Transcriptomic Response of Daphnia Magna to Nitrogen or Phosphorus Limited Diet

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Abstract

Background: Effects of nutrient-imbalanced diet on the growth and fitness of zooplankton were widely reported. However, little is known about the molecular mechanisms driving the physiological changes of zooplankton under nutrient stress.

Results: In this study, we investigated the physiological fitness and transcriptomic response of *Daphnia magna* when exposed to nitrogen (N)-limited or phosphorus (P)-limited algal diet (*Chlamydomonas reinhardtii*) compared to regular algae (N and P saturated). *D. magna* showed higher ingestion rates and over expression of genes encoding digestive enzymes when fed with either N-limited or P-limited algae, reflecting the compensatory feeding. Under P-limitation, both growth rate and reproduction rate of *D. magna* were greatly reduced, which could be attributed to the downregulated genes within the pathways of cell cycle and DNA replication. Growth rate of *D. magna* under N-limitation was similar to normal group, which could be explained by the high methylation level (by degradation of methionine) supporting the body development.

Conclusions: Phenotypic changes of *D. magna* under nutrient stress were explained by gene and pathway regulations from transcriptome data. Generally, *D. magna* invested more on growth under N-limitation but kept maintenance (e.g. cell structure and defense to external stress) in priority under P-limitation. Post-translational modifications (e.g. methylation and protein folding) were important for *D. magna* to deal with nutrient constrains. This study reveals the fundamental mechanisms of zooplankton in dealing with elemental imbalanced diet and shed light on the transfer of energy and nutrient in aquatic ecosystems.

Background

Ecological stoichiometry, a branch of ecology that considers how the balance of energy and elements influences the living systems [1], has been widely studied in both land and aquatic systems where stoichiometric imbalances between predator and prey can affect trophic interactions and ecosystem functions. Elemental compositions (e.g. C : N : P, short for carbon : nitrogen : phosphorus) of phytoplankton can vary easily and frequently following hydrographic conditions of ambient water. Nutrient limited algae usually are deficient in certain constituents (e.g., certain lipids and amino acids) and are considered as low-quality prey for herbivorous zooplankton (lack of food preference) [2]. The performances (e.g. ingestion, growth and reproduction rates) of zooplankton in response to low-quality diet, which have consequences for both phytoplankton and zooplankton population dynamics and energy flow, is important to the understanding of ecological stoichiometry in aquatic systems.

“All life is chemical”, and certain chemical elements are critical to organisms for building and maintaining basic biological structures and core functions [1, 3]. For example, nitrogen is an essential component for synthesizing nucleic acids (i.e. DNA and RNA) and amino acids (or protein), while phosphorus is needed for producing nucleic acids and phospholipids. Transcriptome study (expressed mRNA) provides a way to identify the key genes and metabolic pathways that are directly related to the stress response of

organisms. For example, genes involved in P uptake, transport and assimilation expressed remarkably in response to nutrient constrains [4]. Apart from pathways related to the use of the constrained nutrients, some central pathways (e.g. carbon metabolism) were also significantly influenced, as a way to maintain homeostasis [5]. Previous transcriptomic studies have well explained the effects of N-limited or P-limited diet on the phenotypic performance of bacteria, phytoplankton, land plants and vertebrates [6, 7], whereas little is known about the transcriptomic response of zooplankton to nutrient constrains and their trade-off strategy (among growth, reproduction and other activities) .

Daphnids, which filter particles in the size range of 0.5 - 50 μm including algae and bacteria, are key aquatic herbivores and model species to study aquatic ecology. Daphnids may often experience nutrient-limited diet coming from (1) intracellular biochemical difference among ingested food, e.g. green eukaryotic algae, cyanobacteria and fungi, (2) movement of daphnids between heterogeneous food patches, and (3) seasonal succession in the taxonomic composition of phytoplankton [8]. Much work has been done to study the phenotypic response of daphnids, especially on the growth and reproduction rates, to the nutritional constraints induced by diet, which showed that food elemental compositions (particularly N and P) have profound effects on daphnids population dynamics. For instance, P-deficient food can significantly reduce the growth rate and egg production of daphnids, and the growth rate of daphnids correlates well with algal P content [2, 9]. N-limited diet can also affect the performance of daphnids, but the negative effects were not as severe as P-limited algae in some studies [2, 9]. However, except two studies reporting the transcriptomic response of daphnids to P-limitation using gene microarrays [3, 10], the metabolic mechanisms (in terms of gene regulation and involved pathways) of daphnids' physiological changes in response to nutrient limitation remain unclear.

In this study, we sequenced the mRNA extracted from *Daphnia magna* (one kind of daphnids species widely distributed in freshwater) fed by N- or P-limited green algae (*Chlamydomonas reinhardtii*) for 7 days and compared that to those on normal diet (N and P sufficient *C.reinhardtii*). Through gene and pathway enrichment analysis, we found that *D. magna* over expressed genes encoding digestive enzymes under both N-limited and P-limited diets, which explains their higher ingestion rate and compensatory feeding behavior. Their growth and reproduction were severely impaired under P-limitation, which can be attributed to the down-regulation of genes controlling cell cycle and DNA replication. Under N-limitation, gene regulation related to methionine metabolism maintained the body growth but reduced reproduction of *D. magna*. Our results suggest that post-translational modifications (e.g. methylation and protein folding) were important for *D. magna* to deal with nutrient imbalance and stress.

Results

C : N : P of algae as food for *D. magna*

Manipulation of media nutrient contents yielded significantly different elemental composition in *Chlamydomonas reinhardtii*. Cells grown in N-limited medium had the lowest N content (1.95%±0.15%, atomic ratio) and higher C : N ratio (16.7±0.1), while cells grown in the P-limited medium had the lowest P

content (0.12%±0.01%, atomic ratio) and higher C : P ratio (467.7±89.8), as compared with normal culture (C : N = 7.5±0.2, C : P = 65±6.1, $p < 0.01$, by Welch two sample *t*-test) (Table S1).

Physiological characteristics of *D. magna* under different diet conditions

During the 7-day experiment, *D. magna* with P-limitation diet had the shortest body length compared to that with N-limitation or nutrient sufficient diet (Fig. S1a). After 3 days, *D. magna* exhibited higher ingestion rate but lower egg production rate when fed with N- or P-limitation diet than that with nutrient sufficient diet (Fig. S1b, c). Significant difference of the physiological performances of *D. magna* between two treatments at each sampling point was shown in Table S2. For the entire experiment period, taking ages into consideration, both N- and P-limitation had significant influences on the ingestion and egg production rates of *D. magna* (Table S3). P-limitation had a significant effect on the growth rate (i.e. slow increase in body length) of *D. magna* while this effect was weak for *D. magna* under N-limitation (Table S3, Fig. S1). Here, we focused on the comparison between nutrient limited group and nutrient sufficient group on the 7^{th} day when transcriptomic samples were collected. As shown in Fig. 1, on the 7^{th} day, *D. magna* had higher ingestion rate fed with either N-limited (64.32±10.83, 10^4-cells of algae per *D. magna* per day) or P-limited algae (69.64±21.7, 10^4-cells of algae per *D. magna* per day) than that fed with nutrient sufficient (Normal) algae (41.22±5.93, 10^4-cells of algae per *D. magna* per day) (N-limitation vs Normal: df = 3.85, $p = 0.0024$; P-limitation vs. Normal: df = 3.47, $p = 0.021$, Welch two sample *t*-test) (Fig. 1a). Growth rate of *D. magna* was lower under P-limitation (151.43±10.17, mm day^{-1}) than N-limitation (177.44±16.93, mm day^{-1}) or Normal (176.27±9.34, mm day^{-1}) (Fig. 1b). An average of 20±9.00 eggs were produced for each *D. magna* fed with normal algae on the 7^{th} day, which was much higher than *D. magna* fed N-limited algae (7.58±5.05 eggs per *D. magna*, df = 20.98, $p < 0.001$) and P-limited algae (5.14±5.43 eggs per *D. magna*, df = 21.37, $p < 0.001$) (Fig. 1c). Similar to egg production, *D. magna* fed with nutrient limited diet had a smaller number of newborns (1.89±0.58 and 1.01±0.32 newborns per *D. magna* with N- and P-limited algae, respectively) compared with nutrient sufficient diet (3.43±0.71 newborns per *D. magna*) (N-limitation vs. Normal: df = 4, $p = 0.047$; P-limitation vs. Normal: df = 4, $p = 0.01$) (Fig. 1d).

Transcriptome sequencing and assembly

Transcriptome libraries were constructed using total mRNA and subjected to Illumina deep sequencing (about 18 G per sample). After quality control, clean reads were assembled to contigs: number of contigs in each sample ranged from 227920 to 306664, with N_{50} ranged from 1459 bp to 1597 bp. On average, 105191 unique genes of *D. magna* were predicted after sorting and redundancy filtration, representing 89.2% of the total genes in the transcriptome libraries (Table S4).

Principal component analysis (PCA) using all unique genes showed that the biological replicates of each treatment (N-limitation, P-limitation and Normal) were close to each other and far from other treatments, supporting the reliability of RNA-seq data. Difference between each experimental group and control group

was significant by analysis of similarity (ANOSIM) test ($p < 0.01$), suggesting the significant effects of low-quality diet on the gene expression of *D. magna* (Fig. 2a, b).

Differential expression analysis

Using a cut-off criteria of $|\log_2(\text{fold change})| > 1$ and $p < 0.05$, a total of 3804 and 2690 differentially expressed genes (DEGs) were identified for *D. magna* under N-limitation and P-limitation, respectively, with the Normal as comparison. 1253 DEGs were shared by the two nutrient deficient conditions. With either N-limitation or P-limitation diet (compared with Normal), more genes of *D. magna* were down-regulated (2470 and 1599 DEGs, respectively) than up-regulated (1337 and 1361 DEGs, respectively) (Fig. 2c).

GO and KEGG enrichment of DEGs

All DEGs in this study were assigned to GO terms with three categories: biological process (BP), molecular function (MF) and cellular component (CC). To relate them to the physiology of *D. magna*, we further grouped the GOs at two levels: a higher level (i.e. parent terms) (Fig. 3a) which is an enrichment category, and the finest level (which is the direct annotation of genes) with some representatives (Fig. 3b). For *D. magna* under either N-limitation or P-limitation, metabolic process (132 and 105 DEGs, respectively) and catalytic activity (both 134 DEGs) were highly enriched. Notably, much more DEGs were affiliated to multicellular organism development (GO:0007275) in N-limitation than P-limitation. For instance, several GOs related to body development, e.g. regulation of cell maturation (GO:19034214), ecdysis (GO:0018990) and tissue remodeling (GO:0048771), were highly up-regulated ($\log_2\text{FC} > 4$) in the condition of N-limitation, but not significantly changed under P-limitation.

KEGG enrichment of DEGs showed that three pathways (cysteine and methionine metabolism, retinol metabolism, and drug metabolism) were significantly enriched for *D. magna* under N-limitation (q-value < 0.05) (Table 1). In the pathway of cysteine and methionine metabolism, genes of *metK* and *ahcY*, which encode S-adenosylmethionine synthetase (EC:2.5.1.6) and adenosylhomocysteinase (EC:3.3.1.1) respectively, were up-regulated (Fig. 4). For *D. magna* under P-limitation, pathways of DNA replication, cell cycle, glutathione metabolism and protein processing in endoplasmic reticulum (ER) were significantly enriched ($q < 0.05$). DEGs involved in DNA replication and cell cycle were all down-regulated ($n = 6$ and 7, respectively) while DEGs involved in protein processing in ER were all up-regulated ($n = 9$).

Table 1. Significantly enriched pathways of *D. magna* fed by N- or P-limitation diet.

KEGG pathway	No. DEGs (up, down)	*q*-value
N-limitation vs Normal		
Cysteine and methionine metabolism	10 (3, 7)	0.0019
Retinol metabolism	4 (1, 3)	0.044
Drug metabolism	6 (2, 4)	0.045
P-limitation vs Normal		
Protein processing in ER	9 (9, 0)	0.006
DNA replication	6 (0, 6)	0.049
Cell cycle	7 (0, 7)	0.001
Glutathione metabolism	6 (3, 3)	0.048

Normal: nutrient sufficient diet. ER: endoplasmic reticulum. Pathway with *q*-value < 0.05 means it's significantly enriched by differentially expressed genes.

Validation of genes by qPCR

To validate the DEGs identified in the RNA-seq data in this study, the expression of 7 selected DEGs (see detail information in Table S5 and S6), which were mainly from enriched KEGG pathways, was confirmed by qPCR. As shown in Fig. S2, all qPCR results were in agreement with the RNA-seq analysis (Spearman correlation r = 0.7, $p < 0.05$). For example, under N-limitation, expression of genes encoding S-adenosylmethionine synthetase (*metK*) and adenosylhomocysteinase (*achY*) increased significantly compared with the Normal group (both $p < 0.05$, Welch two sample *t*-test).

Discussion

Effects of low-quality diet on *D. magna* physiology: why the effect of P-limited diet is more severe?

Our results were in accord with previous studies reporting the effects of low-quality diet on the growth and reproduction of daphnids, with a conclusion that phosphorus-limited algae is a much poorer food for *D. magna* [2, 9]. Firstly, we found that growth rate of *D. magna* with N-limited diet did not change significantly compared with Normal. As proteins form the main nitrogen pool in metazoans (~ 80%), stoichiometric compositional changes associated with N-limitation should be mostly concentrated in the metabolism of amino acid, and therefore the profile of amino acid could be an important indicator of the nutritional status and closely related to the performance of zooplanktons [11, 12]. From the transcriptome data of our study, cysteine and methionine metabolism was the most enriched pathway of *D. magna* under nitrogen deficiency. One of the up-regulated genes was *metK*, encoding S-adenosylmethionine synthetase (EC: 2.5.1.6) which produces S-adenosylmethionine (SAM) from methionine. SAM is the principal biological methyl donor made in the cytosol of every cell, including methylation of proteins,

nucleic acids and lipids. SAM is also required in other important processes, including synthesis of polyamines which are essential for various cellular functions affecting growth and development [13]. Another up-regulated gene was adenosylhomocysteinase (*achY*, EC:3.3.1.1) which hydrolyzes S-adenosylhomocysteine (SAH) to homocysteine (Hcy). The over-expression of the two genes (*metK* and *achY*, from both qPCR and RNA-seq results) would increase the ratio of SAM: SAH which is frequently used as an indicator of cellular methylation capacity (higher ratio means higher capacity) [14]. Methylation is an important way for *Daphnia* to response and adapt to environmental stress [15]. It is also closely related to cell growth and tissue differentiation of metazoans and could be induced by nutrient restriction [16]. Therefore, in our study, the increased degradation of methionine and SAM: SAH ratio may promote the methylation level and stimulate the development (or growth) of *D. magna* with N-limited diet.

Methionine is necessary for the increase in fecundity of arthropods (e.g. fruit fly and copepods) and plays an important role in controlling the lifespan of animals [17]. Methionine is easy to be degraded during the hydrolysis process. Therefore, decrease of methionine, at the same time, may lead to the reduced reproduction of *D. magna* under N-limitation in our study.

Secondly, *D. magna* fed with P-limited algae had the lowest growth rate and fewest number of newborns in our study. *Daphnia* species have higher requirement for phosphorus than other crustacean zooplankton and their juveniles constantly have higher specific P content than adults [18]. Effects of low-P diet on the phenotypic plasticity (e.g. growth and reproduction) of *Daphnia* were well described in previous studies [2, 9, 18], however, little is known about the underlying mechanism especially on the transcriptomic level. In one study using microarrays of RNA hybridization, down-regulated genes in tryptophan metabolism (tryptophan is essential for body development but lack in P-limited algae *Scenedesmus*), tRNA synthesis (control of protein synthesis) and hormone metabolism (delaying sexual maturity) were explained for the lower growth and reproduction rate of *Daphnia pulex* under P-limitation [3]. The comparison of transcriptome responses of ancient and modern *Daphnia* genotypes to dietary P-supply showed that different transcriptomic mechanism could result in similar phenotypes [10], pointing out the needs for more studies in order to unveiling the fundamental mechanisms of dietary-induced phosphorus constrains on zooplankton.

In our study, DEGs (annotated by KEGG) involved in DNA replication and cell cycle were all down regulated in *D. magna* fed with P-limited diet. This result was in accord to a previous study where genes related to nuclear structure, replication, recombination and repair were down-regulated for daphnids fed with low-P diet [10]. Inhibition of P-limitation on nucleic acid metabolism and cell division could strongly affect the growth rate of phytoplankton, yeast and bacteria [19-21]. Thus, our result suggested similar mechanisms may exist in zooplankton, leading to low growth rate of daphnids population.

Cell cycle in metazoans is controlled by a number of mechanisms on the gene level. Effects and mechanisms of nutritional limitation on cell cycle and growth rate were well demonstrated for phytoplankton and yeast in previous studies [19, 21, 22] while little is known about zooplankton. For

instance, the decrease of growth rate was closely related to the number of cells arrested at the G0/G1 phase for yeast [19] and G2+M phase for marine diatom *Thalassiosira pseudonana* [22]. In our study, for *D. magna* under P-limitation, down-regulated genes within cell cycle were detected linking to all 4 phases of cell cycle, which may explain why daphnids under P-limitation usually grow much slower. Although several genes (*CHEK2*, *APC1* and *STAG1*) of *D. magna* under N-limitation were down-regulated in the G2 and M phase, growth rate did not reduce, suggesting the different effects and mechanisms of nitrogen limitation between zooplankton and phytoplankton [12]. Though several genes within the cell cycle pathway were down-regulated under N-limitation as well, none DEGs co-occurred in this pathway under both conditions (N- and P-limitation). The same pattern was also observed in the pathway of DNA replication, suggesting the distinct effects and regulation mechanisms between N-limitation and P-limitation for *D. magna*.

How do *Daphnia* deal with stoichiometric constrains? Lessons from transcriptomic data

We employed a modified framework of the dynamic energy budget (DEB) model [23] to track metabolic pathways of elemental nutrients from the aspects of maintenance, growth and reproduction. To investigate how *D. magna* deal with diet-induced elemental constrains, we focused on the investment of nutrients to the three above aspects and related molecular metabolisms. We mainly focused on the genes within the significantly enriched pathways although other central pathways (e.g. carbon metabolism) are important and essential in dealing with nutrient constrains for daphnids.

Increasing in feeding rate, which is also called compensatory feeding, is one strategy for herbivorous zooplankton to deal with specific nutritional constrains in diet. While previous study showed that daphnids increased their ingestion rate when fed low-quality algae [24], no significant difference in feeding rates between daphnids fed P-sufficient and P-depleted algae was also reported [18]. These contrasting results suggest that compensatory feeding varies with changes in diet including food abundance, digestibility, and the elemental ratio in food. In our study, ingestion rate of *D. magna* fed with either N-limited or P-limited algae was higher on the 7^{th} day than that fed with normal diet, indicated the compensatory feeding of *D. magna* under nutrient constrains. The results were the same after taken body size into consideration (dividing ingestion rate by body length). The increased ingestion rate of *D. magna* under P-limitation (or N-limitation) could be reflected in the up-regulation of genes related with digestion, such as tripeptidase activity (GO:0034701) and sterol esterase activity (GO:0004771), which is an important mechanism of physiological adaptation of daphnids to nutritionally changing environments [8]. Besides, expression of alkaline phosphatase (APA, GO:0004035, a biomarker for P-limitation in zooplankton) was up-regulated under P-limitation ($Log_2FC = 1.004$, $p < 0.001$, not shown in figures due to its relatively lower fold change value), suggesting the accelerated P acquisition by *D. magna.*

Transcriptomic data allows us to investigate the pathways involved in sequestering limiting elements and the trade-off strategy of zooplankton under nutrient stress [3]. As discussed above, up-regulation of genes involved in methionine metabolism explained why growth rate of *D. magna* under N-limitation kept similar to normal ones. This suggests that N (e.g. amino acid and protein) absorbed from diet could be mainly

allocated to the development of somatic tissues of *D. magna* under N-limitation, rather than reproduction or maintaining the fitness of body (e.g. defense to bacteria and toxin transportation). Meantime, the increased ecdysis (GO:0048771) is not only a necessary step for arthropods to have a lager body size but also contributes to the balance of nutrient after taking in excessive low-quality food (a way to discharge excess carbon) [25].

We showed that under N-limitation, the highly up-regulated genes ($log_2FC > 3$, in GO terms) in metabolisms of *D. magna* were related to amino acids and protein (e.g. proteasome assembly, de novo protein folding and ornithine metabolic process) which are necessary for the body development of zooplankton. We attributed the unaffected growth rate of *D. magna* under N-limitation to the high methylation level supported by methionine degradation. Thus, for *D. magna* under N-limitation, the assimilated nitrogen should be mainly assigned to body growth (i.e. development), rather than reproduction. This result is in agreement with the report that copepods under N-limitation were unable to utilize dietary N efficiently for egg production due to the N demands for maintenance (including growth) [26].

Under P-limitation, although both growth rate and reproduction rate decreased significantly, a remarkable number of up-regulated genes of *D. magna* were detected, which may represent the mechanisms in dealing with phosphorus constrains. Regulation of protein process in endoplasmic reticulum (ER) is one of the post-translational modifications (PTMs, e.g. phosphorylation, monoubiquitination, and glycosylation) which are important ways for organisms to enhance nutrient acquisition and utilize efficiency [27]. In our study, within the pathway of protein process in ER, all DEGs were up-regulated under P-limitation. These genes were mostly distributed across the whole process of protein folding (from entering ER to correctly folded and exported to Golgi). For instance, proteins are translocated to ER through a protein-conducting channel which is formed by a conserved, heterotrimeric membrane-protein complex, the Sec61 (including Sec61 subunit alpha, Sec61A) complex [28]. Heat shock proteins (e.g. HSP90) help protein recognition by luminal chaperones and contributes to minimize protein aggregation, repair and protect cellular proteins from stress damages. Calreticulin (encoded by CALR) can promote the efficient folding of glycoproteins in several ways. VIP36 (encoded by *LMAN2*) is an intracellular lectin cycling with the ability to recognize high-mannose type glycans and transport various glycoproteins from the ER to the Golgi apparatus [28]. Thus, our results reflected the accelerated process of protein folding and transporting under phosphorus constrain, without any DEGs detected in the pathway of unfolded protein response. This is different from the transcriptomic response of *Daphnia* to toxic algal diet where unfolded proteins were accumulated and unfolded protein response was activated to keep protein homeostasis.

Several highly expressed genes of *D. magna* ($log_2FC > 3$, e.g. membrane raft assembly GO:0001765, myosin III complex GO:0042385 and microtubule GO:0005874) can be related to the accelerated process of protein process in ER and indicates the enhanced maintenance of fundamental structure and functions of cells under P-limitation. For instance, membrane ensures cell survival upon nutritional stress in eukaryotes. P-starvation can induce membrane remodeling (e.g. replace phospholipid in membrane by

non-P lipid) and recycling in phytoplankton as a way to save and accumulate phosphorus [29]. The up-regulation of genes within post-translation process and cell structure (e.g. membrane) biogenesis was also mentioned in a study comparing ancient and modern daphnids under P-limitation [10]. Nevertheless, the underlying P-related molecular pathways and gene regulation mechanisms need further investigation.

A recent study showed that low-quality diet (cyanobacteria) could reduce the output of *Daphnia* gut parasites [30]. Besides, food with high C : P ratios can significantly reduce bacterial infection rates in daphnids [31]. Similarly, in our study, genes of *D. magna* related with bacterial infection (e.g. antimicrobial humoral response GO:0019730, and positive regulation of phagocytosis GO:0060100) were highly up-regulated ($log_2FC > 3$) under P-limitation. This could be related to the significantly enriched pathway of glutathione (GSH) metabolism in *D. magna* under P-limitation, as GSH plays an important role in the resistance of organisms to different biotic challenges [32]. In contrast, the highly down-regulated genes of defense response to bacterium (GO: 0009816, $log_2FC = 10.34$) and toxin transporter activity (GO:0019534, $log_2FC = 4.74$), together with a significant change in pathway of drug metabolism (4 of 6 DEGs were down-regulated), suggested decreased immune responses of *D. magna* under N-limitation. It is reasonable because immune responses require high quantities of N in forms of proteins to defend against infection [33]. These results suggest that *D. magna* invested most of the ingested N to body growth (or development) instead of keeping fitness under N-limitation.

Conclusions

In this study, using transcriptomic data, we investigated the metabolic response of *Daphnia magna* to nutrient stress caused by low-quality diet. *D. magna* exhibited higher ingestion rate when fed with either N-limited or P-limited algae, suggesting a strategy of compensatory feeding to meet the demand of essential elements that is deficient in the diet. This could be achieved by the over expression of genes encoding digestive enzymes. Down-regulation of genes corresponding all phases of cell cycle were detected for *D. magna* with P-limited diet, suggesting a severe negative impact of P-stress on cell proliferation which are essential for the growth and reproduction of metazoans.

Our study showed that *D. magna* dealt nutrient constrains through post-translational modification mechanisms. For instance, regulation of methionine metabolism under N-limitation may enhance the methylation level, which supports the somatic growth and development. With P-limited diet, protein folding in endoplasmic reticulum was accelerated, and this may allow *D. magna* to keep the basic cellular structure and function. We concluded that *D. magna* tended to invest more on growth (i.e. body development) under N-limitation but keep maintenance (e.g. cell structure and defense to external stress) in priority under P-limitation. These findings provide an important link between *Daphnia*'s metabolism, physiology and demography. However, the genes (and involved pathways) focused in our analysis is a very small percentage of the total number of the annotated genes. Other enriched metabolic pathways in our study, such as retinol metabolism which is essential for the reproduction of zebrafish and mammals [34], are well studied and proven important for the development and homeostasis maintenance of

vertebrates but remain unclear for most invertebrates. Their relationships with the phenotypic responses of *D. magna* to nutrient stress require further investigation.

Methods

Culture of algae

Green algae *Chlamydomonas reinhardtii* (CC1690) was grown in 1-liter vessels with BG11 medium [35]. P-limited algae were obtained by reducing K_2HPO_4 by 10-fold from 40 mg/L to 4 mg/L, while N-limited algae were obtained by reducing the concentration of $NaNO_3$ by 30-fold from 1.5 g/L to 0.05 g/L. No modification of medium was made for the Normal group. Algae were cultured in a chamber under constant temperature of 23.5°C and a 14:10 light/dark cycle (20 μmol m^{-2} s^{-1}). Concentration of algae was measured daily by a Beckman Coulter Z2 Cell and Particle Counter (Beckman Coulter Inc., FL). Cellular carbon (C) and nitrogen (N) of algae in three conditions (i.e. Normal, N-limited and P-limited) were quantified by a FlashSmart CHNS Elemental Analyzer (Thermo Fisher, USA). Cellular P was analyzed as orthophosphate after acidic oxidative hydrolysis with 1% HCl. The concentration of PO_4^{3-} was measured manually according to a previous report [36].

Measurement of ingestion, growth and reproduction rates of *D. magna*

Before this experiment, *D. magna* was cultured in the creek water and fed with sufficient *C. reinhardtii*. During the experiment, newborns of *D. magna* were collected within 12 h after birth and transferred to 3 glass beakers (volume = 5L) with 0.45 μm filtered fresh water. Three kinds of algae (i.e. N-limited, P-limited and nutrient sufficient *C. reinhardtii* as described above) was added to each beaker respectively as prey for *D. magna*. Ingestion rate, growth rate and reproduction rate (numbers of egg in the brood and daily newborns) of *D. magna* in each treatment were measured on the 1^{st}, 3^{rd}, 5^{th} and 7^{th} day during the experiment.

For ingestion rate measurement, on each sampling day, 10 individuals of *D. magna* were taken from the beaker and put in a 600 mL polycarbonate bottle (Nalgene), with 3 replicates. Algae concentration (as food) was kept constantly to 100,000 cells/mL (i.e. ~2.5 mg C L^{-1}) which was above the incipient limiting level according to a pre-experiment. Grazing experiment was conducted for 24h and ingestion rate (*I*, cells $daphnids^{-1}d^{-1}$) was calculated following the previous studies [37]:

$$I = \ln(Ct'/Ct) \times (V/nt) \times [C]$$

Where Ct' and *Ct* (cells mL^{-1}) are the prey (*C. reinhardtii*) concentrations at the end of incubation in control and experimental bottles, respectively. *V* is the volume of the culture (mL), *t* is the incubation time (day) and *n* is the number of *D. magna* used. *C* is the prey concentration in the experimental bottles

averaged over the incubation period. Prey (fixed by acidic Lugol's solution) concentration was counted using an inverted microscope (Olympus CK30).

To measure growth rate, on each sampling day, 15 individuals of *D. magna* were randomly taken from the beaker for each treatment. Body length was measured from the anterior margin of the eye to the base of the tail spine of *D. magna* using a microscope (Olympus IX51). Daily increase in body length was used to represent growth rate.

To measure reproduction rate, 10 individuals of *D. magna* (with triplicates for each treatment) were taken from the beaker on the first day and cultured in a 600 mL polycarbonate bottle (Nalgene) for 7 days with the same medium in the beaker. On each sampling day (i.e. 1^{st}, 3^{rd}, 5^{th} and 7^{th} day), number of eggs and newborns in the brood pouch of *D. magna* were calculated using an inverted microscope (Olympus CK30). Newborns were removed immediately after calculating. Daily increase in egg production and newborns were used as reproduction rate.

RNA extraction

On the 7^{th} day, for each type of diet, 50 individuals (rinsed by Milli-Q water for 3 times. No starvation step was conducted as it will cause extra transcriptomic response) were taken from the beaker and pooled as one sample. Triplicate samples were collected for each type of diet. Body tissues were grinded manually in a PCR tube and kept in RNAlater solution at -80^{o}C. RNA was isolated using TRIzol reagent (Takara, Japan) and RNA Mini kit (Thermo Fisher, USA) in accordance with the manufacturer's instructions. Genomic DNA was removed using the Turbo DNA-free DNase kit (Ambion), and mRNA was isolated with the MicroPoly (A) Purist kit (Ambion). RNA integrity was evaluated using an Agilent 2100 bioanalyzer (Agilent Tech., CA).

Sequencing and transcript assembly

Each replicate mRNA was pooled and sequenced with Illumina HiSeq2500 (Novogene, China), generating 150 bp paired end reads. FastQC (Babraham bioinformatics) was used to assess the quality of raw reads and quality control (length > 140 bp, without ambiguous "N", and average base quality > 20) [38]. SRC_c method [39] was employed to sort reads affiliated to *D. magna* from the whole metatranscriptome (mainly include reads from *D. magna*, bacteria, fungi and *C. reinhardtii*). For this step, library of *D. magna* was built using RNA-seq datasets from a previous study [40]. Then, *D. magna* reads were assembled to contigs using Trans-ABySS v2.0.1 with k-mer set from 32 to 92, with a step of 5 [41]. Open reading frames were predicted from the assembled contigs by TransDecoder v5.3.0 [42] and coverage information of predicted genes were revealed by Bowtie v2.2.9 [43] and SAMtools v1.9 [44]. Lastly, the MEGAN software (Community Edition, v. 6.15.1) [45] was used as a second step to sort the genes affiliated to *D. magna* (database file: "prot-acc2tax-Jul2019X1.abin", all genes within the taxonomy "Pancrustacea" was used).

Differential expression genes analysis, GO and KEGG enrichment

A gene abundance (distributions in 9 samples, i.e. 3 triplicates for 3 kinds of diet, respectively) table was built for downstream analysis. Differentially expressed genes (DEGs) were estimated using “edgeR” in the “Bioconductor” package in R (v 3.4.1) [46]. Gene showing $|\log_2(\text{fold change})| > 1$ and *p*-value < 0.05 at any of the two comparison groups (N-limitation *vs*. Normal, and P-limitation *vs*. Normal) was defined as DEG [47]. DEGs were further annotated by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). Higher level of GO terms and KEGG pathways were enriched by DEGs using TBtools [48], and corrected Benjamini *p*-value (i.e. *q*-value) < 0.05 as cut-off was used to define significantly enriched KEGG pathways.

Validation of RNA-seq by qPCR

To validate the RNA-seq results, 7 DEGs, mainly within the enriched KEGG pathways, were randomly selected to confirm the expression profiles in our study. Primers were designed by Primer Premier v6.00. For each sample, 500 ng of extracted RNA were used for reverse translation by HiScript III RT SuperMix for qPCR kit (Vazyme Biotech, China). After the synthesis of cDNA, qPCR was performed with Faststart Universal SYBR Green Master (Roche, Germany) on LightCycler 384 (Roche, Germany). The qPCR reaction followed: an initial hold at 50℃ for 2 min and 95℃ for 10 min followed by 45 cycles of 95℃ for 15 s and 60℃ for 1 min. The relative abundances of genes were calculated by a $2^{-\Delta\Delta Ct}$ method, with β-actin as an internal control [47].

Statistical analysis

Statistical analysis was conducted in R software (v 4.0.0). Welch two sample *t*-test was used to test the significant differences in ingestion rate, body length and numbers of eggs in the brood pouch of *D. magna* on each sampling day (i.e. Day1, Day3, Day5 and Day7). Measured physiological parameters of *D. magna* on the 7^{th} day were presented as the mean ± SD derived from the biological replicates. Because age of *D. magna* can have great effects on the physiological performances as a covariate, two-way ANOVA (analysis of variance) was used to testing the effects of nutrient limitation (i.e. N- and P-limitation, respectively) on *D. magna* across the entire experiment period. Principle component analysis (PCA) was used to cluster transcriptome samples according to the abundance of genes (Hellinger-transformed), and difference between two clusters of samples was tested by analysis of similarities (ANOSIM) using the “anosim” function.

Lists Of Abbreviations

N: nitrogen; P: phosphorus; Normal: nutrient sufficient; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DEG: differentially expressed genes; FC: fold change; ANOVA: analysis of variance; PCA: principle components analysis; ANOSIM: analysis of similarity; df: degree of freedom; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; ER: endoplasmic reticulum.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree the publication of this manuscript.

Availability of data and materials

Transcriptome sequencing data was deposited in GenBank (Sequence Read Archive) and is available under the BioProject PRJNA597965.

Competing interests

The authors declare no competing interests.

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Authors' contributions

ZX and YL designed and conducted this study. ZX wrote the manuscript. ML and HL provided comments and suggestions on this study and revised the manuscript. All authors approved the final submitted manuscript.

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Figures

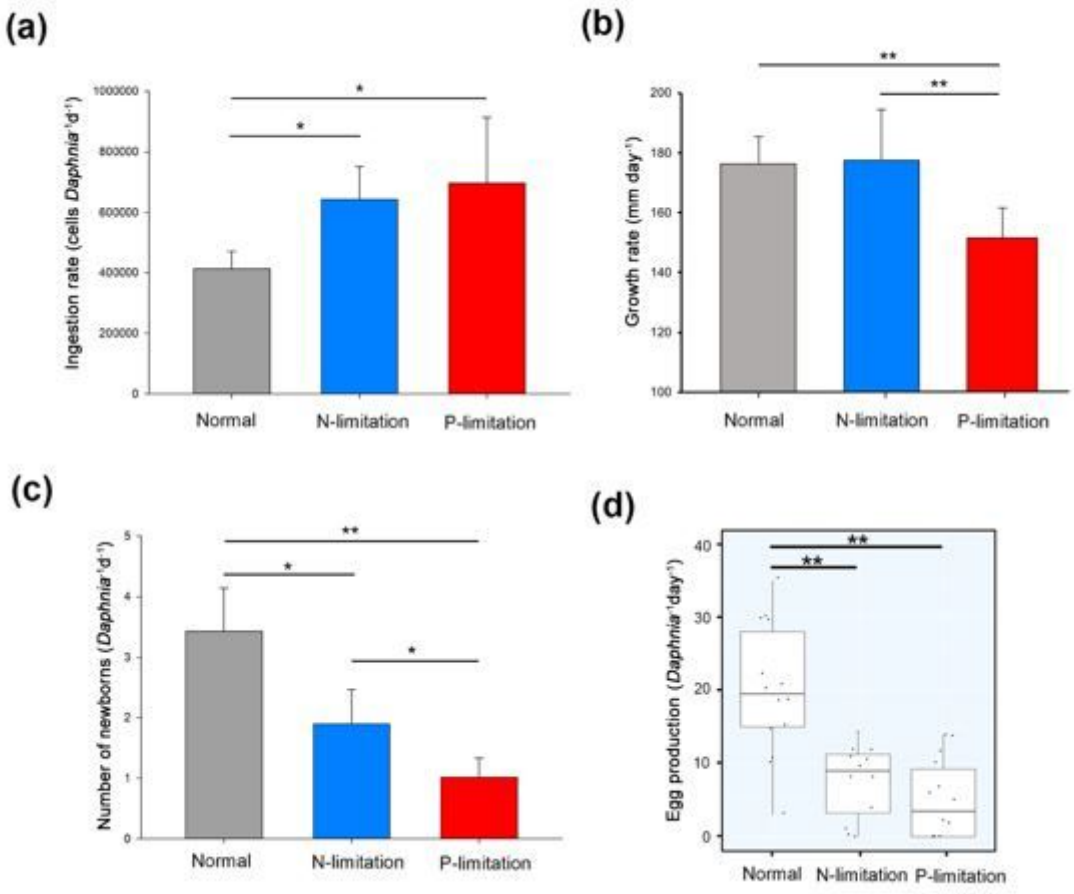

Figure 1

Performances of Daphnia magna fed with different types of algae on the 7th day. (a) shows the average (with standard deviation, SD) ingestion rate of one individual D. magna; (b) shows the average (with SD) increase in body length as an index of growth rate for D. magna; (c) is the average number (with SD) of newborns of one individual D. magna; and (d) is the egg production rate of each D. magna. Significant difference between groups is marked with asterisk (*: $p < 0.05$, **: $p < 0.01$, by Welch two sample t-test).

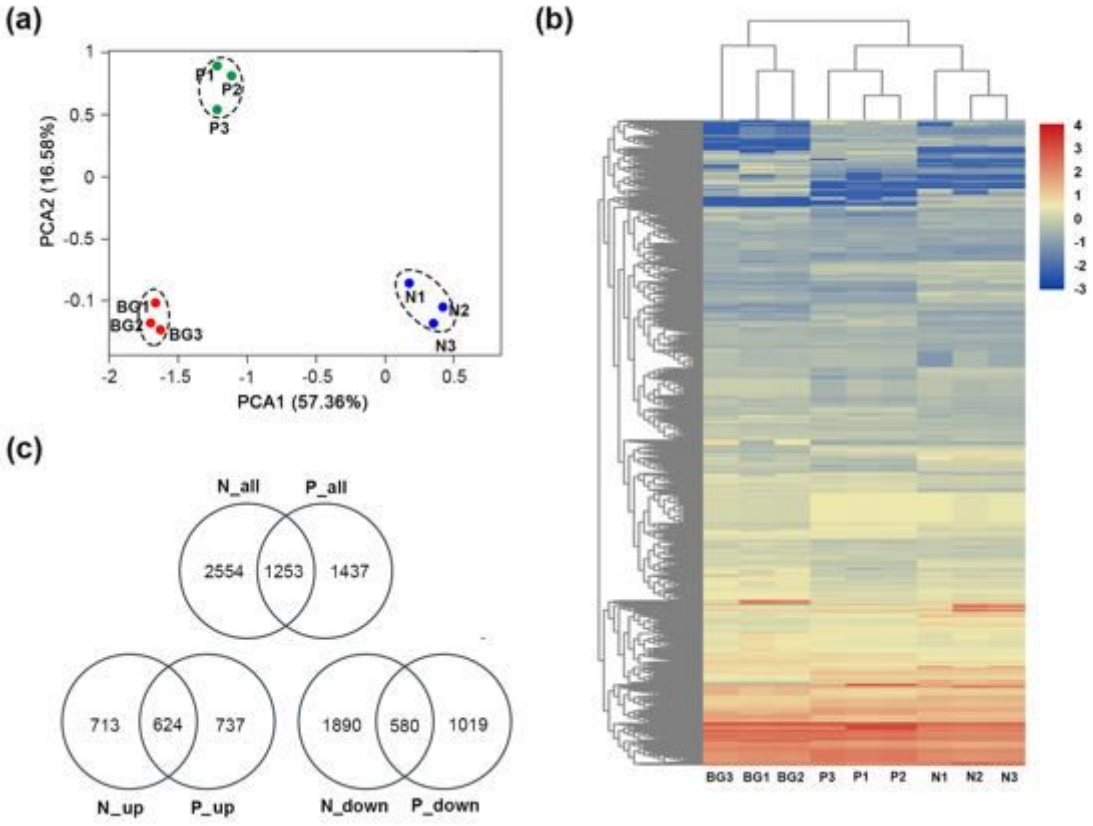

Figure 2

Overview of D. magna transcriptome data and genes distributions in each sample. (a) Principal component analysis (PCA) of the RNA-seq data. Each dot represents one sample. BG is the Normal group (i.e. nutrient sufficient). N stands for N-limitation and P stands for P-limitation (Number 1, 2 and 3 represent triplicates). Samples near to each other are grouped by dashed circles. (b) Heatmap showing the distribution of unique genes in each sample using normalized depths of genes. (c) Venn diagram showing the DEGs in the comparisons among the three treatments.

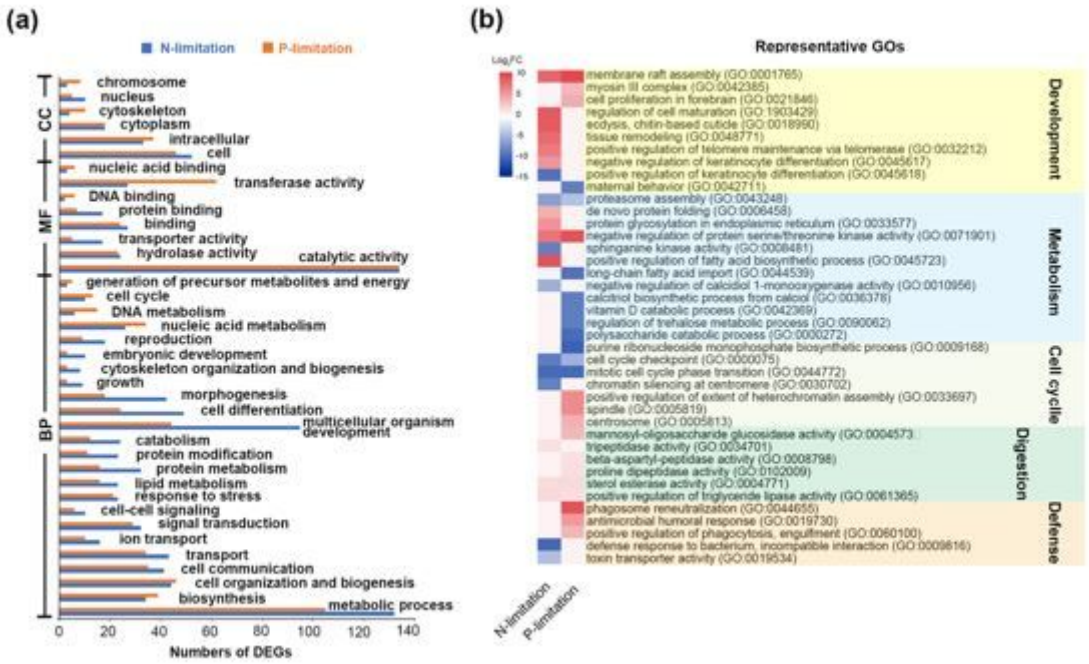

Figure 3

Gene ontology (GO) enrichment of DEGs. (a) GO terms enriched at a high level. BP: biological process, MF: molecular function, CC: cellular component; (b) heatmap of some representatives of the finest GO terms.

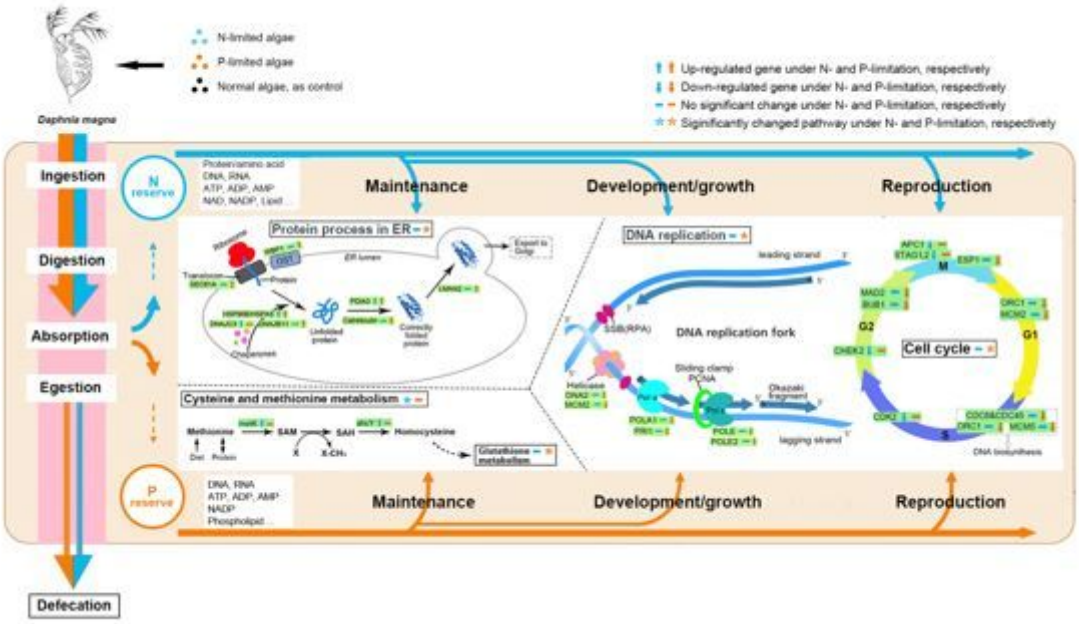

Figure 4

Framework showing the biological pathways of D. magna influenced by low-quality diet. Incoming food from environment is ingested, digested and assimilated into two nutrient reserves (N and P) which will

support the performance (maintenance, growth and reproduction) of D. magna. Five significantly enriched KEGG pathways are illustrated to explain the changes in the performance of D. magna under nutrient constrains, with names of pathways in black solid lines. DEGs (some representatives) within these pathways were in green boxes. Detail information of these DEGs are in Table S5. ER: endoplasmic reticulum; OST: oligosaccharyl-transferase complex; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; SSB: single-stranded DNA-binding proteins; RPA: replication protein A; Pol α (or ε): DNA polymerase α (or ε). G1, S, G2 and M are the four stages of cell cycle.

Supplementary Files

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