Journal of Animal Ecology

British Ecological Society

Journal of Animal Ecology 2016, **85,** 817–828 doi: 10.1111/1365-2656.12505

Parasite infection alters nitrogen cycling at the ecosystem scale

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Summary

1. Despite growing evidence that parasites often alter nutrient flows through their hosts and can comprise a substantial amount of biomass in many systems, whether endemic parasites influence ecosystem nutrient cycling, and which nutrient pathways may be important, remains conjectural.

2. A framework to evaluate how endemic parasites alter nutrient cycling across varied ecosystems requires an understanding of the following: (i) parasite effects on host nutrient excretion; (ii) ecosystem nutrient limitation; (iii) effects of parasite abundance, host density, host functional role and host excretion rate on nutrient flows; and (iv) how this infection-induced nutrient flux compares to other pools and fluxes. Pathogens that significantly increase the availability of a limiting nutrient within an ecosystem should produce a measurable ecosystem-scale response.

3. Here, we combined field-derived estimates of trematode parasite infections in aquatic snails with measurements of snail excretion and tissue stoichiometry to show that parasites are capable of altering nutrient excretion in their intermediate host snails (dominant grazers). We integrated laboratory measurements of host nitrogen excretion with field-based estimates of infection in an ecosystem model and compared these fluxes to other pools and fluxes of nitrogen as measured in the field. Eighteen nitrogen-limited ponds were examined to determine whether infection had a measurable effect on ecosystem-scale nitrogen cycling.

4. Because of their low nitrogen content and high demand for host carbon, parasites accelerated the rate at which infected hosts excreted nitrogen to the water column in a dose–response manner, thereby shifting nutrient stoichiometry and availability at the ecosystem scale. Infection-enhanced fluxes of dissolved inorganic nitrogen were similar to other commonly important environmental sources of bioavailable nitrogen to the system. Additional field measurements within nitrogen-limited ponds indicated that nitrogen flux rates from the periphyton to the water column in high-snail density/high-infection ponds were up to 50% higher than low-infection ponds.

5. By altering host nutrient assimilation/excretion flexibility, parasites could play a widespread, but currently unrecognized, role in ecosystem nutrient cycling, especially when parasite and host abundances are high and hosts play a central role in ecosystem nutrient cycling.

Key-words: metabolism, stoichiometry, trematode, metacercaria, nutrients, biogeochemistry, phosphorus, nitrogen, parasite

Introduction

Ecosystems are organized around the availability and cycling rates of crucial macronutrients, namely carbon (C), nitrogen (N) and phosphorus (P) (Elser *et al.* 2000). Nutrient stoichiometry and cycling rates affect many properties of communities and ecosystems, including food chain lengths, primary and secondary production and species diversity (Elser *et al.* 2000; Cross *et al.* 2006; Hall 2009). Importantly, however, individual species also have

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the potential to regulate ecosystem-level nutrient cycling by regulating crucial pathways for limiting nutrients (Croll *et al.* 2005; Bardgett *et al.* 2006; Kurten *et al.* 2008). Single-species control over ecosystem-level nutrient cycling occurs when species have unique physiological or life-history traits that affect the release or accumulation of a limiting nutrient in the ecosystem – such as nitrogen-fixing plants (Kurten *et al.* 2008), stress-inducing predators (Hawlena & Schmitz 2010), or through high abundances (Hall, Tank & Dybdahl 2003).

Despite repeated calls, the effects of parasites on ecosystem processes remain largely unexplored (Raffel, Martin & Rohr 2008; Loreau 2010; Hatcher, Dick & Dunn 2012). Yet, their potential importance is strongly suggested by the ways in which introduced pathogens can alter communities and ecosystems [e.g. chestnut blight (Anagnostakis 1987), rinderpest (Sinclair 1979) and amphibian chytridiomycosis (Kilpatrick, Briggs & Daszak 2010)]. While such invasive pathogens can drive obvious change, the 'hidden' roles of endemic parasites within their native systems are harder to isolate – although they may often be just as important. Beyond their direct effects on host mortality and population growth, parasites may alter ecosystem processes through more subtle changes in host physiology (Bernot & Lamberti 2008), behaviour (Lafferty & Morris 1996) or nutrient excretion (Becker 1980; Bernot 2013). Sato *et al.* (2012), for instance, found evidence for indirect effects of parasitic nematomorph worms on nutrient cycling in streams through host behaviour modification. Infected crickets were more likely to fall into streams (where the worms breed) and be eaten by trout, with cascading effects for benthic invertebrates and autotrophs. Similarly, acanthocephalan infection of aquatic isopods reduced host consumption of leaf litter by nearly 47%, ultimately slowing litter decomposition rates (Hernandez & Sukhdeo 2008). Parasite effects on ecosystem-level energy and nutrient flows are likely to vary depending on host functional role(s), the relative size of infection-induced nutrient fluxes and existing ecosystem nutrient demands.

One parasite group that is particularly likely to influence nutrient cycling in aquatic ecosystems is the digenetic trematodes. Not only are these organisms extremely common in terrestrial–aquatic ecotones, with a biomass density fivefold larger than other parasite groups (and comparable to that of insects) (Kuris *et al.* 2008; Preston *et al.* 2013), but growing evidence indicates that larval trematodes often cause significant changes in host metabolism and nutrient excretion stoichiometry in their aquatic snail intermediate hosts (Becker 1980; Tunholi *et al.* 2011). For instance, Bernot (2013) found that infected snails' excreta had higher N : P ratios than did uninfected snails. Such snails can be dominant consumers in benthic ecosystems and regulate nutrient flows through both grazing effects and excretion/egestion (McCormick & Jan-Stevenson 1989; Hall, Tank & Dybdahl 2003; Evans-White & Lamberti 2005). To date, however, few studies have evaluated the potential for parasites to regulate nutrient fluxes via their effects on host metabolism and nutrient processing.

Combining ecological stoichiometry with metabolic theory provides a helpful framework to evaluate parasite effects on ecosystem-level energy and nutrient flows through host–parasite interactions (Allen & Gillooly 2009). For many consumers, there is often an optimum body nutrient stoichiometry (C : N : P) for growth, maintenance and reproduction (Sterner & Hessen 1994; Hall 2009). This body stoichiometry determines the ratios at which consumers assimilate various nutrients from their food and release nutrients through their excreta/egesta (McCormick & Jan-Stevenson 1989; Elser *et al.* 1995; Evans-White & Lamberti 2005). It is expected that parasites assimilate nutrients from their hosts at ratios sufficient for their own growth and development, regardless of host stoichiometry. Therefore, any observed changes in host nutrient excretion stoichiometry caused by the onset of infection should be consistent with the host–parasite stoichiometric mismatch. The addition of parasites is also expected to units (MUs – mitochondria, ribosomes, etc.) in infected hosts [organism mass $\propto$ 1/MU density; (Allen & Gillooly 2009)], thus increasing demand for metabolically available C and P [P-rich MUs – growth rate hypothesis; (Elser *et al.* 2003)] in the modified host–parasite phenotype. The stress of infection may also exact a metabolic cost, similar to predator-induced stress (Hawlena & Schmitz 2010).

Here, we combined field, experimental and modelling approaches to investigate the direct links between endemic parasite infections and N cycling. By linking field data from 18 N-limited (Mischler, Taylor & Townsend 2014) eastern Colorado ponds (harbouring varying parasite loads) with host-level excretion assays (using field-caught snails), we evaluated the mechanisms through which endemic parasites contribute to ecosystem-scale N cycling. These data were then used to parameterize an empirical model comparing estimated parasite-induced N fluxes (per volume) to other pools and fluxes of N to evaluate the magnitude at which parasites could conceivably alter ecosystem-level N cycling in our study ponds. Our results indicate that parasite-induced host N release can be equivalent to water column N fixation (and in some cases hydrologic input), even in agriculturally impacted ponds.

Materials and methods

STUDY SITE

Data were collected within adjacent groups of shallow ponds in Eastern Colorado – Andrick Ponds State Wildlife Area/The Teal Hunting Lodge (40° 22′16.77″ N, 104° 06′24.89″ W) and the Brush Prairie Ponds State Wildlife Area (40° 12′46.68″ N, 103° 38′37.53″ W). These shallow ponds are replenished via irrigation ditches. Aside from parasite load, the 18 ponds included in the

study are biotically/abiotically similar (Mischler, Taylor & Townsend 2014). The ponds fill to capacity in the late spring and gradually evaporate throughout the summer with occasional water inputs from the ditch. Field measurements were constrained to dates between the end of May and the end of August (2010, 2011 and 2012) because of Colorado Division of Wildlife access restrictions.

PARASITE LIFE CYCLE AND INFECTION DATA

We focused on the metacercaria stage of the digenean trematode *Cotylurus flabelliformis* (Cort, Brackett & Olivier 1944), because they are native and abundant and parasitize dominant benthic consumers (aquatic snails). *Cotylurus flabelliformis* has a complex life cycle. Eggs hatch into miracidia which infect aquatic lymnaeid snails. The parasite develops into a sporocyst within the snail (the parthenitae stage), reproduces asexually and generates free-swimming cercariae. These short-lived cercariae exit the snail and penetrate a second intermediate host, which is also a lymnaeid snail, and develop into metacercariae through a period of rapid growth and maturation, absorbing and sequestering host-derived nutrients (Cort, Brackett & Olivier 1944). Once developed, the metacercariae continue to metabolize and excrete within the second intermediate host until ingested by the definitive host [waterfowl Campbell (1973a)], develop into mature worms, reproduce sexually and lay eggs that are passed via faeces into water bodies.

During August 2010 and 2011, we collected lymnaeid snails around the perimeter of each of the 18 ponds in our study area. Snails were collected using 1-m dip net sweeps every ~20 m around the circumference of each pond to determine snail densities. Fifty snails were randomly selected per pond for evaluation of trematode infection. In 2011, if fewer than 50 snails were collected, we haphazardly collected additional snails by hand so that at least 50 snails were collected per pond. Snails were dissected within 36 h of collection by crushing the shell and carefully teasing apart the tissue. *Cotylurus flabelliformis* metacercariae loads were determined using a dissecting microscope. Mature parthenitae infections from other trematodes were also noted.

We collected *Stagnicola elodes* snails (n = 115) in August 2012 from a single pond at Andrick to determine how variations in *C. flabelliformis* metacercariae abundances within snails (0 to 450) affected snail nutrient storage and excretion. Snails were hand-selected within the same size class by length (mean = 22·22 ± 2·33 mm) and immediately placed individually within acid-washed and DI-rinsed 60-mL centrifuge tubes with 40 mL of Whatman GF/F-filtered pond water. We maintained water-filled tubes csontaining snails and snail-free controls (water only) at epilimnetic temperatures (21–25 °C) in the dark for 3 h and then removed the snails. Water was filtered (GF/F) and analysed for dissolved nutrients [total dissolved organic carbon (TDOC), total dissolved nitrogen (TDN), dissolved inorganic nitrogen (DIN), soluble reactive phosphorus (SRP) and total dissolved phosphorus (TDP); Supporting Information (SI)], while snails were dissected to quantify infection abundance as above. Snails co-infected with metacercariae and parthenitae were omitted from the analyses to avoid confounding effects of parthenitae on snail excretion. Additionally, snail foot tissue and metacercariae were retained for nutrient measurements (%C, %N, %P; SI). Faeces egested during the 3 h excretion experiment were also analysed for nutrient content (SI).

NUTRIENT ANALYSES

We filtered weekly surface water samples (GF/F) from pond benthic areas into acid-washed polypropylene containers to determine the evolution of nutrients in individual ponds over the 2011 growing season and measured conductivity, pH, temperature and Secchi depth in situ at each pond. Periphyton biomass was calculated based on the mass of periphyton collected from three 5-cm-diameter pvc pipes placed randomly in each pond and allowed to accrue periphyton for 30 days. Periphyton samples for nutrient analyses were obtained by placing movable substrates (wood, macrophytes, dead reeds) and associated periphyton (collected every 30 m) into plastic bags with DI water and shaking them for 30 s to force periphyton into suspension. For ponds with circumferences larger than 300 m, we collected 10 equally spaced samples around the perimeter. Periphyton was filtered onto a GF/F, dried and evaluated for %C, %N and %P (SI). Single 1·5-L composite seston samples from each pond (pre-filtered with 153-μm Nitex mesh) were filtered onto GF/Fs, dried and also analysed for %C, %N and %P (SI). Snail foot tissues were ground to a powder and analysed for %C, %N and %P (SI). We collected metacercariae from snails, washed them in DI water and divided them into three different-sized subsamples (371, 209 and 94 metacercariae) and analysed each for %C, %N and %P (SI). Different-sized subsamples were used to ensure the data fell within the standard curve. Snail faeces were ground and analysed for %C and %N (SI). Water containing snail excretions was analysed for TDOC, TDN, SRP and DIN (SI).

We evaluated pond nutrient limitation using a nutrient-enrichment bioassay experiment modelled after Elser *et al.* (2009) in which nitrogen, phosphorus and nitrogen and phosphorus together were added to 250-mL bottles filled with pond water (4 replicates each, 16 per pond; SI). Bottle assays were incubated in the laboratory at a constant temperature (25 °C to 26 °C) under a 16-h light : 8-h dark cycle for 5·5 days, after which all seston was filtered and evaluated for Chl *a*. Additionally water column N fixation rates were measured via the acetylene reduction method (Flett, Hamilton & Campbell 1976) by incubating pond water (30–40 μmol m^{-2} s^{-1} at 25 °C to 26 °C) within polypropylene syringes (3 replicates, 1 control) for 3 to 4 h (Scott, Doyle & Filstrup 2005; Bradburn, Lewis & McCutchan 2012) and measuring ethylene concentrations (SI).

ANALYSES

Our analyses were intended to: (i) determine the limiting nutrient in these ponds; (ii) identify the effects of infection on host nutrient storage and excretion; and (iii) compare observed fluxes of the limiting nutrient from infection-altered host excretion to other pools (water column DIN) and fluxes (water column N fixation, hydrologic delivery via the ditch) to evaluate the impact of *C. flabelliformis* on nutrient cycling at both the level of small patches of benthic habitat (<1 m^2) as well as the ecosystem level. We compared organism-level nutrient excretions to ecosystem-level nutrient pools and fluxes on a per volume basis (mg L^{-1}). All statistical analyses were conducted in R 3.0.1 (R Development Team 2013). We applied transformations as needed to satisfy assumptions of equal variance and normality. Uncertainties are ±1 SE about the mean.

Assessment of nutrient limitation

We determined which nutrients were likely limiting in these pond systems by comparing field-derived values to nutrient thresholds from the literature [water column DIN : TDP – (Morris & Lewis 1988); periphyton and seston N : P – (Hillebrand & Sommer 1999)] and by conducting nutrient addition bioassays (SI). Bioassay results were analysed using a two-way ANOVA and subsequent post hoc pairwise comparisons to determine significant responses to N and/or P nutrient addition as well as interactions ($\alpha = 0{\cdot}05$) following Elser *et al.* (2009) as described in Mischler, Taylor & Townsend (2014).

Analysis of laboratory excretion trials

We constructed three general linear models to determine how well metacercariae abundance could predict: (i) host TDOC excretion (square root transformation, normal error distribution); (ii) host DIN excretion (normal); and (iii) host SRP excretion (gamma error distribution). Each of these models included snail shell length as an additional explanatory variable to take into account how slight variations in snail size may affect nutrient excretion. Similarly, we constructed general linear models with normal error distributions to determine how well metacercariae abundance could predict snail foot %C, %N and %P as well as snail faecal %C, %N and C : N, again using snail shell length as an additional explanatory variable. These data were analysed as continuous variables and displayed as categorical variables (high and low infection determined by median metacercariae abundance) to more easily illustrate the underlying statistical relationships. Snail and parasite tissue data were analysed using a one-way ANOVA and subsequent post hoc pairwise comparisons to determine significant differences in tissue stoichiometry between uninfected snails, lightly infected snails (below median), heavily infected snails (above median) and the parasites themselves ($\alpha = 0{\cdot}05$). Sources of variation among these snail-based data include the following: the original periphyton nutrient content consumed by snails, snail size, environmental temperature (varies snail excretion rates and parasite development rates) and the developmental state of the parasite (SI).

Comparison of nutrient fluxes

Using our pond-averaged values for benthic water column DIN concentrations, benthic water column N fixation rates, hydrologically supplied DIN from the ditch, and DIN excretions from infected snails, we constructed a simple empirical model comparing DIN fluxes through a typical benthic water volume for each of the ponds. All fluxes were converted to per volume (mg L^{-1}) fluxes; we used our field-derived and pond-averaged metacercariae abundances (0 to 16 parasites per snail per pond in 2011) and individual dip net snail densities (0 to 414 snails per m^2). Changes in N excretion rates per parasite were estimated using the linear model developed in the excretion trials ($9{\cdot}6 \times 10^{-05}$ mg of DIN per metacercaria per day). Snails per unit area measurements were converted to snails per unit volume by applying a uniform benthic water depth of 0·1 m, consistent with measurements made during field collection.

Standing stock DIN was parameterized as the mean of DIN across ponds for the entire season ($0{\cdot}1 \pm 0{\cdot}008$ mg L^{-1} DIN) and daily fluxes of N from N fixation (0·0009 mg N L^{-1} day^{-1}) and the irrigation ditch (early season = 0·014 mg N-DIN L^{-1} day^{-1}, late season = 0·0022 mg N-DIN L^{-1} day^{-1}) were also included. We estimated ditch DIN fluxes per volume by assuming that 80% of total evaporation (~22% of water volume per month) is replaced by ditch water with known DIN concentrations. This is a conservative estimate based on the gradual decline of pond volumes throughout the season and the evaporation rate derived from the May–September average of pan measurements from a nearby site. The daily N flux (per volume) from N fixation was estimated by taking laboratory-derived N fixation rates and assuming a daily 10 h window of daylight.

Pond-scale field patterns

Initial data collected during 2010 were analysed using multiple regression to determine the relationship between the distribution of *C. flabelliformis* metacercariae and various environmental variables expected to affect infection (water column DIN, total dry snail biomass per m^2 and total pond area). We used a full-factorial modelling approach (with average metacercariae load per snail per pond as the response variable), to select the most favourable model (based on Akaike weights). These results were used to guide 2011 field collections and analyses. In 2011, mean periphyton %N was measured in addition to the variables measured in 2010 and analysed via a multiple regression, this time using the ratio of water column DIN : periphyton %N (log base-10-transformed) as the response variable and metacercariae abundance and snail biomass as explanatory variables; we again applied a model selection procedure. In addition, we combined 2010 and 2011 data in a linear mixed-effects model (normal error distribution, site = random effect) to evaluate whether any variables (average metacercariae load per snail, year, mean snail biomass per area and mature parthenitae prevalence) had a consistent effect on water column DIN (log-10 transformation) across years. Periphyton biomass was compared to snail density, periphyton %N, water column DIN and abundance of metacercariae via Pearson's correlation coefficient.

Results

NUTRIENT CYCLING

Results of field measurements of pond nutrient conditions and short-term assays indicated that ponds were generally N-limited. In most ponds, the ratio of DIN : TDP decreased strongly throughout the season (Fig. 1a) due to a decline in DIN concentrations (Fig. 1b), indicating the development of stronger N limitation conditions. Seston and periphyton commonly exhibited luxury uptake (Hillebrand & Sommer 1999) of excess P (Fig. 1c), which further supports an assessment of N limitation. In experimental bottle assays, 12 of the 13 ponds that responded to nutrient addition exhibited some degree of N limitation. N fixation rates averaged $0{\cdot}065 \pm 0{\cdot}004$ μ L^{-1} day^{-1} across ponds, consistent with adjacent water bodies (Bradburn, Lewis & McCutchan 2012). Further information is described by Mischler, Taylor & Townsend (2014).

Water column nutrient conditions were consistent in indicating N limitation/N and P colimitation (Fig. 1a,b). DIN : TDP ratios of ditch supply water were within N

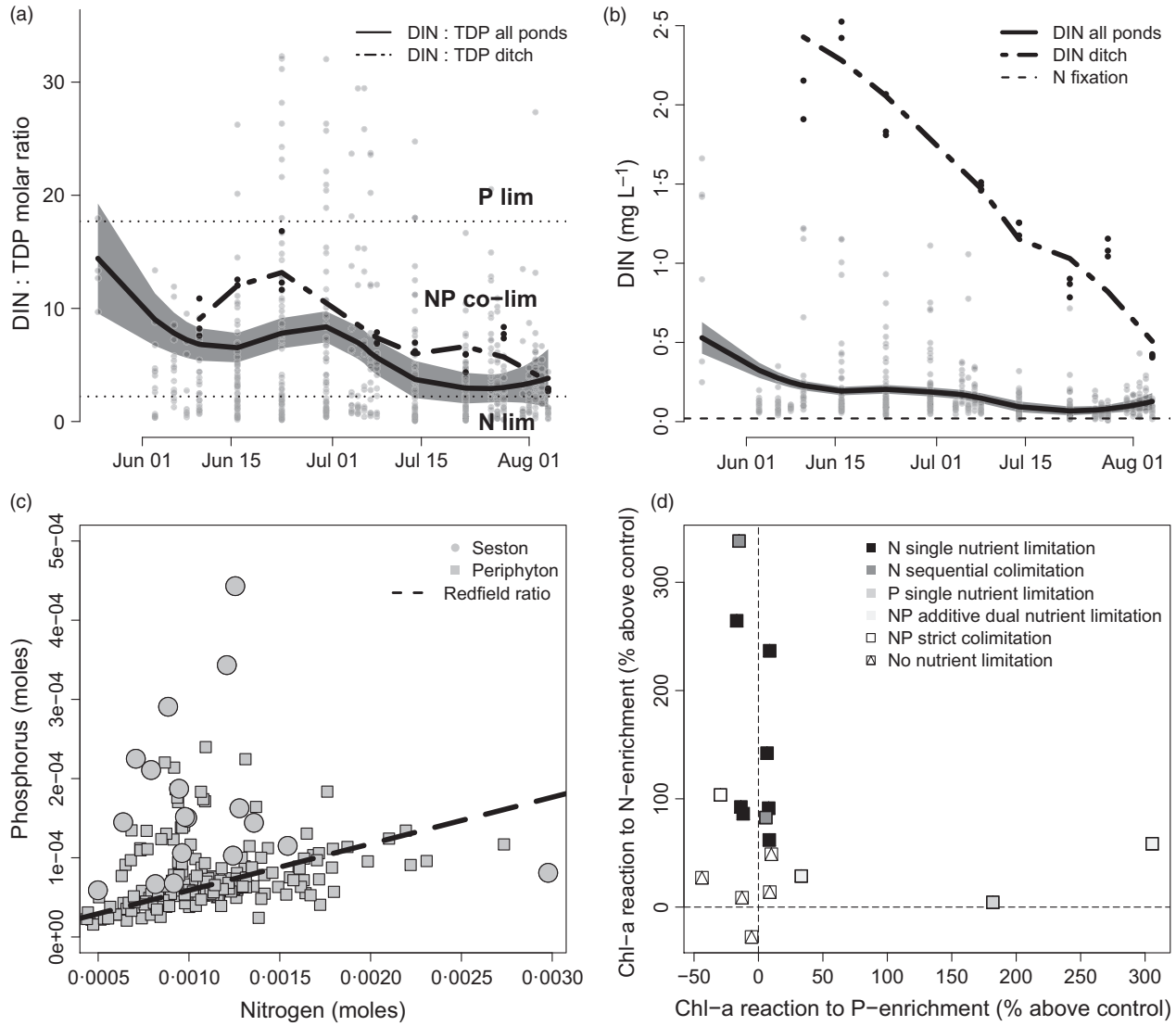

Fig. 1. Evidence indicating widespread high nitrogen demand across our study ponds. The four lines of evidence represented emerge from (a) water column DIN : TDP stoichiometry (N = 573), (b) water column DIN concentrations (N = 573), (c) periphyton and seston N : P stoichiometry (N = 190; N = 18), and (d) the results from nutrient addition bottle assays (N = 26). In (a) water column DIN : TDP stoichiometries (molar ratio) from each pond (black line with grey shading denoting ±2 SEM) and the ditch (dashed line) across the growing season (JJA) are compared to nutrient limitation thresholds from Morris & Lewis (1988) (thin black horizontal dashed lines). These comparisons indicate ponds are either experiencing NP colimitation (DIN : TDP between 18 and 2·2) or N only limitation (DIN : TDP less than 1) across the growing season. In (b) water column DIN concentrations from each pond (black line with grey shading denoting ±2 SEM) and the ditch (dashed line) across the growing season are compared to the nitrogen fixation threshold determined by Bradburn, Lewis & McCutchan (2012) in adjacent systems (thin black dashed horizontal line); nitrogen fixation is suppressed by DIN concentrations above 0·02 mg L^{-1}. DIN concentrations steadily decrease in both the ponds and the ditch due to high biological N demand. In (c) periphyton (squares) and seston (circles) molar N : P stoichiometries are compared to the modified Redfield ratio (dashed red line) of Hillebrand & Sommer (1999) illustrating that P is in relative excess compared to N in seston and periphyton. In (d) the results of a nutrient addition bioassay using water from 18 of these ponds indicates widespread stimulation of seston by either N or N and P addition according to the methods of Elser *et al.* (2009). Data contained in this figure are from Mischler, Taylor & Townsend (2014).

and P colimitation thresholds throughout the growing season (Fig. 1a) and in most ponds the DIN : TDP ratios strongly decreased throughout the season (Fig. 1a) driven by a decline in DIN concentrations (Fig. 1b), indicating the development of stronger N limitation conditions. Seston and periphyton commonly exhibited luxury uptake (Hillebrand & Sommer 1999) of excess P (Fig. 1c). In experimental bottle assays, 12 of the 13 ponds that responded to nutrient addition exhibited some degree of N limitation. N fixation rates averaged 0·065 ± 0·004 μmol L^{-1} day^{-1} across ponds, consistent with adjacent water bodies (Bradburn, Lewis & McCutchan 2012). Further information is described by Mischler, Taylor & Townsend (2014).

FIELD INFECTION DATA

Metacercariae were stoichiometrically distinct from their snail hosts, with metacercariae tending to be C-rich and N-poor compared to snail hosts (parasite C : N : P = 100 : 13 : 1, host C : N : P = 80 : 16 : 1; Fig. 2a–c). Per cent N was significantly higher in uninfected snails vs. metacercariae while C : N was significantly lower in all snails compared to metacercariae (uninfected snail N, C : N = 11·1 ± 0·4, 3·7 ± 0·05; parasite N, C : N = 7·0 ± 0·7, 7·8 ± 0·2; $P < 0·05$; Fig. 2b,c). Among infected snails, host foot tissue %N decreased with increasing metacercariae abundances (Fig. 2b – R^2=0·09, $P = 0·007$, slope = −0·004258 ± 0·001, residual SE = 1·8), inducing a corresponding increase in infected snail tissue C : N with increasing infection (Fig. 2c – $R^2 = 0·12$, $P = 0·001$, slope = 0·000129 ± 0·000048, residual SE = 0·06). The within pond distributions of snails were patchy, with densities of over 400 snails per m^2 in some productive benthic areas. Average snail densities ranged from 0 to 123 snails per m^2 per pond. The average number of metacercariae per snail per pond ranged from 0 to 16 in 2011, with one pond surveyed in 2012 reaching 165 metacercariae per snail; metacercariae prevalences averaged almost 40%, while parthenitae prevalences (the first-stage parasite within the intermediate host) averaged less than 10%.

In the excretion trials, higher *C. flabelliformis* metacercariae abundances corresponded to decreased excretion of C (as total dissolved organic C; Fig. 3a – $R^2 = 0·09$, $P = 0·005$, slope = −0·0006 ± 0·0002, residual SE = 0·20) and increased host N excretion rates (as dissolved inorganic N; Fig. 3b – $R^2 = 0·09$, $P = 0·005$, slope = $3·8 \times 10^{-06} \pm 1·2 \times 10^{-06}$, residual SE = 0·001), resulting in significantly lower C : N ratios in the excreta of highly infected snails (Fig. 3c – $R^2 = 0·10$, $P = 0·004$, slope = −0·0017 ± 0·0004, residual SE = 0·58). There were no significant effects of infection on faecal %C, %N and C : N (Fig. 3d–f); faecal C : N was 11% higher than periphyton C : N. Host shell length was not significantly associated with host tissue, excretion or faecal nutrients in any of the GLMs, nor did it interact with infection status to influence the response variables. Phosphorus (as soluble reactive phosphorus) excretion was also reduced with increasing infection (Fig. S1, Supporting Information, $R^2 = 0·06$, $P = 0·02$).

EFFECT OF PARASITISM ON ECOSYSTEM-WIDE NUTRIENT CYCLING

In 2011, additional daily DIN released via infection was estimated to be up to 6% of mean pond DIN standing stock in some benthic areas (based on average pond infection abundances and per dip net snail densities) (Fig. 4), with considerable spatial variability within ponds. Parasite-induced N fluxes were over two and a half times that of the ditch late in the season and over six times that of water column N fixation for the highest dip net sweeps.

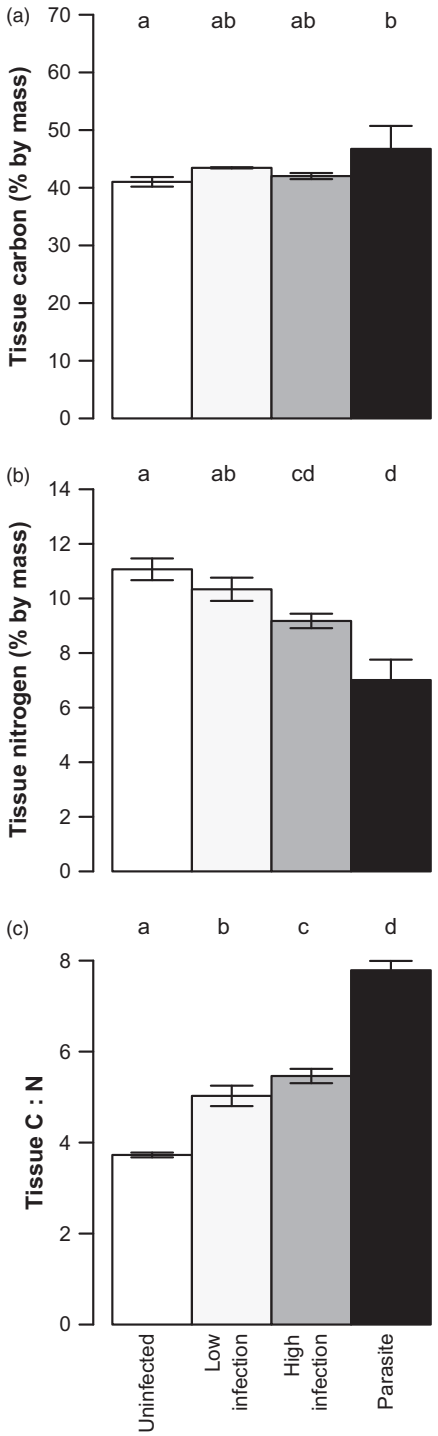

Fig. 2. Nutrient data indicating a stoichiometric mismatch between host snails and *Cotylurus flabelliformis* metacercariae as well as the effect of infection on host stoichiometry. Columns represent the mean (±1 SEM) nutrient stoichiometry values for the following: (i) uninfected snails (N = 10); (ii) hosts with parasite abundances less than the median (N = 58); (iii) hosts with parasite abundances greater than the median (N = 34), and the parasites themselves (N = 3 pooled samples, 674 parasites total) for carbon (a), nitrogen (b) and the C : N ratio (c). Letters indicate significant differences between groups as determined by a one-way ANOVA with associated post hoc tests ($P < 0·05$). All percentages are based on dry mass and all ratios have been converted to molar ratios.

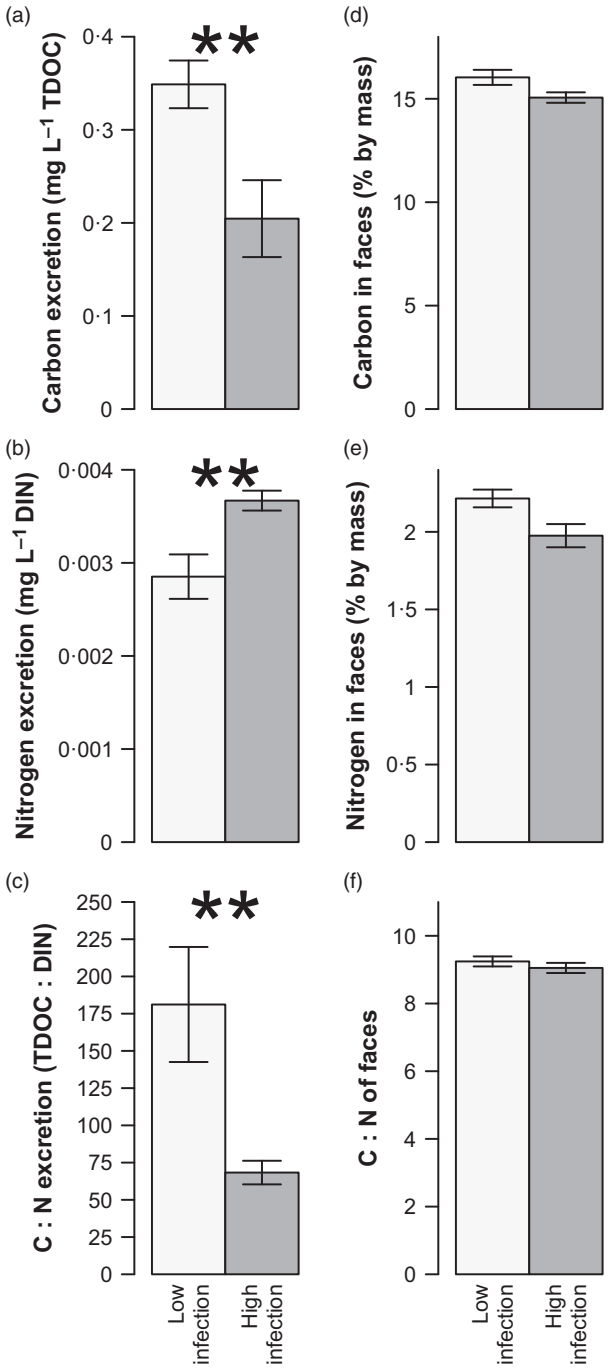

Fig. 3. Nutrient data indicating the effects of high and low infection on mean (± 1 SEM) host excretion and egestion. Nutrient excretion (a–c) was measured as total dissolved organic carbon (a), dissolved inorganic nitrogen (b) and TOC : DIN (c), excreted per snail per day. Mean (± 1 SEM) nutrient egestion through faeces (d–f) for high- and low-infection groups was measured as %C (g), %N (h), and C : N (i). Excretion and egestion were collected during the 3 h excretion trial by snails in the high- (N = 34) and low-infection (N = 58) groups (split by median parasite abundance). All percentages are based on dry mass, and all ratios have been converted to molar ratios. Asterisks indicate significant differences (**$P < 0{\cdot}01$) as determined by general linear models applied to the full data sets.

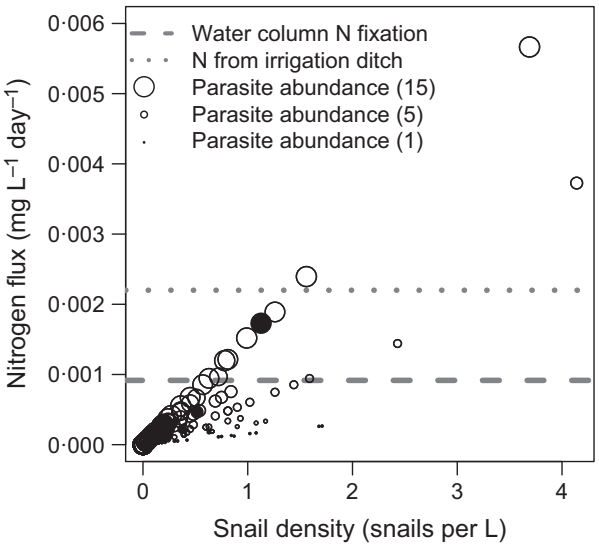

Fig. 4. Modelled N fluxes from infection for a hypothetical benthic volume of water. Comparison of nitrogen fluxes within a hypothetical volume of water taking into account the following sources: water column N fixation (horizontal dashed grey line), hydrologic delivery via the irrigation ditch (horizontal dotted line), and additional nitrogen delivered to the water column due to infection with *Cotylurus flabelliformis* metacercariae via host snail excretion (circles) as estimated via field data. Snail densities for individual dip net sweeps (open circles, N = 330) and pond-averaged snail densities (black solid circles, N = 18) were combined with pond-averaged metacercariae abundances and observed N excretion rates to determine 'hotspot' as well as pond-averaged N contributions to the water column due to infection. Model results illustrate the potential variation in additional N liberated by excretion within shallow benthic ecosystems, which is dependent on both the average infection abundance per snail within the benthic area as well as snail density.

When averaged across all dip net sweeps, parasite-induced N fluxes were 17 ± 3% that of water column N fixation. For even higher metacercarial abundances [e.g. up to 165 metacercariae per snail in 2012, up to 1000 per snail in the literature (Campbell 1973b)] infection-induced changes in DIN excretion are expected to be much greater.

In 2010, mean metacercariae abundance and water column DIN were significantly correlated ($P = 0{\cdot}001$, $r = 0{\cdot}68$; Fig. 5a). While this correlation was strong, it was unclear whether N concentrations were forcing parasite abundances or whether parasite abundances were somehow affecting water column N concentrations. With the additional collection of periphyton data in 2011, we determined that the cycling of N between the periphyton and the water column (negative relationship, $P = 0{\cdot}018$, $R^2 = 0{\cdot}28$, Fig. S2, Supporting Information) is dependent on both snail biomass and metacercariae abundance (and their interaction) based on our model selection procedure (periphyton biomass had no effect). The model with both main effects (snails and infection) and the interaction term was the best supported ($P = 0{\cdot}03$, $R^2 = 0{\cdot}36$, >3 ΔAIC_c units lower than any other models); the interaction term *P*-value

was 0·04 (see SI for candidate models). Breaking the data into high and low snail biomass (using a median split), we found that snail infection affects DIN/periphyton %N only at high biomass ($P = 0{\cdot}007$, $R^2 = 0{\cdot}62$); at low snail biomass there was no effect of snail infection ($P = 0{\cdot}349$) (Fig. 5b). The initial finding that metacercariae infection increased water column DIN was consistent across years, with only infection and the interaction between infection and year having a significant effect ($P = 0{\cdot}03$, $R^2 = 0{\cdot}44$; Fig. 5a); snail biomass, parthenitae prevalence and the interaction between snail biomass and infection had no effect.

Discussion

Vitousek (1990) outlined the conditions under which a single species is likely to affect ecosystem-level processes through alterations in nutrient cycling. First, that species must have physiological or life-history traits that affect an element limiting to plant or microbial activity, and secondly, the species must have a large enough effect on the distribution and/or the cycling rate of the limiting element in order to be biologically significant. These conditions can occur either when organisms reach high densities/biomasses or when organisms affect key aspects of ecosystems that have a disproportionate effect on nutrient cycling (Schmitz, Hawlena & Trussell 2010). How and when these criteria will be met for parasitic organisms remains an open question, particularly for endemic parasites that do not cause obvious disease. Based on estimates from our empirical model (Fig. 4), parasite-induced N fluxes within benthic habitats can be on the same scale as other major sources of N in freshwater systems (water column N fixation, hydrologic inputs). In this study, *C. flabelliformis* metacercariae infection accelerated snail N excretion rates (Fig. 3b), likely causing enhanced fluxes of N from the periphyton (the snails' food resource) to the water column (Fig. 5a,b) where N is in high biological demand (Fig. 1; Mischler, Taylor & Townsend 2014).

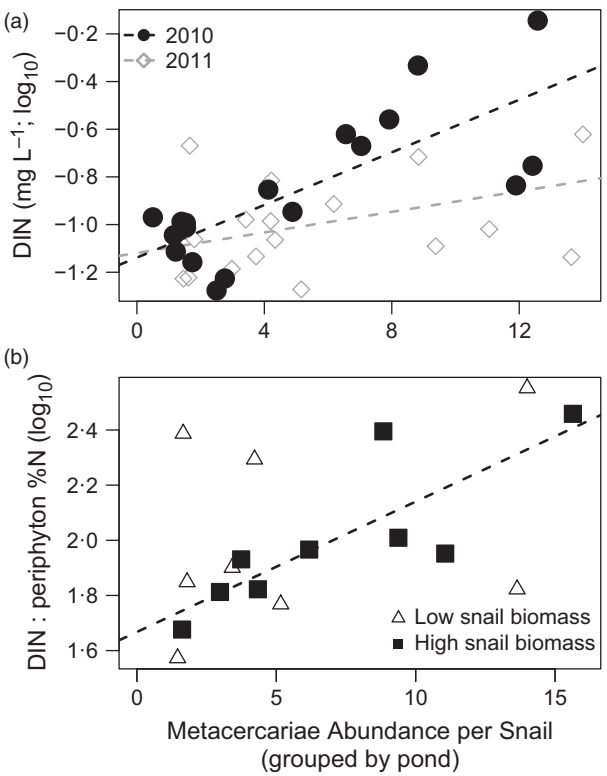

Fig. 5. Data from 2010 (solid circles, N = 18) and 2011 (open diamonds, N = 18) were analysed using a linear mixed-effects model with pond as the random variable (a). These data indicate a significant relationship between water column N and infection ($P = 0{\cdot}03$, $R^2 = 0{\cdot}44$, significant interaction between infection and year). The dashed lines are linear best fit lines for comparison. Further investigation with the 2011 data (b) indicates the portion of N in the water column (as DIN) vs. the %N content in the periphyton is dependent on the interaction between snail biomass per m^2 and average metacercariae abundance per snail ($P = 0{\cdot}03$, $R^2 = 0{\cdot}36$). As parasite load increases, the N concentration in the water column vs. the periphyton %N increases due to increased host N excretion, but only for ponds with high snail biomasses ($P = 0{\cdot}01$, $R^2 = 0{\cdot}62$, solid squares). Data were transformed to satisfy assumptions of equal variance and normality. The dashed line is a linear best fit line based off the high biomass data only.

ORGANISM-LEVEL EFFECTS

To alter N cycling, infection must change the ways in which N is stored and excreted from host organisms. Such changes can be caused by the following: (i) differential nutrient demand between parasite and host (Fig. 2a–c); (ii) increased stress/metabolic demand; and (iii) utilization of alternative metabolic pathways to sustain parasite and host. *C. flabelliformis* metacercariae (C : N : P = 100 : 13 : 1; Fig. 2a–c) have an enhanced demand for C and a reduced demand for N compared to the snail host (C : N : P = 80 : 16 : 1) based solely on stoichiometric differences (Cross *et al.* 2005), contrary to studies on free-living animals (Fagan *et al.* 2002). High levels of C-rich carbohydrates are expected in long-lived larval parasite stages for maintenance and rapid maturation within the definitive host (Richards, Pascoe & James 1970). In fact, Metacercariae contain comparatively more glycogen and lipids and less protein than adult worms (Siddiqui & Nizami 1981). Additionally, while protein incorporation into metacercariae tends to end within the first few days of infection, glucose incorporation for maintenance and storage continues indefinitely in many species (Lowenberger, Chadee & Rau 1994). Also, adult trematode reproductive structures contain sclerotinizing proteins that are not present in the metacercariae (Smyth & Clegg 1959). High parasite C : N ratios compared to the host is also corroborated by previous studies (Calow & Jennings 1974; Burky & Hornbach 1979; Siddiqui & Nizami 1981; Bernot 2013).

This host–parasite stoichiometric mismatch shifts the host–parasite phenotype towards a higher C : N ratio compared to the snail alone (Fig. 2c). Our nutrient excretion data corroborate this shift with increased parasite abundance leading to decreased host C excretion (enhanced assimilation) and increased N excretion (Figs 3b and 6a,b).

However, ecological stoichiometry theory predicts that host N excretion should increase due to decreased N assimilation by the host–parasite phenotype, therefore host tissue N content should remain relatively constant. Instead, host tissue N decreases with increasing parasite abundance (Fig. 2b) resulting in an increase in snail tissue C : N with infection (Fig. 2c). Additionally, faecal %N and C : N were unchanged by metacercariae abundances, indicating the observed increase in N excretion was not caused by an infection-induced decrease in host N assimilation rates (Figs 3d–f and 6a,b).

The metabolic theory of ecology (Allen & Gillooly 2009) predicts that infection should increase the metabolic rate of the host–parasite phenotype compared to the host alone, which in turn leads to exhaustion of host carbohydrates and the catabolism of host proteins. During protein catabolism, non-metabolically relevant N-rich components are excreted, which accounts for the elevated N excretion with increasing metacercariae abundance observed in this study (Fig. 3b). This effect is only compounded by stress, which has been shown to increase organism C use at the expense of N reserves (Hawlena & Schmitz 2010). The host must contend with the immunological stress of infection (Osnas & Lively 2006), metabolic stress and stress in coping with the parasite's potentially toxic excretory products (Becker 1980), parasite-related internal damage/feeding (Dawes 1963; Crews & Esch 1987) and parasite growth (Margolis & Boyce 1969). Together these changes in C and N assimilation/excretion result in significant alterations in the C : N ratio of snail nutrient regeneration to the water column (Fig. 3c).

ECOSYSTEM-LEVEL EFFECTS

Taken together, our results indicate that: (i) sampled ponds tend to be N-limited (Fig. 1); (ii) metacercariae within snails have a significantly lower N content (higher C : N) relative to their hosts (Fig. 2); and (iii) snails with higher infection loads have higher N excretion rates (lower C : N of excreta) and lower body %N (higher C : N of body tissue) (Figs 3b,c and 2b,c). These observations lead us to our ecosystem hypothesis: increased infection results in accelerated host-induced N fluxes from the periphyton to the water column, and hence higher water column N concentrations and lower periphyton N content. To explore this hypothesis, we used a simple empirical model to investigate the fluxes of N to a hypothetical benthic volume of water, based on field-derived values of metacercariae abundances, host DIN excretion rates, snail density, ditch hydrologic nutrient delivery and water column N fixation (Fig. 4). For 2011, pond-averaged metacercarial abundances and pond-averaged snail densities resulted in estimates of parasite-induced N flux rates to the water column approaching (and exceeding) that of water column N fixation; snail densities from single dip net sweeps produced estimates of parasite-induced N exceeding N fixation and, in some cases, hydrologic supply from the ditch (Fig. 4). While the overall effect will undoubtedly vary with snail density, infection abundance and fluctuations in metabolic activity, our calculations strongly suggest that metacercariae infection of aquatic snails have the potential to substantially alter the N cycle both in biogeochemical 'hotspots' as well as pondwide.

These model-derived estimates are corroborated by our field data. At the pond scale, water column DIN increased with infection load in 2010 and 2011 (Fig. 5a). Additionally, periphyton %N was inversely related to water column DIN (Fig. S2, Supporting Information), suggesting that allochthonous nutrients are not driving infection through N accumulation in snail forages, but instead, a plausible explanation is that N is being

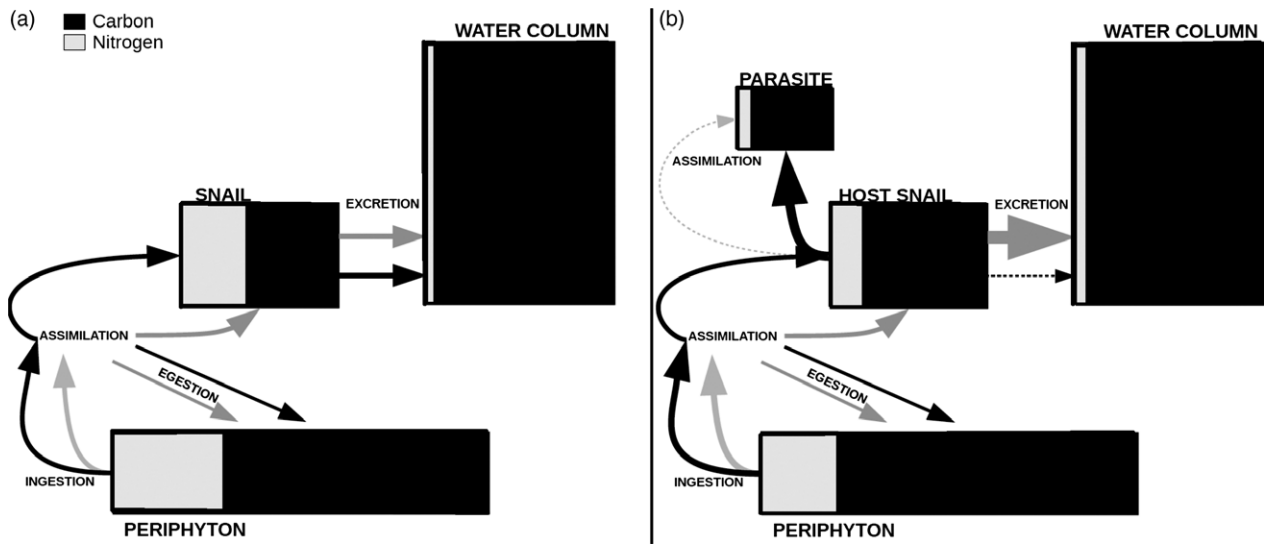

Fig. 6. Diagram for parasite alteration of nutrient pools and fluxes; (a) represents no infection and (b) includes parasites. Ratio of black : grey within boxes qualitatively represents C : N, while arrow colour/thickness represents nutrient/magnitude. Relative magnitudes/stoichiometries depend on parasite abundance and host–parasite relationship.

translocated from the periphyton pool to the water column pool at a rate altered by infection. As expected, the water column DIN : periphyton %N ratio is dependent on the interaction between metacercariae load and snail density, with a strong positive relationship between parasites and DIN : periphyton %N ratios at high snail densities (snails translocating N from periphyton to the water column), and no relationship at low snail densities (Fig. 5b).

We openly acknowledge that these are correlative field data for which the causal pathways could be reversed or be driven by additional, unmeasured variables. In the absence of large-scale manipulations, our proposed mechanisms remain hypothetical. Nonetheless, the weight of evidence and overall consistency among the stoichiometric analyses, excretion measurements, empirical modelling and comparative pond sampling collectively favour the explanation that infection – via its effects on nitrogen excretion from host snails – drives changes in patch- and pond-level nitrogen cycling. If, for instance, greater bird activity at certain ponds (e.g. due to more perching habitat) was driving both higher N inputs (via bird faeces) and greater infection (via deposition of trematode eggs), we would expect nutrient concentrations to correlate more strongly with bird-derived infectious stages (i.e. parthenitae), rather than with metacercariae (which is not the case).

Another possibility is that higher N availability/supply in ponds increased periphyton biomass and/or periphyton %N, which in turn could increase metacercariae prevalence and abundance either through: (i) snails infected with parthenitae releasing more cercariae through longer life spans or increased cercarial shedding rates; (ii) enhanced development and survival of metacercariae; or (iii) increases in snail densities. We have found no relationship between periphyton biomass and water column N indicating that periphyton may be largely relying on sediment-derived sources of N (VonSchiller *et al.* 2007). Additionally, we have not found any relationship between periphyton biomass and snail density or parasite prevalence/abundance; our data do not support allochthonous N supply driving infection through changes in periphyton biomass. Similarly, our data do not support allochthonous N supply or seepage from sediments driving infection through increases in periphyton %N as water column N and periphyton %N are inversely related (Fig. S2, Supporting Information).

While we cannot completely eliminate these alternative hypotheses in the absence of manipulations, they are inconsistent with the observed data. Instead, our interpretation is that the parasites are driving the observed patterns of N cycling between the water column and the periphyton through their hosts' excretions. This interpretation is consistent with our data indicating that snails with more metacercariae tend to store less N (Fig. 2b) and excrete more N (Fig. 3b) into the water column. While not strictly causal, these data are thus consistent with infected snails 'pumping' N (Evans-White & Lamberti 2005) from the periphyton to the water column faster than uninfected snails (Fig. S3, Supporting Information).

How widespread might these parasite-induced changes in nutrient cycling be? *C. flabelliformis* is found throughout North America and often achieves high abundances within aquatic systems (Campbell 1973b). Increased N excretion appears to be a common response of snails to the stresses of trematode infection in the first intermediate host (Becker 1980; Bernot 2013) as well as the second intermediate host (the present study). This study is the first to document this effect in metacercariae, and it may apply to other parasite genera beyond trematodes. Additionally, in other systems, pathogens can have similarly strong effects on nutrient/energy cycling. For example, in marine systems, viruses have the potential to drastically affect carbon cycling (Suttle 2007), while parasitic plants have been shown to indirectly increase below-ground rates of N cycling (Bardgett *et al.* 2006). While N flux scaled with host/parasite abundance in this study, many parasites may alter host-induced nutrient cycling at low abundances, especially in cases where parasites are large compared to the host. Understanding fundamental feedbacks between pathogens and nutrient cycling is an important frontier for ecosystem science in the future. However, predicting the effects of parasites on nutrient cycling demands a detailed understanding of the prevalence and abundance of the parasite, its developmental stage, how this parasite affects host vital rates and nutrient regeneration, and ultimately how parasite-induced nutrient fluxes compare to other pools and fluxes of nutrients in the ecosystem (Fig. S3, Supporting Information).

Because of the pervasiveness of parasites in a variety of ecosystems (Kuris *et al.* 2008), many such systems may contain 'hidden' effects of parasites on nutrient cycling. This study provides an initial framework for parasites' roles in ecosystem-level nutrient cycling (Fig. S3, Supporting Information), but more detailed work quantifying the mechanisms behind this framework is needed to fully understand this potentially important and largely overlooked role of parasites in shaping ecosystem structure and function.

Acknowledgements

We are grateful to Pete Walker, Devon Lane and Todd Cozad of the Colorado Division of Wildlife for facilitating access to field sites, and to Phil Taylor, Will Wieder, Hanna Ding, Christine Seibold and Samantha Weintraub for their analytical expertise. Stephaine Hayden, Emily Oliver and Rebecca Woythal provided valuable assistance in both the laboratory and the field. JM was supported by the National Science Foundation Graduate Research Fellowship under Grant No. DGE-0802270 and the U.S. Environmental Protection Agency's Science to Achieve Results (STAR) programme. PTJJ was supported by a grant from the National Science Foundation (DEB-1149308) and a fellowship from the David and Lucile Packard Foundation.

Data accessibility

Raw data underlying our analyses are available from the Dryad Digital Repository http://dx.doi.org/10.5061/dryad.mt240 (Mischler *et al*. 2016).

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Received 13 July 2015; accepted 8 February 2016
Handling Editor: Spencer Behmer

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. Phosphorus content of metacercariae, host excretions, and snail body tissues.

Figure S2. Inverse relationship between water column DIN and periphyton %N.

Figure S3. Diagram of the ecological framework for parasite-altered ecosystem level nutrient cycling.

Table S1. Candidate models for AIC analysis.