Transcriptome sequencing of a keystone aquatic herbivore yields insights on the temperature-dependent metabolism of essential lipids

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Abstract

Background: Phytoplankton dietary quality is the major determinant of the trophic transfer efficiency at the plant-herbivore interface in freshwater food webs. In particular, the phytoplankton's content of the essential polyunsaturated omega-3 fatty acid eicosapentaenoic acid (EPA) has repeatedly been shown to determine the secondary production in the major zooplankton herbivore genus Daphnia. Despite extensive research efforts on the biological model organism Daphnia and the availability of several Daphnia genomes, little is known on the molecular mechanisms underlying the limitation of Daphnia by dietary EPA availability. Results: Here, we used RNAseq to analyse the transcriptomic response of Daphnia magna fed with diets with or without EPA to specifically analyse gene-networks and pathways that are driven by dietary EPA availability. Since EPA is – among other functions – critical to the adaptation of poikilothermic organisms to low environmental temperatures, we ran our experiment at two temperature levels to investigate potentially constrained physiological responses to EPA availability at lower temperatures. Here, trade-offs between food quality and homeoviscous adjustments of biological membranes are expected through the incorporation of polyunsaturated fatty acids. Conclusions: Our highly controlled eco-physiological experiments revealed an orchestrated response of genes involved in transformation and signalling of the essential fatty acid, including eicosanoid-signalling pathways with potential immune functions. We provide an overview of downstream-regulated genes, which contribute to enhance growth and reproductive output. We also identified numerous EPA-responsive candidate genes of yet unknown function, which constitute new targets for future studies on the molecular basis of EPA-dependent effects at the freshwater plant-herbivore interface.

Background

Primary producer biomass is typically of poor quality to herbivores, which limits the trophic transfer of energy through food webs to higher trophic levels (trophic transfer efficiency, [1]. In aquatic environments, the photosynthetic base of the food web consists of small unicellular phytoplankton that is consumed by herbivorous zooplankton. Several constraints on algal food quality have been demonstrated, as algae can be hard to either ingest or digest by herbivores [2, 3] they can provide an unbalanced supply (stoichiometry) of nutrients [4], and – in many cases – their biochemical composition does not meet the herbivores' demands in essential nutritional compounds, such as essential fatty acids [5, 6], sterols [7, 8], vitamins [9] or amino acids [10, 11].

In freshwater ecosystems, crustacean zooplankton of the genus *Daphnia* are the major pelagic herbivores and form a crucial link between primary producers and consumers [12]. Beyond their key role in freshwater food webs, daphnids are a well-established model system of environmental toxicology, experimental ecology and evolution due to their ecological importance combined with an exceptionally high level of phenotypic plasticity [13-16]. The genomes of several *Daphnia* species have been sequenced and it thus is one of the few animal genera on which extensive ecological and genomic information are available [17, 18]. Interestingly, all the complex and plastic responses of daphnids are generated from a

relatively small genome [17, 19]. This makes *Daphnia* an excellent animal model for gene expression studies in response to environmental cues (e.g. [20-22]).

Daphnids have been repeatedly shown to be particularly affected by diets with an inappropriate supply in essential fatty acids, as they are unselective filter-feeders that cannot preferentially take up algal cells rich in particular lipids [23]. A lack or limiting availability of certain omega-3 (ω3, and to a lesser degree ω6) polyunsaturated fatty acids (PUFAs) has been shown to constrain somatic growth, reproduction and population growth in several *Daphnia* species [24-26]. This is due to the fact that ω3 and ω6 PUFAs typically can only be synthesized by primary producers, but not by animals [5, 27-29]. These 'families' of PUFAs can therefore be considered as essential dietary constituents for most animals, including *Daphnia* [30].

Beyond their role in growth and reproduction, it is well-known that PUFAs are a critical component of the so-called 'homeoviscous adaptation' of biological membranes to low temperatures [31]. This concept implies an incorporation of more highly unsaturated fatty acids with 'bent' alkene chains (versus 'straight' chains of saturated alkanes in saturated fatty acids) to maintain high flexibility of cellular lipid bilayers at low temperatures with concomitant low molecular motion [32]. It has been shown that low temperatures increase *Daphnia*'s demand for dietary PUFAs to allow the maintenance of normal physiology [33, 34] and behaviour [35]. Thus, food quality and temperature constitute intertwined factors that challenge the expression of different phenotypes, to achieve best possible performance through plastic acclimatory responses.

In particular, the availability of the highly unsaturated ω3-PUFA eicosapentaenoic acid (EPA, C-20:5 ω3) was repeatedly shown to be crucial for *Daphnia* growth and reproduction via controlled PUFA supplementation experiments [5, 6, 36]. Understanding the molecular mechanisms how EPA availability affects food webs is of global importance, since aquaculture studies have already shown reduced fitness and increased inflammatory responses from higher trophic levels, such as fish, when ω3 fatty acids are limiting [37, 38]. Alarming prospects in connection with future availability of EPA on larger scale were already proposed in a meta-analysis in connection with higher temperatures resulting in reduced primary production of long chain PUFAs due to climate change [39].

Despite the growing body of evidence for the importance of dietary PUFAs in general and of EPA in particular, our understanding of the molecular physiology underlying the PUFA/EPA metabolism and gene networks responsive to the absence or availability of this critical dietary compound are still very limited. Heckmann et al. [40] conducted an in-silico analysis of the genome of *Daphnia pulex,* which yielded first insights on potential mechanisms that are affected by ω6 – eicosanoids. Proposed candidate genes are involved in signalling pathways deduced from the ω6-PUFA arachidonic acid (ARA, C-20:4 ω6), affecting prostaglandin and leukotriene signalling. These candidates were confirmed in follow-up gene expression studies [41-43]. However, it is important to emphasise that ω6 PUFAs (e.g. ARA) are generally believed not to be inter-convertible into ω3 PUFAs (such as EPA) in metazoans [30], although this has been questioned [44].

In this study, we hypothesise that dietary availability or absence of EPA will affect specific gene networks connected to lipid metabolism, cellular signalling and immune-regulating pathways similar to what has been demonstrated for eicosanoids derived from ω6-PUFAs [40-42]. We aim to unravel such gene networks specific to dietary EPA availability using a single genotype (clone) of *Daphnia magna* as a model system. Since EPA is crucial for acclimation to low temperature in *D. magna* (e.g. [35]), such gene networks may become particularly visible at lower temperatures. We thus employed a strictly controlled EPA supplementation experiment at two temperatures to characterise gene expression patterns in *D. magna* using RNAseq and discuss these results in connection with the animals' respective growth performance and fatty acid composition. While an earlier study [6] had focused exclusively on single target genes with differential expression dependent on dieatry EPA availability, we here look for larger scale transcriptomic adjustments driven by different food types and EPA, which should yield insights on PUFA-dependent gene regulation networks. Due to the high level of control on the experimental factors, this yields insights on the genetic basis underlying EPA (and more generally ω3 PUFA) dependent metabolism in this keystone herbivore that will have repercussions for herbivore ecology and physiology in general.

Results

Physiological effects at whole animal level

In our experiment, we fed *D. magna* with two different basal diets (GA – green alga, CY –cyanobacteria) that do not contain any long-chain (i.e. > C-18) polyunsaturated fatty acids to monitor physiological and transcriptomic effects of controlled supplementations with the essential C-20 ω3 PUFA EPA.

Somatic growth rates (SGR), which constitute a good fitness proxy in cladocerans [45], were strongly affected by EPA-availability (2way-ANOVA, $F_{5, 48}$ = 411.318, $p \leq 0.001$) and temperature (2way-ANOVA, $F_{1, 48}$ = 2295.402, $p \leq 0.001$; Fig. 1). Combined effects were detected, as well (2way-ANOVA, $F_{5, 48}$ = 28.779, $p \leq 0.001$).

In general, growth rates were much lower at 15°C reaching only 56.8 – 61.7 % of the performance at 20°C. EPA had a positive effect on *D. magna* growth when fed with the green alga *Acutodesmus obliquus* at both experimental temperatures (GA + EPA $p \leq 0.001$). Similarly, EPA improved the SGR when fed with the cyanobacterium *Synechococcus elongatus* (CY + EPA), at 20°C ($p \leq 0.001$) and 15°C ($p = 0.014$).

Somatic growth rates were higher in all CY-treatments than in respective GA-fed cultures. As stated below (see Material and Methods), cyanobacterial diets were further supplemented with cholesterol and alpha linoleic acid to support reaching maturity (time point of sampling) on this poor diet, which may have supported growth rates to the observed levels.

At both temperatures, the supplementation with empty control liposomes (GA + C) had no effect on SGR (20°C $p=0.593$; 15°C $p \leq 0.881$), as similar growth rates were observed when raising *D. magna* on

supplement-free food or the respective supplementation of control liposomes to the same basal diet. Although lower growth rates were determined at 15°C, the animals were up to 26.8% heavier in absolute body mass (data not shown) than the individuals kept at 20°C.

EPA incorporation and fatty acid composition

The supplementation of EPA and the natural differences in fatty acid composition in basal diets were considered as main drivers for observed growth performances and subsequently for the detected expression profiles at respective temperatures.

D. magna in EPA treatments accumulated supplemented EPA (Fig. 2A + B). Tissue EPA content of *D. magna* was significantly higher at 20°C compared to 15°C.

The two different basal diets resulted in different tissue fatty acid compositions of *D. magna* (Fig. 3) with respect to the proportions of different fatty acid species (state of saturation). No significant differences were seen for saturated fatty acids (SAFAs), either from basal diets or from the applied treatment conditions. However, monounsaturated fatty acid (MUFA) proportions differed significantly between diets. Within 15°C, higher contents were found in CY-fed daphnids (for CY vs GA, GA+C and GA+E $p<0.001$; for CY+E vs GA, GA+C $p<0.001$; and CY vs GA+E $p=0.002$). Similarly, higher contents were detected at 20°C (for CY as well as for CY+E vs GA, GA+C and GA+E $p<0.001$). A temperature effect of differing MUFA level was only detectable in the treatment CY+E with $p<0.001$.

Polyunsaturated fatty acids (PUFAs) were found to be significantly higher in GA-food sources (all single GAs vs all single CYs $p<0.001$) with a tendency of higher recruitment at lower temperature, although not significant.

Differential gene expression overview

The total sequencing output of all samples was about 1,540.9 million reads with an average read amount of 51.4 million reads (± 4.1 SD) per sample. We did not detect differences in the total expression output among treatments or temperatures. With a mapping success of 79.89% (± 0.69% SD) we calculated FPKM values for further analyses for each replicate. To broadly compare expression profiles in terms of differential expression driven by food composition and culture temperature, we used the ArtNOG annotation to analyse the transcript diversity among different treatments (Fig. 4) by functional COG (categories of orthologous groups) assignments.

We distinguished differential responses by altered transcripts in *D. magna* fed either GA or CY. In general, the total amount of altered transcripts (driven by EPA, temperature and combined effects) was slightly different, with 3,688 and 4,305 transcripts for GA and CY, respectively. However, temperature-sensitive transcripts were much more pronounced when *D. magna* were raised on cyanobacteria (3,385 temperature-specific sequences), whereas on the green algal diet much less transcripts (2,001 sequences)

were altered. The opposite trend was seen for transcripts that displayed EPA-sensitivity: GA treatments yielded 1,635, CY treatments only 175 differently expressed transcripts. Similarly, combined effects were more pronounced in GA diet (669 transcripts; CY: 128 transcripts). For both basal diets, altered expression levels were most prominently detected in categories (with known functions) T and O, i.e. 'Signal transduction mechanisms' and 'Posttranslational modifications', in connection with the factors temperature and EPA availability. Further changes in cellular processes and signalling categories were seen for 'Cytoskeleton' (Z) and 'Intracellular trafficking, secretion and vesicular transport' (U). Affected metabolic functions concerned 'Carbohydrate- '(G), 'Amino acid-' (E), 'Lipid-' (I) and 'Inorganic ion transport' (P) as well as 'Secondary metabolite biosynthesis, transport and catabolism' (Q). These alterations were paralleled by changes in the 'Transcription machinery' (K), but also alterations in 'Translational-' (J) and 'RNA processing' (A) transcripts, which were stronger affected by the factor temperature.

Core response profiles of affected transcripts

To provide a more detailed overview of the large set of responsive genes depicted in Fig. 4, we further analysed genes in the respective categories to extract common responses in connection with the factors temperature, EPA availability and combined effects of both factors. From the most prominent categories (see above), we cross-matched congruently regulated transcripts in GA and CY treatments to obtain basal diet – independent gene expression patterns (Table 1, Supplementary Files).

In total, we found 381 transcripts with a specific functional artNOG assignment to be affected by temperature (details in Supplementary File 1). In the cluster of 'Information storage and processing', strongest altered gene expression was detected indicating a transcriptomic remodelling driven by temperature. Most of the genes were up-regulated at 15°C compared to 20°C. This may not only be provoked by the necessity of different functions, but by compensation to maintain efficient reaction norms through increased transcript amounts at lower temperatures. To a lesser extent, this holds also for the COG clusters 'Cellular processes and signalling' as well as for genes in 'Metabolism' with more complex patterns. Here, functional changes became visible that were not thoroughly connected to compensation strategies. The overall increments in gene expression profiles varied also with the applied basal diet, often with higher expression levels in GA diets than in CY. Interestingly, most temperature-responsive genes of all clusters display a generally higher expression level when EPA was available (see Supplementary File 1). Specific expression profiles will be detailed and discussed below with respect to the functional patterns.

Much less genes were detected for a common response to EPA (15 candidates) or in connection with combined effects (5 candidates; see Table 2 and Supplementary File 2). The selection of shared altered transcripts between basal diets did include candidates with extremely opposing levels in transcript amount.

Many EPA-influenced genes displayed a down-regulation with supplementation, in particular on the GA diets. An exception to this are the transcripts of the carboxylic ester hydrolase and the aromatic-L-amino-acid decarboxylase, which were expressed with highest levels in animals on GA diets supplemented with EPA. The first may be attributed to lipid metabolism (although jet assigned with artNOG category “R – functional prediction only”, the second is part of amino acid metabolism and is involved in cell communication and signalling as this enzyme catalyses the production of dopamine, serotonine, tryptamine and histamine.

In animals fed CY-EPA diets, the highest expression levels were observed for endo-beta-1.4-mannanase, animal haem peroxidase and THAP domain-containing protein, which are involved in fructose-mannose metabolism, cyclooxygenase activity and the regulation of transcription, respectively. Here, a contrasting regulation of transcripts between the different basal diets and EPA supply becomes very explicit.

Genes regulated congruently in both basal diets were Myosin–IB, an uncharacterized protein (KZS03735.1), Angiopoetin-1 receptor-like protein and Glycerol ether metabolic process (protein) with a down-regulation while EPA is available.

Our statistical analysis followed by a cross-match of significant genes between diets yielded six genes that display combined effects of temperature and EPA availability (see Supplementary File 2). The highest expression level was detected for Cytochrome P450. At higher temperature this enzyme was upregulated in CY+EPA, at lower temperature in GA+EPA diet. Transcripts of (putative) Trypsin-7, Endo-beta-1.4-mannanase, as well as Opsin Rh6 were regulated similarly with higher levels at lower temperature in CY+EPA diets, and were repressed at high temperatures in GA+EPA diets.

Discussion

We studied transcriptomic effects of dietary EPA availability in combination with temperature to disentangle responsive gene networks behind the beneficial effects of this long chain ω3-PUFA on a physiological level. We underpin these effects by quantifying somatic growth rates as a fitness proxy together with the animals’ fatty acid composition. This allowed us to discriminate gene expression patterns indicative for a complex interplay between resource availability and temperature responses in the aquatic model herbivore *Daphnia magna*.

Physiological performance and fatty acid composition

As for most animals, the fatty acid composition of *Daphnia* sp. reflects the composition of their diet [47]. In nature, the occurrence of PUFA-rich phytoplankton in lakes at cooler temperatures in spring matches the nutritional demand of zooplankton at the beginning of the season providing high proportions of PUFAs for growth and reproduction [48] but also membrane remodelling [32]. Seasonal shifts in temperature and food availability should therefore be mirrored in altered transcript expression with signatures that are particularly attributable to these factors.

In our analysis, responses in life history traits in connection with EPA availability at different temperatures showed that *Daphnia* cultivated at 15°C displayed a higher demand for EPA than specimen at 20°C by impaired growth when EPA was limiting (Fig. 1), which is in line with an earlier study [49]. However, EPA levels of *D. magna* were higher at 20°C (Fig. 2), contrary to the assumption that more EPA should be required at 15°C for homeoviscous adaptation. This has been been reported before in the same temperature regime [49, 50].

The total amount of EPA in *D. magna* is higher at the low compared to the higher temperature as a consequence of higher total body mass at the lower temperature. Nevertheless *D. magna* may have been ultimately limited by EPA availability due to the enhanced PUFA demand at lower temperatures. A higher amount of EPA accumulation in somatic tissue at 20°C compared to tissue amounts at 15°C is further supported by a recent study [50].

Nevertheless, when we analysed the daphnids' fatty acid composition with respect to saturation state (SAFAs, MUFAs and PUFAs; see Fig. 3) almost no temperature-effects became visible within the different food types (except for MUFAs in the CY + EPA treatment). Consequently it is likely, that the applied thermal difference of 5°C was not severe enough to alter the animals' FA contents.

Gene expression

By assessing gene expression profiles in *D. magna* under strictly controlled experimental conditions, we were able to attribute particular functional changes specifically to temperature and EPA availability. In general, temperature elicits large responses connected to RNA and DNA related processes ("Information storage and processing" see Figure 4) which are represented by a complex network of genes involved and replication as well as transcription and translation machinery. The key abiotic factor provoked also the alteration of transcripts affecting signal transduction mechanisms, posttranslational modification as well as carbohydrate-, amino acid- and lipid transport mechanisms and inorganic ionic transport processes.

Although the effect size of EPA altered transcripts was lower than the temperature-induced effects, this dietary constituent nevertheless is a major driver of improved growth at the physiological level (see above).

The transcriptomic responses so far analysed in connection with long chain polyunsaturated fatty acids rely on studies of enzymes that are involved in eicosanoid synthesis of the "arachidonic pathway" [51]. These enzymes are known to convert eicosanoids like into important signalling molecules like prostaglandins or leukotrienes in invertebrates, but also in mammals [40, 52]. In our study, EPA provoked various functional changes in translation and transcription, but also signal transduction mechanisms, changes in intracellular trafficking as well as altered transcript levels for cahbohydrate-, amino acid and lipid metabolism that are detailed below.

Information storage and processing

In this cluster the strongest thermal effects are seen for the categories ‘RNA processing and modification’ (category A); ‘translation, ribosomal structure and biogenesis’ (J) and K (‘transcription’). Adjustments in the transcriptome become visible here, as these functions are modulated as first response to altered conditions. A high proportion of maintenance costs is attributed to this gene regulation, which compensate effects of bio-physical reaction norms [53-55]. Generally higher expression values were observed at colder temperature and were more enhanced than in other functional classes, like ’cellular processes and signalling’ or ’metabolism’ (see Supplementary File 1). This effect is known as compensatory effect and was previously shown to vary between clones of *D. pulex* due to local adaptation [56]. Many candidates attributed in this cluster through artNOG annotation showed high transcript levels at both lower temperature and dietary EPA availability. This indicates adjustments of the transcriptome by both factors.

Signalling

Numerous G-protein signalling transcripts as well as serine/threonine kinases and opsins were found to be thermally sensitive and were elevated with dietary EPA availability. Such candidates are potential mediators of anti-inflammatory processes and are connected to healing and growth of cells in mammals [57]. Further, RAs and Ran- transcripts (RAs-related nuclear protein) that are factors involved in G-protein signalling affecting gene expression cascades involved in cell growth, differentiation and survival [58] were upregulated. It is likely that Ras and Ran transcripts mediate sensing and signalling cascades for growth in *Daphnia* or other invertebrates. Similarly, the production of resolvins and protectins, molecules derived from EPA as well as from the longer docosahexaenoic acid (DHA, 22:6 ω3), are involved in cytokine and leukotriene signalling via G-proteins [59]. Transcripts of signalling cascades involving stimulators like dopamine or serotonin (products of the aromatic-L-amino-acid decarboxylase) were found to be upregulated in the EPA treatments. It remains to be investigated whether such products do function as neurotransmitters or if they serve other endocrine functions in invertebrates. First indication for the utilization of dopamine in *Daphnia* sp. was found in connection with predatory stress [60].

We also identified transcripts of cytochrome P450 in connection with the combined effects of temperature and EPA availability (Supplementary file 2). This is an important indicator for the biotransformation of EPA. Potential mechanisms are the conversions of EPA into five regioisomeric epoxyeicosatetraenoic acids (EETeTrs) and ω/(ω-1)- hydroxyeicosapentaenoic acids (19- and 20-HEPE) [61] which mediate (at least in mammals) a delicate balance between pro- and anti-inflammatory responses [62].

Numerous gene families of chytochrome P450 as well as pseudogenes have been identified across the animal kingdom [63]. For *Daphnia*, 75 functional CYP genes and 3 pseudogenes belonging to 4 clans, 13

families, and 19 subfamilies were identified so far [64]. However, the particular functional implications for many of these genes are still to be determined.

Cellular structure and metabolism

The higher growth rates were paralleled by higher expression of genes for cytoskeletal structures accompanied by induced growth factor receptors and fibronectin, which were expressed at higher levels at 15°C when EPA was available.

The different profiles of carbohydrate metabolic transcripts (G, but also in E) maltase, amylase and alpha-glucan branching enzyme indicate a different quality of the basal food sources, but also different energetic demands at both temperatures. While sugars seem to be stronger metabolized at 20°C, glycogen anabolism becomes effective at 15°C. This may be due to the fact that a faster metabolism is connected to a higher temperature accompanied with a higher demand for sugars, which is also mirrored by higher growth rates at physiological level. Carbohydrate metabolism was also differentially regulated when *Daphnia sp.* were challenged with diets of different qualities in terms of nutrient stoichiometry [65], which may indicate that this is a very general response to food quality alterations. Genes in carbohydrate metabolism involved in inflammatory processes (SAPA) or connected to chitin and moulting (chitotriosidase) were regulated as well in a temperature-dependent manner, but also do reflect a higher variation that may mirror the variability of individuals in sample pools.

Despite low transcriptional levels at GA-diet, peptidases like trypsin or chymotrypsin, aminotransferases as well as metallopepdiases were up-regulated in CY diets, especially at the lower temperature (E). This was also mirrored in the expression of Eip55E (Ecdysteroid-inducible polypeptide 55 subunit E), which is involved in sulphur amino acid metabolic processes like cysteine and glutathione biosynthesis.

The different expression profiles in carbohydrate and amino acid metabolism indicate a recruitment of different enzymes to extract energetic compounds like sugars or amino acids from the different basal diets [66]. Different digestive efforts are indicated (for CY-diets) through high expression levels of metallopepdidases, trypsins and aminotransferases as well as by chaperones like T-complex proteins. Thus, different basal diets provoke different phenotypes to handle and digest the different food items.

In the ‘lipid metabolism’ category (I), high levels of acyl-CoA dehydrogenases were expressed at 20°C, especially when EPA was available. This indicates the transcription of RNAs related to degradation of fatty acids. Transcript levels for transporters and intracellular transport structures associated with the transport mechanisms of long chain fatty acids were up-regulated when EPA was absent. This may be a mechanism to cover the higher demand for long chain PUFAs under EPA limitation. The higher expression levels of fatty acid transporters was accompanied by the expression of a transporter in the category ‘inorganic ion transport and metabolism’ (P) as well as by ABC transport proteins (ATP-Binding Cassette sub-family C/ member 4) and cytochrome P450 305a1, which are involved prostaglandin-mediated signalling (Q, secondary metabolites).

High vitellogenin levels were pronounced in GA+EPA diets accompanied by the expression of glycerol-3-phosphate acyltransferase and acyl-CoA-binding domain-containing protein 7 with slightly higher (maybe compensatory) levels at 15°C, which may be involved in the biogenesis of vitellogenin, as observed before [43]. Also, a secretory phospholipase A2 was induced indicating higher effort to digest liposomal supplemented diets.

High levels of dynein, myosin and tubulin (Z) indicate a remodelling of the cytoskeleton at lower temperature. As the solubility and viscosity of the cytosol seems to be affected, a structural remodelling is indicated by the latter transcripts that are further supported by EPA availability. In this context, higher levels of fibronectin and endothelial growth factor receptor indicate a mediation of processes involved in cell division and growth [67].

Expressed candidate genes connected to ‘inorganic ion transport and metabolism` (P) were up-regulated in animals feeding on cyanobacteria at 20°C. Further gene upregulation occurred at 15 °C, which indicates dynamic adjustments of the osmotic balance at the lower temperature. Especially Ca^{2+}- as well as a serotonin transporters were expressed more strongly at the lower temperature in the GA- diet supplemented with EPA. This matches the observed pattern for G-protein transcripts and conjoined candidates in category T, which may contribute to the same messaging pathway [68]. Similarly, cytochrome P450 305a1 transcript displayed the same pattern in the category Q ‘secondary metabolites…‘, which may also indicate a conjoined function in a signalling pathway. Interestingly, other Cytochrome P450-like proteins seem to be highly temperature sensitive and were expressed with low or high levels in GA+EPA diets at 20°C and 15°C, respectively.

Cytochrome P450 transcripts and subsequent proteins seem to play an important role in the metabolisation and potential tansformation of EPA into signalling. Potential pathways for a transformation of the long chain polyunsaturated fatty acid EPA into other endocrine signalling molecules were proposed earlier by [40]&[52]: the cyclooxygenase (COx)- pathway, 2) the lipoxygenase (LOX)- pathway or 3) the cytochrome P450 - pathway. Recent expression studies, however, have shown that COx expression is not affected by EPA-availability [43, 51], and so far, no LOX genes have been found in *Daphnia* species [43]. Altogether, our study delivers a profound insight into EPA-connected metabolism and indication that a transformation into endocrine signalling may rely on Cytochrome P450-based conversions, which need to be detailed by further studies.

Conclusions

With our study we demonstrate the plastic transcriptional responses in *Daphnia magna* to different food types and temperatures. The expression patterns in different phenotypes were highly dependent on the dietary availability of the ω3-polyunsaturated fatty acid EPA. Our results suggest a distinct cascade utilizing different forms of cytochrome P450 to mediate sensing the essential compound by transformation and subsequent G-protein signalling. Affected target genes then stimulate further

transcription, transport mechanisms of intermediates, cellular growth, and reproduction. Altogether, this promotes a positive overall physiological performance. This is the first time that the orchestrating gene response of *Daphnia* was recorded to this explicit stimulus. Our study thus reveals some of the molecular mechanisms underlying the positive effects of a particular dietary omega-3 fatty acid and constitutes a profound resource of transcriptional patterns even for genes with poor or no annotation. Many new candidates for future investigations and characterization of EPA-related pathways are hidden in the so far uncharacterized genes (see expression profiles: Supplementary File 3), which can be now be annotated to be EPA-responsive.

As cladoceran zooplankton forms the link in the trophic transfer of matter and energy between primary producers to higher levels in the food chain, this is an important step for our understanding of the resource-driven limitation at the aquatic plant-herbivore interface.

Methods

Food cultures

The green alga (GA) *Acutodesmus obliquus* and the cyanobacterium (CY) *Synechococcus elongatus* served as poor-quality food sources to monitor effects of the fatty acid EPA via supplementation, as they do naturally not contain long chain (> 18 C) ω3 PUFAs [49, 69, 70].

Respective culture conditions of all used food – organisms are listed in Table 2.

Animals and experimental design

Daphnia magna clone P132.85 originating from the pond Driehoek in Heusden (The Netherlands; N51°44'01", E5°08'17") were pre-cultured at either 15°C, or 20°C in aged, aerated and sterile-filtered (45 µm) tap water under dim light conditions for at least 2 months. The animals were kept at a maximum density of 15 individuals L^{-1} under non-limiting food conditions by feeding them 2 mg carbon $L^{-1}$of *A. obliquus* during pre-culture every second day.

Juvenile *D. magna* for experiments originated from mothers that carried the third clutch of parthenogenetic offspring. Neonates (within 8 h after release from the mothers' brood pouch) were randomly distributed to jars containing 600 ml tap water and respective experimental food conditions. Experimental specimens were than fed on a daily basis (20°C) or every other day (15°C) by transferring them in fresh glasses with food and supplements.

Food treatments comprised a green algal (GA), or cyanobacterial (CY) basal diets, as well as supplemented treatments with EPA liposomes (GA + EPA, CY + EPA). To control that liposomes themselves had no effect we included a control (GA + C) supplementing the empty vector. All conditions were replicated with n=5. Each replicate comprised 15 individuals.

Liposomes were prepared as according to [73]. All *D. magna* individuals were raised until they deposited the first clutch of eggs into their brood pouch (reaching maturity) before sampling. On pure cyanobacterial diets, *D. magna* do not produce offspring, as neither needed sterols nor significant amounts of PUFAs are available [8]. Therefore, all CY treatments were supplemented with at least cholesterol and alpha linoleic acid (ALA, C-18:3 ω3) as otherwise the production of eggs would have been impaired. All liposome supplementations were standardized to 320 µl L^{-1} for desired PUFAs and sterols. After reaching maturity, *Daphnia* were caught, rinsed twice with deionized water before blotting them dry with lint-free tissue. Whole animal samples were shock-frozen in liquid nitrogen and stored at -80°C thereafter until further experimentation.

From each replicate, two individuals were used for somatic growth rate analysis; the remaining individuals were deep frozen for lipid composition monitoring and expression analyses.

Daphnia fitness parameters

Somatic growth rates were monitored using the mean dry weight of two adult individuals from each treatment when reaching maturity. As a reference we used the mean dry weight of 40 neonates at the beginning of the respective experimental temperature. The animals were rinsed with deionized water and dried for 24 hours at 60° C in pre-weighed aluminium boats before measuring their dry masses. Somatic growth rates (SGR) were determined in accordance to Lampert et al. [45] to monitor whole animal fitness by the formula:

$$SGR = [(\ln(m_t) - \ln(m_0)]/t.$$

As *Daphnia* grows with an exponential rate, the natural logarithms were applied to final weights m_t from each replicate and to the starting weight m_0 to calculate the net weight gain by their difference. Growth rates were than put into relation to the time period t, the number of days until the specimen deposited their first clutch of eggs in their brood pouch, which is typically considered as the onset of maturity in *Daphnia* spec. [74].

Fatty acid composition

The analysis of fatty acid composition was done by means of fatty acid methyl esters (FAME), which were subsequently quantified by gas chromatography (GC). For each treatment 3 mature *D. magna* from each replicate (previously stored at -80) were first extracted over night at 4°C with 5 ml extraction reagent (ExR, dichloromethane/methanol (2:1, v:v)) and sonicated for a minute in an ultrasound bath. The extract was than pooled with a second round of extraction using 3 ml ExR and sonication. Prior first sonication, two standard FAMEs were added for quantification: 10 µg heptadecanoic acid methyl ester (C-17:0 ME) and 5 µg tricosanoic acid methyl ester (C-23:0 ME). Cellular debris was removed by centrifugation at 5.000 rpm for 5 min at room temperature. After evaporation of solvent the sample was transesterified at

70 °C for 20 min using 5 ml of 3 N methanolic HCl to built FAMEs. These were in turn extracted by adding ~2 ml isohexane, which dissolves the FAMEs into the upper liquid phase which was transferred to a fresh glass vial. A second round of isohexane extraction was done to minimize loss of material that was not transferred in the first round, leaving a small portion of the upper phase in the vial. After evaporation of isohexane, FAMEs were washed from the glass tube in a volume of 3 x 100 µl. After re-applied evaporation, FAMEs were dissolved in 50 µl isohexane to concentrate and standardize samples for the GC measurement. Each measurement was conducted using 1 µl FAME-extract on a 6890-N GC System (Agilent Technologies, Waldbronn, Germany) equipped with a DB-225 capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness, J&W Scientific, Folsom, CA, USA). Instrument settings were as follows: injector and FID temperatures 200 °C; initial oven temperature 60 °C for 1 min, followed by a 120 °C/min temperature ramp to 180 °C, then a ramp of 50 °C/min to 200 °C followed by 10.5 min at 200 °C, followed by ramp of 120 °C/min to 220 °C followed by 10.5 min at 220 °C; helium with a flow rate of 1.5 ml/min was used as the carrier gas. Quantification of the fatty acids was performed by referring to the internal standards and to response factors determined for each FAME from mixtures of known composition [5, 75]. The correlation coefficient for the response factors was > 0.98 for each individual FA calibration curve. Single fatty acid contents were related to the carbon content of the body tissue using the previously determined carbon to dry mass conversion factor for body tissue, 0.41 µg carbon (µg dry mass)$^{-1}$ [76].

Nucleic acid preparation and sequencing

Based on their different fitness at the physiological level, which was monitored by means of somatic growth rates, we selected sample pools for RNA extraction. From both experimental temperatures we prepared RNA from 4 treatment groups for sequencing with 3 replicates each. We did not include the GA (only food) treatment, as there were no differences to GA + C on physiological level (SGR) or in PUFA composition. We pooled the material of 5 specimens for each replicate to ensure appropriate amounts for RNA extraction and comparability. Total RNA was extracted using the NucleoSpin RNA extraction kit (Machery und Nagel, Düren/Germany) according to the manufacturer´s instructions. In addition, traces of remaining genomic DNA were removed using the Turbo DNA-free Kit (Invitrogen, Karlsruhe/Germany) in line with the manufacturer´s manual. The quality of the isolated RNA was monitored using capillary electrophoresis on a Bioanalyzer (Agilent Technologies, Santa Clara/USA). Only samples with $OD_{280}/OD_{260} \geq 2.0$, $OD_{280}/OD_{230} \geq 2.0$ and RIN ≥ 8.0 were used for sequencing. Clean-up of mRNA, cDNA library construction as well as sequencing was conducted at the University of Cologne's 'Cologne Centre for Genomics'. Respective cDNA libraries were than sequenced on an Illumina HiSeq 4000 platform in the paired end 75 bp -mode using two lanes for the above described 30 sample pools (5 treatment groups with 3 replicates at two temperatures). Raw sequencing data were clipped and trimmed using Trimmomatic [77] to remove sequencing adapters and low quality regions at the beginning and end of sequences.

Data analysis and processing

We sequenced mRNA of pools of 5 *D. magna* individuals from 4 different food treatments (GA + C, GA + EPA, CY, CY + EPA) originating from either 15°C, or 20°C with 3 replicates each. We used FastQC [78] to monitor sequence quality at a critical threshold score > 30, which was passed for samples. Reads originating from single treatment replicates were mapped to *Daphnia magna* genome (NCBI BioProject accession No. PRJNA298946, comprised of ~ 130 million bases) to gather single specific expression profiles. Single reads of treatment replicates were mapped to the *Daphnia magna* genome assembly version 2.4 (NCBI BioProject accession No. PRJNA298946), containing 26.646 translations (open access since April 2016). Mapping was conducted using RSEM v.1.2.31 [79] connected to bowtie2 [80] to generate FPKM outputs for transcript analysis.

To gather a more functional overview, we assigned the translations of genomic sequence data with orthologous groups using the eggNOG v. 4.0 database [81]. In particular, we assigned sequences with artNOGs (arthropod Non supervised Orthologous Groups, composed of 21 species in this group), which are grouped in COG (Categories of Orthologous Groups). For assignments we used BLASTp at an e-value cut-off $\leq 10^{-3}$ and a HSP cut-off length $\geq$33 bases to provide additional information for categorization.

For differential gene expression analysis we used MeV software v. 4.8.1 [82] connected to edgeR [83]. Two-Way ANOVA was used on the basis of normalized FPKM values to identify differently expressed genes driven by EPA-availability and temperature at respective basal food sources.

In addition, t-tests were conducted between treatments within temperatures, or vice versa characterize gene expression profiles at a critical threshold of $p \leq 0.01$. Responsive transcripts were analysed via cross-match (Hulsen, de Vlieg, & Alkema, 2008) to gather consensus lists.

Abbreviations

+ C Supplementation with control liposomes

+ E or EPA Supplementation with EPA liposomes

ALA Alpha linoleic acid, C-18:3 ω3

ANOVA Analysis of variance

ARA Arachidonic acid, C-20:4 ω6

artNOG Arthropod non-supervised orthologous groups

BLAST Basic Local Alignment Search Tool

bp Base pair

C-17:0 ME Standard for FAME analytics, heptadecanoic acid methyl ester

C-18 Fatty acids with total length of 18 carbon molecules

C-20 Fatty acids with total length of 20 carbon molecules

C-23:0 ME Standard for FAME analytics, tricosanoic acid methyl ester

cDNA Complementary DNA

COG Categories of orthologous groups

COx Cyclooxygenase

CY Cyanobacterium (here in particular of the species *Synechococcus elongatus*)

Cyano Cyanophycea medium, used for the culture of *Synechococcus elongatus*)

CYP Cytochrome p-450

DHA Docosahexaenoic acid, C-22:6 ω3

DNA Deoxyribonucleic acid

EETeTrs Epoxyeicosatetraenoic acids

eggNOG Database for orthologous groups hosed by the EMBL

EPA Eicosapentaenoic acid, C-20:5 ω3

ExR Extraction reagent for lipids extraction

FA Fatty acid

FAME Fatty acid methyl esters

FastQC Quality assessment software for sequencing data

FPKM Fragments per kilobase of exon model per million reads mapped

GA Green alga (here in particular of the species *Acutodesmus obliquus*)

GC-FID Gas chromatography-flame ionization detector

HEPE Hydroxyeicosapentaenoic acids

HSP High scoring pairs

LOX Lipoxygenase

MeV Multi Experiment viewer

MUFA Monounsaturated fatty acid

NCBI National Centre for Biotechnology Information

PUFA Polyunsaturated fatty acid

Ran Ras-related Nuclear protein

Ras Protein family involved in G-Protein signalling

RNA Ribonucleic acid

RNAseq Ribonucleic acid sequencing

rpm Rotations per minute

SAFA Saturated fatty acid

SAG International acronym for Culture Collection of Algae at Göttingen University, Germany

SAPA Substrate-associated protein A

SD Standard deviation

SEM Standard error of means

SGR Somatic growth rate

THAP Thanatos Associated Protein, indicates death-domain in protein

v:v Percent of volumes

Z/4 Name of the medium used for *Acutodesmus obliquus* culture

ω3 Fatty acid species with unsaturation (carbon double bond) at the n-3 position

ω6 Fatty acid species with unsaturation (carbon double bond) at the n-6 position

Declarations

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Availability of data and materials

RNAseq data are available at the NCBI sequence read archive under accession number SRP109969 with sample accessions SAMN07259933 - SAMN07259947 (20°C) and SRS2947545 - SRS2947559 (15°C). A detailed description and overview of the respective sampling material is available under the BioProject accession number PRJNA391248. The results of the RNAseq experiment are also accessible under the GEO entry GSE107545.

Authors' contributions

PF and HW conceived and conceptualised the experiment. HW conducted the experiments and analysed physiological, fatty acid and transcriptomic data and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable, as animal experiments on invertebrates (except cephalopods and higher crustaceans, which does not apply here) do not require regulatory approval according to § 8 TierSchG of the national regulations of Germany.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1: Overview of altered transcripts in COG regulated independent from basal diets. Two-way ANOVA results of *D. magna* expression profiles were cross-matched [46] between GA and CY diets to determine common gene expression patterns in functional COG groups. Resulting transcript numbers are given in connection with the respective factors.

COG cluster	COG	Category description	Temperature	EPA	Interactions
Information storage and processing	J	Translation, ribosomal structure and biogenesis	31	2	0
	A	RNA processing and modification	54	0	0
	K	Transcription	27	0	0
Cellular processes and signalling	T	Signal transduction mechanisms	50	2	1
	Z	Cytoskeleton	48	1	0
	U	Intracellular trafficking, secretion, and vesicular transport	9	1	0
	O	Posttranslational modification, protein turnover, chaperones	44	1	0
Metabolism	G	Carbohydrate transport and metabolism	17	0	0
	E	Amino acid transport and metabolism	39	1	1
	I	Lipid transport and metabolism	22	0	0
	P	Inorganic ion transport and metabolism	20	1	0
	Q	Secondary metabolites biosynthesis, transport and catabolism	20	0	1
Poorly characterised	R	General function prediction only	100	2	1
	S	Function unknown	97	2	0
	X	No match in artNOG	104	2	1
Total			682	15	5

Table 2: Algal and cyanobacterial culture conditions and respective growth media.

Organism	Strain	Medium	Culture conditions	Temperature and light conditions
green alga *Acutodesmus obliquus*	SAG 276-3a	Z/4 [71]	5 L semi-continuous batch culture with a 20% Vol. replacement with fresh sterile medium every other day	20°C and a constant light intensity
cyanobacterium *Synechococcus elongatus*	SAG 89.79	Cyano [72]	chemostat at a dilution rate of 20% d^{-1}	of ~ 60 µmol photons m^{-2} s^{-1}

Additional File Legends

Supplementary file 1: temperature significant gene expression profiles

Supplementary file 2: EPA significant gene expression profiles and combined effects

Supplementary file 3: Expression profiles of “poorly characterized” genes responsive to temperature, EPA and combined effects

Figures

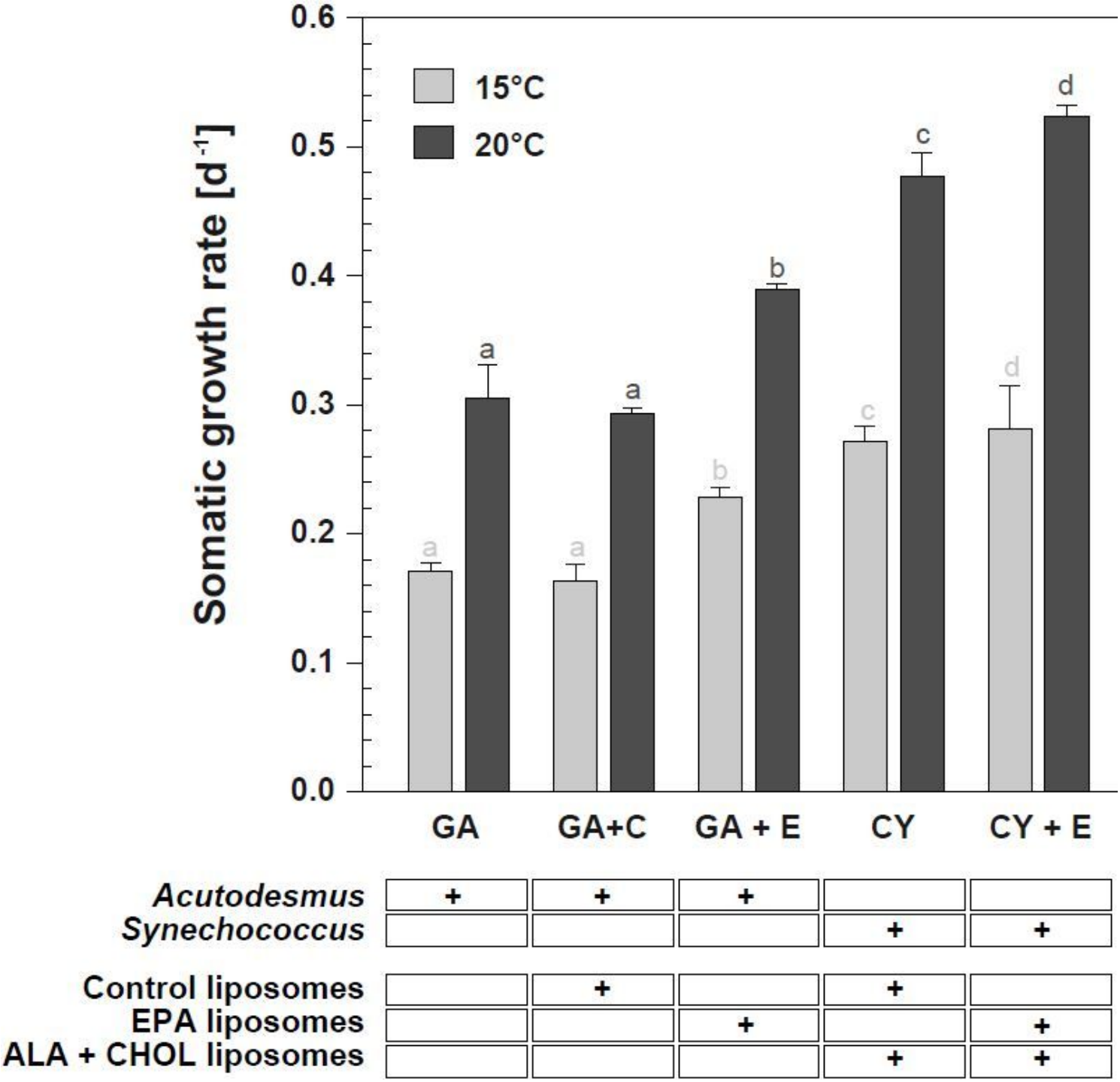

Figure 1

Juvenile somatic growth rates of D. magna (means ± SD of n = 5) in response to different food sources and EPA-supplementation via liposomes at two experimental temperatures. Different letters indicate significant differences within each temperature regime (Tukey's HSD following two-way ANOVA).

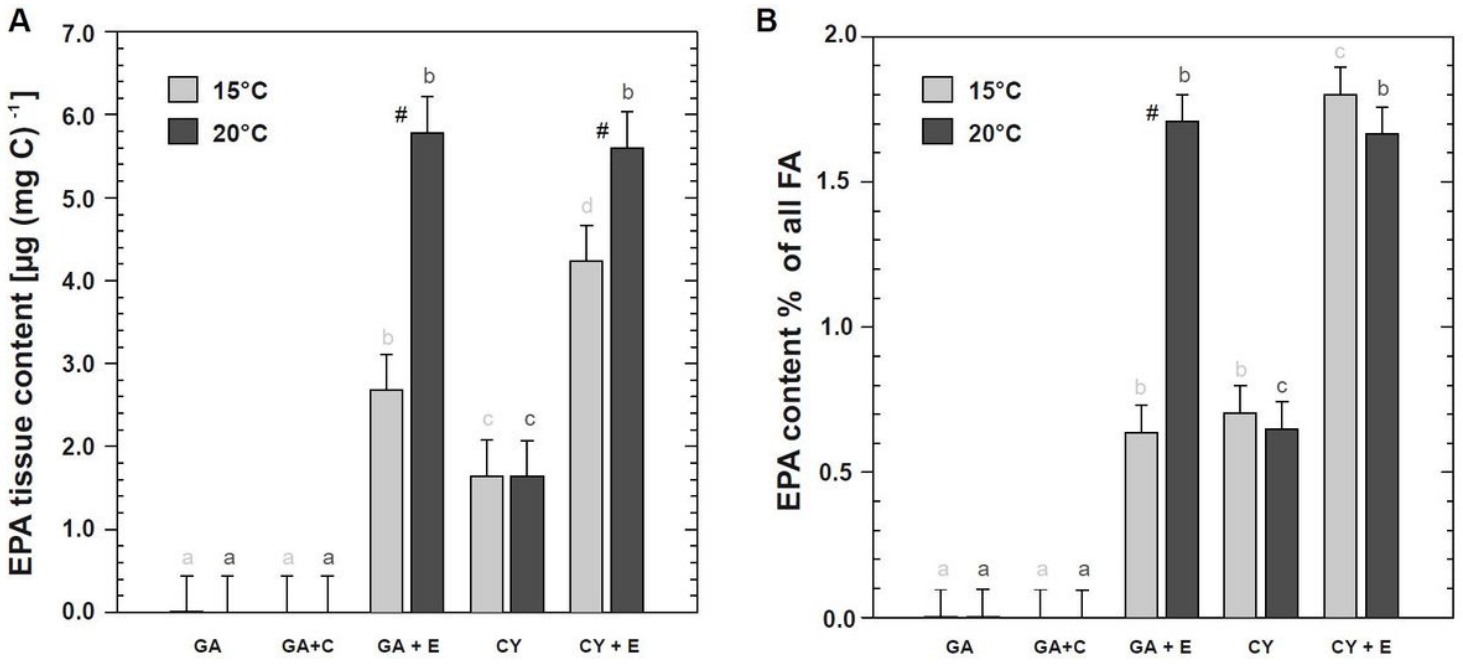

Figure 2

EPA levels in D. magna. A) Mean tissue EPA concentrations (± SEM of n = 3 independent replicates consisting of 3 individuals each); B) mean EPA proportion (± SEM) of all fatty acids. Treatment effects are indicated by different letters, temperature effects by hash keys as determined by two-way-ANOVA followed by Tukey´s HSD post-hoc comparisons. Treatment codes indicate basal food item (GA = Acutodesmus obliquus, a green alga; CY = Synechococcus elongatus, a cyanobacterium) and the supplements (C = control liposomes, E = EPA liposomes).

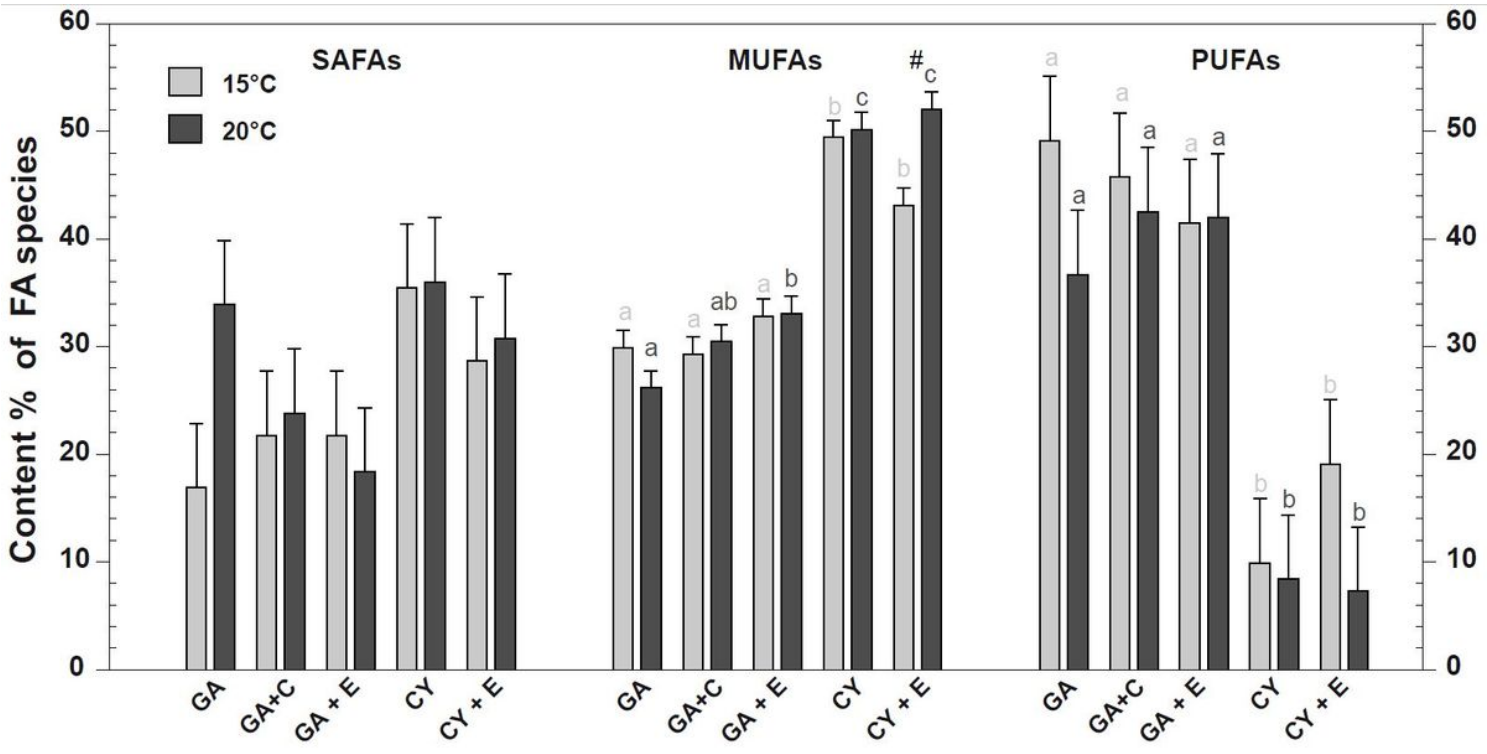

Figure 3

Fatty acid composition of D. magna in the experiment. The three groups display the mean (± SEM of n = 3) proportion of saturated (SAFAs), monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs) in D. magna in the respective treatments. Different letters indicate significantly different means within temperatures according to Tukey's HSD following two-way ANOVA, hash key indicates significant temperature effects. Treatment codes indicate basal food item (GA = Acutodesmus obliquus, a green alga; CY = Synechococcus elongatus, a cyanobacterium) and the supplements (C = control liposomes, E = EPA liposomes).

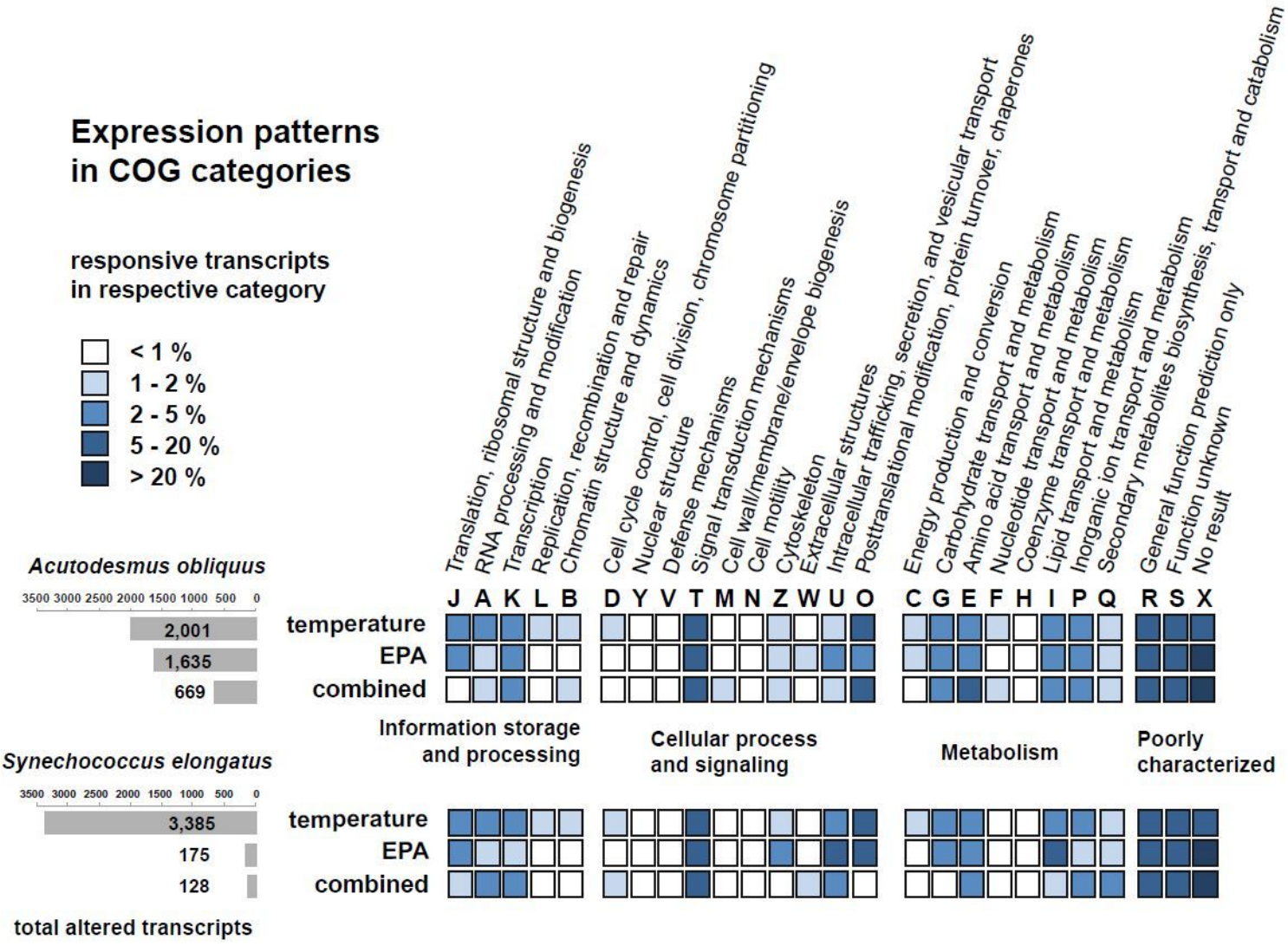

Figure 4

Differential gene expression analysed within basal diets. Display shows a result summary of a two-way ANOVA (at significance level of p = 0.01) among expression profiles with the factors ± EPA and ± temperature within basal food types. Grey bars on the left show the amount of significantly different expressed transcripts that were found to be modulated either by temperature, food or combined effects. The total amount of the respective transcripts was then functionally annotated by the ArtNOG categorisation (given in % of the total response). Colour coding indicates the abundance of transcripts in each category.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.pdf
- supplement2.pdf
- supplement3.pdf