

Transgenerational effects of poor elemental food quality on *Daphnia magna*

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Received: 11 June 2009 / Accepted: 10 November 2009 / Published online: 2 December 2009
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Abstract Environmental effects on parents can strongly affect the phenotype of their offspring, which alters the heritability of traits and the offspring's responses to the environment. We examined whether P limitation of the aquatic invertebrate, *Daphnia magna*, alters the responses of its offspring to inadequate P nutrition. Mother *Daphnia* consuming P-poor algal food produced smaller neonates having lower body P content compared to control (P-rich) mothers. These offspring from P-stressed mothers, when fed P-rich food, grew faster and reproduced on the same schedule as those from P-sufficient mothers. In contrast, offspring from P-stressed mothers, when fed P-poor food, grew more slowly and had delayed reproduction compared to their sisters born to control mothers. There was also weak evidence that daughters from P-stressed mothers are more susceptible to infection by the virulent bacterium, *Pasteuria ramosa*. Our results show that P stress is not only transferred across generations, but also that its effect on the offspring generation varies depending upon the quality of

their own environment. Maternal P nutrition can thus determine the nature of offspring responses to food P content and potentially obfuscates relationships between the performance of offspring and their own nutrition. Given that food quality can be highly variable within and among natural environments, our results demonstrate that maternal effects should be included as an additional dimension into studies of how elemental nutrition affects the physiology, ecology, and evolution of animal consumers.

Keywords Ecological stoichiometry · Maternal effects · Life history · Growth rate · Phosphorus

Introduction

A mother's environment can strongly affect her offspring's viability and fitness (Rossiter 1996; Mitchell and Read 2005). Transgenerational effects have received considerable attention, especially those mediated by the epigenome and that result in altered patterns of gene expression in the offspring generation (Pembrey 2002; Gluckman and Hanson 2004; Lillycrop et al. 2005; Feil 2006). Maternal effects in animals are typically viewed from an evolutionary standpoint as an adaptive trait, which allows parents to transfer information about the environment to their offspring and thereby increase their fitness (e.g., Alekseev and Lampert 2001). An alternative mechanism for maternal effects involves the transfer of material resources to offspring during prenatal development (Boersma 1995; Rossiter 1996). The net effect of variable resource transfer from differentially nourished mothers would depend upon whether impoverished parents improve offspring quality through increased resource provisioning or transfer nutrient stress and produce lower quality offspring. In both cases,

Communicated by Ulrich Sommer.

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transgenerational environmental effects from mother to offspring would partly disconnect an offspring's phenotype from its own gene-environment interactions.

One aspect of the maternal environment that could affect an offspring's phenotype is food quality. Inadequate intake of certain dietary elements, such as N and P, strongly affects key physiological processes and life history traits in many animal consumers (Sterner and Elser 2002; Frost et al. 2005). Despite compensatory mechanisms that increase the acquisition of the limiting element (i.e., Raubenheimer and Simpson 1993; Fink and Von Elert 2006), consumers of element-deficient food grow more slowly, exhibit delayed and reduced reproduction, and experience elevated mortality rates (Sterner and Elser 2002). Other consequences of elemental limitation on aquatic invertebrates are reduced mass and altered elemental composition of offspring (DeMott et al. 1998; Urabe and Sterner 2001; Boersma and Kreutzer 2002), which provide a potential avenue by which nutritional effects on mothers would be transferred to subsequent generations.

The nature of maternal nutritional effects could depend upon the offspring's own environment (Boersma and Kreutzer 2002). Transferred nutritional stress from mothers to offspring might manifest itself only if offspring are themselves nutritionally challenged. Under this circumstance, altered maternal provisioning would either be a net benefit or cost to the offspring depending upon the direction of the effect. Alternatively, offspring from poorly nourished mothers but themselves receiving food of good quality may compensate for a maternally imposed deficit by increasing their own resource allocation to growth, reproduction, and metabolism. If so, poor maternal nutrition may appear to have minimal fitness consequences on well-nourished offspring. On the other hand, increased investment in somatic growth in these offspring may be accompanied by reduced resource allocation to other physiological processes necessary for maintenance and defense (Frost et al. 2005). For example, increased growth rate has been found to be accompanied by lower immunocompetence in a variety of animal taxa (Zera and Harshman 2001). As such, resource-based maternal effects may be most apparent when comparing multiple life history traits (e.g., growth and immunocompetence) in animals grown under contrasting nutritional environments.

There is growing evidence that the aquatic invertebrate, *Daphnia*, is especially susceptible to the negative effects of high food C:P ratios in its algal food. In laboratory (Sterner 1993; Urabe and Sterner 2001; Becker and Boersma 2003) and field studies (Elser et al. 2001; Urabe et al. 2002), high food C:P ratios reduce *Daphnia*'s juvenile growth rates and reproductive rates. P limitation of laboratory-grown *Daphnia* also reduces the mass and P content of their neonates (DeMott et al. 1998; Urabe and Sterner 2001;

Boersma and Kreutzer 2002). However, whether physiological changes in offspring born to differentially P-nourished mothers alter their life history responses to food P quality and their risk of infectious disease remains unknown. Here we tested for transgenerational effects of P-poor nutrition of mothers on offspring performance in *Daphnia magna* and determined whether these effects are modified by C:P ratios of the offspring's own food.

Materials and methods

Algal food culturing

We used the green alga, *Scenedesmus acutus* (University of Toronto Culture Collection strain 10), as the food source for *Daphnia magna* in this study. Algal food was grown in multiple semi-continuous culture jars that were diluted daily with COMBO media (Kilham et al. 1998). Algal P content was adjusted by altering the P concentrations in media and culture dilution rates (ranging from 0.1 to 0.5 day⁻¹ between the P-poor and P-rich cultures) provided to separate algal culture flasks (Frost et al. 2008). Algae were grown at three P levels to produce P-rich (molar C:P ratio ~100; all ratios hereafter given in molar ratios), P-medium (C:P ratio ~400), and P-poor cells (C:P ratio ~900). All algal cultures were grown at 20°C in environmental chambers under a 16:8-h light:dark cycle. Irradiance was provided by two 400-W Lumalux bulbs (LU400/ECO; Sylvania, Mississauga, ON, Canada) at a light intensity of ~150 μmol s⁻¹ m⁻² near the culture jars' surfaces. After centrifuging collected algae at 5,000 rpm, resuspended subsamples were collected on glass fiber filters (Whatman GF/C), dried for >24 hr at 60°C, and weighed on a microbalance. Algal filters were subsequently digested with persulfate and P was assessed using molybdate-blue reaction (American Public Health Association 1992). These data on algal mass and P content were used to mix the original three separate algal cultures to produce food having desired C:P ratios. This mixing procedure allowed us to generate multiple food C:P ratios that remained relatively constant during the culturing of mother *Daphnia* and during subsequent daughter experiments, which together lasted a total of 4–6 weeks. Additional filters were analyzed for C and N with an Elementar analyzer (Vario EL; Elementar). These data were used to calculate more precisely the nominal food C:P ratios fed to experimental animals.

Animal culturing

From birth, mother *D. magna* were raised in groups of ten individuals in jars (400 ml) of P-free, COMBO media

(Kilham et al. 1998) and provided algal food at non-limiting quantities (2–6 mg C l⁻¹). Within a single experiment, we grew groups of second-generation monoclonal *Daphnia* on two to three nominal food C:P ratios. These C:P ratios varied among experiments but typically included foods having low (100–200), medium (300–500) and high (700–900) C:P ratios. We maintained a different number of jars for mothers growing on different food C:P ratios to compensate for the smaller number of offspring produced from P-limited mother *Daphnia*. Food was provided every other day and the ration was raised through the course of each experiment. COMBO media was changed every fourth day to remove animal waste and to limit the build-up of non-consumed algal material. Despite this frequent exchange of food and culturing media, food C:P ratios may have changed across each 4-day period. Consequently, the gradient of nominal food C:P ratios established in this study should be viewed from a qualitative rather than a quantitative perspective as typical of the range of food nutritional conditions that would exist among natural lake systems (e.g., Guildford and Hecky 2000). Only neonates born after the second clutch were used in experiments. During each of the three experimental runs (see below), daughters born to mothers in the same food treatment were pooled from all jars for use in subsequent experiments. This pooling was necessitated by the larger number of offspring needed (particularly for the reproduction and infection experiments) than was provided by a single jar of mother *Daphnia* on any particular day. Additional mothers were grown on a range of nominal food C:P ratios (~100–900) to generate daughter *Daphnia* for analysis of their body dry mass and P content. *Daphnia* P content was measured on dried specimens as described above. For this additional analysis, groups of neonates ($n = 10$) were analyzed from replicate jars ($n = 3–6$) housing mothers that consumed food C:P ratios across a gradient of nominal food C:P ratios (85, 220, 440, 800).

Growth rate experiments

Daughters harvested from the different maternal food C:P ratios were fed mixed algal food having a range of food C:P ratios (~100–900) for 6 days and their mass-specific growth rate estimated. On the first day of each experimental run, neonates from each different maternal food C:P content were placed into separate tubes containing 20 ml of COMBO media. Daughters within each maternal food C:P ratio level were assigned to one of three different food C:P ratios with from nine to 14 replicate animals per food type. Throughout the experiment, *Daphnia* were fed high quantities of algae every other day with the ration increasing throughout the experiment from 2 to 6 mg C l⁻¹. Additional neonates were saved from each maternal type, dried

for >24 h at 60°C, and weighed with a microbalance to estimate their dry mass. For this growth experiment, food was replenished on the second and fourth day. On the sixth day, each animal was removed from its tube, placed into pre-weighed aluminum cups, dried for >24 h at 60°C, and weighed on a microbalance. Mass-specific growth rates (MSGR) were calculated as:

$$\text{MSGR} = \frac{\ln(B_2) - \ln(B_1)}{\text{time}}$$

where B_2 is the measured post-mass and B_1 is the average mass of neonates (day 0). These growth experiments were repeated on three separate dates (July–August 2007, October–November 2007 and February–March 2008) with a total of eight independent maternal food C:P ratios.

Reproduction experiments

Daughters from different maternal food C:P ratios were obtained on their day of birth and placed individually into 40 ml of COMBO media. Animals were assigned to different food C:P ratio treatments (between 15 and 20 animals per food C:P ratio) produced by the algal mixing treatment (as described above) and were maintained for the course of the experiment. In these experiments, daughter *Daphnia* were fed every other day and were placed into fresh COMBO media on every fourth day. Animals were checked every day for the presence of offspring. When offspring were found, we recorded the day of reproduction and counted the number of neonates. Individual *Daphnia* were maintained until each had produced three broods, after which they were removed from the experiment. This period varied depending upon the P diet of the *Daphnia*. It was as short as 14 days for P-sufficient animals and as long as 30 days for P-limited animals. These reproduction experiments were repeated on three separate occasions (July–August 2007, October–November 2007 and February–March 2008) and yielded results from nine different maternal food C:P ratios.

Infection experiments

Infection rates of *Daphnia* by an endoparasite, *Pasteuria ramosa*, were estimated in daughters from differentially P-nourished mothers that were consuming a range of algal food C:P ratios (~100–700) with three separate experiments. In general, daughters from each maternal food C:P ratio were collected on the day of their birth and singly placed into 20 ml of COMBO media. We assigned 50 daughters from each maternal line to each daughter food C:P ratio and maintained these food C:P treatments throughout the experiment. Animals were exposed for the first 6 days of the experiment to 5,000 *Pasteuria* spores, a

dose (250 spores/ml) known to produce intermediate infection rates (30–50%) of *Daphnia* (Regoes et al. 2003). Spores were obtained from frozen infected *Daphnia* by homogenizing whole bodies with a pestle. The density of spores in this homogenate was estimated based on cell counts with a hemocytometer and appropriate spore aliquots were added to each *Daphnia* tube. We assumed there would be no appreciable effects of spores on *Daphnia* nutrition given the very low amount of mass (~ 0.004 mg C/l) added in bacterial spores. After 6 days, animals were transferred into 40 ml of spore-free COMBO media and maintained for 2 additional weeks. After day 15, animals were checked daily for signs of infection (red color and lack of eggs in brood pouch). On day 20, we dissected all animals that had failed to show definitive signs of infection to check for bacterial spores. We conducted this experiment in August 2007, October 2007, and April 2008.

Statistical analysis

For mother and daughter body P content, we assessed the effects of mother food quality using one-way ANOVA. Post hoc differences between individual food C:P ratio levels within each group (i.e., mother and daughters) were determined with Tukey's honest significant difference test. To illustrate the nature of daughter responses to maternal food quality, we graphically present growth and reproduction of daughters to changes in their own food C:P ratios for animals born to P-sufficient (nominal food C:P ~ 100) and P-stressed mothers (nominal food C:P ~ 700) with data collected from two separate experiments (August and October 2007). To statistically assess the effects of maternal P nutrition on juvenile growth rates and adult reproductive measures, we first modeled the relationship between each response variable (i.e., mass-specific growth rate, day of first reproduction, and average brood size) to changes in daughter food C:P ratios for each maternal food C:P ratio separately using simple linear regression. To meet the assumption of normality, we natural log transformed the average brood size data and performed a reciprocal transformation on the day of first reproduction data. Normality was tested by use of the Shapiro–Wilk's test. We assessed the assumption of homoscedasticity by visual inspection of residuals and found no heteroscedasticity. The slopes of response variable–daughter food C:P ratio relationships were subsequently regressed against mother food C:P ratios. We used this analysis to guarantee independence among mother food C:P ratios and to allow the detection of mother–daughter food quality interactions. Following this approach, we interpreted a significant effect of maternal food C:P ratio on daughter response slopes as evidence that daughter responses to food quality varied due to maternal diet. We repeated this analysis for the infection

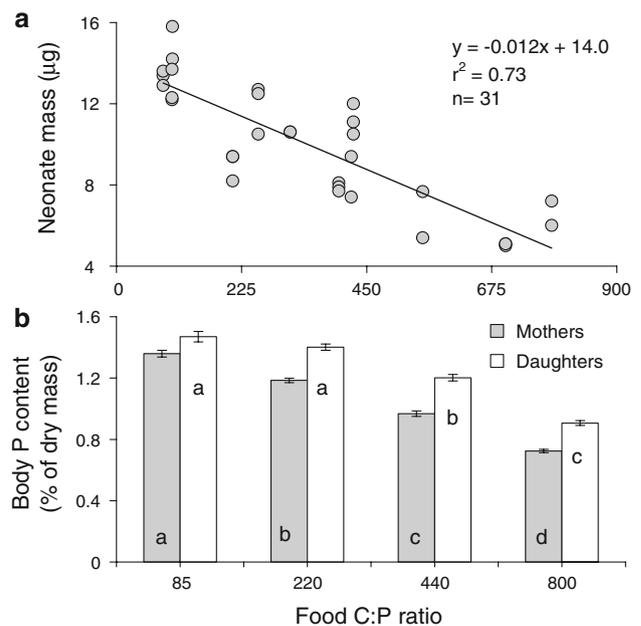


Fig. 1 **a** Mass of *Daphnia* neonates born to mothers consuming different nominal food C:P ratios. Each point is the average mass of ten neonates from one replicate jar. **b** Effects of nominal food C:P ratios on *Daphnia* mother and neonate body P content (mean \pm SE) based on six (C:P ratios 85 and 800) and 12 (C:P ratios 220 and 440) replicate jars. Bars within a category (mother and daughter) with different letters were found to be significantly different ($P < 0.001$) from other nominal food C:P ratios based on Tukey's honest significant difference tests

experiment except that we first used logistic regression to assess the relationship between the proportion of infected individuals and daughter food C:P ratio. We subsequently examined how the slopes and intercepts of these relationships varied among maternal food C:P ratios. All regression statistics were estimated using the R 2.8.0 statistical program (The R Foundation 2008).

Results and discussion

Mother *Daphnia* fed high C:P ratio food produced newly born offspring that were smaller (Fig. 1a), a result which contrasts with previous reports of increased neonate mass from *Daphnia* mothers grown under low food-quantity conditions (e.g., Ebert 1993; Boersma 1997). The smaller neonates produced by P-stressed *Daphnia* seen here may be partly attributable to the smaller size of their mothers (Boersma 1997). The linear reduction in neonate mass with increasing mother P limitation was accompanied by a reduction in offspring body P content (Fig. 1b). The reduction in the P content of offspring born to P-stressed *Daphnia* is qualitatively similar to that seen in previous studies (DeMott et al. 1998; Boersma and Kreutzer 2002).

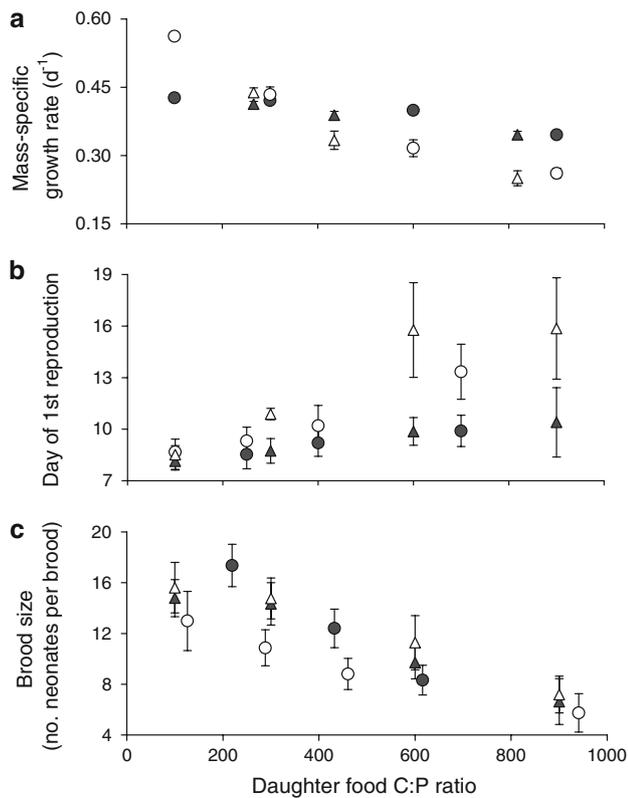


Fig. 2 **a** Mass-specific growth rate, **b** day of first reproduction, and **c** average brood size (mean ± SE) of *Daphnia* born to mothers consuming low nominal food C:P ratios (~100, closed symbols) and high nominal food C:P ratios (~700, open symbols) in two separate experiments (denoted by triangles and circles)

We also found daughters to have higher P content than their mothers (regardless of maternal food C:P ratio), which suggests that reproduction may be a significant P sink for mothers (Boersma and Kreutzer 2002).

Maternal P nutrition altered the relationship between growth rates of juvenile *Daphnia* in the daughter generation and their own food P nutrition (Figs. 2a, 3a). For daughters consuming low food C:P ratios, initial differences in mass created by high food C:P ratios in the maternal diet were largely erased by the sixth day of growth (data not shown). *Daphnia* juveniles born to P-stressed mothers apparently increase their growth rates to compensate for their initial lower mass and P content when they were fed food with low C:P ratios. For daughters consuming high food C:P ratios, maternal P limitation resulted in smaller juveniles and reduced growth rates after 6 days of growth (Fig. 2a). These contrasting effects of maternal food C:P ratios among daughters grown on different food C:P ratios resulted in a greater negative slope between daughter growth rate and food C:P ratio for *Daphnia* born to P-stressed mothers (Fig. 3a). Thus the transfer of negative effects of P limitation from mother

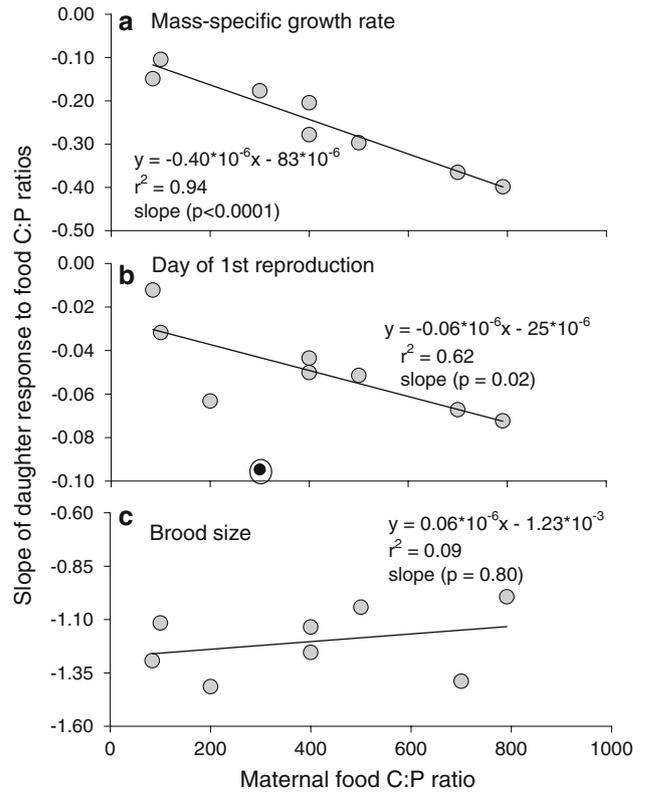


Fig. 3 Regressions of **a** mass-specific growth rate, **b** day of first reproduction (inverse transformed), and **c** average brood size (natural log transformed) between *Daphnia* daughters and maternal nominal food C:P ratio. Note that one point (marked by a dark circle) has been excluded from the day of first reproduction analysis presented in **b**

daphnids to daughters was strongest in poorly P-nourished offspring.

Reproductive responses of daughters showed somewhat weaker responses to maternal P nutrition compared to effects seen on daughter growth rates (e.g., Fig. 2a vs. c). The average number of days to first reproduction of daughter *Daphnia* consuming high food C:P ratios increased from 11 days for those born to P-sufficient mothers to 17 days for those born to P-stressed mothers. In contrast, the day of first reproduction did not differ significantly between daughter *Daphnia* from different mother food C:P ratios but who were consuming low C:P ratio algal food. Together this produced a greater negative effect of elevated maternal food C:P ratios on time to reproduction in daughters consuming high algal C:P ratios (Fig. 3b). As the statistical significance of this relationship was largely determined by one outlier (mother C:P 300; $P = 0.15$ with versus $P = 0.02$ without), we tenuously conclude that maternal food C:P ratios alters the time to first reproduction in daughters and that this effect interacts with daughter food P content. The slopes of the relationship between the brood size of daughter *Daphnia* and their food C:P ratios were relatively invariant. Consequently, there was no

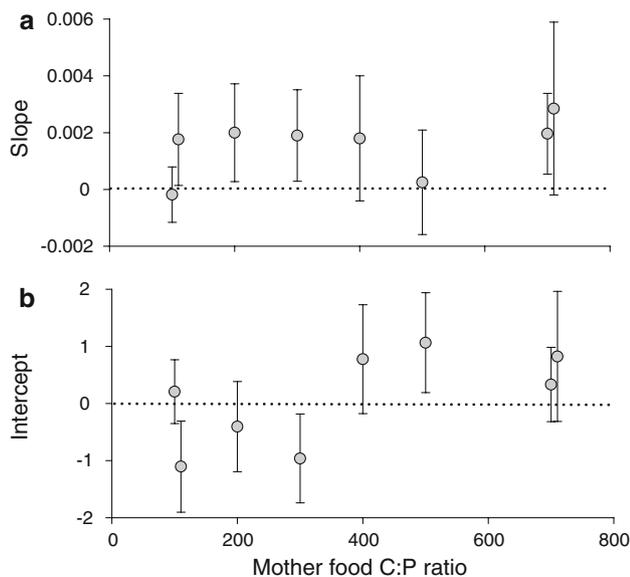


Fig. 4 **a** Slope and **b** intercept of regressions between infection rate (proportion of infected individuals) by *Pasteuria ramosa* and daughter food C:P ratios against maternal food C:P ratios. Shown are the derived regression statistics along with their 95% confidence intervals. Note that points for food C:P ratios 100 and 700 have been slightly offset for visual clarity

relationship between the slope of brood size responses to daughter food C:P ratio and maternal food C:P ratio (Figs. 2c, 3c). Nevertheless, given the observed delay in their first reproduction, *Daphnia* born to P-stressed mothers and consuming high food C:P ratios should experience reduced fitness and strongly constrained population growth rates (Sibly and Hone 2002).

In a previous study, we found a strong negative effect of dietary food P content on the infection of offspring derived from P-rich mothers but did not evaluate the potential importance of transferred maternal effects (Frost et al. 2008). In this study, we repeatedly found a positive relationship between infection rate (i.e., % of individuals becoming infected) and daughter food C:P ratio (positive slope; Fig. 4a), which is opposite of our previous results (Frost et al. 2008). This difference in infection rate responses of *Daphnia* to increasing food C:P ratios is difficult to explain but may be linked to differences in the type of water that we used to raise the *Daphnia* in the two studies (river water vs. COMBO). The effects of artificial growth media on *Daphnia* infection and the mechanisms underlying these effects are thus in need of further study.

Nevertheless, we found weak evidence in this study from three sets of experiments conducted over the course of a year that the infection risk in daughters was affected by maternal food P content. This effect was not overtly present as neither the slope nor the intercept of the logistic regressions was strongly related to maternal food C:P ratio

(Fig. 4a, b). However, close inspection of intercepts from the infection rate-daughter food C:P ratio regressions shows a clumping of negative values at low maternal C:P ratios and positive values for higher maternal food C:P ratios (Fig. 4b). As there was no difference among derived regression slopes attributable to maternal food C:P ratios, the greater intercepts translate into an increased infection intensity for a given daughter food C:P ratio. An elevated risk of bacterial infection in daughters born to P-stressed mothers would be consistent with our hypothesis that reduced resource allocation to daughters by P-limited mothers results in a steeper trade-off between growth and defensive metabolism. However, given the considerable variability in slopes and intercepts (as indicated by the 95% confidence intervals) within and among the maternal C:P ratios examined in this study (Fig. 4a, b), this conclusion should be treated, without additional experimentation, with caution. There is thus the need to fully characterize the relationship between maternal nutrition of *Daphnia magna* and the establishment of bacterial infection in differentially nourished daughters.

We found effects of poor elemental food quality in the maternal diets of *Daphnia* on offspring growth and reproduction. One mechanism that could account for this transferred nutritional effect is condition-dependent phenotypic plasticity in the offspring. This type of phenotypic plasticity appears to be controlled by internal physiological mechanisms that regulate metabolism and resource allocation and depend upon the organism's current internal and external environment (Schlichting and Smith 2002). Alternatively, mother *Daphnia* may alter the genotypic expression of offspring directly through genetic changes or via epigenetic regulation. There is evidence that *Daphnia* (even though asexual) can generate genetic variability in offspring after as few as two generations with direct effects on the life history traits studied here (i.e., growth rate and reproduction; Gorokhova et al. 2002). Alternatively, animal nutrition can alter the epigenome and produce trans-generational environmental effects (Oommen et al. 2005). However, it is unclear whether P-stress can alter the genome or epigenome of invertebrates and thereby gene expression in their offspring. The molecular and physiological mechanisms for this phenomenon should thus be determined both in *Daphnia* and other animal taxa that transfer effects of poor elemental food quality to their offspring. In any case, our results indicate that adaptive maternal effects can be strongly constrained by poor nutrition, which limit the physiological capabilities of the offspring to respond to the environmental cues provided by the mother.

Our results also suggest that daughters of P-stressed *Daphnia* mothers increased growth rates and were more susceptible to bacterial infection when feeding upon P-rich

food. The life history of *Daphnia*, in particular, appears predicated on rapid growth and reproduction (Elser et al. 2000), which may necessitate trade-offs between growth and defensive metabolism. Given the high P demands of fast growth (Elser et al. 2000; Sterner and Elser 2002), transferred P stress may result in less resource allocation to immunocompetence in r-selected animals (sensu MacArthur and Wilson 1967). Metazoans that grow more slowly, having lower P requirements, would potentially suffer less fitness costs from transgenerational P limitation and may not exhibit the associated change in infectious disease risk.

Transgenerational effects of P nutrition in *Daphnia* would potentially have long-lasting consequences on lake food webs and will complicate efforts to study food quality effects on aquatic consumers. Transferred effects of elemental food quality across generations could create inconsistent demographic responses of natural (and laboratory) populations of *Daphnia* to food of the same C:P ratio. Such blurring of P-limitation effects across generations would thus decouple direct relationships between algal food C:P ratio and inferred *Daphnia* nutritional status. Nutritional stresses on aquatic consumers, even if short lived, could produce alternative trajectories for their population sizes, alter the competitive interactions of their offspring, and change relationships between food elemental composition and the ratios of nutrient release. The importance of maternal effects, as shown here, in natural ecosystems would depend, in part, upon the frequency that mothers consume food that differs in C:P ratios from their daughters. As relatively little is known about how much seston C:P ratios vary within lakes across fairly short time scales (5–15 days), this remains an area in need of additional study (but see Sommer 1989; Urabe 1993). Cross-generation stoichiometric dynamics should thus be considered in future studies examining the prevalence and effects of elemental constraints on aquatic invertebrates in lake ecosystems.

Acknowledgments We thank S. McCarthy and M. Kingsbury for their assistance in the laboratory. We also thank Thomas Fabbro for his valuable comments that improved an earlier version of this manuscript. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada and Trent University. The experiments described in this manuscript comply with the current laws of Canada.

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