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262

Sanni Aalto

A Stoichiometric Perspective on Host-Parasite Interactions

JYVÄSKYLÄN YLIOPISTO

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ABSTRACT

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Parasites cause significant harm to their host, but are also important members of food webs affecting trophic dynamics. Anthropogenic nutrient enrichment has been proposed to explain the growing worldwide emergence of parasites, suggesting that even the prevailing seasonal nutrient concentrations may alter parasite prevalence patterns in nature. Parasites share the same limited resources as their hosts. Thus changes in host food quality may alter parasite growth and virulence and consequently alter the parasite-driven changes in host population dynamics and stoichiometry reflected in the whole food web. In this thesis, associations between environmental stoichiometry (carbon:nitrogen:phosphorus ratios) and host-parasite interactions were examined under natural conditions in a lake and under nutrient enrichment in a mesocosm experiment. The effect of stoichiometric food quality of the host on parasite growth and virulence was investigated experimentally, as well as the effect of infection on the stoichiometrical content, stable isotope values and physiology of the host under different food quality and quantity regimes. The results revealed that under natural conditions the interactions between nutrient concentrations and parasite epidemics were diverse, as some parasites showed positive and some negative associations with either environmental nutrients or host nutrient contents. In the laboratory, parasite infection changed host stoichiometry, energy allocation and physiology, implying that parasites may induce changes both in host population dynamics and in carbon and nutrient recycling by hosts. Experimental evidence also suggests that the stoichiometric demands of both the host and the parasite may affect the outcome of host-parasite interactions under nutrient deficiency. The data presented in this thesis suggest that nutrient regimes may affect parasite infection patterns, but the interactions between nutrients and disease are dependent on the host-parasite system studied.

Keywords: *Daphnia*; ecological stoichiometry; environmental quality; nutrient loading; parasite prevalence; parasite virulence.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original articles, which will be referred to in the text by their Roman numerals I-IV. I have contributed significantly to planning, data collection and analyses as well as writing each article.

I Aalto S.L., Ketola T. & Pulkkinen K. 2013. No uniform associations between parasite prevalence and environmental nutrients. Manuscript.

II Aalto S.L., Kaski O., Salonen K. & Pulkkinen K. 2013. Responses of algae, bacteria, *Daphnia* and natural parasite fauna of *Daphnia* to nutrient enrichment in mesocosms. *Hydrobiologia*. Published online doi: 10.1007/s10750-012-1261-3

III Aalto S.L. & Pulkkinen K. 2013. Food stoichiometry affects the outcome of *Daphnia*-parasite interaction. *Ecology and Evolution* 3: 1266–1275.

IV Pulkkinen K., Aalto S.L. & Nykänen H. 2013. Stable carbon and nitrogen isotope values in *Daphnia magna* fed with a single food source: effects of parasitism and food shortage. Manuscript.

1 INTRODUCTION

1.1 Ecological and biological stoichiometry

Ecological stoichiometry is the study of the balance of energy and nutrients in ecological interactions and processes, whereas biological stoichiometry focuses on energy and nutrients in biological systems (Sterner & Elser 2002). Usually energy is defined as carbon (C), which is both the main component of biomolecules involved in the energy metabolism of organisms (e.g. lipids and carbohydrates) and also generally used to quantify the flux of matter and energy in ecosystems (Sterner & Elser 2002). In a stoichiometric context, the most important nutrients are nitrogen (N) and phosphorus (P), because both are constituents of compounds essential for the function and structure of organisms and limit production in both terrestrial and aquatic ecosystems (Elser et al. 2000a).

Every organism has its own unique elemental composition and consequently unique demands for elements. However, in addition to their quantity, the ratio between energy (C) and nutrients acquired also regulates the growth and reproduction of organisms. Fast growing organisms commonly have low C:P and N:P ratios (i.e. high P content; Main et al. 1997, Elser et al. 2000b), suggesting that P is especially important for growth. Indeed, one central idea in biological stoichiometry, the growth rate hypothesis (GRH), proposes a positive association between growth rate, P content and RNA content of organisms, meaning that fast growing organisms have high P content due to high allocation to P-rich ribosomal RNA (rRNA), which is needed in protein synthesis and growth (Elser et al. 1996, 2000). Evidence from diverse biota supports GRH; for example, RNA may comprise as much as ~49 % of total body P in fast growing crustaceans and in bacterial cells as much as ~82% (Elser et al. 2003, Acharya et al. 2004a, Vrede et al. 2004). The high rRNA allocation demands high RNA production and it seems that faster growing organisms have longer rRNA intergenic spacers (IGSs) and thus greater potential for transcription of rRNA (Elser et al. 2000c, Weider et al. 2004). However, the coupling between P content and growth is less important when P is not limiting (Elser et al. 2003) or if there is

some other stressor than P-limitation depressing growth rate (Lukas et al. 2011). Moreover, the availability of N may also affect specific growth rates of organisms, since N is a building block for amino acids and consequently important in protein synthesis (Hessen et al. 2007). Overall, GRH implies that life-history strategy and specific growth rate of an organism defines its energy and nutrient requirements.

In nature, organisms differ significantly in the degree to which they can tolerate imbalanced ratios of acquired C and nutrients and in their need to maintain internal stoichiometry. Autotrophs exhibit large variation in C:N:P ratios under differing light intensity and nutrient concentrations (Hessen et al. 2002, Urabe et al. 2002a) and producers with high C:nutrient ratios are common (Sterner & Elser 2002, Sterner et al. 2008). Herbivores, on the other hand, are more stoichiometrically homeostatic and have to maintain internal C:N:P equilibrium (Sterner & Elser 2002). They typically have high demands for essential nutrients for optimal growth and reproduction (Elser et al. 2000a, Hessen 2008). Thus herbivores commonly face nutrient limitation, as the resource C:nutrient ratio exceeds their optimum value, leading to reduced growth efficiency for C (produced biomass/ingested C) and impairing growth, reproduction and survival (Fig. 1; Sundbom & Vrede 1997, Urabe et al. 1997, Acharya et al. 2004b, Koch et al. 2009). Furthermore, elemental deficiency may lead to decreased herbivore biomass (Urabe et al. 2002a, DeMott & Van Donk 2013), reducing grazing pressure towards autotrophs.

However, the balance between C:nutrient ratios of autotrophs and herbivores also alters the rates and ratios of limiting nutrients released by herbivores. This consumer-driven nutrient recycling (CNR) has been reported especially in aquatic ecosystems, where zooplankton grazers, such as *Daphnia*, have a significant role in nutrient recycling (Sterner 1986, Elser & Urabe 1999). *Daphnia* have the highest P content and lowest body N:P of all zooplankton taxa and they suffer from P-deficiency when food C:P ratio exceeds ~250, a common condition in lakes (Sterner & Hessen 1994, Elser et al. 2000a). Under P-limitation, *Daphnia* increase absorption and assimilation efficiency for P, and consequently decrease the efflux of P, retaining a higher proportion of P in biomass (DeMott et al. 1998, He & Wang 2008). Simultaneously they dispose of excess C in dissolved organic form (DOC) or via increase in respiration (Darchambeau et al. 2003, Jensen & Hessen 2007) raising C release rates as compared to P (Fig. 1; Sterner et al. 1992, Frost et al. 2004). Consequently, P-limitation of herbivores leads to lower nutrient input to autotrophs (Elser & Urabe 1999). However, in certain conditions, P-limited herbivores may improve P supply to autotrophs through their grazing and nutrient recycling activities (Urabe et al. 2002b).

This indicates that herbivore community composition and the distinct stoichiometric demands of different herbivore species affect stoichiometry of food webs. Indeed, Elser et al. (1988) demonstrated that shifting zooplankton

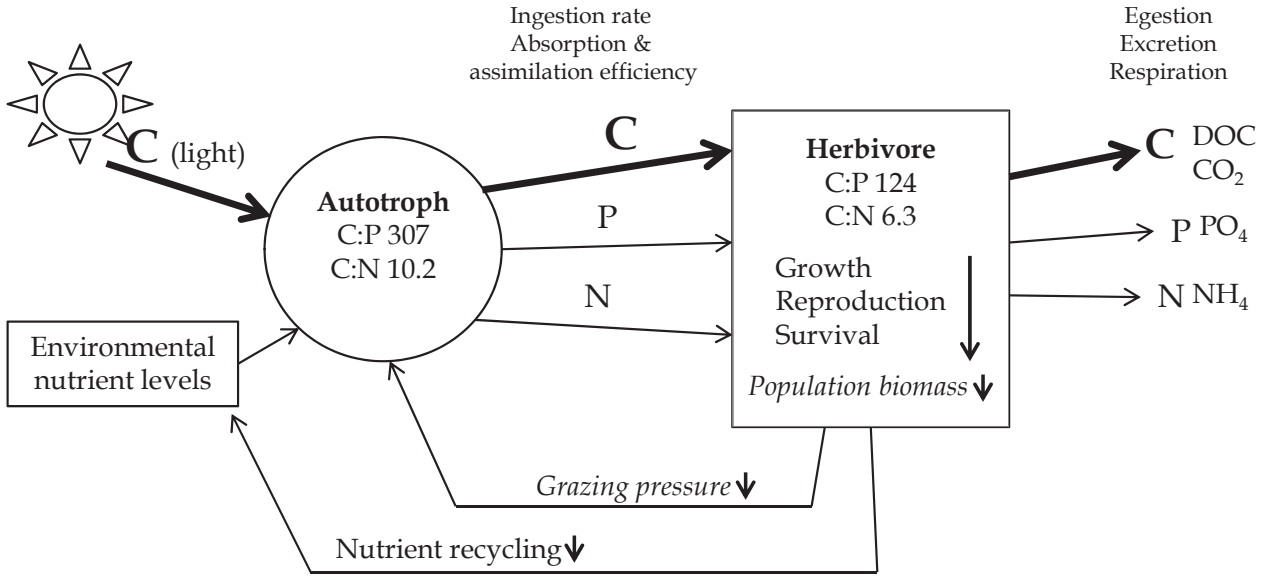

FIGURE 1 Schematic diagram of carbon and nutrient cycling in an autotroph-herbivore-system illustrating both individual and population-level effects. The figure is modified from Frost et al. (2005).

community composition from *Daphnia* dominance to copepod dominance resulted in changes in environmental stoichiometry (environmental C:N:P ratios). As copepods have higher N:P and thus lower requirements of P than *Daphnia*, their dominance lead to N-limited instead of P-limited phytoplankton growth.

In addition to consumer-prey relationships, environmental stoichiometry may also affect species interactions, as different taxa might have similar stoichiometric demands and thus compete for limited available nutrients (Sterner & Elser 2002, Moe et al. 2005). Overall, it seems that variation in environmental nutrient concentrations may partly explain the observed variation in population dynamics and genotypic diversity of species within communities and further in community composition in different biota (Andersen et al. 2004, Seidendorf et al. 2007, Jeyasingh et al. 2009).

1.2 Parasitism and host-parasite interactions

A parasite is an organism which exploits another organism, the host, to obtain its energy and nutrients thereby causing harm to its host (Price 1977). Parasites can be grouped into micro- and macroparasites. Microparasites, or pathogens, replicate directly within the host and their virulence (the negative impact of a parasite on host fitness) is strongly dependent on host (immunological) response. To sustain itself in a host population, a parasite has to transmit from an infected host individual to an uninfected susceptible host individual. Microparasites transmit either horizontally —with a direct contact or mediated via vectors — or vertically —from parent to offspring. Macroparasites, which are considered to be the "typical" parasites, develop within the host and produce infective propagules (e.g. eggs), which are then released and transmitted to new hosts. The virulence of macroparasites is density-dependent, as their negative impact on the host increases with the number of parasites in the host. Both micro- and macroparasites may have different life cycles: some species infect only a single host species, while others have complex life cycles and have to go through several intermediate hosts before finally completing the life-cycle in a definitive host (Anderson & May 1979, May & Anderson 1979).

In nature, parasite infection patterns are commonly coupled with host population dynamics (Hudson et al. 1998) meaning that increase in host density and thus the availability of susceptible hosts promotes transmission and increases parasite prevalence (the proportion of infected individuals in the host population). Due to the harmful nature of infection on life-history traits of host individuals, this leads to a decrease in growth and reproduction and consequently population density of the host, which in turn decreases parasite transmission and prevalence. In addition to host density, other host characteristics, such as size distribution (Stirnadel & Ebert 1997, Hall et al. 2007), genotype and genotypic diversity (Little & Ebert 1999, Decaestecker et al. 2003, Altermatt & Ebert 2008) and nutritional status (Pulkkinen & Ebert 2004, Frost et

al. 2008a, Seppälä et al. 2011) may affect the establishment of parasite epidemics, as well as parasite growth and virulence.

Parasites, in turn, affect host population dynamics (Anderson & May 1978) via deteriorating vital life-history traits of the hosts and modifying host behaviour (Lefèvre et al. 2009). Parasites also have an important role in shaping host evolution via driving gene-selection within host populations (Haag & Ebert 2004, Duffy & Sivars-Becker 2007, Yin et al. 2012).

Environmental factors directly affect both host and parasite dynamics, but also the quality and strength of host-parasite interactions. Anthropogenic disturbances, such as climate change and eutrophication, have been suggested to promote diseases globally (Marcogliese 2001, Harvell et al. 2002, Johnson et al. 2007, McKenzie & Townsend 2007, Johnson et al. 2010a, Paull & Johnson 2011) either directly by enhancing parasite transmission success and virulence (Bruno et al. 2003, Mitchell et al. 2005, Voss & Richardson 2006, Vale et al. 2008) or indirectly by increasing the abundance of hosts (Johnson et al. 2007). Pollutants and habitat alterations have also been proposed to alter the prevailing disease dynamics (Lafferty & Kuris 1999).

However, parasite prevalence patterns commonly show seasonal fluctuations even under prevailing undisturbed conditions (May & Anderson 1979), which has been explained by changes in host-population dynamics, but also by seasonality of the weather (Altizer et al. 2006) or habitat characteristics (Cáceres et al. 2006, Johnson et al. 2009). In addition to abiotic factors, trophic interactions between hosts and non-hosts (species in which the parasite is not able to develop) may affect prevalence patterns. Predation can reduce the infection prevalence if predators are feeding selectively on infected prey (Duffy et al. 2005, Pulkkinen & Ebert 2006) or enhance disease spread if a predator causes a release of infective propagules from the host while feeding (Cáceres et al. 2009) or by indirect effects of predation on the host (Duffy et al. 2011). Community composition and biodiversity may also alter transmission and emergence of pathogens and parasites. As diverse communities contain a higher proportion of non-host species, increase in biodiversity may reduce transmission either directly or indirectly (Keesing et al. 2006, Johnson & Thieltges 2010, Johnson et al. 2012).

1.3 Parasites in food webs

Parasitism is the most common lifestyle among organisms, yet the significant role of parasites in food webs has been acknowledged only quite recently (Kuris et al. 2008, Lafferty et al. 2008). As every free-living organism possibly has parasites and parasites interact not only with their host but with the food sources and predators of their hosts and also with other parasites sharing the same host, parasites increase chain length, connectance and nestedness of food webs (Lafferty et al. 2006, Amundsen et al. 2009). Since parasites shape host physiology and behaviour, they consequently influence the trophic interactions between host

and non-host species and drive trophic cascades in food webs. They may affect competition via weakening competitively dominant hosts or alter predator-host dynamics and intraguild predation (Hatcher et al. 2006). In addition, trait-mediated indirect effects of parasites on hosts may lead to reduced grazing pressure of herbivore hosts on autotrophs and affect the whole community structure (Mouritsen & Poulin 2005, Wood et al. 2007). Overall, parasites may enhance biodiversity if their host is a common dominant species in an ecosystem or may reduce biodiversity via inducing trophic cascades which may lead to extirpation and extinction of non-host species (reviewed in Hatcher et al. 2012).

In addition to trophic interactions, parasites have a significant role in regulating energy flow, since they may comprise a large proportion of the biomass and production in ecosystems (Kuris et al. 2008, Preston et al. 2013). Trophically-transmitted parasites, which modify host behaviour and vulnerability to predation, may enhance conversion of biomass from one trophic level to another (Lafferty & Morris 1996, Thomas & Poulin 1998). In addition, the consumption of parasites, either free life-stages or those consumed concomitantly with an infected host organism, will alter energy flow in ecosystems (Johnson et al. 2010b).

1.4 A stoichiometric perspective on host-parasite interactions

Consumers commonly face low stoichiometric quality of environmental resources and concurrently are hosts to numerous parasite species. Both of these stressors separately impair the functioning of an organism, which is further cascaded up to alterations at population and community level. Thus it may be assumed that consumers encountering both nutrient deficiency and infection may suffer even more. However, as parasites live within their hosts sharing the same limited pool of nutrients, nutrient deficiency of the host may also worsen parasite growth and transmission. Smith (1993) proposed that the resource competition may influence the outcome of host-parasite interactions by affecting parasite virulence. The degree of the nutrient competition, and thus the effect on host-disease dynamics, may depend on the stoichiometric demands of both the host and the parasite. If the parasite has similar nutritional requirements as the host, the nutrient competition is strong and the parasite may show higher virulence than in a situation where host and parasite are less limited by the same element and nutrient competition is weaker. However, the effect of resource competition on parasite virulence is also dependent on the epidemiology, feeding site and behaviour of the parasite, although information concerning these factors is poor.

A few stoichiometric parasite studies have focused especially on the effect of P-limitation of the hosts on the host-parasite dynamics. Generally, GRH predicts that, in addition to the growth of the host, host P-limitation should also depress the growth of the parasite. Indeed, poor resource quality of the host has been shown to alter the parasite transmission rate and to decrease the production

of reproductive propagules within a P-rich *Daphnia* host in laboratory conditions (Frost et al. 2008a, Hall et al. 2009). Concomitantly, increase in parasite virulence under host P-limitation has also been demonstrated to lead to higher reproductive losses of *Daphnia* than under high quality food (Frost et al. 2008a).

If environmental stoichiometry affects host-parasite interactions, can parasites also affect environmental nutrient concentrations? In fact, parasites have been shown to alter C:nutrient ratio of the hosts (Forshay et al. 2008, Frost et al. 2008a). Changes in host stoichiometry might be attributable to the growing parasitic biomass comprising a large proportion of the total host biomass, but also to parasites directly altering host stoichiometry via exploiting the energy and nutrient reserves and affecting physiological functions of the host. Changes in host stoichiometry may be reflected in rates of C and nutrient release from the host. Hence, taking into account the essential role of parasites in food webs, parasites may have an important role in C and nutrient recycling in ecosystems.

1.5 Aims of the study

The significance of environmental nutrient levels in driving disease dynamics has been acknowledged recently (e.g. McKenzie & Townsend 2007, Johnson et al. 2010a). However, the studies have mainly focused on disturbances in food web dynamics (eutrophication) and how they might promote pathogen infection in aquatic ecosystems (Lafferty & Holt 2003, Johnson et al. 2007). Less attention has been paid to interactions between prevailing seasonal nutrient concentrations and parasite prevalence patterns in nature. The stoichiometric quality of environmental resources used by the host may partly explain the observed parasite infection patterns, as parasites are exploiting host resources. Thus nutrient deficiency of the host may, in addition to deteriorating the growth of the host, also alter the growth and virulence of the parasite and affect the outcome of host-parasite interactions (Smith 1993). On the other hand, parasites may also alter the physiology and elemental allocation of hosts, affecting host stoichiometry and the rates of C and nutrient recycling by the host.

The aim of this thesis was to investigate these hypothesized interactions between environmental stoichiometry and host-parasite interactions. First, the interactions between seasonal host-parasite dynamics and environmental nutrient concentrations were examined in natural conditions (I). Concomitantly, the effect of nutrient enrichment on the density and stoichiometry of the food sources and of the host, and further on the prevalence and intensity of the parasite fauna was investigated (II). In addition, it was studied how changes in host food quality and quantity may shape host-parasite interactions (III, IV). The effect of stoichiometric food quality of the host on parasite growth and virulence, i.e. the negative effect of parasite on the life-history traits of a host was investigated (III). The mechanisms by which infection may alter the stoichiometric content and elemental allocation of hosts under different food quality and quantity regimes were also studied (III, IV).

2 MATERIALS AND METHODS

The interactions between nutrient concentrations and host-parasite dynamics were studied in Lake Mekkojärvi (I, II). The diversity and incidence of parasites in the *Daphnia* population were monitored in summer 2007. To explore the long-term dynamics of both nutrients and host-parasite interaction, a 3-year field survey was carried out during 2008–2010 (I).The responses of *Daphnia* and its parasites to nutrient enrichment were studied in enclosures in Mekkojärvi between July and August 2008 (II). The effect of food quality or quantity and parasite infection on life history traits (III), stoichiometry (III, IV) and physiology (III, IV) and stable isotope values of *Daphnia* (IV) were studied experimentally in the laboratory in the Department of Biological and Environmental Sciences in University of Jyväskylä in 2011.

2.1 *Daphnia*

Members of the genus *Daphnia* (Crustacea: Cladocera) are herbivorous zooplankters. *Daphnia* reproduce parthenogenetically, and also sexually by producing ephippia (resting eggs), depending among other factors on the availability of resources. Under sufficient food supply, *Daphnia* reach maturity within 5–10 d and are easy to maintain as monoclonal lineages in the laboratory. *Daphnia* have been widely used in stoichiometric studies, since they have high requirements for P as compared to other zooplankton taxa and they commonly suffer P-limitation in nature (Sterner & Hessen 1994, Elser et al. 2000a). In addition, *Daphnia* also serve as hosts to numerous endoparasites and epibionts (e.g. Green 1974, Stirnadel & Ebert 1997, Ebert 2005), which have been demonstrated to impair their growth, survival and reproduction (reviewed in Ebert 2005). Furthermore, *Daphnia* have wide distribution from freshwater lakes to saline ponds and have a major impact on food web structure and energy flow from lower to upper trophic levels in freshwater ecosystems (Sterner 1986, Elser & Urabe 1999). Thus *Daphnia* are good model organisms in studies which aim to

provide information on environmental stoichiometry and host-parasite interactions both under natural conditions and the laboratory.

2.2 Field studies (I, II)

2.2.1 Study site

Lake Mekkojärvi is a small, shallow and highly humic lake, located in the Evo Forest Area in Southern Finland (61°13´N, 25°8´E). It has steep temperature, oxygen and nutrient gradients during the open water season. Due to high concentration of coloured dissolved organic carbon (DOC; 20–45 mg C l^{-1}; Taipale *et al.* 2008), the euphotic zone is only 0.5–1 m thick. The annual primary production of phytoplankton is below 10 mg C m^{-2} and decreases from spring to autumn (Salonen et al. 2005), whereas the bacterial production remains rather constant throughout the year (Taipale et al. 2009a).

The cladoceran *Daphnia longispina* (O. F. Müller) is the dominant zooplankton species in Lake Mekkojärvi (Salonen & Lehtovaara 1992). In addition to phytoplankton, *Daphnia* population relies heavily on bacterial food, especially on methanotrophs, on which they feed during short trips to the oxic–anoxic boundary layer (Salonen & Lehtovaara 1992, Taipale et al. 2008). Predation pressure on *D. longispina* is low, because the lake lacks zooplanktivorous fish and the main predators, *Chaoborus* larvae and *Notonecta* sp. are sparse.

In 2007, the parasite community was surveyed and four endoparasite species infecting *D. longispina* were found from Lake Mekkojärvi. Subsequently one bacterium (*Pasteuria ramosa*) and one microsporidian (*Larssonia obtusa*) species were identified. Two parasite species infecting gut epithelial cells remained unidentified (Fig. 2). The unidentified small gut parasite (USGP) is probably a microsporidian since it forms clusters with varying number of spores (15–30 spores per cluster) inside the gut epithelial cells. The unidentified large gut parasite (ULGP) is oval and approximately 20 µm long. In addition, one epibiont, *Vorticella* sp., was found colonizing *D. longispina*. See Table 1 for further description of the parasites.

2.2.2 Field sampling

In the enclosure experiment (I), samples were collected once per week during the 6 weeks of the experiment. In the longer survey (II), Lake Mekkojärvi was sampled during the ice-free period from May until September or November between 2008 and 2010 every 2 to 4 weeks varying between years and months.

TABLE 1 Characteristics and average prevalence of parasite species found infecting *Daphnia longispina* in Lake Mekkojärvi. References in which the species have been described are provided.

	Size of infective stages (µm)	Site of infection	Transmission mode	Average prevalence	Reference
Endoparasites					
Microsporidia					
USGP	~2	Gut wall	?	13.7 %	
Larssonia obtusa	4.3-4.6 x 2.6-3	Fat body, intracellular	intermediate host?	1.9 %	Vidtmann & Sokolova 1994
Bacteria					
Pasteuria ramosa	1-10	Haemocoel	Horizontal	0.6 %	Ebert et al. 1996
Unknown classification					
ULGP	20	Gut wall		42.9 %	
Epibiont					
Ciliata					
Vorticella sp.	20–25 x 70–150	Carapax/ filter apparatus	Horizontal	21.4 %	Green 1974

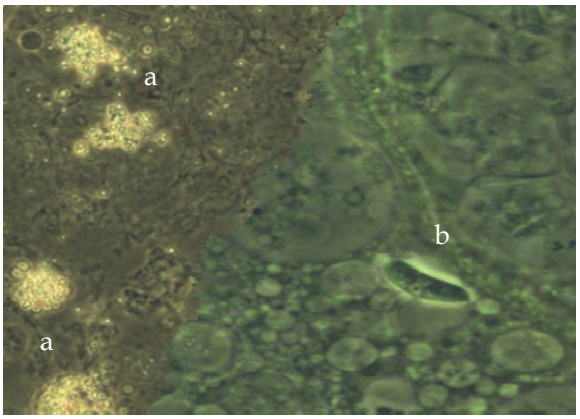

FIGURE 2 The spore clusters of an unknown small gut parasite (USGP; left, a) and a specimen of an unknown large gut parasite (ULGP; right, b) inside the gut of *Daphnia*. Combined photos, taken by Katja Pulkkinen.

2.2.3 Parasite species richness, prevalence and intensity

To assess the presence of parasites, 50–150 live adult females per sampling date were screened (as in Stirnadel & Ebert 1997, Decaestecker et al. 2005, Ebert 2005). First *Daphnia* were screened for visible signs of parasite infections and *Vorticella* sp. individuals attached to the carapace were counted. Infections with *P. ramosa* or *L. obtusa* could be recognized as a whitish mass either in the body cavity or in

the neck of hosts, and the presence was further verified by examination of the dissected animal under phase-contrast microscopy. The gut of the animal was dissected from the body and the number of epithelial cells infected by USGP or the number of individual ULGP was counted. The species richness was estimated as number of parasite species per infected female. The prevalence of a parasite species was calculated as the number of females infected per total females examined and the overall parasite prevalence was calculated as in Decaestecker et al. (2005). Intensity of infection was estimated for ULGP and *Vorticella* sp. as number of specimens and for USGP as number of infected gut epithelial cells per infected female.

2.3 Laboratory studies (III, IV)

The model system used in the laboratory experiments consisted of a freshwater crustacean *Daphnia magna* Straus (Crustacea: Cladocera) and a microsporidian *Glugoides intestinalis* Chatton (Microspora: Glugeidea; (Larsson et al. 1996). *G. intestinalis* is an intracellular horizontally transmitted parasite, which infects host gut epithelial cells through waterborne spores (Ebert 1995). It reproduces directly and produces sphorophorous vesicles containing 20–30 spores. The number of vesicles increases exponentially during the early infection (by more than one order of magnitude from day 7 to day 13 after infection) and they become visible inside host gut cells on day 10–13 after the establishment of the infection (Ebert 1994, 1995, Katja Pulkkinen unpubl.). Spores released from ruptured gut cells either reinfect other epithelial cells or are transmitted to other *Daphnia* individuals via faeces.

The food alga, *Scenedesmus gracilis*, was grown in semibatch cultures in modified WC medium (Guillard & Lorenzen 1972, without vitamin solution). To create low quality food (P-limited algae), the P-content of the medium was reduced to 10 % of the original.

2.3.1 Life table experiment (III)

Uninfected and infected *Daphnia* were fed with either P-limited or P-sufficient alga, and their growth, reproduction and survival were recorded for 26 d. In addition, the development of parasite infection was examined by dissecting the infected hosts and counting the spore vesicles at separate time points during the experiment.

2.3.2 Stoichiometric & isotope experiment (IV)

This experiment tested how parasite infection, food shortage and their combined effect altered the stoichiometry and stable C and N isotope values of the host. Uninfected and infected *Daphnia* were fed either sufficiently (2 mg C l^{-1}) or suffered food shortage (0.5 mg C l^{-1}). Stoichiometry and isotope values of hosts

were recorded at age of 14 and 28 d to follow if the progress of parasite infection intensified the negative effect of the parasite.

2.4 Stoichiometric and stable isotope analyses of food sources and *Daphnia*

The C and N content (%C, %N) and stable C and N isotope values ($\delta^{13}C$ and $\delta^{15}N$) of *Daphnia* (I-IV) and algae (III, IV) were determined with a Carlo-Erba Flash 1112 series Elemental Analyser connected to a DELTAplus Advantage IRMS (Thermo Fisher Scientific Corporation, Waltham, MA, USA). Animals or samples of algae were picked into tubes, freeze-dried and weighed into tin cups. Generally, two replicates were prepared from one sample, each with a dry weight approximately 0.5 mg for *Daphnia* and 1 mg for algae. Samples were run against IAEA standard NBS-22 using dried and homogenized fish muscle for *Daphnia,* and dried and powdered potato leaves for algae as internal laboratory working standards. $\delta^{13}C$ values were corrected for lipids (Syväranta & Rautio 2010, II, IV).

For determining P content of *Daphnia* (I-III) and of algae (III, IV), freeze-dried animals or subsamples of algae were combusted (450 ^{o}C, 4h), diluted in 0.2N H_2SO_4 and measured with a QuickChem 8000 analyser (LaChat instruments, Loveland, CO, USA). To measure C per litre (mg C l^{-1}) for algae, subsamples of algae were filtered on preweighed GF/C-filters, dried at 60 ^{o}C and weighed with an analytical balance.

2.5 Statistical analyses

Several different statistical analyses were used in the study (Table 2).

2.6 Other analyses

Several physico-chemical and biological analyses were conducted in the study (Table 3).

TABLE 2 The statistical analyses used in the study.

Statistical analysis	Description	Reference
GLM	I	
Wilcoxon signed ranks test	I	
t-test	II	
Mann-Whitney U-test	II, III	
Repeated-measures ANOVA	II	
Kruskal Wallis H-test	II	
Two-/Three-way ANOVA	III, IV	
Cox regression	III	
ANOVA on ranks	IV	Zar 1984
The adjusted ranks transformation test	IV	Leys & Schumann 2010

TABLE 3 Physico-chemical and biological analyses conducted in the thesis.

Analysis	Description	Reference
Chl *a*	I, II	
pH	I, II	
Alkalinity, conductivity, water colour	II	
NH_4, NO_2+NO_3, PO_4	I, II	
seston TP, TN	I, II	
Bacterial biomass & abundance	II	Kankaala et al. 2010
Zooplankton abundance & diversity	I, II	
P ingestion rate	III	Lampert & Taylor 1985
Daphnia respiration rate	IV	Salonen 1981

3 RESULTS & DISCUSSION

3.1 The interactions between environmental nutrient levels and host-parasite dynamics

In the long-term field survey (I), the prevalence patterns of the four endoparasite and the one epibiont species infecting *Daphnia* varied seasonally and between species. Two of the endoparasite species, the unknown small gut parasite USGP and unknown large gut parasite UGLP, and the epibiont *Vorticella* sp. were prevalent throughout the study period, while *Larssonia obtusa* and *Pasteuria ramosa* were found sporadically and infected only a small proportion of the *Daphnia* population. In contrast to earlier surveys concerning the natural parasite fauna of *Daphnia* (Stirnadel & Ebert 1997, Ebert et al. 2001, Decaestecker et al. 2005, Johnson et al. 2009), *Daphnia* density and parasite prevalence patterns were not found to be connected, which might be due to low prevalence of the virulent species (*L. obtusa* and *P. ramosa*). It may also imply the possibility of an intermediate host for some species (e.g. *L. obtusa*; Refardt et al. 2002). The findings agree with previous results from Lake Mekkojärvi, which suggested that *Daphnia* density in the lake is regulated by food quantity as well as by the availability of different phytoplankton and bacteria food sources (Taipale et al. 2008, 2009b). However, parasite epidemics were linked with changes in host fecundity, as there was a negative interaction between overall parasite prevalence and number of eggs per fecund female. Furthermore, infections with *L. obtusa* and the epibiont *Vorticella* sp. reduced the fecundity found when the egg production of infected and uninfected *Daphnia* was compared.

The overall parasite species richness and the prevalence of the epibiont *Vorticella* sp. increased when the P content of *Daphnia* decreased. The negative association between species richness and P content of the *Daphnia* might have been due to parasite-driven changes in host stoichiometry, as at least *P. ramosa* is known to induce changes in C:N:P of *Daphnia* (Frost et al. 2008b). This finding may also be related to the host being more susceptible to infection when food quality is poor (Frost et al. 2008a, Hall *et al.* 2009), but without laboratory

experiments and exact information on life-cycles and transmission patterns of all the species, causal relationships between parasite prevalence patters and host resource quality cannot be distinguished. *Vorticella* sp. is known to compete for food with *Daphnia* (Kankaala and Eloranta 1987), and thus the decline in P content of *Daphnia* under higher prevalence might imply resource competition between the host and the epibiont and this was further seen as lower reproductive output of *Daphnia*.

USGP prevalence had a positive relationship with seston TP and TN, which can be used as proxies of the stoichiometrical quality of the food sources in Lake Mekkojärvi (Järvinen & Salonen 1998). Host resource quality might have affected transmission or growth of the USGP, which has previously been shown with other parasite species infecting *Daphnia* (Frost et al. 2008a, Hall et al. 2009). Increase in USGP prevalence was also recorded when *Daphnia* fed proportionally more on bacterial food, as indicated by $\delta^{13}C$ values (Taipale et al. 2008). If *Daphnia* migrated down in the water column to feed on bacterial food, transmission of USGP spores from sediment might have been facilitated, as microsporidian spores have been shown to remain infective in the sediment (Decaestecker et al. 2004). The other gut parasite, ULGP, was most prevalent when *Daphnia* had high N content and were consuming mainly phytoplankton, as indicated by less negative $\delta^{13}C$ values and positive association between parasite prevalence and chlorophyll *a* concentration. This might indicate that resource quality of *Daphnia* affects the transmission of ULGP or that it causes changes in the physiology and elemental allocation, and thus in the stoichiometric content of *Daphnia*, but again, laboratory studies are needed to confirm this.

In the mesocosm study (II), nutrient enrichment did not increase the biomass of phytoplankton or bacteria as in the earlier studies (Jansson et al. 1996, 2001, Elser et al. 2001). However, the epilimnetic seston N and P concentrations were higher in treated mesocosms, indicating higher resource quality for *Daphnia*. It seems that if nutrient enrichment induced any increase in the biomass of the phytoplankton or the bacteria, they were readily consumed by *Daphnia*, which was seen as higher nutrient content and lower C:nutrient ratios of *Daphnia*. The incorporation of added nutrients in the food web was further seen as a decrease in stable N isotope values of *Daphnia.* The added nitrogen compound had a lower $\delta^{15}N$ value than lake seston, which was reflected in the isotopic composition of the food sources and consequently of the *Daphnia*. Nutrient enrichment did not change *Daphnia* density, but there was more weekly variation in the proportions of juveniles and mature *Daphnia* in treated mesocosms, which may have been due to parasite-driven instability in population dynamics (Ebert et al. 2000, Pulkkinen & Ebert 2006). Unfortunately, it was not possible to study the effect of the observed parasites on life-history traits of *Daphnia*, since the infection could not be maintained under laboratory conditions.

Earlier studies have reported higher percentage of infection and/or production of infective propagules in *Daphnia* receiving high quality food (Frost et al. 2008a, Hall et al. 2009). One of the four observed endoparasite species,

USGP, was more prevalent in enriched mesocosms, while there was no difference in the prevalences of the other parasite species between treatments. In addition, the infection intensities of parasites were unaffected. It seems that the transmission of the parasites, which was connected to elemental content and stoichiometry of Daphnia in the whole lake survey (I), was not affected by nutrient addition, although USGP, which was more directly associated with host resource quality, did respond to nutrient enrichment. This may indicate that the nutrient addition was so moderate that while it was incorporated into the food web, the induced changes in stoichiometry of *Daphnia* were not large enough to alter transmission patterns or promote growth of parasites within the hosts.

The results of the long-term field survey (I) and nutrient enrichment study (II) suggest that environmental nutrient concentrations may affect parasite prevalence patterns by altering the quality and/or quantity of food sources and thus the stoichiometric content of hosts, rather than their density. However, the connections between nutrients and parasite dynamics were diverse and parasite species-specific.

3.2 Host-parasite interactions under qualitatively and quantitatively different host food regimes

3.2.1 The effect on parasite growth

In the experiment concerning the effect of food quality on parasite growth (III), the mean number of spores did not differ between P-limited and P-sufficient animals. Based on the assumptions of GRH (Elser et al. 1996), host P-limitation should depress the growth of parasites, but the similarity in the spore loads between food treatments in the experiment indicates that parasite infection could develop and progress even in hosts suffering from severe P-stress. This implies that parasite growth was mainly limited by some element other than P.

3.2.2 The effect of parasite infection on host life-history traits

The effect of parasite infection differed between P-sufficient and P-limited treatments. In agreement with previous experimental evidence (Ebert et al. 2000), in P-sufficient conditions infection impaired host survival and further growth, leading to lower body mass at maturity and lower overall reproductive output than for uninfected animals (III, IV). Under P-limitation, host survival was equally poor between uninfected and infected hosts and animals did not reproduce before death. This indicates that extreme P-limitation severely disrupted the physiological functions of *Daphnia*, as has been shown in previous studies (e.g. Sundbom & Vrede 1997, Urabe et al. 1997, DeMott 2003, Acharya et al. 2004b, Jeyasingh et al. 2011). However, under P-limitation, infected animals had higher juvenile growth rate than uninfected individuals. Under P-limitation, *Daphnia* acquire excess C which they have to dispose of in order to maintain their

internal stoichiometric balance. If the parasite was mainly using this excess C rather than the host P supply, it might have facilitated the host by decreasing the surplus C, enabling infected hosts to allocate more energy to growth than their uninfected counterparts. The view of parasite growth being dependent on the C supply of the host was supported by the host growth being equally poor between uninfected and infected hosts under host food shortage, implying that also parasites might have been deprived under low C supply (IV).

3.2.3 The effect of parasite infection on the elemental composition, physiology and stable isotope values of the host

Parasite infection did not affect P content or C:P ratio in this study (III, Fig. 3), which is in contrast to earlier work in which parasite infection has been reported to alter especially P content of *Daphnia* (Forshay et al. 2008, Frost et al. 2008b). In study IV, infected hosts having high food supply had less C and N, and lower C:N, than uninfected hosts. However, under food deprivation infection did not affect the host C content, which may have been attributable to low C input to the host depressing the growth of the parasite. Thus it seems that the parasite was mainly exploiting C and/or N storage of the host.

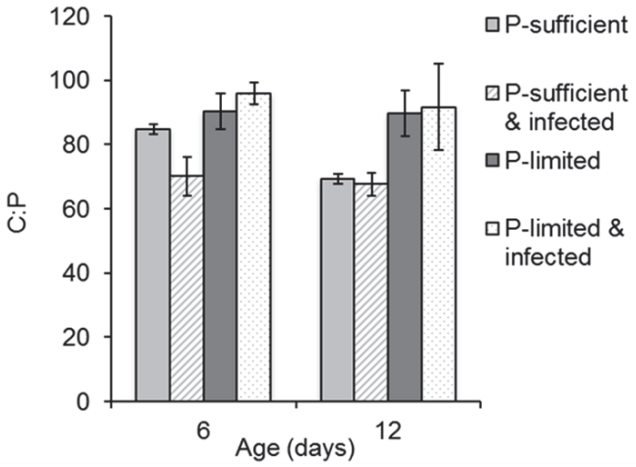

FIGURE 3 Mean (± SE) body C:P ratio of *Daphnia* in response to infection status and food quality.

Differences in filtration rates of uninfected and infected hosts have been proposed to explain the parasite-induced changes in host elemental content (Forshay et al. 2008). However, in the current study system, the filtration rates of uninfected and infected animals did not differ (III), nor did their respiration rates (IV). These results imply that the parasite altered some other part of the metabolic pathway, such as assimilation or excretion. The stable isotope results (IV) suggest that the parasite was inducing changes in host energy allocation. Since the experimental animals were fed with the single food source, the differences in $\delta^{13}C$ and $\delta^{15}N$ values between uninfected and infected *Daphnia*

reflected the parasite-driven changes in the host physiology. Infected animals had higher $\delta^{13}C$ values and lower C:N indicating that they had lower lipid storage than uninfected individuals (DeNiro & Epstein 1977, Post et al. 2007). Under food deprivation, the pattern was similar, which might have been caused by both infection and energy shortage. Under high food supply, infected *Daphnia* had higher $\delta^{15}N$ values than uninfected individuals. These results imply that the parasite might have exploited host N storage, forcing the host to use all the nutrition available and leading to increase in $\delta^{15}N$ (Adams & Sterner 2000, Vanderklift & Ponsard 2003). Under food shortage there was no difference in $\delta^{15}N$ values between treatments, as both uninfected and infected *Daphnia* suffered from the low food, and consequently, low nutrient input. The low C and N supply might have also depressed the growth of the parasite.

3.2.4 The effect of parasite infection on the host

Overall, the results gained from laboratory experiments (III, IV) suggest that the parasite used in the experiment had lower requirements for P than the *Daphnia* host, as it did not induce any changes in host P content (III). Based on stoichiometric and stable isotope results (IV), the parasite was inducing changes mainly in energy (C) or N metabolism. However, in the life-table experiment (III), where the input of N was constant, the effect of infection on host growth was different between food treatments, suggesting that the parasite was particularly changing energy allocation by the host. As there were no differences in filtration rates (III) or in respiration rates (IV) between uninfected and infected hosts, parasite infection might have been altering either absorption, assimilation or excretion rates of C and N (He & Wang 2006).

Stable isotope results implied that the parasite was inducing changes in the lipid content of the host. Indeed, microsporidians use host energy storage and especially lipids to produce spores (Biderre et al. 2000, Rivero et al. 2007). Spores are then released from the gut, which might explain the observed decrease in C content of infected *Daphnia* (IV). Furthermore, parasite-induced changes in lipid accumulation might have explained the intriguing higher growth rate of infected than uninfected hosts under P-limitation. Under P-limitation, *Daphnia* gain a large amount of excess C which they have to excrete to maintain somatic stoichiometry (Sterner & Elser 2002). Although *Daphnia* are capable of storing some excess C as lipids (Sterner et al. 1992, Tessier et al. 1983), they have to actively dispose of most leftover C via increasing respiration and excretion of DOC (Darchambeau et al. 2003, Anderson et al. 2005, Jensen & Hessen 2007, He & Wang 2008, Hessen & Anderson 2008). It seems that under low food quality infected *Daphnia* were able to convert a larger amount of excess C into lipids and possibly also had decreased costs of excreting the extra C, since the parasite was exploiting host lipid storage. In contrast, under high quality food *Daphnia* did not acquire excess C and the parasite was directly reducing C available for host somatic growth. This view is supported by the finding that the parasite was able to produce comparable numbers of spore clusters in extremely P-limited and P-

sufficient hosts, which again implies the parasite was less dependent on host P content.

In earlier studies the loss of egg production by infected individuals has been suggested as one explanation for the observed differences in host stoichiometry (Forshay et al. 2008, Frost et al. 2008b). However, parasite species used in these earlier works, the bacterial parasite *P. ramosa* (Frost et al. 2008a,b) and the chytridiomycete *Polycaryum laeve* (Forshay et al. 2008), are highly virulent and they castrate the host soon after the establishment of infection (Ebert et al. 2000, Johnson et al. 2006). The microsporidian parasite, *Glugoides intestinalis*, used in this study is a more benign parasite and impairs host reproduction only slightly, as was observed in study III and earlier by Ebert et al. (2000). Thus it seems that the parasite was inducing changes in host stoichiometric content both directly by exploiting C-rich lipid storage and indirectly by affecting energy and nutrient allocation via changing host reproduction and growth.

4 CONCLUSIONS

The results of this thesis indicate that host-parasite interactions are under the influence of environmental stoichiometry In natural conditions, parasite prevalence patterns were connected to either resource quality (N & P) or to host stoichiometry, suggesting that the seasonal variation in the availability of environmental nutrients may drive the fluctuations in parasite prevalence patterns commonly observed in nature (Altizer et al. 2006). However, the effects of nutrients on parasite dynamics were diverse, as some parasites showed positive and some negative associations with N and P of the environment or the host. The results of the nutrient enrichment study further demonstrated that the effect of nutrient loading on parasite epidemics is dependent on the interactions between environmental resources, parasites, hosts and other members of the food web. Thus knowledge of the epidemiological traits of parasites and of food web structure is needed to distinguish the causal relationships between environmental nutrient levels and host-parasite dynamics in nature.

The results from the laboratory experiments imply that the stoichiometric demands of both the parasite and the host affect the outcome of host-parasite interactions under different food quality and quantity regimes. It seems that lower dependence of the parasite on *Daphnia* P might facilitate less destructive exploitation of the host. Possibly, when parasite and host have different stoichiometric demands, with the parasite being less P-limited than the host, the parasite may show moderate virulence compared to parasites having similar stoichiometric demands as their host. In fact, several virulent species infecting P-rich *Daphnia* have been suggested to be nutrient limited (Forshay et al. 2008, Frost et al. 2008b). However, the patterns between host food quality and parasite virulence are complex and further experimental evidence is needed.

This thesis provides valuable information concerning the role of parasites in regulating the stoichiometric content of their host. The hosts used in the thesis, *Daphnia,* have an important role in regulating the stoichiometry of the environment and food resources in lake food webs (Sterner 1986, Elser & Urabe 1999). The results of this thesis demonstrate that parasites, even rather benign ones, are able not only to impair the vital life-history traits, but also to alter the

stoichiometric content of *Daphnia*. This implies that parasites may, in addition to affecting *Daphnia* population dynamics, also change the rates of C and nutrient recycling by *Daphnia*. Consequently, and considering their importance and ubiquity in food webs (Lafferty et al. 2008), parasites might be important drivers of stoichiometry also in other autotroph-herbivore-systems, and may affect the trophic cascades observed both in terrestrial and aquatic ecosystems.

Overall, the results obtained in this thesis suggest that one might expect the worldwide anthropogenic alterations of C and nutrient loading (Bennett et al. 2001, Monteith et al 2007, Elser et al. 2009, 2010) to affect parasite infection patterns. Indeed, eutrophication has been proposed to explain disease emergence in humans and wildlife (Johnson et al. 2010a). However, the results also imply that robust global generalizations about the effects of nutrient enrichment on prevailing parasite dynamics will prove hard to develop, since the interactions between nutrient and epidemics are diverse and strongly dependent on the host-parasite system studied.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Stoikiometrinen näkökulma lois-isäntäsuhteeseen

Jokainen eliö tarvitsee kasvaakseen ja lisääntyäkseen tietyn määrän energiaa (hiiltä) ja ravinteita (typpeä ja fosforia). Luonnossa on yleistä, että tuottajien hiili:ravinne-suhde on korkea ja vaihtelee ympäristöolojen, kuten valon määrän, mukaan. Kasvinsyöjät taas tarvitsevat yleensä paljon ravinteita verrattuna hiileen ja kärsivät usein ravinnepuutoksesta, joka heikentää niiden kelpoisuutta ja vähentää niiden kierrättämien ravinteiden määrää ja vaikuttaa edelleen ravintoverkon toimintaan ja hiili:ravinne-suhteisiin.

Loiset ovat eliöitä, jotka ottavat kaikki tarvitsemansa energian ja ravinteet isännästään heikentäen samalla isännän kasvua ja lisääntymistä. Loisten esiintyminen isäntäpopulaatiossa on yleensä kausittaista. Siihen vaikuttavat isäntäpopulaation tiheyden ja isännän ominaisuuksien lisäksi myös isännän ja muiden eliöiden väliset vuorovaikutussuhteet sekä useat ympäristötekijät.

Ihminen on muuttanut merkittävästi hiilen ja ravinteiden kiertoa vaikuttaen niiden saatavuuteen ja sitä kautta ravintoverkkojen ja eliöyhteisöjen toimintaan. On esitetty, että ihmisen aikaansaama vesistöjen ravinnekuorman kasvu voi olla yhteydessä maapallon laajuisesti havaittuun loisten ja taudinaiheuttajien määrän lisääntymiseen. Myös luonnossa normaalisti esiintyvä ravinteiden saatavuuden kausittainen vaihtelu voi osaltaan selittää loisten esiintymisen kausivaihtelua isäntäpopulaatiossa, mutta aiheesta on tehty vain muutamia tutkimuksia. Isännän saaman ravinnon laatu voi säädellä loisen esiintyvyyttä, kasvua ja isännälle aiheutuvan haitan suuruutta, koska loiset jakavat saman energia- ja ravinnevaraston isännän kanssa. Loiset voivat myös muuttaa isännän stoikiometriaa (hiili:ravinne-suhdetta) ja sitä kautta vaikuttaa ympäristön hiili- ja ravinnekiertoihin. Tämän väitöskirjan tarkoituksena oli tutkia ympäristön hiili:ravinne-suhteiden ja lois-isäntäsuhteen välisiä vuorovaikutuksia.

Ensimmäisessä osatyössä (I) selvitettiin, ovatko luonnon ravinnepitoisuuksien muutokset yhteydessä lois-isäntäsuhteen muutoksiin. Tulokset osoittivat, että isäntien tiheys oli yhteydessä ravinnon määrään ja laatuun, mutta ei loisten esiintymiseen, mikä johtui todennäköisesti vahingollisimpien loisten harvinaisuudesta tutkimusajanjakson aikana. Tulokset osoittivat myös, että joidenkin loislajien esiintyvyys kasvoi ja joidenkin laski ympäristön typpi- ja fosforipitoisuuden kasvaessa. Esimerkiksi yhdessä *Daphnia*-isännässä esiintyvien loislajien määrä oli suurimmillaan, kun isännän fosforipitoisuus oli alhaisimmillaan, kun taas suolessa esiintyvän USGP-loisen esiintyvyys oli suurimmillaan isännän resurssien laadun ollessa korkeimmillaan.

Osatyössä II tutkittiin, miten typpi- ja fosforilisäys vaikuttaa ravintokohteiden ja isännän runsauteen sekä stoikiometriseen laatuun ja sitä kautta loisten esiintyvyyteen ja isäntien loiskuormiin. Ravinnelisäys paransi sekä ravintokohteiden että isännän laatua, mutta ei lisännyt niiden tiheyksiä. Vain yksi neljästä tutkitusta loisesta oli yleisempi ravinnelisäyksen saaneissa tutkimusaltaissa, mutta muiden loisten esiintyvyydet ja isäntien loiskuormat

pysyivät samanlaisena käsittelyiden välillä, joten ravinnelisäys ei ollut riittävän suuri vaikuttaakseen kaikkien loisten esiintymiseen. Osatöiden I ja II tulosten perusteella näyttää siltä, että vuorovaikutussuhteet loisten esiintymisen ja ympäristön ravinnepitoisuuden välillä ovat loislajikohtaisia joidenkin reagoidessa ennemmin isännän alkuainekoostumukseen kuin ympäristön ravinteisuuteen. Lisäksi on tunnettava sekä tutkittavien loisten epidemiologia että ravintoverkon rakenne, jotta voidaan tarkemmin selvittää ympäristön ravinnekuorman ja loisinnan välisiä vuorovaikutusmekanismeja.

Osatyössä III tarkoituksena oli selvittää, vaikuttaako isännän kärsimä ravinnepuutos loisen kasvuun ja loisen isännälleen aiheuttaman haitan suuruuteen. Tulosten mukaan isännän ravinnepuutos ei merkittävästi heikentänyt loisen kasvua. Kärsiessään pelkästään ravinnepuutoksesta isäntä kasvoi hitaammin ja kuoli aiemmin kuin runsasravinteista ruokaa saanut isäntä. Loisinfektio vaikutti eri tavalla erilaista ruokaa saaneisiin isäntiin. Runsasravinteisella ravinnolla ruokituissa isännissä loisinfektio heikensi selviytymistä ja lisääntymistä, kun taas vähän ravinteita sisältävällä ravinnolla ruokittujen loisittujen ja loisettomien isäntien kuolevuus oli sama eikä kumpikaan ryhmä ehtinyt lisääntyä. Runsasravinteista ruokaa saaneissa isännissä loisinfektio heikensi kasvua, mutta ravinnepuutoksesta kärsivissä isännissä loisen vaikutus oli päinvastainen ja loisitut isännät kasvoivat nopeammin.

Osatöissä III ja IV tutkittiin ravinnon laadun tai määrän ja loisinnan yhteisvaikutusta paljon fosforia sisältävän isännän alkuaine- ja vakaiden isotooppien koostumukseen sekä fysiologiaan. Tulosten perusteella isännän ravinnon laatu vaikutti huomattavasti isännän fosforipitoisuuteen ja suodatusnopeuteen. Loinen muutti sekä isännän alkuaine- että isotooppikoostumusta, mutta ei vaikuttanut isännän suodatus- tai hengitysnopeuteen. Näiden tulosten perusteella tutkimani loinen käyttää isännän isännälle tärkeän fosforin sijasta isännän hiilipitoisia rasvavarastoja. Isännän stoikiometrisen koostumuksen ja elinkierron piirteiden muuttuminen loisinfektion myötä johtunee sekä tästä isännän rasvavarastojen käytöstä että loisen aiheuttamista muutoksista isännän energian ja ravinteiden käytössä. Osatöiden III ja IV tulokset antoivat viitteitä siitä, että sekä loisen että isännän ravinnevaatimukset vaikuttavat loisen kasvuun ja haitallisuuteen isännälle isännän kokiessa ravinnepuutosta. Näyttää myös siltä, että loislajit, jotka käyttävät enimmäkseen jotain muuta kuin isännälle tärkeää alkuainetta, voivat olla isännälleen vähemmän haitallisia kuin loislajit, jotka kilpailevat samasta alkuaineesta isännän kanssa. Saatujen tulosten perusteella näyttää siltä, että loiset voivat vaikuttaa isännän alkuainekoostumukseen ja sitä kautta koko ravintoverkon ja yhteisön hiilen ja ravinteiden kiertoon sekä maa- että vesiekosysteemeissä.

Tämän väitöskirjan tulosten perusteella voidaan päätellä, että ihmisen aikaansaamat muutokset hiilen ja ravinteiden kierrossa maapallolla vaikuttavat myös lois-isäntäsuhteisiin, mutta vaikutukset ovat monimuotoisia ja ovat voimakkaasti riippuvaisia tutkittavasta lois-isäntä-systeemistä.

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ORIGINAL PAPERS

I

NO UNIFORM ASSOCIATIONS BETWEEN PARASITE PREVALENCE AND ENVIRONMENTAL NUTRIENTS

by

Sanni L. Aalto, Tarmo Ketola & Katja Pulkkinen

Manuscript

II

RESPONSES OF ALGAE, BACTERIA, *DAPHNIA* AND NATURAL PARASITE FAUNA OF *DAPHNIA* TO NUTRIENT ENRICHMENT IN MESOCOSMS

by

Sanni L. Aalto, Outi Kaski, Kalevi Salonen & Katja Pulkkinen 2013

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CLADOCERA

Responses of algae, bacteria, *Daphnia* and natural parasite fauna of *Daphnia* to nutrient enrichment in mesocosms

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Abstract Understanding responses of parasites to changes in nutrient regimes is necessary for prediction of their role in aquatic ecosystems under global change in nutrient loading. We studied the response of the natural parasite fauna of *Daphnia longispina* to nutrient enrichment in mesocosms in a small humic lake. We measured the concentrations of inorganic phosphorus and nitrogen in the water, total nutrients in the seston, algal and bacterial biomass, *Daphnia* population dynamics, *Daphnia* stoichiometry, *Daphnia* stable isotope values and the presence and abundance of parasites in treated mesocosms as compared to three control ones. Incorporation of the nutrient enrichment in the food web was seen as increased nutrient concentrations in the epilimnion and as a decrease in carbon: nutrient ratios and δ^{15}N values in *Daphnia*. Nutrient enrichment did not significantly influence algal, bacterial or *Daphnia* biomass. One of the four parasite species observed, unidentified small gut parasite, had a higher prevalence (percentage of *Daphnia* infected) in treated mesocosms, but its intensity (number of parasites per infected host) remained the same among treatments. Our results suggest that the effect of nutrient enrichment on host–parasite dynamics is dependent on complex interactions within food webs and on the epidemiological traits of parasites.

Keywords *Daphnia longispina* · Host–parasite interaction · Enclosure · Stoichiometry · Food web dynamics

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Introduction

In addition to predation and competition, parasitism is one of the key biological interactions affecting the fitness of organisms. Parasites impair host growth, reproduction and survival, and thus affect host population dynamics and their role in food webs (Lafferty et al., 2008). Parasites and pathogens themselves are affected by both biotic (e.g. food web structure, Lafferty et al., 2008) and abiotic (e.g. temperature; Marcogliese, 2001) factors. The significance of nutrient availability in driving disease dynamics has been recognized only recently (Marcogliese, 2001; McKenzie & Townsend, 2007; Johnson et al., 2010). In freshwater ecosystems, parasite prevalence and abundance have been shown to respond positively to enrichment of the major nutrients, nitrogen (N) and phosphorus (P) (Lafferty, 1997; Lafferty & Holt, 2003; McKenzie & Townsend, 2007).

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Nutrient enrichment can directly enhance the growth (Bruno et al., 2003; Voss & Richardson, 2006) or virulence (Wedekind et al., 2010) of pathogens. In addition, elevated resources can increase pathogen effects indirectly via increase in host density (Johnson et al., 2007; McKenzie & Townsend, 2007).

Distinguishing the ultimate cause behind changes in disease dynamics is difficult, as host–pathogen interactions depend on several environmental and biological variables. Biotic factors, such as predation pressure on hosts (Duffy, 2007; Hall et al., 2010), can also modify the parasite infection pattern. Thus, increases in N and P loading may not always have a visible effect on disease if the ecosystem is complicated, i.e. the number of interactions between and within trophic levels is high (McKenzie & Townsend, 2007).

Cladocerans, especially *Daphnia,* form a key link in energy and nutrient mobilization between primary producers and higher trophic levels in many aquatic ecosystems (Sterner & Hessen, 1994; Jansson et al., 2007; Lampert, 2011). They utilize both phytoplankton and bacterioplankton as food sources (Kankaala, 1988; Taipale et al., 2007), and their elemental requirements are well studied (Sterner & Elser, 2002). Due to their high P demand they commonly suffer "quality starvation" (i.e. do not meet their optimal elemental demands for growth) in natural conditions as the P concentration of food sources is typically low (Hessen, 2008). By recycling of nutrients, *Daphnia* can significantly affect phytoplankton production (Elser & Urabe, 1999; Sterner & Elser, 2002).

Natural populations of *Daphnia* host numerous parasite species, which reduce their fecundity and survival (Ebert, 2005). By taking energy from the host, parasites may change the host metabolic demands and food intake (Moore, 2002; Schmidt-Hempel, 2011). This could alter assimilation and excretion of nutrients in infected hosts. These effects could cascade on to other trophic levels. Experiments with different *Daphnia* parasites have indicated increased production of infective propagules in response to increased P content of *Daphnia* food (Frost et al., 2008; Hall et al., 2009a). Paradoxically, enhanced quality and quantity of food seems to lead to decreased transmission, possibly via decreasing the clearance volume of *Daphnia* and the probability of ingestion of infective propagules of parasites (Hall et al., 2007, 2009a). Thus, understanding and predicting the effects of complicated interactions between nutrient stoichiometry and parasite infections in aquatic ecosystems remain challenging.

In this study, we tested the effect of nutrient enrichment on food web dynamics and on the epidemiology of *Daphnia* parasites and epibionts in experimental mesocosms in a small pond. The aim of the nutrient addition was to increase phytoplankton biomass and change the stoichiometric composition of the food sources available for *Daphnia*. We hypothesized that the improved nutritional status of *Daphnia* would increase their value as a resource for parasites and subsequently lead to increases in parasite load. For parasite prevalence, we had no a priori expectations, since parasite transmission might be affected differently by changes in density, foraging behaviour, or filtration rate of *Daphnia*.

Methods

Study site

Lake Mekkojärvi is located in Southern Finland in the Evo Forest Area (61°13′N, 25°8′E). The lake is small (0.35 ha) and shallow (mean depth 3 m), with steep vertical temperature, oxygen and nutrient gradients. Due to high concentration of dissolved organic carbon (DOC; 20–45 mg C l^{-1}; Taipale et al., 2008), the euphotic zone is only 0.5–1 m thick. Total P concentration is 10–15 μg P l^{-1} in the epilimnion and 25–35 μg P l^{-1} in the hypolimnion. The values for total N are 500–1,000 and 800–1,100 μg N l^{-1}, for the epilimnion and hypolimnion, respectively (Taipale, 2007). The annual primary production of phytoplankton is below 10 mg C m^{-2} and decreases from spring to autumn (Salonen et al., 2005). The bacterial biomass increases from spring to autumn and is highest in the hypolimnion in late summer (Taipale et al., 2009). Autotroph production is co-limited by N and P (Järvinen & Salonen, 1998).

The zooplankton of the lake is dominated by a cladoceran, *Daphnia longispina*, whose biomass fluctuates between 1,000 and 20,000 individuals m^{-2} during the open water season and is responsible for more than 90% of the metazoan zooplankton biomass at midsummer (Salonen & Lehtovaara, 1992; Salonen et al., 1994). These *Daphnia* rely heavily on bacterial food, especially on methanotrophs, on which they feed

during short trips to hypoxic metalimnion (Salonen & Lehtovaara, 1992; Taipale et al., 2008). Predation pressure on *D. longispina* is low, because the lake lacks zooplanktivorous fish and the main predators are *Chaoborus* larvae at densities ca. 300 ind m^{-2} and *Notonecta* sp. (Salonen & Lehtovaara, 1992).

In a preliminary survey, four parasite species infecting *D. longispina* were found from Lake Mekkojärvi: one bacterium (*Pasteuria ramosa*), one microsporidian (*Larssonia obtusa*) and two unidentified endoparasites living in the gut (Aalto & Pulkkinen, unpublished data). The smaller of the two endoparasites (unidentified small gut parasite, hereafter referred to as USGP) is most likely a microsporidian. It forms clusters with varying number of spores (15–30 spores per cluster) inside the gut epithelial cells. The larger endoparasite (unidentified large gut parasite, hereafter referred to as ULGP) is oval and ca 20-µm long. It can be found either interstitially or within the epithelial cells. In addition, we recorded the occurrence of an epibiont (*Vorticella* sp.).

Experimental design

A mesocosm experiment was performed in late summer (14 July–18 August 2008). We chose this time period for our experiment because a preliminary study indicated that the prevalence and intensity of parasites increases from spring/early summer towards late summer. At the same time, the concentration of phytoplankton and dissolved inorganic P (PO_4) has significantly decreased in the shallow epilimnion since the spring bloom (Järvinen & Salonen, 1998). The experiment was made in cylindrical mesocosms (diameter 2 m, height 4 m) constructed of 0.2-mm polyethylene film and extending from the surface to the sediment in the middle of Lake Mekkojärvi. The mesocosms enclosed natural *D. longispina* populations, densities of which were checked to be similar among mesocosms by quantitative samples counted visually at the field and returned to mesocosms (Table 1). The movement of zooplankton into and out of the mesocosms was prevented by the anoxic sediment. Three mesocosms received weekly additions of P (10 µg P l^{-1} $week^{-1}$ as KH_2PO_4) and N (100 µg N l^{-1} $week^{-1}$ as NH_4NO_3) and three mesocosms acted as controls. Nutrients were adjusted to approximately double the P concentration of the epilimnion. The P:N addition ratio of 1:10 was based on earlier addition experiments in humic lakes (Jansson et al., 2001), and increased the mean total N concentration 1.2 times. Nutrient addition was made to the epilimnion, which has the highest primary production (Kuuppo-Leinikki & Salonen, 1992).

Physical, chemical and biological measurements

The mesocosms were sampled each week before nutrients were added. Water samples were taken from the epilimnion (0–0.6 m), metalimnion (0.6–1.2 m) and hypolimnion (1.2–3.0 m) using a Limnos tube sampler (height 0.6 m, volume 4.25 l). From the epilimnion and metalimnion, two samples were pooled and from the hypolimnion a pooled sample was derived from three samples collected from discrete depths (1.2–1.8, 1.8–2.4 and 2.4–3.0 m). Samples were passed through a 100-µm mesh plankton net to retain zooplankton for quantification. Chemical determinations and measurements of chlorophyll concentrations and bacterial biomasses were made from the water passed through the net. Additional zooplankton samples were taken as vertical hauls from 3 m to the surface in each mesocosm using a 100 µm plankton net for determination of nutrient and stable isotope values of *Daphnia* and for counting their parasites from fresh samples. Oxygen and temperature were measured from one treated and one control enclosure at depths of 0, 0.5, 1, 2 and 3 m using a YSI 55 probe (Yellow Springs Instruments).

Samples for inorganic N (ammonium; NH_4, nitrite plus nitrate; NO_{2+3}) and P (PO_4) were filtered through glass fibre filters (Whatman GF/F) and measured with QuickChem 8000 flow injection analyzer (Lachat instruments). Samples for total nitrogen (TN) and total phosphorus (TP) were determined after digestion by alkaline persulphate. Alkalinity, pH, conductivity and water colour were determined using the validated routine methods of the Finnish Standard Association (www.SFS.fi). For chlorophyll *a* (Chl *a*) and bacteriochlorophyll *d* (BChl *d*) determinations, a 0.5 l water sample from each layer was filtered through a glass fibre filter (Whatman GF/F). Pigments were extracted in ethanol and their absorptions measured with a spectrophotometer (Shimadzu UV-240) at 665 and 750 nm for Chl *a* and 655 nm for BChl *d*. The concentration of Chl *a* was calculated according to Lorenzen (1967). The 665 and 655 absorption ratio was used to indicate the changes in relative proportions of algal and

Table 1 Comparison of initial (week 0) water chemistry, algal and bacterial biomasses, nutrient ratios and stable isotope values in *Daphnia*, their population parameters and prevalence and intensity of the two most common parasites between control mesocosms and those allocated to nutrient treatment (N + P) after the first sampling

	Control	N + P	*t* test significance
NH_4 (μg N l^{-1})	14 ± 2.0	18 ± 7.2	0.62
NO_{2+3} (μg N l^{-1})	41 ± 7.6	26 ± 0.7	0.4*
PO_4 (μg N l^{-1})	1.3 ± 0.33	1.7 ± 0.33	0.7*
TN (μg N l^{-1})	684 ± 46	670 ± 13	0.78
TP (μg N l^{-1})	18 ± 1.2	19 ± 0.3	0.61
Chl *a* (mg m^{-3})	11 ± 5.1	17 ± 5.5	0.21
PhyBio (mg m^{-2})	520 ± 178	445 ± 153	0.8*
BacDen (10^6 cell ml^{-1})	17 ± 5.1	15 ± 5.0	0.71
BacBio (μg C ml^{-1})	271 ± 31	221 ± 49	0.20
C:P	154 ± 22	155 ± 39	0.97
C:N	5.2 ± 0.11	5.2 ± 0.19	0.87
$\delta^{13}C$ (‰)	−40 ± 0.88	−40 ± 0.49	0.85
$\delta^{15}N$ (‰)	3.8 ± 0.10	4.0 ± 0.38	0.4**
DapDen (10^3 ind m^{-3})	12 ± 1.7	11 ± 4.7	0.87
DapLeng (mm)	0.95 ± 0.05	1.0 ± 0.09	0.22
USGPprev (%)	4.7 ± 2.3	7.8 ± 1.7	0.13
USGPint	8.6 ± 6.5	9.0 ± 3.9	0.93
ULGPprev (%)	61 ± 8.3	62 ± 14	0.98
ULGPint	5.2 ± 0.9	4.6 ± 1.9	0.65

Ammonium (NH_4), nitrite plus nitrate (NO_{2+3}), phosphate phosphorus (PO_4), total nitrogen (TN), total phosphorus (TP) and chlorophyll *a* (Chl *a*) concentrations, phytoplankton biomass (PhyBio), bacterial density (BacDen) and biomass (BacBio), *Daphnia* carbon:phosphorus (C:P), *Daphnia* carbon:nitrogen (C:N) ratios, stable isotope values of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in *Daphnia*, *Daphnia* density (DapDen), *Daphnia* mean length (DapLeng), USGP prevalence (USGPprev) and intensity (USGPint), ULGP prevalence (ULGPprev) and intensity (ULGPint) in *Daphnia* populations. Nutrient concentrations as well as Chl *a* concentration are given for the epilimnion. All other values are given for the whole water column. Values are mean ± SE ($n = 3$)

* Nitrite plus nitrate and phosphate values and phytoplankton biomass analyzed with Mann–Whitney *U* test

** For $\delta^{15}N$ values equal variances not assumed

bacterial chlorophyll. According to Arvola et al. (1992), a 665/655 ratio greater than 0.8 indicates the dominance of Chl *a* and a ratio below 0.5 indicates the dominance of BChl *d*.

For determination of phytoplankton biomass, bacterial biomass and bacterial density, 200 ml subsamples of water were fixed with 1 ml of Lugol's iodine solution. The species composition and biomass of phytoplankton were determined with an inverted microscope using a settling chamber technique (Utermöhl, 1958). Subsamples (50 ml) were sedimented for 24 h and 25 random fields were counted in each sample at ×200 magnification. The biovolumes were estimated from the linear dimensions measured for each taxon. For analysis of bacterial density and biomass, subsamples (1 ml) were decolourized with thiosulphate and then stained with DAPI-solution (1 μl ml^{-1}; 4,6-diamino,-2-phenylindole 171 dihydrochloride, Sigma) on black polycarbonate filters (Osmonics, pore size 0.2 μm). Five to ten randomly taken fields per filter were photographed with an epifluorescent microscope (Olympus BX60-microscope, Y-CMAD3-camera) at ×1,000 magnification and were analyzed with analySIS-program (analysis 3.1 Soft Imaging System). Bacterial biomass was estimated based on length and width of bacteria measured by the programme. Bacterial cell volumes were converted to carbon by a factor 0.36 pg C μm^{-3} (Tulonen, 1993). Bacterial density and biomass were determined at the beginning and on the second and last weeks of the experiment.

Zooplankton were preserved in 70% ethanol. The presence and number of other invertebrate species

were recorded. The number of *Daphnia* individuals and eggs or juveniles in the brood pouch were counted. The lengths of 100 *Daphnia* individuals were measured from the top of the head to the base of the tail spine under a preparation microscope (Leica L2, ×3/ Olympus SZX9, ×40 magnification). Mean *Daphnia* density and length and mean number of eggs and juveniles per adult female (>1.24 mm) were estimated for each mesocosm. For the determination of the elemental ratios (C:P, C:N) and N (δ^{15}N) and carbon (δ^{13}C) stable isotope values of *Daphnia*, animals were rinsed with deionized water, picked into tubes and stored in a freezer at −80°C. Two replicates from freeze-dried samples were weighed in tin cups before the determination with a Carlo-Erba Flash 1112 series elemental analyser connected to a DELTAplus Advantage mass spectrometer (Thermo Fisher Scientific Corporation) and run against IAEA standard NBS-22 using dried and homogenized fish muscle as an internal laboratory working standard. Values of δ^{15}N give information on the inorganic N source used by the *Daphnia* (Matthews & Mazumder, 2007) while δ^{13}C values are indicators of the food sources (Vander Zanden & Rasmussen, 1999). In Lake Mekkojärvi, the bacteria have lower δ^{13}C values than phytoplankton and thus higher δ^{13}C values in treated mesocosms would indicate less feeding on bacteria in the hypoxic metalimnion (Taipale et al., 2008).

Live samples of *D. longispina* were investigated for the presence of parasites (as in Stirnadel & Ebert, 1997; Decaestecker et al., 2005; Ebert, 2005). To avoid observer bias, one person screened all the samples unaware of the origin of the sample. From each mesocosm and sampling date, 50 individuals were studied, making a total of 1,800 of *D. longispina* individuals examined. Infections in juveniles are generally in an early phase and therefore difficult to detect (Stirnadel & Ebert, 1997; Ebert, 2005); thus, only adult females were included in the screening. First, *Daphnia* were screened for visible signs of parasite infection under preparation microscope with transmitted light illumination. *Vorticella* sp. individuals attached to the carapace were also counted. The presence of the endoparasite *L. obtusa* could be seen as altered transparency of the head and body cavity of the hosts. The presence of endoparasite spores was further verified by examination of the dissected animal under phase-contrast microscope (Leitz Biomed, ×400 magnification). As this parasite usually fills the entire body cavity, the number of spores was not enumerated. The other endoparasite *P. ramosa* found in the preliminary survey was not encountered from the mesocosms. The gut of the animals was dissected apart from the body and checked for the number of epithelial cells infected by USGP or for the presence of individual ULGP. The prevalence for all parasite species was calculated as the number of infected females per total females examined. Intensity of infection was estimated for ULGP and *Vorticella* sp. as number of specimens and for USGP as number of infected gut epithelial cells per infected female.

Data analysis

All mesocosms were sampled once before nutrient additions. Data from the first week was analyzed separately using independent samples *t* tests, comparing mean values in those mesocosms subsequently allocated to nutrient treatment to mean values in those mesocosms remaining in the control group (see Tables 1, 2). For the remaining 5 weeks, a repeated-measures analysis of variance (RM-ANOVA) was used to analyze if fixed factor (nutrient addition and/or time) had an effect on the dependent variables (NH_4, NO_{2+3}, PO_4, TN, TP, Chl *a*, algal biomass, bacterial biomass and density, *Daphnia* C:P, C:N, δ^{13}C and δ^{15}N values, mean sum of eggs and neonates of adult *Daphnia* female, *Daphnia* population density, mean length and CV of length in *Daphnia*, prevalence and intensity of USGP, ULGP, *L. obtusa* and *Vorticella* sp.). As the circularity assumptions of the variance–covariance matrix were not met, we used Greenhouse-Geisser-corrected significance levels. The normality of the data was tested with Shapiro–Wilks test and homogeneity of variances using Levene's test. In case of non-normality and/or heteroskedasticity of the data, the Mann–Whitney *U* test was used for studying the effect of nutrient addition and the Kruskal–Wallis *H* test for the effect of time. Spearman's rank correlation was used to study the relationship between *Daphnia* density and parasite prevalence. All values are presented as mean ± SE, for the untransformed data. The programme PASW (version 18.0, IBM Corporation, Armonk, NY, USA) was used for statistical analysis.

Results

Initial conditions and physical parameters in mesocosms

No differences were detected between the mesocosms in the initial conditions in any of the measured variables (Table 1). During the experiment, no major changes occurred in the environmental conditions. Temperature in the epilimnion varied between 14 and 19°C and oxygen between 4 and 6 mg l^{-1} during sampling. For other physical and chemical variables ranges were: pH 5.1–5.6, alkalinity 0.04–0.06 mmol l^{-1}, conductivity 36–43 µS cm^{-1} and water colour 400–1,400 mg Pt l^{-1}.

Nutrient concentration in lake water and in seston, and algal and bacterial biomass

Following the nutrient addition, inorganic nutrient concentrations in the epilimnion increased significantly as compared to control mesocosms (Fig. 1; Table 2). The mean ammonium (NH_4) concentration (Fig. 1a) was over two times higher and the mean nitrite plus nitrate (NO_{2+3}) concentration (Fig. 1b) was almost three times higher in treated than in control mesocosms. The mean phosphate (PO_4) concentration was nearly three times higher in treated than in control mesocosms (Fig. 1c; Table 2). N and P concentrations in the epilimnetic seston also increased in the treated mesocosms. Both the mean TN (Fig. 1d) and TP

Table 2 RM-ANOVA and Mann–Whitney *U* tests of water chemistry, algal and bacterial biomasses, nutrient ratios and stable isotope values in *Daphnia*, their population parameters and prevalence and intensity of the two most common parasites between control and treatment (N + P) mesocosms

	Control	N + P	Test statistics	Significance
NH_4 (µg N l^{-1})	32 ± 5.8	74 ± 7.1	$U = 31$	**<0.001**
NO_{2+3} (µg N l^{-1})	34 ± 2.7	99 ± 9.4	$U = 8$	**<0.001**
PO_4 (µg N l^{-1})	1.9 ± 0.25	5.2 ± 0.52	$U = 18$	**<0.001**
TN (µg N l^{-1})	723 ± 6.3	843 ± 9.4	$F_{1,4} = 60$	**0.002**
TP (µg N l^{-1})	16 ± 0.36	23 ± 0.70	$U = 2$	**<0.001**
Chl *a* (mg m^{-3})	13 ± 1.7	11 ± 1.7	$U = 96$	0.51
BacDen (10^6 cell ml^{-1})	220 ± 21	185 ± 16	$F_{1,4} = 4.2$	0.11
BacBio (µg C ml^{-1})	16 ± 0.99	13 ± 0.91	$F_{1,4} = 89$	0.40
C:P	137 ± 4.9	123 ± 2.7	$U = 61$	**0.03**
C:N	5.2 ± 0.06	5.0 ± 0.05	$U = 47$	**0.01**
$\delta^{13}C$ (‰)	−40 ± 0.24	−40 ± 0.36	$F_{1,4} = 4.2$	0.53
$\delta^{15}N$ (‰)	3.0 ± 0.14	1.9 ± 0.23	$F_{1,4} = 4.2$	**0.004**
EggNeo	0.3 ± 0.1	0.1 ± 0.0	$U = 162$	**0.04**
DapDen (10^3 ind m^{-3})	14 ± 2.1	14 ± 1.8	$U = 107$	0.84
DapLeng (mm)	1.2 ± 0.03	1.2 ± 0.03	$U = 95$	0.49
CV	1.3 ± 0.07	1.6 ± 0.11	$U = 63$	**0.04**
USGPprev (%)	26 ± 3.2	35 ± 3.4	$F_{1,4} = 4.2$	**0.02**
USGPint	17 ± 2.2	17 ± 1.7	$F_{1,4} = 0$	0.96
ULGPprev (%)	43 ± 3.9	44 ± 3.2	$U = 112$	0.97
ULGPint	3.1 ± 0.19	3.5 ± 0.31	$F_{1,4} = 4.2$	0.06

Ammonium (NH_4), nitrite plus nitrate (NO_{2+3}), phosphate phosphorus (PO_4), total nitrogen (TN), total phosphorus (TP) and chlorophyll *a* (Chl *a*) concentrations, bacterial density (BacDen) and biomass (BacBio), *Daphnia* carbon:phosphorus (C:P), *Daphnia* carbon:nitrogen (C:N) ratios, stable isotope values of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in *Daphnia*, mean sum of eggs and neonates per adult *Daphnia* female (EggNeo), *Daphnia* density (DapDen), *Daphnia* mean length (DapLeng), mean of the coefficient of variation in *Daphnia* length (CV), USGP prevalence (USGPprev) and intensity (USGPint), ULGP prevalence (ULGPprev) and intensity (ULGPint) in *Daphnia* populations. Values are mean ± SE ($n = 15$). *P* values in bold denote significant effect

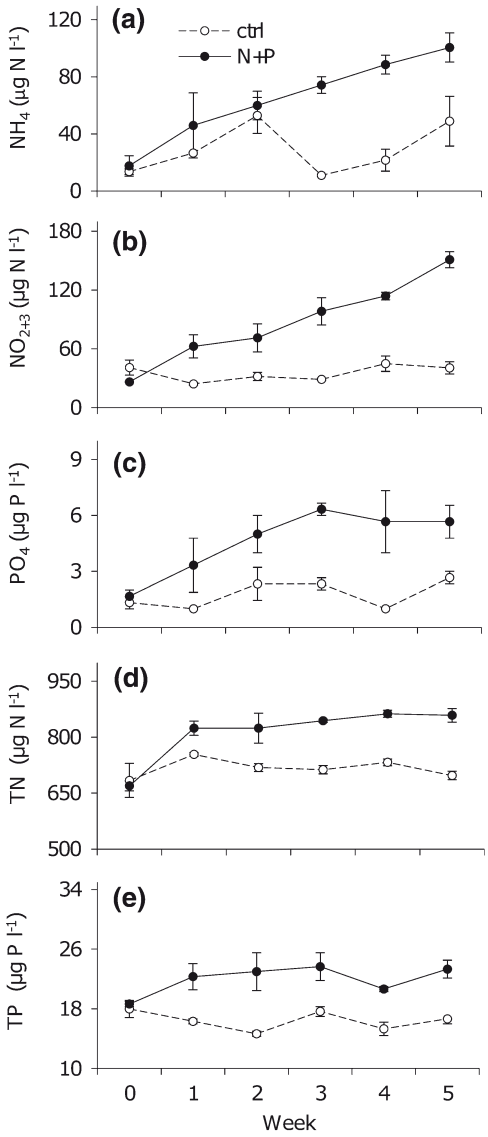

Fig. 1 Inorganic and seston nutrient concentrations in the epilimnion of control (*open circles*, *dashed line*) and treated (*filled circles*, *solid line*) mesocosms during the 6-week experiment: **a** ammonium, **b** nitrite plus nitrate, **c** phosphate, **d** seston TN and **e** seston TP. *Symbols* represent mean ± SE ($n = 3$). Note that on week 0-treated mesocosms had not received nutrients yet

concentrations (Fig. 1e) were higher in treated than in control mesocosms (Table 2).

There was temporal variation in epilimnetic Chl *a* concentration during the experiment (Fig. 2a), but the mean concentration did not differ between the treated and control mesocosms (Table 2). Nutrient addition did not shift the ratio between Chl *a* and BChl *d*, which remained less than 0.8 during the whole experiment in both treated and control mesocosms. The algal biomass did not significantly change in the treated mesocosms during the experiment (Kruskal–Wallis, $H = 0.2$, d.f. $= 2$, $P = 0.80$; Fig. 2b). In the control mesocosms, there was, however, a consistent fourfold peak in the biomass on week 3 as compared to any other week (RM-ANOVA, $F_{2,4} = 14.4$, $P = 0.015$; Bonferroni corrected pairwise comparisons between week 3 and week 2 or 5, $P < 0.05$ for both; Fig. 2b). This peak was caused almost entirely of an increase in the biomass of two species of *Mallomonas* (82% of total algal biomass in control mesocosms). Other algal taxa encountered from the mesocosms were *Monomastix* sp., *Cryptomonas* sp., *Chlamydomonas* sp., *Botryococcus* sp., *Tabellaria* sp., *Closterium* and *Scourfeldia* sp.

There was no temporal change in the bacterial density or biomass between the second and last week of the experiment (RM-ANOVA, $F_{1,4} = 0.01$, $P = 0.94$, for density; $F_{1,4} = 0.30$, $P = 0.61$, for biomass; Fig. 2c, d). Neither bacterial density nor biomass differed between the treatments (Fig. 2c, d; Table 2).

Responses of *Daphnia* stoichiometry and population dynamics to nutrient addition

Nutrient addition affected the stoichiometry of *Daphnia*. The mean C:P was significantly lower in *Daphnia* from treated mesocosms than in *Daphnia* from control mesocosms (Table 2), the differences becoming evident during the last 3 weeks of the experiment (Fig. 3a). The mean C:N ratio was also significantly lower in treated than in control mesocosms (Table 2), and again the differences became evident during the last 3 weeks of the experiment (Fig. 3b). There was no difference in δ^{13}C values (Fig. 3c; Table 2), implying that there were no changes in the feeding of the *Daphnia* among treatments. However, *Daphnia* individuals in treated mesocosms had lower δ^{15}N values than those in controls (Fig. 3d; Table 2), which indicates incorporation of the added N.

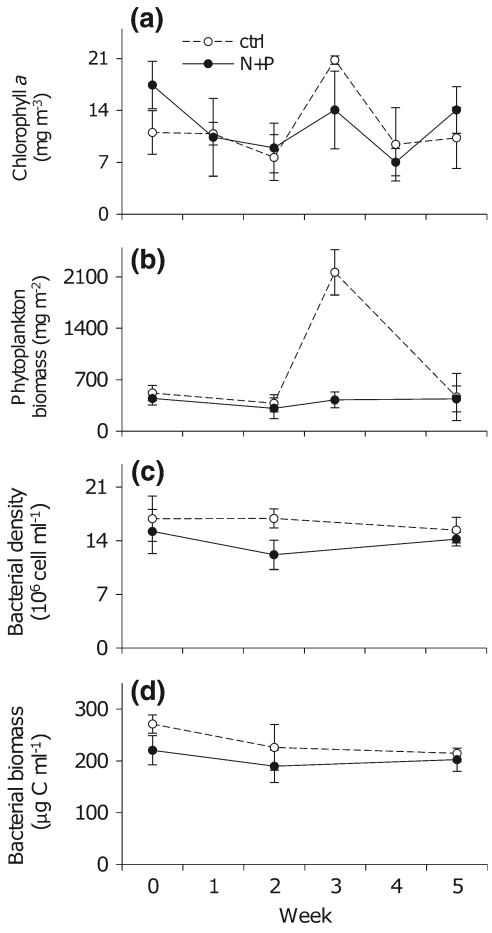

Fig. 2 Algal and bacterial quantifications in control (*open circles*, *dashed line*) and treated (*filled circles*, *solid line*) mesocosms during the 6-week experiment: **a** Chl *a* in epilimnion, **b** phytoplankton biomass, **c** bacterial density and **d** bacterial biomass in whole water column. *Symbols* represent mean ± SE ($n = 3$). Note that on week 0-treated mesocosms had not received nutrients yet

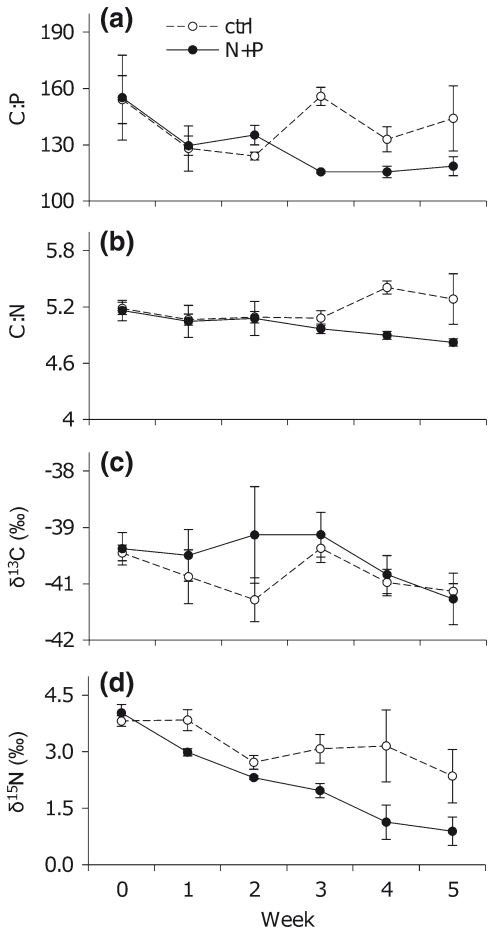

Fig. 3 Stoichiometry and stable isotope values in *Daphnia* in control (*open circles*, *dashed line*) and treated (*filled circles*, *solid line*) mesocosms during the 6-week experiment: **a** C:P, **b** C:N, **c** δ^{13}C and **d** δ^{15}N. *Symbols* represent mean ± SE ($n = 3$). Note that on week 0-treated mesocosms had not received nutrients yet

The overall mean sum of eggs and neonates in the brood pouch per adult *Daphnia* female were significantly lower in treated than in control mesocosms (Fig. 4a; Table 2), which was due to high egg/neonate production during the last 2 weeks of the experiment in control mesocosms. The pattern was the same among the *Daphnia* screened for parasites (data not shown). The *Daphnia* population density in the mesocosms varied during the experiment (Kruskal–Wallis, $H = 13.3$, d.f. $= 4$, $P = 0.01$), but there was no difference between the treatments (Fig. 4b; Table 2). The mean length of the *Daphnia* was similar in controls and treated mesocosms (Fig. 4c; Table 2). However, the mean of the coefficient of variation (CV) in length was higher in treated than in control mesocosms (Fig. 4d; Table 2), indicating that during

the experiment there was more variation in the length distribution of the treated mesocosms than in that of the control ones.

Other invertebrate species found in the mesocosms were *Polyphemus pediculus*, *Scapholeberis mucronata*, *Chironomus* sp. and copepod species, all occurring at low densities. *Chaoborus* larvae were found sporadically from all mesocosms.

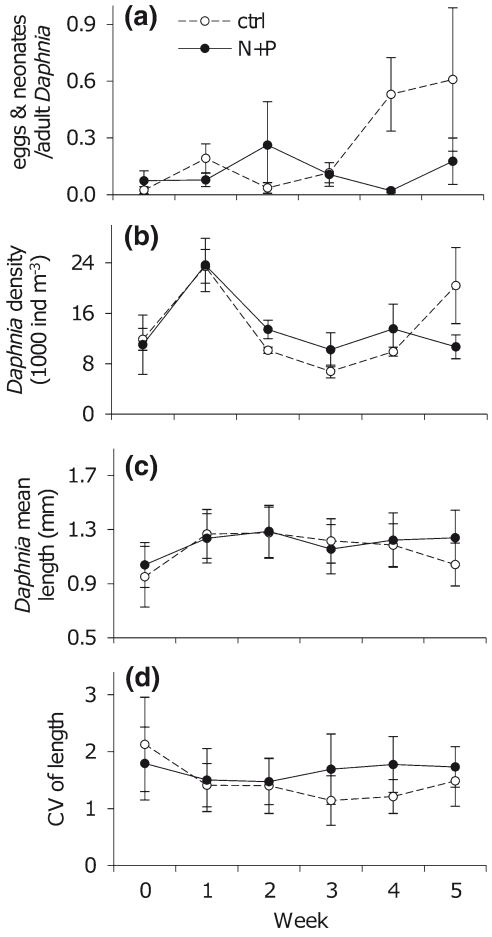

Fig. 4 *Daphnia* population parameters in control (*open circles*, *dashed line*) and treated (*filled circles*, *solid line*) mesocosms during the 6-week experiment: **a** eggs and neonates/adult *Daphnia* female, **b** *Daphnia* density, **c** mean length and **d** coefficient of variation of length. *Symbols* represent mean ± SE ($n = 3$). Note that on week 0-treated mesocosms had not received nutrients yet

Parasite prevalence and intensity

The microsporidian *L. obtusa* occurred in the control mesocosms only for 1 week after the beginning of nutrient addition at prevalences below 2% (Fig. 5a). In treated mesocosms, *L. obtusa* was discovered also on weeks 3 and 4 at similarly low prevalences. The epibiont *Vorticella* sp. appeared on week 2 both in control and treated mesocosms at prevalences around 60–70%, but with only a few individuals per infected *Daphnia*. It then declined dramatically and remained at very low levels especially in control mesocosms (Fig. 5b). Neither of these species occurred at frequencies sufficient to allow statistical comparisons to be made.

Two unidentified parasite species, the microsporidian USGP and the large protozoan-like ULGP, were both found throughout the experiment. At the beginning of the experiment, USGP prevalence was below 10% in all mesocosms, but steadily increased both in control and treated mesocosms to over 40% (RM-ANOVA, time: $F_{4,16} = 31.6$, $P < 0.001$). USGP prevalence was significantly higher in treated than in control mesocosms (Table 2), but with no significant interaction between time and treatment ($F_{4,16} = 0.61$, $P = 0.59$; Fig. 5c). The mean intensity of USGP varied during the experiment, mostly between 10 and 20, and no differences between the treatments were detected (Table 2). In contrast to USGP prevalences, ULGP prevalences declined during the experiment from around 60% at the beginning to around 30–40% at the end of the experiment (Kruskal–Wallis, $H = 13.3$, d.f. $= 4$, $P = 0.01$). In this case, there was no difference between the control and treated mesocosms (Fig. 5d; Table 2). The mean intensity of ULGP varied between 2 and 5 parasites per host, with no statistical difference between the control and treated mesocosms (Table 2).

There was no significant positive relationship between *Daphnia* density and USGP/ULGP prevalence (Spearman's rank correlation, $r = -0.09$, $P = 0.59$; $r = 0.15$, $P = 0.36$, for USGP and ULGP, respectively). Nor was any relationship found between USGP/ULGP prevalence and *Daphnia* density in the previous week ($r = -0.11$, $P = 0.56$; $r = 0.28$, $P = 0.13$, for USGP and ULGP, respectively).

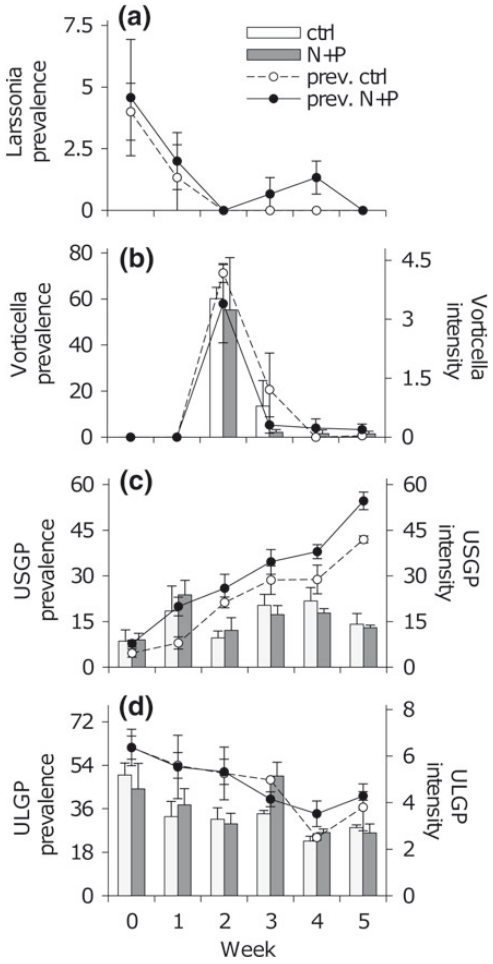

Fig. 5 Parasite prevalence (%; control: *open circles* and *dashed line*, treated: *filled circles* and *solid line*) and intensity (control: *open bars*, treated: *filled bars*) in *Daphnia* in treated and control mesocosms during the 6-week experiment: **a** *L. obtusa*, **b** *Vorticella* sp., **c** USGP and **d** ULGP. *Symbols* represent mean ± SE ($n = 3$). Note that on week 0-treated mesocosms had not received nutrients yet

Discussion

The results of our experiment demonstrate complex interactions between trophic levels. As evidenced by decreases in δ^{15}N values and carbon:nutrient ratios of *Daphnia* in treated mesocosms, nutrient addition was clearly incorporated into the food web. In spite of this, no detectable effects on algal and bacterial biomasses or *Daphnia* density or size were seen. Contrary to our hypothesis, improved nutritional quality of *Daphnia* did not increase their parasite load. One of the four parasites found from *Daphnia* during the experiment had a higher prevalence in the mesocosms receiving nutrients.

In contrast to earlier experiments (Jansson et al., 1996; Elser et al., 2007), our nutrient addition did not increase algal and/or bacterial biomass. Previously, it has been found that addition of N + P stimulated bacterial production in humic lakes only when DOC concentrations were lower than 15 mg l^{-1} (Hessen et al., 1994; Jansson et al., 1996, 2001), due to binding of phosphate to iron–humic complexes at high DOC concentrations. Consequently, 20–45 mg C l^{-1} DOC concentration in Lake Mekkojärvi might have limited the availability of added nutrients for primary producers. However, as the measured reactive PO_4 concentration was finally at least twice as high in treated mesocosms, we may assume that there was proportionally more free P available than in control mesocosms. There was a fourfold increase in algal biomass in the control mesocosms of the third week of the experiment caused by two species of *Mallomonas*. The peak followed a week with the highest temperature in the epilimnetic water. However, differences in any physical or chemical parameters or in *Daphnia* density between the control and treated mesocosms do not offer an explanation for this phenomenon. Possibly *Mallomonas* were able to multiply in the control mesocosms because they may need less nutrients and they are more resistant to *Daphnia* grazing than other algal taxa present (Salonen & Arvola, 1988).

Alternatively, any increases in primary producer biomass in response to nutrient addition were readily consumed by the *Daphnia* population. This is implied by the decreased carbon:nutrient ratios in *Daphnia*. The seston of Lake Mekkojärvi is known to correspond to the stoichiometry of phytoplankton, bacterioplankton and rotifers (Järvinen & Salonen, 1998). Thus, higher nutrient concentration of seston indicates higher N and P concentration of phytoplankton and bacterioplankton, which is passed on to *Daphnia*. This idea is further supported by the lower δ^{15}N values in *Daphnia* in treated mesocosms. Their N isotopes are acquired from phytoplankton and bacteria, whose δ^{15}N values reflect those of their inorganic N source (Matthews & Mazumder, 2007). The added N

compound (NH_4NO_3) had lower $\delta^{15}N$ value (0.87 ± 0.29‰) than lake seston-POM (2.5–5‰; Taipale et al., 2008). As the $\delta^{13}C$ values in *Daphnia* did not show differences among the treatments, there is no evidence of any change in the proportions of phytoplankton and bacterioplankton consumed by *Daphnia* due to nutrient addition. However, egg ratio, which is conventionally used as an indicator of improved nutrition in *Daphnia* (Hessen, 1990; references therein), was lower in treated mesocosms, and does not at first sight support improved nutritional conditions. A more careful inspection reveals that the higher egg production in control mesocosms was probably induced by the peak in algal biomass on week 3, which was accompanied by an increase in C:P ratios of *Daphnia.* This suggests that increased egg ratio was induced by increased C availability rather than improved nutrition in terms of N and/or P acquisition.

Despite the improved nutrition suggested by body stoichiometry and $\delta^{15}N$ values, the nutrient addition did not increase the abundance of *Daphnia.* This finding could have resulted from increased predation pressure cutting down any increases in *Daphnia* population density. Due to univoltine life cycle of the invertebrate predator *Chaoborus* (Sæther, 1997), increases in larval densities due to nutrient addition during the experiment can be excluded. However, it is possible that *Chaoborus* grew bigger if they had more *Daphnia* to eat in treated mesocosms, becoming thus more efficient predators.

Another explanation for no changes in *Daphnia* density in the treated mesocosms may be that *Daphnia* population dynamics was affected by increase in parasite prevalence due to nutrient addition. Several previous studies have demonstrated negative effects of parasites on *Daphnia* populations (Decaestecker et al., 2005; Johnson et al., 2009; Hall et al., 2011). Parasites are known to cause instability in *Daphnia* host populations (Ebert et al., 2000; Pulkkinen & Ebert, 2004), which might explain the higher weekly variation in the proportions of juveniles and mature individuals in nutrient-enriched mesocosms compared to control ones. Unfortunately, we could not verify potential negative effects of the parasites on vital rates of *Daphnia* experimentally, as we did not manage to maintain infections in the laboratory.

The frequency of *Daphnia* infected with one of the parasites, USGP, was higher in the populations in the treated mesocosms. However, no interaction between time and treatment was found; thus, the result could reflect initial differences in prevalence (4.7 in controls vs. 7.8% in treated mesocosms at week 0), as the statistical power to detect initial differences was low. No correlation between *Daphnia* density and USGP prevalence was found, indicating that USGP transmission is not density dependent. Previously, Frost et al. (2008) have reported higher percentage of infection in *Daphnia* receiving high quality food. On the other hand, Hall et al. (2007, 2009a) found that higher food quality and quantity diminished transmission in *Daphnia* infected with a fungus, because higher food quantity led to diminished clearance rate and subsequent intake of parasite spores. In our experiment, the higher prevalence of infection is thus consistent with higher quality rather than quantity of algal food for *Daphnia*. Also *Chaoborus* predation on infected *Daphnia* can facilitate dispersal of spores (Cáceres et al., 2009), but due to the univoltine life cycle of *Chaoborus* (Sæther, 1997), we may exclude this explanation for increased USGP prevalence, as changes in *Chaoborus* density due to nutrient addition were unlikely.

Contrary to earlier findings with increased propagule production in well-fed hosts (Frost et al., 2008; Hall et al., 2009a, b), intensity of USGP (the number of infected epithelial cells) was not larger in treated mesocosms. It seems that even if *Daphnia* had higher nutrient content, they did not offer qualitatively or quantitatively enough resources to increase production of USGP spores. Another possibility is that hosts exceeding some level of USGP intensity died and were thus excluded from our data.

The other two endoparasites, ULGP and *L. obtusa*, did not respond to nutrient enrichment. This suggests that the epidemiology of the different parasites found from *Daphnia* in Lake Mekkojärvi is mediated by different factors. Possibly their life cycles include intermediate hosts (Refardt et al., 2002). Also the status of ULGP as a parasite remains currently unclear. The epibiont *Vorticella* sp. perhaps should not be considered as a parasite, as it does not utilize host resources directly, although dense bunches may impair host swimming or filtration ability (Green, 1974). Interestingly, *Vorticella* in Lake Mekkojärvi has been shown to compete for food with *Daphnia* (Kankaala & Eloranta, 1987). The peak prevalence of *Vorticella* was following the *Daphnia* peak density in the previous week, indicating a dependency on *Daphnia* density.

Ours is the first study of changes to a natural parasite fauna in response to changes in host nutrient regime. Our aim of improving the nutritional status of *Daphnia* was accomplished as evidenced by lower C:nutrient ratios. However, contrary to our hypothesis, this did not lead to increases in parasite load. We observed higher prevalence of one parasite species in treated host populations, which could reflect increased transmission for this species, but could also be caused by higher initial prevalences in mesocosms allocated to nutrient addition treatment. Combined with the previous reports (Frost et al., 2008; Hall et al., 2009a), our results show that different species of parasites respond to increased nutrient loading in different ways, possibly depending on their epidemiology. Thus, the outcome of host–parasite dynamics under different nutrient regimes cannot be predicted without knowledge of the food web dynamics and epidemiology of parasites under concern. Considering the prevalence of parasites in aquatic ecosystems (e.g. Lafferty et al., 2008) and their ecological significance in driving changes in host populations (Decaestecker et al., 2005; Ebert, 2005; Johnson et al., 2007), understanding host–parasite interactions would be crucial for prediction of their effects under changing nutrient loading in fresh water systems (Bennett et al., 2001; Monteith et al., 2007; Elser et al., 2009).

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III

FOOD STOICHIOMETRY AFFECTS THE OUTCOME OF *DAPHNIA*-PARASITE INTERACTION.

by

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Food stoichiometry affects the outcome of *Daphnia*–parasite interaction

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Keywords
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Abstract

Phosphorus (P) is an essential nutrient for growth in consumers. P-limitation and parasite infection comprise one of the most common stressor pairs consumers confront in nature. We conducted a life-table study using a *Daphnia*–microsporidian parasite model, feeding uninfected or infected *Daphnia* with either P-sufficient or P-limited algae, and assessed the impact of the two stressors on life-history traits of the host. Both infection and P-limitation negatively affected some life-history traits tested. However, under P-limitation, infected animals had higher juvenile growth rate as compared with uninfected animals. All P-limited individuals died before maturation, regardless of infection. The numbers of spore clusters of the microsporidian parasite did not differ in P-limited or P-sufficient hosts. P-limitation, but not infection, decreased body phosphorus content and ingestion rates of *Daphnia* tested in separate experiments. As parasite spore production did not suffer even under extreme P-limitation, our results suggest that parasite was less limited by P than the host. We discuss possible interpretations concerning the stoichiometrical demands of parasite and suggest that our results are explained by parasite-driven changes in carbon (C) allocation of the hosts. We conclude that the impact of nutrient starvation and parasite infection on consumers depends not only on the stoichiometric demands of host but also those of the parasite.

Introduction

Parasites are significant biotic stressors detrimentally affecting individuals and populations of host species. In contrast to other stressors (predation, competition, pesticides), parasites have a reciprocal relationship with the target organism, as parasites drain their energy from the host. Thus the presence of other stressors that negatively affect the host can also indirectly mediate the progress of infection or development of the parasite itself (Lafferty and Kuris 1999; Duffy et al. 2011). Subsequently, other stressors may alter the virulence of the parasite, that is, the fitness cost imposed on the host by the parasite (Jokela et al. 2005; Johnson et al. 2006a, 2007; Coors and De Meester 2008; Coors et al. 2008).

Host nutrition has an important role in host–parasite interactions. Previous studies have mainly concentrated on the effect of food quantity on host physiological functions (e.g., immune defense) and the development of the parasite infection. For example, food deprivation decreased parasite load in the water flea *Daphnia magna* (Pulkkinen and Ebert 2004) and in the snail *Lymnaea stagnalis* (Seppälä et al. 2011). Fewer studies have considered the effect of food quality on host–parasite interactions. In the caterpillar *Spodoptera exempta*, survival of bacterially infected larvae was lower with decreasing dietary protein-to-carbohydrate ratio (Povey et al. 2009). Recently, studies have (Frost et al. 2008; Hall et al. 2009) demonstrated poor nutritional condition (low food phosphorus P concentration) of the host to decrease parasite within-host reproduction.

Nutrient competition between the host and the parasite may determine the outcome of infection (Smith 1993) and could be expected to depend on the stoichiometric demands of both partners. If parasite and host are competing for the same elemental nutrient, parasite effects on host physiological status might be more drastic and it might thus express higher virulence than in a

situation where stoichiometric demands of the parasite and the host differ. The strength of nutrient competition could be expected to vary among different host–parasite combinations.

Phosphorus is an important component for numerous key molecules, including nucleid acids (RNA and DNA) and energetic nucleotides (ATP) and as such, essential for organism growth and function (Sterner and Elser 2002). Consumers have high demand for P for optimal growth and reproduction (Elser et al. 2000; Hessen 2008). However, producer carbon:phosphorus (C:P) ratios vary widely following environmental nutrient concentrations (Sterner and Elser 2002; Sterner et al. 2008) and autotrophs with high C:P are common (Elser et al. 2000, 2010). This leads to a stoichiometric mismatch, where consumers (notably grazers) are limited by P which implicitly means C is in excess for (nearly) homeostatic consumers. Thus P-limitation will cause reduced growth efficiency in terms of C or energy, and it may consequently change the life-history parameters, behavior or physiological status of organisms (Urabe et al. 1997; Sterner and Elser 2002; Frost et al. 2005, 2010). Considering the frequency of P-limitation and ubiquity of parasites in nature, they constitute a powerful combination of mutual stressors.

In this study, we address the impact of P-limitation on parasite virulence and their combined effect on key life-history traits of host. The host, water flea *D. magna* (Fig. 1), has high requirements for nutritional phosphorus (~1% P; Main et al. 1997) and P-limitation has major negative impact on growth and reproduction (Sundbom and Vrede 1997; Jeyasingh et al. 2011). The parasite, *Glugoides intestinalis*, is a microsporidian endoparasite, which infects host gut epithelial cells and thus shares host energy and nutrient supply (Ebert 1995; Larsson et al. 1996). It is rather avirulent (Ebert et al. 2000) as compared with parasite species used in previous studies concerning P-limitation and parasite infection (Frost et al. 2008; Hall et al. 2009).

We conducted a life-table experiment, feeding uninfected or infected *Daphnia* either with P-sufficient or P-limited algae. Key life-history traits were recorded over one life cycle in order to see at which stage P-limitation alters parasite virulence. We also followed the progress of the parasite infection in order to estimate the growth of the parasite. On the basis of earlier knowledge (Hessen et al. 2002; DeMott 2003), we expected strong negative effects of P-limitation on *Daphnia* survival, growth, and reproduction. Similarly, we expected negative fitness effects on hosts fed P-sufficient food when infected (Ebert et al. 2000). Regarding the interaction between P-limitation and infection, we considered two alternative outcomes. If the parasite had high requirements for P, parasite spore production and its virulence would decrease in the P-limited host. However, if the parasite was less limited by P than the *Daphnia* host, parasite would be able to grow and even increase in virulence under P-limitation.

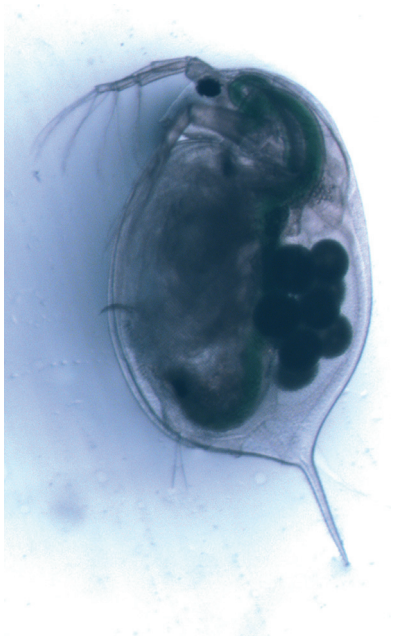

Figure 1. Water flea *Daphnia magna*.

Materials and Methods

Study system

The model used in the experiment consisted of a freshwater crustacean *D. magna* Straus (Crustacea: Cladocera) and its obligatory parasitic microsporidian *G. intestinalis* Chatton (Microspora: Glugeidea; Larsson et al. 1996). *G. intestinalis* is an intracellular parasite infecting host's gut epithelial cells through waterborne spores. It reproduces directly and transmission is horizontal between hosts (Ebert 1995). Hosts do not recover from infection (Ebert et al. 2000). *G. intestinalis* produces spherical clusters of 20–30 spores (Ebert and Mangin 1997). The number of clusters increases exponentially during the early infection (by more than one order of magnitude from day 7 to day 13 after infection; Ebert 1994, 1995). The *Daphnia* clone (DK-35-9) used in the experiment has been maintained in the laboratory for several years. It is the original host for the parasite *G. intestinalis*.

Life-table experiment

The food algae, *Scenedesmus gracilis*, was grown in semi-batch cultures in modified WC medium (Guillard and Lorenzen 1972, without vitamin solution) with a biweekly renewal corresponding to a dilution rate 0.2 per day. P-sufficient algae was grown in medium containing 50 μmol P/L, yielding a stable C:P ratio of approximately 200. For P-limited algae, the P-concentration was reduced to 5 μmol P/L, which resulted in C:P ratios ranging between 900 and 1100. The cultures were grown at least 2 weeks before starting the experiment to attain steady state. The cell density was calculated for each batch of algae used for feeding. As a proxy of C content we used cell numbers and C concentrations (mg C per cell) from a preliminary algae growth experiment (S. L. Aalto and K. Pulkkinen unpubl. data).

Prior to the experiment, *Daphnia* females were transferred to glass jars filled with 200 mL ADaM (Klüttgen et al. 1994; modified by using only one twentieth of the SeO_2 concentration) in groups of 10–20 animals. They were fed ad libitum with a suspension of the P-sufficient food algae. Experiments were started with neonates from at least second brood of these mothers born within 12 h. Neonates were distributed randomly in groups of 10 into 100 mL of ADaM. Half of the neonates were exposed to parasite infection by cohabitation with five *D. magna* females infected with *G. intestinalis* for 24 h. Control animals were treated similarly with females from uninfected cultures. During exposure, experimental animals were fed with P-sufficient algae at 2 mg C/L. After 24 h, animals were transferred individually into 50 mL of ADaM. The females used for infection were dissected and checked for presence of infection in order to verify exposure of all experimental animals in the infected treatment.

In the experiment, 48 replicates of infected and 20 of uninfected were fed with P-sufficient algae and 58 and 40, infected and uninfected, respectively, with P-limited algae. Of these, 25 randomly selected infected individuals fed with P-sufficient algae and 15 fed with P-limited algae were allocated only for following the progress of parasite infection (see later). Individuals in both treatments received 1 mg algal C/L per day on first 6 days and subsequently 2 mg C/L per day. Every other day, individuals were transferred with a pipette in a small volume of ADaM onto a glass slide and photographed with video camera (GO-5-CLR-12, QImaging, Surrey, Canada) attached to research stereo microscope (SZX9, Olympus, Hamburg, Germany). Then they were transferred to fresh media and fed. Juveniles released were counted and discarded.

The photographs were analyzed with ImageJ (Image Processing and Analysis in Java; version 1.44) to measure the length of *Daphnia* (top of the helmet to the base of the caudal spine) and to detect the eggs in brood chamber. Lengths were converted into dry weights using separate preestablished relationships for all four treatments. For P-sufficient treatment: Dry weight (μg) = 10.4* length (mm)$^{3.03}$, r^2 = 0.89, Dry weight (μg) = 8.4* length (mm)$^{2.28}$, r^2 = 0.78, for uninfected and infected animals, respectively. For P-limited treatment: Dry weight (μg) = 10.2* length (mm)$^{0.84}$, r^2 = 0.32, Dry weight (μg) = 9.9* length (mm)$^{1.39}$, r^2 = 0.54, for uninfected and infected animals, respectively.

Growth (g) was calculated from individual dry weights (W_1 and W_2) at successive times (t_1 and t_2) according to Lampert and Trubetskova (1996):

$$g = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}.$$

We calculated two different indices: juvenile growth (g_j) was calculated as a difference in individual dry weight at day 6 (W_2) and day 0 (W_1). To calculate the growth until maturation (g_m), W_2 was defined individually as the day when first clutch of eggs were released into the brood pouch. In uninfected animals this age varied between 8 and 12 days and in infected between 10 and 16 days. Experiment was ended when remaining animals had produced their third clutch (day 26).

To follow the progress of parasite infection during the experiment, randomly chosen individuals fed with P-sufficient algae were dissected at ages of 13, 21, and 26 days. In addition, all remaining animals from the life-history experiment at day 26 were dissected. The first time point was chosen based on earlier knowledge that parasite infection is fully developed and spore clusters are visible inside host gut cells on days 10–13 after the establishment of the infection (Ebert 1994, 1995; K. Pulkkinen unpubl. data). Due to high mortality, animals fed with P-limited algae were dissected already at ages of 9 and 13 days and infection could not be followed further. Spore loads of the animals were determined from freshly dissected guts by enumerating the number of spore clusters within epithelial cells under microscope (Leitz Biomed, Leica Microsystems, Wetzlar, Germany) using 100–400 × magnification with phase contrast (Ebert and Mangin 1997).

In order to calculate C:P ratios of the algae, subsamples were collected and freeze dried (Alpha 1-4 LD Plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). Particulate C was analyzed on Carlo-Erba Flash 1112 series Elemental Analyser (Thermo Fisher Scientific, Waltham, MA). Particulate P was measured with QuickChem 8000 flow injection analysis system (LaChat instruments, Loveland, CO). To measure carbon per liter (mg C/L), subsamples of algae were filtered on preweighed CF/C filters, dried in 60°C and weighed with

analytical balance (ED224S, Sartorius AG, Göttingen, Germany).

Phosphorus content and ingestion rate of *Daphnia*

Neonates born within 24 h from at least second brood of mothers kept in standard conditions were distributed randomly in groups of 20 into 100 mL of ADaM and exposed either to infection or treated as uninfected controls as described above for the life-table experiment. Eleven replicates of infected and 11 replicates of uninfected animals (20 per replicate) were subsequently fed with P-sufficient algae, and 12 and 11, infected and uninfected, respectively, with P-limited algae.

Four replicates of each treatment were analyzed for body P content at the age of 6 days and the remaining replicates at age of 12 days. Some P-limited replicates suffered from high mortality, leaving fewer replicates for analyses at age of 12 days. Animals were rinsed with deionized water, collected into glass scintillation vials, and freeze dried. Samples were combusted (450°C, 4 h), diluted in 0.2N H_2SO_4, and analyzed with QuickChem 8000 analyzer.

To measure ingestion rate (1000 cells ind^{-1} h^{-1}), nine replicates of infected and nine replicates of uninfected animals (ten per replicate) were fed with P-sufficient algae, and eight infected and eight uninfected replicates with P-limited algae. A batch of P-sufficient algae was radiolabelled with 1.5 MBq of carrier-free radioisotope ^{33}P. After 48 h, the algae were centrifuged (1500 rpm, 5 min) to remove any dissolved radioactive isotope and resuspended into ADaM. Unlabeled subculture of algae was treated in the same way and analyzed for C:P ratios and algal cell concentrations as in the life-table experiment (see above). Ingestion rates were measured from four replicates of infected and four replicates of uninfected P-sufficient animals, and three infected and three uninfected replicates of P-limited animals at the age of 6 days and from the remaining replicates at the age of 12 days. In each ingestion trial, 1–3 animals were allowed to feed with labeled P-sufficient algae for 8 min, then rinsed with ADaM and transferred to scintillation vials. To measure algal and background activities, 2 mL of the feeding solution used in the ingestion trial as well as 2 mL of solution filtered through 0.2 μm Nucleopore filter was put in separate scintillation vials. The sample volume in *Daphnia* samples was adjusted to 2 mL with ADaM. Samples were solubilized with 1 mL of Solvable™ (Perkin Elmer, Waltham, MA) at room temperature overnight. Next day, activity of the ^{33}P was determined with scintillation counter (Rackbeta, Perkin Elmer) using 10 mL of scintillation cocktail (HiSafe3, Perkin Elmer), channel 50–190 and counting time of 10 min, resulting in 100–3000 counts per minute for *Daphnia* and 1000–8000 for algae samples. Activity in filtrate samples was close to background values. Ingestion rate was calculated following Lampert and Taylor (1985).

Statistical analyses

All statistical analyses were conducted using PASW (version 18.0, IBM Corporation, Armonk, NY). Two-way analysis of variance (two-way ANOVA) or three-way analysis of variance (three-way ANOVA) was used if the data met or could be transformed to meet the normality assumptions. Otherwise, nonparametric tests (Mann–Whitney *U*-test) were used. Differences in the survival of individuals during the experiment were analyzed with Cox regression analysis using a time-dependent covariate and interaction of food treatment and infection status as covariates, and food treatment and infection status as categorical covariates. Growth curves were analyzed with a nonlinear regression model. The model was based on von Bertalanffy growth equation for weight ($W_{age} = W_{max} \times (1-e^{(-K \times (age-t_0))})^3$), where W_{max} is the estimated asymptotic size, K is curvature index, and t_0 is age of size 0. Curves with nonoverlapping confidence intervals of the asymptotic sizes were determined to be statistically significantly different.

Results

P-limitation inhibited the growth of *Daphnia* at juvenile stage (g_j) relative to P-sufficient animals (Fig. 2A). The interaction between food and infection was statistically significant (two-way ANOVA $F_{1,102} = 172$, $P < 0.001$), such that in P-limited treatment infected individuals had higher growth rate compared with uninfected ones ($F_{1,102} = 36$, $P < 0.001$), but vice versa in P-sufficient treatment ($F_{1,102} = 140$, $P < 0.001$). As all P-limited animals died before maturation, growth until maturation (g_m) was calculated only for P-sufficient animals, in which the infection impaired the growth statistically significantly (two-way ANOVA, $F_{1,32} = 96$, $P < 0.001$, Fig. 2B). Infected maturing animals had lower specific growth rates than juvenile animals (paired *t*-test, $t = 3.1$, $P = 0.008$), but there was no difference within uninfected animals (paired *t*-test, $t = -0.4$, $P = 0.68$).

These results were further confirmed by nonlinear regression models for the growth curves, which showed that the asymptotic weight of individuals fed with P-limited food (17 ± 3 μg ind^{-1}; mean ± 95% confidence intervals) was substantially lower than those fed with P-sufficient food (1046 ± 666 μg ind^{-1}; Fig. 3). The asymptotic weight did not differ between uninfected and

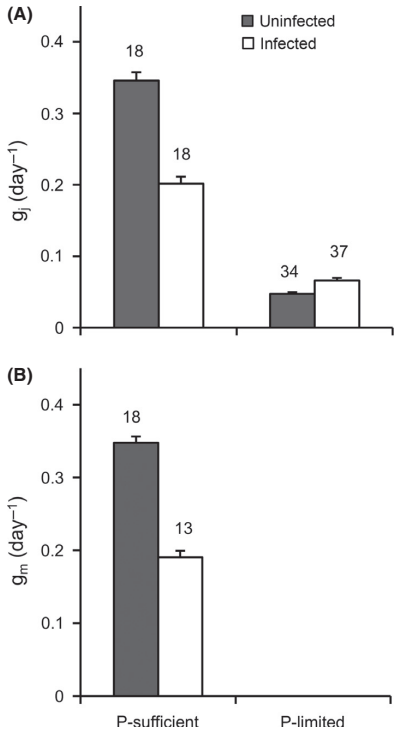

Figure 2. Mean (±SE) juvenile growth rate (g_j) (A) and growth until maturation (g_m) (B) in response to infection status and food quality. Sample sizes (number of *Daphnia* measured) are indicated above the bars.

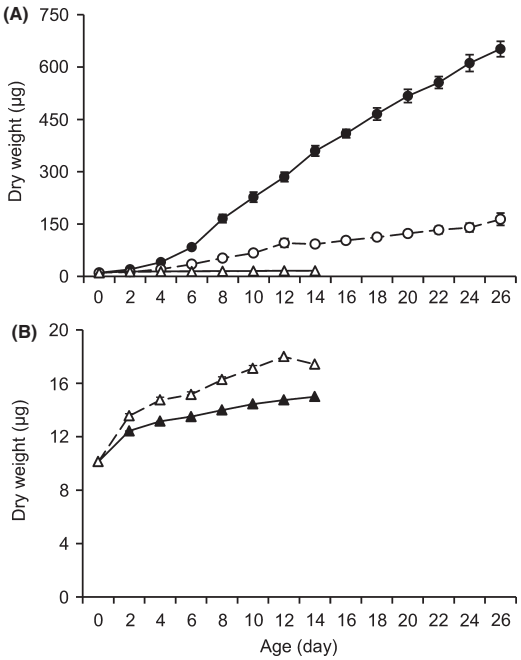

Figure 3. The growth of *Daphnia magna* over time for P-sufficient (filled dots), infected P-sufficient (open dots), P-limited (filled triangles), and infected P-limited (open triangles) treatments (A). Due to the large scale in panel (A) hiding the difference between the P-limited treatments, the growth curves for P-limited (filled triangles) and infected P-limited (open triangles) are presented (B). Means ± SE are depicted.

infected P-limited individuals (17 ± 2 μg and 20 ± 6 μg ind^{-1}, respectively), while infected individuals in the P-sufficient treatment (195 ± 52 μg ind^{-1}) reached lower weight than uninfected individuals (850 ± 70 μg ind^{-1}).

All P-limited animals died before maturation, thus we could compare reproductive parameters only between infected and uninfected *Daphnia* fed with P-sufficient food. Body mass at maturity was lower for infected (107 ± 3 μg) than for uninfected *Daphnia* females (365 ± 14 μg; ANOVA for sqrt-transformed values, $F_{1,34} = 450$, $P < 0.001$). Infected females produced their first brood later than uninfected females (Mann–Whitney *U*-test, $U = 93$, $P = 0.029$, Fig. 4A). Similar trend was seen within the next clutches (second clutch: $U = 81$, $P = 0.05$; third clutch: $U = 41$, $P = 0.03$). The average clutch size was smaller in infected animals, but the difference was not statistically significant within each clutch ($U = 128$, $P = 0.29$; $U = 86$, $P = 0.11$; $U = 66$, $P = 0.36$; for first, second, and third clutch, respectively, Fig. 4B). However, the cumulative number of neonates born during the experiment (of the first three clutches) was smaller in infected than in uninfected females (ANOVA, $F_{1,34} = 9.93$, $P = 0.003$).

According to Cox regression, survival of *Daphnia* consuming P-limited food was lower compared with animals fed with P-sufficient food (Fig. 5, Table 1). The effect of infection alone was not statistically significant. However, as indicated by significant interaction between food and infection, and the negative coefficient estimate in Cox regression, infection decreased survival proportionally less within P-limited animals than within sufficient ones (Table 1).

In P-limited animals, few spore clusters were visible in two of the five animals dissected at day 9. At day 13, the mean number of spores did not differ statistically significantly between P-limited and P-sufficient animals (Mann–Whitney *U*-test, $U = 22$, $P = 0.26$, Fig. 6). Due to low survival, the development of the spore load of P-limited animals could not be followed further. In *Daphnia* consuming P-sufficient food, the spore load remained the

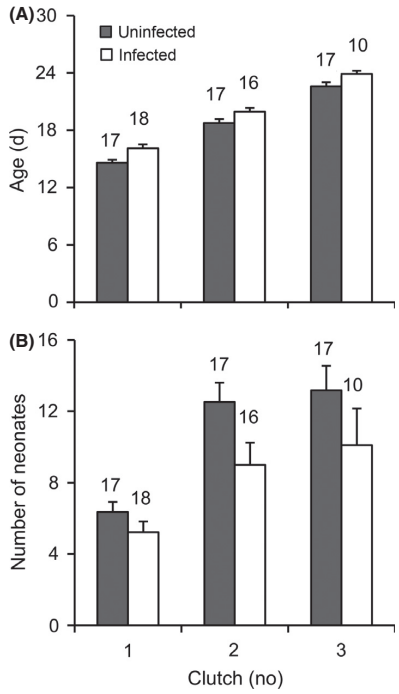

Figure 4. Mean (±SE) production age for each clutch (A) and size of the clutch for each clutch (B) in uninfected and infected P-sufficient individuals. Sample sizes (number of *Daphnia* measured) are indicated above the bars.

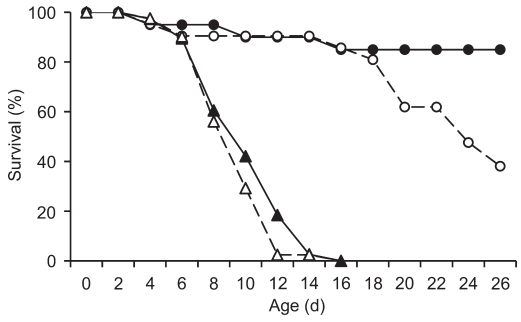

Figure 5. Percent of *Daphnia magna* hosts surviving over time between P-sufficient (filled dots), infected P-sufficient (open dots), P-limited (filled triangles), and infected P-limited (open triangles) treatments.

same between days 13 and 21 (Kruskal–Wallis *H*-test; pairwise comparisons: ages 13 and 21 days, $z = -0.99$, $P = 0.97$, Fig. 6). However, at the end of the experiment (day 26) the remaining animals had significantly lower

Table 1. Coefficient estimates (B), SE values, and test statistics for terms in the final model and terms excluded from the model produced by Cox regression survival analysis.

Terms in final model	B	SE	Wald	df	*P*-value	Exp(B)
Food	4.438	0.463	91.966	1	0.000	84.597
Time*Food*Infection	0.054	0.016	11.544	1	0.001	1.056
Food*Infection	−1.35	0.626	4.614	1	0.032	0.260
Terms not included in final model			Score	df	*P*-value	
Infection			0.141	1	0.707	

Exp(B) is the associated mortality risk, that is, a value greater than 1 indicates an increased mortality risk compared with the baseline group, and a value less than 1 indicates a lower mortality risk. The survival of P-limited animals is contrasted against P-sufficient individuals. In interaction terms, the infected animals are contrasted against uninfected ones within food treatments (and in time).

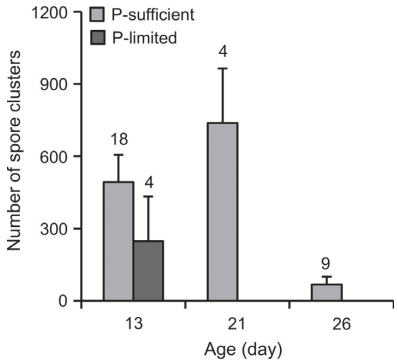

Figure 6. Mean (±SE) spore load in infected P-sufficient and P-limited *Daphnia magna* individuals. Sample sizes (number of *Daphnia* measured) are indicated above the bars.

spore load as compared with day 21 ($\chi = 10.4$, df = 3, $P = 0.02$, pairwise comparisons for ages 21 and 26 days, $z = 15.1$, $P = 0.03$).

P-limited *Daphnia* had lower body P content than P-sufficient animals (three-way ANOVA, $F_{1,30} = 19.9$, $P < 0.001$, Fig. 7A). Juvenile (6 days old) animals had less phosphorus than mature (12 days old) animals ($F_{1,30} = 4.2$, $P = 0.05$). P-limited *Daphnia* had lower ingestion rate than P-sufficient animals (three-way ANOVA, $F_{1,20} = 38.6$, $P < 0.001$, Fig. 7B). Neither body P content nor ingestion rate differed between uninfected and infected animals.

Discussion

The negative effects of both P-limitation and infection with *G. intestinalis* on *Daphnia* growth and survival found

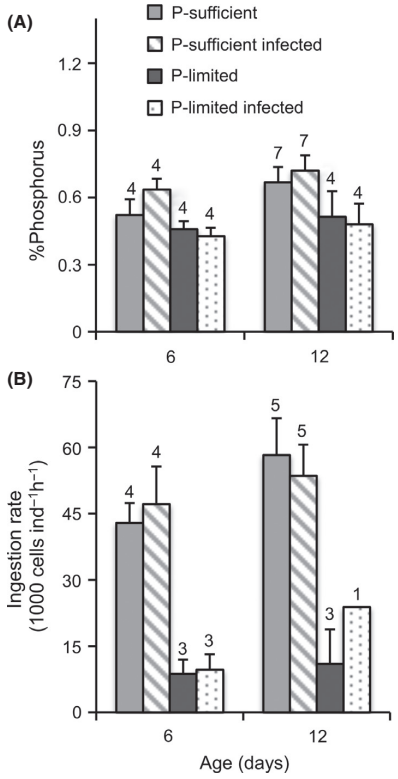

Figure 7. Mean (±SE) body P content (A) and ingestion rate (B) in response to infection status and food quality. Sample sizes (number of replicates measured) are indicated above the bars.

in this study agree with previous findings (e.g., Sundbom and Vrede 1997; Urabe et al. 1997; Ebert et al. 2000; DeMott 2003; Acharya et al. 2004; Jeyasingh et al. 2011). However, when we combined both stressors, we found that juvenile growth rate increased as compared with uninfected animals under severe P-stress. All P-limited individuals infected or not, died before day 16, being not able to reproduce before death. However, the parasite was able to drain enough energy from host to develop and produce spores even under extreme P-limitation, as there was no statistical difference in the number of spore clusters detected in P-limited or P-sufficient individuals by day 13.

Threshold elemental ratio (TER, the minimum needed for somatic maintenance) of C:P (atomic ratio) is suggested to be around ~250 for *Daphnia* (Sterner and Hessen 1994; Elser et al. 2000) and thus our P-deficient treatment (C:P ~ 1000) simulated extreme P-limitation. This resulted in physiological impairments such as molting disruption, with old molts remaining attached to posterior carapace as described previously by Sterner et al. (1993). The decrease in growth efficiency under P-limitation was consistent with the growth rate hypothesis, which states a positive association of body P content with rRNA content and protein synthesis rate, and thus with the growth rate of an animal (Elser et al. 2003). We also found that the ingestion rate and thus food input for the P-limited *Daphnia* was lower than for the P-sufficient animals, which corresponds to the results of He and Wang (2008).

Interestingly, infected animals had higher growth rate than uninfected ones under P-limitation. This could be due to differences in feeding behavior, but we found corresponding ingestion rates in infected and uninfected animals. As part of the body P content of infected animals was incorporated into the parasite, which could not be separated from the host during the P-analysis, and the P contents in uninfected and infected animals were similar, infected animals could have been expected to be even more P-limited in their growth than uninfected ones. Thus, we consider it unlikely that differences in growth rate were due to P-metabolism either. While we cannot rule out the possibility of parasite causing changes in the metabolism of other nutrients important for growth (e.g., nitrogen; Sterner and Elser 2002) that we did not manipulate in the experiment, we suggest that our results are due to the parasite causing changes in carbon (C) allocation of the hosts. The parasite we used, *G. intestinalis*, is a microsporidian, which are known to infect particularly fat tissues of hosts (Wittner and Weiss 1999). Microsporidians do not have their own energy metabolism, but instead exploit host lipid and glucose storages for spore construction, thus being dependent on the host for C (Biderre et al. 2000; Rivero et al. 2007). Under extreme P-limitation, *Daphnia* have to cope with large amounts of excess C in order to maintain somatic stoichiometry. *Daphnia* store some amount of excess C as lipids (Tessier et al. 1983; Sterner et al. 1992), but most of the leftover C is actively disposed via increased respiration and excretion of dissolved organic carbon (DOC) (Darchambeau et al. 2003; Anderson et al. 2005; Jensen and Hessen 2007; He and Wang 2008; Hessen and Anderson 2008). Our hypothesis is that due to exploitation of lipid storage by the parasite, infected *Daphnia* was able to convert a larger amount of excess C into lipids, and possibly also had decreased costs of excreting the extra C. However, after a longer P-limitation, the mismatch between the gain and physiological demands for P led to a premature death of *Daphnia*.

In P-sufficient *Daphnia*, parasite infection impaired growth, which was further reflected as a lower body mass at maturity and lower overall reproductive output as compared with uninfected animals. Similar to P-limited

animals, no difference in ingestion rate or body P content between uninfected and infected individuals was detected. While the decrease in growth rate in infected animals might have been due to drainage of P for parasite growth, this is not supported by the opposite results in P-limited animals. However, also this result is consistent with parasite exploiting host lipids and diminishing C available for host somatic growth. We did not measure lipid contents of our experimental animals, but *Daphnia* body mass and visual lipid content are known to correlate positively (Tessier et al. 1983).

Under sufficient P-supply, spore loads were high between days 13 and 21 and then rapidly declined. Although *G. intestinalis* is considered as a rather benign parasite (Ebert et al. 2000), it produces spores constantly and the number of infected gut cells increases with host age (Ebert 1994, 1995). The decline in the spore loads at the end of the experiment might have been due to death of individuals with the highest spore loads. Individual differences in exposure to parasite at the beginning of the experiment could have subsequently led to differences in the spore load and in the timing of the parasite-induced host death, the risk being highest in hosts with higher spore loads (Ebert 1995).

Several virulent *Daphnia* parasites, for example, *Pasteuria ramosa* (Ebert et al. 2000; Frost et al. 2008), *Polycaryum laeve* (Johnson et al. 2006b; Forshay et al. 2008), and *Metschnikowia bicuspidata* (Ebert et al. 2000; Hall et al. 2009) have been suggested to be nutrient limited (Forshay et al. 2008; Frost et al. 2008; Civitello et al. 2012). On the other hand, several microsporidian parasites, for example, *Hamiltosporidium tvaerminnensis* (formerly *Octosporea bayeri;* Haag et al. 2011), *H. magnivora* (formerly *Flabelliforma magnivora*; Haag et al. 2011), as well as *G. intestinalis,* are rather avirulent to *Daphnia* hosts (Ebert 1995, 2005). Apart from differences in transmission modes, possibly also lower dependence on other elements than carbon of *Daphnia* by the parasite might facilitate the less destructive exploitation of the host. The connections between parasite virulence and their stoichiometric demands would, however, need more experimental evidence.

Our results suggest that the impact of the stressor pair composed of parasite infection and low food nutrient concentration might depend on the nutrient requirements of the parasite in question. During the last decade, the significant role of parasites in food webs and ecosystems has been acknowledged (Lafferty et al. 2008; Amundsen et al. 2009). Only few studies have reported interactions between parasite infection dynamics and nutrients in nature (Johnson et al. 2007; Aalto et al. 2012; Civitello et al. 2012). However, in order to reliably predict the role of parasites in food webs, further knowledge on host–parasite interactions under different nutrient regimes is needed.

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Conflict of Interest

None declared.

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IV

STABLE CARBON AND NITROGEN ISOTOPE VALUES IN *DAPHNIA MAGNA* FED WITH A SINGLE FOOD SOURCE: EFFECTS OF PARASITISM AND FOOD SHORTAGE

by

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