria, C; green algae, G; diatom, D) and tissue type (parasite-containing, P; snail-only, S) on polyunsaturated fatty acid (PUFA) composition

FA measure	*df*	*F*	*p*-value	Pairwise comparisons[b]
PUFA (%)[a]				
Diet				
DPA (docosapentaenoic acid, 22:5n-3)	2	4.50	< 0.05	C > G = D*
Tissue type				
20:2n-6 (eicosadienoic acid)	1	12.75	< 0.005	P > S*
20:3n-6 (dihomo-gamma-linolenic acid)	1	60.16	< 0.005	P > S*
20:3n-3 (eicosatrienoic acid)	1	19.59	< 0.005	P > S*
22:2n-6 (13,16-docosadienoic acid)	1	20.94	< 0.005	P > S*
22:4n-6 (docosatetraenoic acid)	1	18.91	< 0.005	P < S*
PUFA (μg mg^{-1} dry weight)[c,d]				
Diet				
DHA (docosahexaenoic acid, 22:6n-3)	2	5.44	< 0.05	C < D*
Tissue type				
20:3n-6	1	20.60	< 0.005	P > S*
20:3n-3	1	25.10	< 0.005	P > S*
22:2n-6	1	11.45	< 0.01	P > S*

Only significant effects ($p < 0.05$) are shown (see ESM for additional results)

[a] $n = 48$ tissues (parasite = 24, snail = 24; cyanobacteria = 8, green algae = 32, diatom = 8)

[b] For pairwise comparisons, = indicates non-significant differences ($p > 0.05$) among diets or between sample types, with * denoting significant differences. Relatively higher or lower estimated marginal means among comparisons indicated by > or <, respectively

[c] Results exclude data from the green algae diet

[d] $n = 16$ tissues (parasite = 8, snail = 8; cyanobacteria = 8, diatom = 8)

(< 20 carbons) to LC-PUFA is not uncommon in aquatic invertebrates (Brett et al. 2006; De Troch et al. 2012), until recently, only a few organisms (typically primary producers) were known to possess the enzymatic apparatus for de novo biosynthesis of n-3 PUFA. Notably, Kabeya et al. (2018) demonstrated Δ12 (i.e., n-6) and Δ15 (i.e., n-3) desaturase activities responsible for n-6 and n-3 PUFA biosynthesis, respectively, in a wide range of invertebrates, including gastropods. As snail tissues here had far higher PUFA content than the experimental diets upon which they fed, this provides further support for their de novo biosynthesis ability.

We also found high proportions of EFA in snail DGGC tissue containing *Plagiorchis* sp., which is not surprising given that trematode parthenitae derive nutrients directly from their hosts (Kuris 1990; Esch et al. 2002). In fact, there were generally higher EFA levels in trematode-containing tissue compared to snail-only tissue. In the context of food web topology, parthenitae are at a higher trophic level as consumers than their host snails, making them analogous to predators (Lafferty et al. 2008); this means that they are positioned to retain FA from their hosts. Parasite tissues also had higher contents of certain EFA on an absolute basis within the two most nutritionally divergent diets (i.e., cyanobacteria and diatom). Parasites can alter host metabolism, energy reserves (including host lipids), and reproductive effort to support their needs (e.g., Arakelova et al. 2004; Lafferty and Kuris 2009). It is therefore possible that larval parasites could manipulate their snail hosts to increase the biosynthesis of certain EFA. However, as Babaran et al. (2020) recently reported putative sequences in some trematodes for desaturases necessary for de novo n-6 and n-3 PUFA biosynthesis, this possibility cannot be rejected here.

As noted above, snail-only tissue was particularly rich in ARA (~ 15% total composition), and similarly abundant in host tissue containing sporocysts (~ 16%). If this is similar for cercariae developing with the sporocysts, then cercariae could represent a proportionally rich source of ARA. Considering the 1.7–9.3% ARA content reported for various freshwater cladocerans and copepods (e.g., Persson and Vrede 2006; Ravet et al. 2010), this reinforces that cercariae should be considered as nutritionally important members of the zooplankton community (Morley 2012). Notably, these free-living parasite infectious stages could represent key, but heretofore unappreciated, providers and purveyors of critically important ARA within aquatic ecosystems.

In terms of snail diet affecting the composition of their trematode-containing tissue, our findings fit well with those of Narr and Krist (2015), who examined the ability of parthenitae from a plagiorchid species to acquire nutrients from hosts fed algal diets ranging in quality based on P content. They found that "high quality" host diets (i.e., high P content) rarely enhanced elemental P, C, or N composition in

trematode-containing tissue, and P levels in the latter were maintained even if snails were fed low-P diets. This suggests the parasites were able to induce nutrient enrichment in their hosts, or selectively infected nutrient-rich hosts (Narr and Krist 2015). Similarly, we found that key PUFA in snail-only tissue are also seen in their trematode-containing tissue, but can be at higher levels in the latter. This supports previous findings of more EFA in the DGGC of infected vs. uninfected snails (Fried et al. 1993a, c). As such, our results indicate that larval trematodes could be trophic upgraders of EFA, and that they may retain this ability irrespective of host diet quality. If pre-formed EFA are retained in different life cycle stages of *Plagiorchis* sp. (i.e., parthenitae and cercariae), this has various implications for aquatic food web dynamics in terms of EFA transfer through parasite consumption.

The nutritional dynamics of aquatic pelagic zones are heavily regulated by interactions with, and/or contributions from, the benthos, especially in regard to food subsidies for higher trophic-level consumers (Schindler and Scheuerell 2002). McKee et al. (2020) thus suggested a role for cercariae in EFA trophic transfer if they can "unlock" EFA from benthic snail hosts and redistribute these via consumption by pelagic predators. Parthenitae capable of producing thousands of free-living cercariae could therefore represent an unappreciated source of EFA if these compounds are generally maintained at appreciable levels regardless of their snail host's diet, especially if shifting algal communities negatively affect PUFA availability and trophic transfer. For instance, Paerl and Huisman (2008) suggest that nutritionally poor cyanobacteria will likely dominate aquatic primary production due to climate warming and/or eutrophication. Related to this, Gearhart et al. (2017) found that the LC-PUFA and total EFA content of planktivorous fish were negatively impacted in aquatic systems experiencing severe cyanobacteria blooms, presumably through bottom-up effects on zooplankton. If preferred dietary FA sources (e.g., zooplankton) become scarce or nutrient-deficient for such consumers, subsidies from cercariae could prove helpful, and such potential should be explored both qualitatively and quantitatively. Temporal dynamics are also a consideration, with further work needed to determine whether trematode parthenitae remain consistent for key EFA over longer time scales as their host snails experience varied environmental conditions.

To support our current findings, further studies with infected aquatic snails should be performed with additional and more diverse algal diets to reflect the reality that many primary consumers in freshwater systems rely on a combination of food sources, and perform optimally on mixed diets (e.g., Lundstedt and Brett 1991). In addition, our algal cultures were grown under optimal conditions. Temperature can affect algal FA composition (Fuschino et al. 2011; Hixson and Arts 2016), but so can nutrient regimes (Guschina and Hardwood 2009). Further manipulations of host diets (e.g., using nutrient-stressed algae, or algae grown continuously at suboptimal temperatures) are thus warranted to fully understand the temporal dynamics of trophic transfer of nutritionally important EFA from primary producers to host-parasite systems and subsequent consumers, including the effects of environmental variability. The primary producers used here also inherently vary in other respects, such as higher silica content in diatoms, thus possible consequences for snail and trematode FA profiles should be considered.

Future studies should also explore different snail-trematode systems, and address current assumptions that trematode-infected snails conduct FA bioconversion to adapt to both food quality and parasitism. For example, compound-specific isotope analysis of FA (Bec et al. 2011) could assess host snail abilities to synthesize EFA from food sources ranging in nutritional quality by tagging specific FA in their algal diets, as done with other aquatic taxa (e.g., Taipale et al. 2011). If the tagged algal FA are fully incorporated into snail host tissues, this technique may also be used to track the movement of host FA into trematode parthenitae, and ultimately into the cercariae developing within them. This could also be a useful approach to evaluate EFA contributions made by cercariae vs. those of other snail-derived food sources available to consumers (e.g., snail egg masses), as well as the ability of trematode parthenitae to act as "trophic upgraders" of EFA beyond that of their snail hosts.

The zoobenthos are critical to understanding ecosystem-level processes (Covich et al. 1999), and our results suggest that freshwater gastropods can serve as "trophic upgraders" of key EFA such as ALA, EPA, and DHA. Indeed, when these compounds were found to be lacking in the algae-based diets, they were nonetheless consistently present in all snails examined here. Intriguingly, host tissues containing larval trematodes may embody another level of trophic upgrading for such EFA by possessing even higher amounts. The resulting free-living parasite infectious stages could thereby represent a rich source of dietary PUFA, similar to zoospores of chytrid fungi utilizing phytoplankton hosts (Gerphagnon et al. 2019). Given the growing recognition of the negative impacts of anthropogenic disturbances on nutritionally important FA, further research on the nutritional and ecological significance of free-living parasite infectious stages within aquatic food webs will be vital to determine their contributions and roles.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00442-021-04944-5.

Acknowledgements We thank H. Roshon (CPCC), J. Rohonczy (ECCC), K. Puddephatt, R. Goldberg, T. Smith, A. Williams, and B. Schultz for experimental assistance, as well as L.C. Campbell for advice. This work was supported by Discovery Grants from the Natural

Sciences and Engineering Research Council of Canada to MTA and JK (04537-2014 and 05566-2015, respectively). We would also thank the two anonymous reviewers who provided valuable feedback on this manuscript.

Author contribution statement DB, JK, and MA conceived and designed the experiments. DB performed the experiments. DB and CP analyzed the data, and all authors contributed to writing the manuscript. The authors declare that they have no conflict of interest.

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