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Combined effects of dietary polyunsaturated fatty acids and parasite exposure on eicosanoid-related gene expression in an invertebrate model

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ABSTRACT

Eicosanoids derive from essential polyunsaturated fatty acids (PUFA) and play crucial roles in immunity, development, and reproduction. However, potential links between dietary PUFA supply and eicosanoid biosynthesis are poorly understood, especially in invertebrates. Using *Daphnia magna* and its bacterial parasite *Pasteuria ramosa* as model system, we studied the expression of genes coding for key enzymes in eicosanoid biosynthesis and of genes related to oogenesis in response to dietary arachidonic acid and eicosapentaenoic acid in parasite-exposed and non-exposed animals. Gene expression related to cyclooxygenase activity was especially responsive to the dietary PUFA supply and parasite challenge, indicating a role for prostanoid eicosanoids in immunity and reproduction. Vitellogenin gene expression was induced upon parasite exposure in all food treatments, suggesting infection-related interference with the host's reproductive system. Our findings highlight the potential of dietary PUFA to modulate the expression of key enzymes involved in eicosanoid biosynthesis and reproduction and thus underpin the idea that the dietary PUFA supply can influence invertebrate immune functions and host-parasite interactions.

1. Introduction

Polyunsaturated fatty acids (PUFA) are essential lipids required for animal growth and reproduction. Dietary C20 PUFA, such as arachidonic acid (ARA) and eicosapentaenoic acid (EPA), serve as precursors of eicosanoids which are known to modulate reproduction and immunity of vertebrates (Calder, 1998; de Pablo and de Cienfuegos, 2000; Fritsche, 2006; Wathes et al., 2007). In invertebrates, however, eicosanoid biosynthesis has been scarcely linked to the dietary PUFA supply (Schlotz et al., 2012). This is surprising considering the severe physiological and ecological consequences that are associated with an inadequate dietary PUFA provisioning (Fraenkel and Blewett, 1947; Martin-Creuzburg et al., 2012; Müller-Navarra et al., 2000).

In various invertebrate species, the occurrence of eicosanoids has been thoroughly documented (Rowley et al., 2005; Stanley, 2000). Reproductive processes (Machado et al., 2007; Tootle and Spradling, 2008; Wimuttisuk et al., 2013) as well as immune responses have been shown to depend on eicosanoid action (Merchant et al., 2008; Shrestha and Kim, 2010; Stanley-Samuelson et al., 1991). In contrast to eicosanoid biosynthesis in mammals, invertebrate eicosanoid biosynthesis has been suggested to split into two instead of three pathways; an epoxygenase pathway seems to be absent (Heckmann et al., 2008b; Stanley, 2000). The presence of the leukotriene branch has also been doubted as no leukotrienes or orthologs of LOX could be found in a number of invertebrates, including *Daphnia pulex* (Morgan et al., 2005; Yuan et al., 2014). In the biosynthesis of prostanoid eicosanoids (Fig. 1), phospholipases A_2 (PLA2) represent the first step of a chain of consecutive reactions. PLA2 are responsible for the hydrolysis of the *sn-2* ester of phospholipids resulting in the release of free PUFA. Both secretory and cytosolic forms of PLA2 have been shown to be potent in mobilizing eicosanoid precursors and to play a role in host defense against microbial pathogens (Balsinde and Dennis, 1997; Boyanovsky and Webb, 2009; Park et al., 2005; Shrestha et al., 2010). Once in their free form, ARA and EPA compete for the same enzymes for further metabolism (Lands, 1992) and are converted by a cyclooxygenase (COX or PXT) to prostanoids. The following steps of eicosanoid biosynthesis are performed by various enzymes which form the functional eicosanoids

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(Fig. 1). Eicosanoids are suspected to play a regulatory role in reproductive processes and thus reproduction-related genes constitute possible downstream targets of eicosanoid action.

Species of the genus *Daphnia* have become important model organisms in ecology, ecotoxicology and evolutionary biology (Lampert, 2011). Owing to the long history of ecological research, our knowledge of e.g. life history traits or phenotypic plasticity is vast compared to many other model organisms. With the fully sequenced genome of *Daphnia pulex* another major advantage was given to *Daphnia* as a model (Colbourne et al., 2011; Ebert, 2011). However, potential links between eicosanoids, reproduction, and immune defense have been poorly studied in *Daphnia* although the enzymatic machinery for eicosanoid biosynthesis is present and seems to have undergone substantial restructuring (Colbourne et al., 2011; Heckmann et al., 2008b; Yuan et al., 2014). It is well established that the fecundity of *Daphnia* can be increased by supplementing the eicosanoid precursors ARA and EPA, implying that eicosanoid biosynthesis is linked to reproduction (Martin-Creuzburg et al., 2010; Schlotz et al., 2013). Moreover, it has been shown recently that the dietary PUFA supply can improve the performance of *Daphnia magna* under pathogen challenge, suggesting a link between eicosanoid biosynthesis and immunity (Schlotz et al., 2013, 2014). These latter studies also proposed that the presence of eicosanoid precursors (i.e. EPA or ARA) in the diet affects the resource allocation trade-off between reproduction and immune function. Thus, revealing the physiological and genetic mechanisms responsible for these interactions may provide crucial insights into the role of PUFA in modulating host-parasite interactions.

The objective of the present study was to explore whether genes coding for key enzymes of the eicosanoid biosynthesis machinery as well as reproduction-related genes are differentially expressed in female *D. magna* in response to dietary PUFA supply and parasite challenge. Target genes were chosen to cover several steps of biosynthesis starting from substrate release via central conversion to final formation of bioactive eicosanoids. In addition, three genes related to oogenesis were investigated as potential targets of eicosanoid action.

Experimental animals were raised on food sources naturally differing in their C20 PUFA content and composition. In addition, to specifically test for the potential of single PUFA to modulate gene expression, the C20 PUFA-deficient diet was experimentally enriched with either ARA or EPA. To challenge the immune system, half of the animals of each group were exposed to endospores of the parasitic bacterium *Pasteuria ramosa* (Ebert et al., 1996, 2016); subsamples for gene expression analyses were taken 12 and 24 h after parasite exposure.

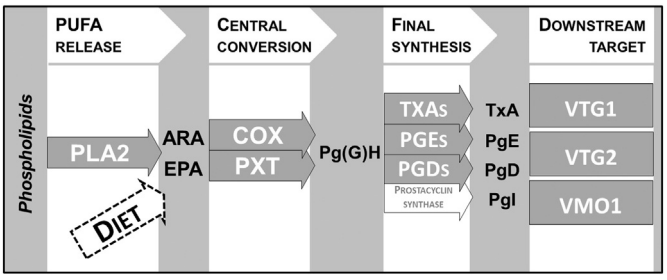

Fig. 1. Schematic overview of the prostanoid pathway based on current evidence from invertebrate models including potential reproduction-related downstream targets. Displayed are key enzymes (arrows) and major metabolites. Gray arrows/boxes indicate genes measured in this study (PLA_2 = phospholipase A_2; COX = cyclooxygenase; PXT = chorion peroxidase; PGES = prostaglandin E synthase; PGDS = prostaglandin D synthase; TXAS = thromboxane synthase; VTG1/2 = vitellogenin 1/2; VMO1 = vitelline membrane outer layer protein 1). A list of target genes and primer sequences is provided in Table 1. The availability of the precursors arachidonic acid (ARA) and eicosapentaenoic acid (EPA), typically released in the first step of eicosanoid biosynthesis by PLA_2, has been manipulated through the diet.

2. Material and methods

2.1. Model system

Under favorable environmental conditions *Daphnia* reproduce parthenogenetically, i.e. no males are produced and all female offspring are clones of their mother ensuring an identical genetic background in all experimental animals. *Daphnia* are unselective filter feeders and do not discriminate between food particles differing in quality (DeMott, 1986). EPA and ARA supplemented via liposomes are efficiently absorbed and incorporated as shown by the presence of dietary PUFA in both soma and eggs following consumption (e.g. Martin-Creuzburg et al., 2010; Schlotz et al., 2013). The use of PUFA-loaded liposomes is thus highly suitable for studying the effects of dietary PUFA on *Daphnia* physiology.

We used *Pasteuria ramosa*, a castrating endoparasitic bacterium, to challenge the experimental animals. The *D. magna - P. ramosa* system has been thoroughly investigated and many aspects of the infection process and the inheritance of resistance have been elucidated (Ebert et al., 2016). The *P. ramosa* clone and the *D. magna* clone used here are known to be compatible (Hall and Ebert, 2012; Luijckx et al., 2011). *P. ramosa* cannot be cultivated outside its host. To harvest the endospores (infective stages of the parasite), infected animals can be crushed and the resulting suspension adjusted regarding the number of spores and subsequently used for infection.

2.2. Food organisms

Two algae differing in their PUFA profiles were used to raise the experimental animals: the green alga *Chlamydomonas globosa* (culture collection of the Limnological Institute, University of Konstanz, Germany), which contains no PUFA of >18 C atoms, and the eustigmatophyte *Nannochloropsis limnetica* (culture collection of the University of Göttingen, Germany; SAG 18.99), which contains considerable amounts of ARA and exceptionally high amounts of EPA. These differing PUFA profiles are well-known to affect the food quality for *Daphnia*. Compared to green algae, *N. limnetica* is of superior food quality. Feeding on *N. limnetica* leads to the accumulation of C20 PUFA in *Daphnia*, resulting in increased somatic growth rates and reproductive output (Martin-Creuzburg et al., 2009, 2010; Schlotz et al., 2013). The algae were each cultured semi-continuously in modified Woods Hole (WC) medium (Guillard, 1975) in aerated 5 L vessels (20 °C; dilution rate: 0.2 d^{-1}; illumination: 100 mmol quanta m^{-2} s^{-1}). Food suspensions were produced by centrifugation of the harvested algae and resuspension in fresh medium. Carbon concentrations were estimated from photometric light extinctions (480 nm) and from previously determined carbon-extinction equations. The carbon–light extinction regressions were confirmed by subsequent carbon analysis of the food suspensions.

2.3. Biochemical analyses

For the analysis of fatty acids in the food suspensions approximately 1 mg particulate organic carbon (POC) was filtered onto precombusted GF/F filters (Whatman, 25 mm). Total lipids were extracted three times from filters with dichloromethane/methanol (2:1, v/v). Pooled cell-free extracts were evaporated to dryness under a nitrogen stream. The lipid extracts were transesterified with 3 M methanolic HCl (60 °C, 20 min). Subsequently, fatty acid methyl esters (FAMEs) were extracted three times with 2 mL of isohexane. The FAME-containing fraction was evaporated to dryness under nitrogen and resuspended in a volume of 20 μL isohexane. FAMEs were analyzed by gas chromatography on a HP 6890 gas chromatograph equipped with a flame ionization detector (FID) and a DB-225 (J&W Scientific, 30 m × 0.25 mm ID × 0.25 mm film) capillary column. Details of GC configurations are given elsewhere (Martin-Creuzburg et al., 2010). FAMEs were quantified by comparison

to an internal standard (C23:0 ME) using multipoint calibration curves determined previously using FAME standards (Sigma-Aldrich). FAMEs were identified by their retention times and their mass spectra, which were recorded with a gas chromatograph-mass spectrometer (GC–MS, Agilent Technologies, 5975C inert MSD) equipped with a fused-silica capillary column (DB-225MS, J&W). Spectra were recorded between 50 and 500 *m/z* in the electron ionization (EI) mode. The limit for quantitation of fatty acids was 10 ng. The absolute amount of each fatty acid was related to the particulate organic carbon (POC), which was determined using an element analyzer (EuroEA3000, HEKAtech GmbH, Wegberg, Germany).

2.4. Liposome preparation

Liposomes prepared according to Martin-Creuzburg et al. (2008) are readily ingested by the non-selective filter feeder *Daphnia* along with the provided food and thus can be used as vehicles for supplementing PUFA. EPA and ARA provided via liposomes are efficiently absorbed and incorporated into soma and eggs of *Daphnia* (Martin-Creuzburg et al., 2010; Schlotz et al., 2013). Liposome stock suspensions were prepared from 3 mg 1-palmitoyl-2-oleoyl-phosphatidylglycerol and 7 mg 1-palmitoyl-2-oleoyl-phosphatidylcholin (Lipoid, Germany) dissolved in an aliquot of ethanol. PUFA-containing liposomes were prepared by adding 3.33 mg PUFAs (i.e., arachidonic acid (20:4n-6, ARA) or eicosapentaenoic acid (EPA, 20:5n-3); both purchased from Sigma) from lipid stock solutions in ethanol. The resulting solutions were further processed as described in Martin-Creuzburg et al. (2008).

2.5. Experimental design

A clone of *D. magna* (HU-HO2, originally isolated in Hungary) was cultivated in filtrated lake water (0.2 μm) containing saturating amounts of *C. globosa* (2 mg C L^{-1}). The experiment was conducted with third-clutch neonates born within 12 h at 20 °C. Animals were kept individually in 80 mL filtrated lake water (<0.2 μm) and randomly assigned to one of the following food regimes: (1) *C. globosa* + control liposomes, (2) *C. globosa* + ARA-containing liposomes, (3) *C. globosa* + EPA-containing liposomes, and (4) *N. limnetica*. Every other day, animals were transferred to new jars containing fresh filtrated lake water (<0.2 μm) and algal food (2 mg C L^{-1}, ad libitum food supply). This procedure ensures equal amounts of carbon for all experimental animals. On day 5, half of the animals in each food regime were exposed to 50,000 endospores of the *P. ramosa* clone C19, a dose known to result in 100% infected animals in this compatible host-parasite combination (Hall and Ebert, 2012; Luijckx et al., 2011); control animals were mock-exposed to the same amount of macerated tissue of healthy daphnids. Twenty-four *D. magna* were pooled to form one replicate, resulting in four replicates for the mock-exposed and four replicates for the parasite-exposed group in each food treatment. 12 and 24 h after parasite exposure animals were sampled (12 individuals per sample) using a cylindrical sieve system (Heckmann et al., 2007) and stored at −80 °C in 400 μL RNA*later*® (Ambion) for subsequent RNA extraction.

2.6. Gene expression analysis

2.6.1. RNA extraction and DNA synthesis

Total RNA was extracted using the RNeasyMini kit with on-column DNase treatment (Qiagen) according to the manufacturer's instructions. RNA concentrations were determined using a NanoDrop™ spectrophotometer (NanoDrop Technologies). Agarose (1.5%) gel electrophoresis was used to verify the quality of the extracted RNA. cDNA was synthesized from 2 μg total RNA using the First Strand cDNA Synthesis Kit (Fermentas) following the manufacturer's instructions. Subsequently, cDNA was diluted 25-fold to a concentration equivalent to 4 ng total RNA μL^{-1} and stored at −20 °C.

2.6.2. Identification of gene homologs, primer design and relative expression of mRNA

The analysis comprised 11 genes (Table 1). To identify gene homologs in *D. magna* a *t*Blast*x* search (Altschul et al., 1990) was performed using 38 genes described as being part of the eicosanoid biosynthesis machinery of *D. pulex* as a query against the genome of *D. magna* (v.2.4, unpublished data). Only best BLAST hits with a score >50 and an e-value $<1*10^{-6}$ were considered for further analysis. In order to define the intron-exon structure of each gene identified in *D. magna*, open reading frames were annotated with the getorf package from EMBOSS (Rice et al., 2000), translated into a protein, and manually checked with a Blast*p* search against the NCBI non-redundant database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were designed using the primer designing tool Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; Ye et al., 2012). Primers were synthesized by biomers.net (Ulm, Germany). Real-time quantitative polymerase chain reactions (qPCR) were conducted on a StepOnePlus™ real-time PCR system (Applied Biosystems) using Power SYBRGreen PCR master Mix (Applied Biosystems). Each reaction was run in triplicate and contained 5 μL of cDNA template (equivalent to 20 ng total RNA) along with 300 nM primers in a final volume of 20 μL. The amplification was performed under the following conditions: 95 °C for 10 min to activate the DNA polymerase, then 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Melting curves were visually inspected to verify a single amplification product. Primer dimers were detected for *sPlA2* and *iPlA2* as well as *PgDs1* and *PgDs2*. However, the signal to noise ratio was large and thus the error caused by the dimer formation was assumed to be small enough not to interfere with target gene measurement.

2.7. Data analysis and statistics

Raw qPCR data were analyzed using Data Analysis for Real-Time PCR (DART-PCR) (Peirson et al., 2003). The calculated reaction efficiencies verified the expected amplification of around 2-fold for all genes. The few outliers detected were removed. The resulting data set was normalized by NORMA-Gene, a method which does not require the use of reference genes (Heckmann et al., 2011). Effects of food and time on the normalized and log-transformed expression of target genes were assessed using two-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc tests. Effects of parasite exposure on gene expression were tested separately for each gene using one-way ANOVA. For illustration, gene expression levels were expressed relative to those recorded in animals consuming *C. globosa* and sampled 12 h post mock or parasite exposure, i.e. the relative gene expressions recorded in animal of these treatments were set to one (Fig. 2). In consequence, food and time effects are shown as fold-changes. To illustrate the influence of parasite-exposure, expression of each gene in parasite-exposed animals was calibrated to the respective gene in mock-exposed animals, i.e. expression levels in the latter were set to one and the exposure effects are shown as fold-changes (Fig. 3). Samples taken 12 and 24 h post exposure were measured in separate runs. However, the efficiencies of the PCR reactions using the same primers were equal for both runs and thus the data were combined for the evaluation of time effects on gene expression. All statistics were carried out using Statistica (v 6.0, StatSoft).

3. Results

3.1. PUFA composition of the food organisms

The food algae differed considerably in their PUFA content and composition. *N. limnetica* contained the eicosanoid precursors ARA and EPA whereas *C. globosa* did not contain PUFA with >18 carbon atoms (Table 2). Supplementation of *C. globosa* with PUFA-containing liposomes provided *D. magna* with 31 μg ARA or EPA mg C^{-1}, respectively (Table 2).

Table 1
Target gene and primer information. Upper and lower sequences represent forward and reverse primers, respectively. Primer sequences for *Pxt*, *Vtg1* and *Vmo1* were taken from Heckmann et al. (2008a); primer sequences for *Vtg2* were taken from Hannas et al. (2011).

Target gene		Gene product function	Primer sequence (5′-3′)	Amplicon size (bp)
sPlA2	Phospholipase A_2 (secretory)	Hydrolysis of the *sn-2* ester of phospholipids, release of free PUFA	GCAATGCGATCCACTTTCCTAC GATGGCGTCGACGGTGAG	79
iPlA2	Phospholipase A_2(Ca^{2+}-independent)		AAGGAAAATCTGACGCCGCT GCCACGTTGATATTTGCCCC	95
Cox	Cyclooxygenase	Catalysis of the first step in the synthesis of prostanoids; conversion of free PUFA to prostaglandin H (PGH)	CACTGGGACGTGATGATGGA AGAGTGCGGCCATATTGGATT	97
Pxt	Chorion peroxidase		CCACCTCGCAAATTGTCTTT GTCCCACGATGGATTCAACT	77
TxAs	Thromboxane A synthase	Formation of thromboxane A (from PGH)	GCAAACTTTTGGAATTGTATGCCC TTGACCACCGTAGATGATGAAGA	74
PgEs	Prostaglandin E synthase	Formation of prostaglandin E (from PGH)	GCGTGCCTTCCTGGATTTTG TGCAGACCAGCCAAGTTGTT	85
PgDs1	Prostaglandin D synthase 1	Formation of prostaglandin D (from PGH)	ACTCGCTATGCTCGATACGG TGGACGAGCAGAACTTTCCC	80
PgDs2	Prostaglandin D synthase 2		TTTGGGCCATTCTTCTCGCT GCTCGACTGGTCCTTCAACA	72
Vtg1	Vitellogenin 1	Egg yolk precursor protein	CTGGCAAATGGGAAATCAAC CCCAGGTGTAAGCCAAACC	93
Vtg2	Vitellogenin 2		CACTGCCTTCCCAAGAACAT ATCAAGAGGACGGACGAAGA	66
Vmo1	Vitelline membrane outer layer protein 1	Integral part of the vitelline membrane	TATTACGCGGTTCAGACGTG GTTGTCCGCCTCACTACCAT	87

3.2. PUFA-induced changes in gene expression

Relative gene expression levels in animals reared on the diet deficient in C20 PUFA (*C. globosa*), the same diet supplemented with ARA or EPA, or the diet naturally containing both of these C20 PUFA (*N. limnetica*) were compared using the deficient diet as calibrator both within the mock-exposed (control) and the parasite-exposed group (Fig. 2, Table 3).

Within the food treatments tested, gene expression levels were either unchanged over time or decreased between 12 and 24 h after mock- and parasite-exposure (Fig. 2). The only exception was the expression of the secretory phospholipase A_2 gene *sPlA2*, which increased over time.

The strongest difference between the C20 PUFA-deficient diet and the C20 PUFA-containing diets was found for the genes *Cox* (Fig. 2e,f) and *Pxt* (Fig. 2g,h). While *Cox*-expression was significantly lower (5-fold) in animals consuming any of the C20 PUFA-containing diets, *Pxt* expression was significantly higher (up to 20-fold). These effects were found for all PUFA-containing diets and thus seem not to be specific for a certain PUFA.

Expression patterns of *iPlA2* (Fig. 2c,d), *TxAs* (Fig. 2i,j), and *PgEs* (Fig. 2k,l) resembled each other. Diet-induced differences in the expression of these genes were moderate (up to around 2-fold) and did not reveal a general PUFA-related pattern, i.e. the ARA- and EPA-supplemented diets did not provoke the same effects. By tendency, animals raised on the ARA-supplemented diet showed the highest levels (after 24 h), while both the EPA-supplemented diet and *N. limnetica* yielded the lowest levels of the respective gene transcripts. *PgDs1* (Fig. 2m,n) and *PgDs2* (Fig. 2o,p) deviated partially from the observations described above. Again, *PgDs1* expression was significantly lower in animals raised on the EPA-supplemented diet and in animals raised on *N. limnetica*, whereas *PgDs2* expression was lower in animals fed the ARA- or EPA-supplemented diets.

Expression of both vitellogenin genes *Vtg1* and *Vtg2* did not differ between mock-exposed (control) animals raised on the different diets (Fig. 2q,s) whereas in parasite-exposed animals the EPA-supplemented diet and *N. limnetica* resulted in lower levels of transcripts (Fig. 2r,t). The expression pattern of the third oogenesis-related gene *Vmo1* (Fig. 2u,v) was unrelated to the vitellogenin gene expression; all PUFA-containing diets yielded a lowered expression.

Differences in the gene expression responses between mock-exposed (control) animals (Fig. 2, left panels) and parasite-exposed animals (Fig. 2, right panels) were attributable to parasite-induced effects, which are described below in more detail (Fig. 3).

3.3. Parasite-induced changes in gene expression

Gene expression levels in animals exposed to the parasite were related to those of mock-exposed (control) animals reared on the same diet. Effects of parasite exposure as well as combined effects of dietary C20 PUFA availability and parasite exposure are summarized in Fig. 1. Parasite-induced up-regulation of gene expression was generally stronger 24 h post exposure. Significant down-regulation of some of the examined genes in response to the parasite was observed only in animals consuming the EPA-supplemented diet 12 h post exposure.

The highest number of induced genes was found in animals consuming the C20 PUFA-deficient diet (6 out of 11 genes; Fig. 3b), followed by the ARA-supplemented diet (Fig. 3d), *N. limnetica* (Fig. 3h) and the EPA-supplemented diet (1 out of 11 genes; Fig. 3f). Genes with the highest fold-changes were *Pxt* (up to 7-fold in the C20 PUFA-deficient diet; Fig. 3b) and *Vtg2* (up to 6-fold in the ARA-supplemented diet; Fig. 3d). Regarding *Pxt*, induction was less pronounced on all C20 PUFA-containing diets compared to the C20 PUFA-deficient diet. *Vtg2* induction was lower on the two EPA-containing diets (Fig. 3f,h) than on the other diets (Fig. 3b,d).

4. Discussion

In the present study, we investigated the hypothesized link between dietary C20 PUFA supply, parasite challenge, and eicosanoid biosynthesis on the transcriptomic level. We explored if the availability of dietary precursors and/or the activation of the immune system result in differential expression of genes coding for key enzymes in the biosynthesis pathway of prostanoid eicosanoids.

The genes *Cox* and *Pxt*, which are central to the prostanoid pathway, were most responsive to the dietary C20 PUFA supply. Although *Cox* gene homologs are missing in insect models, such as *Drosophila melanogaster*, *Aedes aegypti*, and *Bombyx mori*, they are evidently present in other arthropods, including *Daphnia* (Heckmann et al., 2008b; Varvas et al., 2009; Yuan et al., 2014). Nonetheless, in species obviously

lacking COX, prostaglandins have been shown to exert important physiological functions (Vrablik and Watts, 2013). Evidence suggests that another enzyme with COX-like activity, the chorion peroxidase PXT, can exert similar functions. Its crucial role in *Drosophila* follicle maturation corroborates the importance of prostaglandins for reproduction in invertebrate species (Tootle and Spradling, 2008). Thus, both *Cox* and *Pxt* are presumably coding for proteins with supposed COX-like function (note that "*Pxt*" has been termed "*Cox*" in earlier

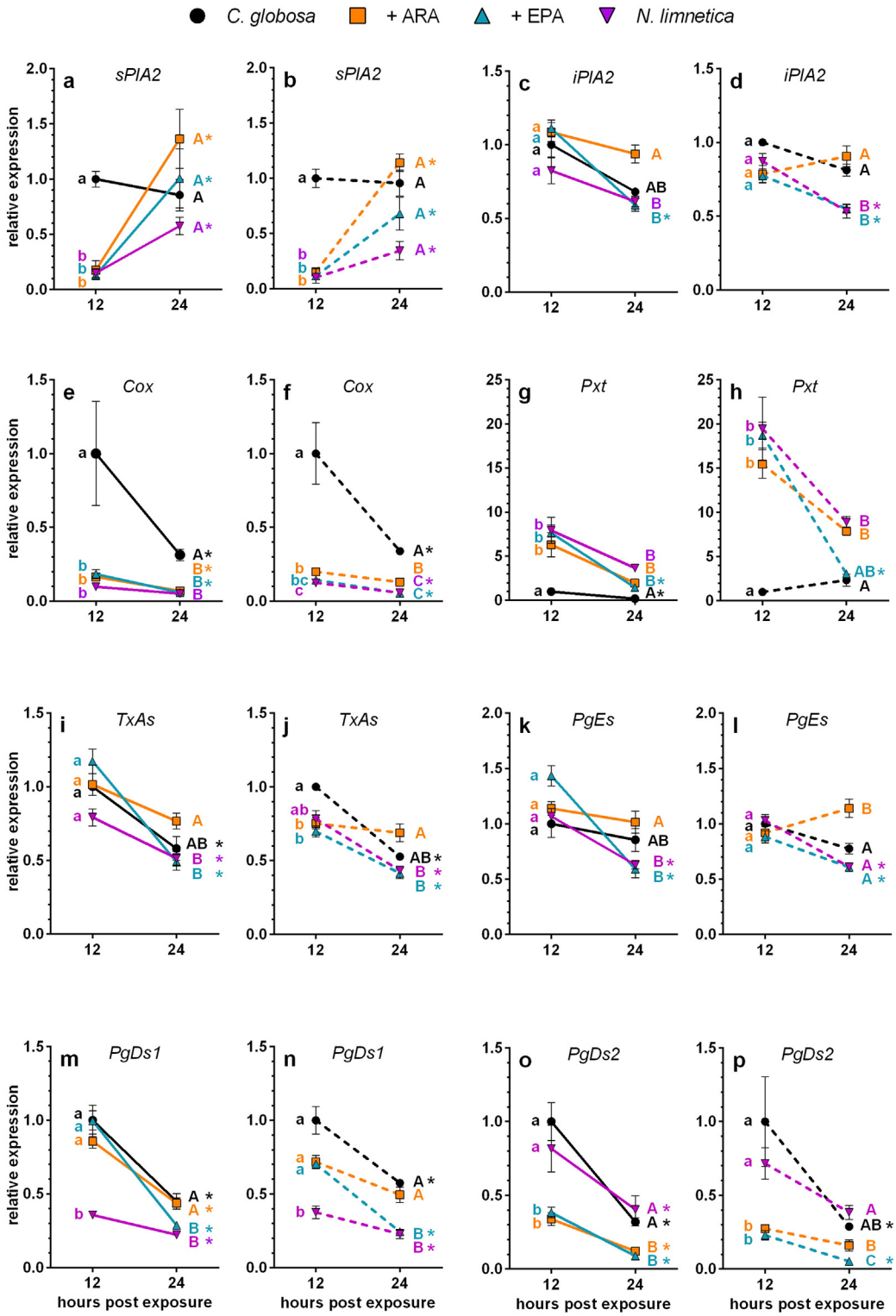

Fig. 2. PUFA-induced effects on relative target gene expression. For each gene, expression levels in mock-exposed animals (panels a, c, e, etc.; solid lines) are shown next to those in parasite-exposed animals (panels b, d, f, etc.; dashed lines). Colors indicate the food treatments: black = *C. globosa*; orange = *C. globosa* + ARA; blue = *C. globosa* + EPA; magenta = *N. limnetica*. For illustration, gene expression in animals reared on the different food sources were calibrated to gene expression in animals reared on *C. globosa* (12 h post exposure). Data are means of four replicates ± standard error of the mean (SEM). Symbols labeled with the same letters do not differ statistically. Food treatment effects 12 and 24 h post exposure were analyzed in a single two-way ANOVA (Table 3); however, differences are pictured separately using lower case letters for 12 h, upper case letters for 24 h. Asterisks indicate significant ($p \leq 0.05$) time effects within the respective food treatment (Tukey's HSD test following two-way ANOVA).

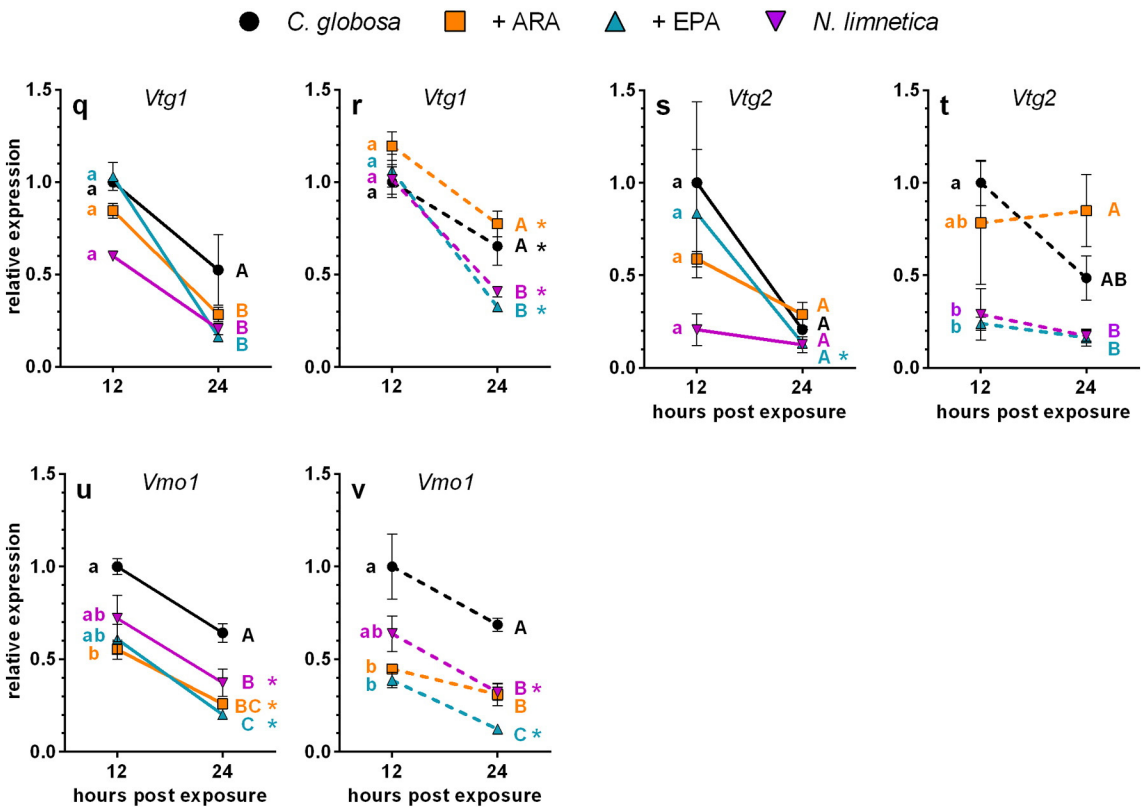

Fig. 2 (*continued*).

gene expression studies (Heckmann et al., 2008a; Schlotz et al., 2012). In the present study, however, the observed expression patterns of *Cox* and *Pxt* in response to the dietary PUFA supply differed clearly, indicating that these genes have distinct functions. While *Cox* expression was significantly reduced in animals feeding on PUFA-containing diets, *Pxt* was up-regulated under these conditions. The increased expression of *Pxt* in PUFA-consuming *D. magna* corroborates previous findings (corresponds to *Cox* expression reported in Schlotz et al., 2012), suggesting that PXT plays a role in prostanoid biosynthesis and reproduction of *D. magna* similar to the PXT in *Drosophila* (Tootle and Spradling, 2008). With our experimental setting we cannot distinguish whether *Pxt* was directly induced by the presence of its substrates ARA and/or EPA or whether the development of *D. magna* was accelerated by the availability of dietary PUFA leading to earlier maturation during which *Pxt* as reproduction-related gene was induced. To clarify this open question, future studies should involve direct measurement of enzyme levels to assess cyclooxygenase activity. The physiological role of COX remains to be elucidated.

Genes coding for proteins upstream and downstream of the central eicosanoid biosynthesis step mediated by COX/PXT showed no or only moderate responses to the dietary PUFA supply. In humans there appears to be an excess of downstream prostaglandin H-metabolizing enzymes relative to COX enzymes (Smith and Murphy, 2002). If this applies also for the prostanoid synthases in *D. magna*, C20 PUFA availability should not induce major changes in the expression of the respective genes, which is in accordance with our findings.

Previous studies have shown that dietary C20 PUFA can influence the performance of *D. magna* under parasite challenge (Schlotz et al., 2013, 2014). Immune responses and eicosanoid regulation presumably occur very rapidly within few hours after parasite or pathogen exposure, as indicated by a rising number of circulating blood cells and early changes in expression of immune genes (Auld et al., 2010; Decaestecker et al., 2011; Heckmann et al., 2008a). In general, we found an increase in transcripts of target genes following parasite challenge. Overall, induction increased from 12 to 24 h after exposure. Looking at the initial step of the eicosanoid biosynthesis pathway we found that the expression of the intracellular phospholipase A_2 gene (*iPlA*$_2$) was induced upon parasite-exposure, but only in animals consuming the C20 PUFA-free diet. Thus, in the absence of dietary C20 PUFA, eicosanoid precursors may have to be mobilized from phospholipids to account for the potentially higher demand for free PUFA during parasite challenge. The expression of *Pxt*, coding for an enzyme with supposed COX-like function, was highly responsive to parasite exposure, which may reflect immune activation and an increased requirement for prostanoid eicosanoids during parasite challenge. Interestingly, animals raised on the C20 PUFA-free diet showed the highest parasite-induced up-regulation of *Pxt* expression. One possible explanation for this finding could be that the expression of *Pxt* in animals reared on the C20 PUFA-containing diets is per se higher than in animals consuming PUFA-deficient diets and thus is less responsive to parasite exposure in the presence of dietary PUFA.

In vertebrates, ARA- and EPA-derived eicosanoids are known to have distinct or even opposing physiological functions (Alcock et al., 2012; Schmitz and Ecker, 2008; Smith, 2005). This suggests that eicosanoid-related gene expression is differentially influenced by the dietary ARA and EPA supply, which is supported by our data. Additive or synergistic effects of ARA and EPA were not evident as the diet which contains both of these PUFAs did not generally induce greater changes in gene expression than one of the precursors alone.

Patterns of parasite-induced gene expression in animals consuming the C20 PUFA-deficient diet, the ARA-supplemented diet, and *N. limnetica*, were qualitatively similar. Significant down-regulation of gene expression was observed only in animals consuming the EPA-supplemented diet 12 h after parasite exposure. In mammals, EPA can effectively decrease the formation of ARA-derived prostaglandins by inhibiting the responsible cyclooxygenase (*Cox-1*; Smith, 2005). Our results suggest that EPA has inhibitory effects on the expression of some eicosanoid biosynthesis enzymes in *D. magna* under pathogen

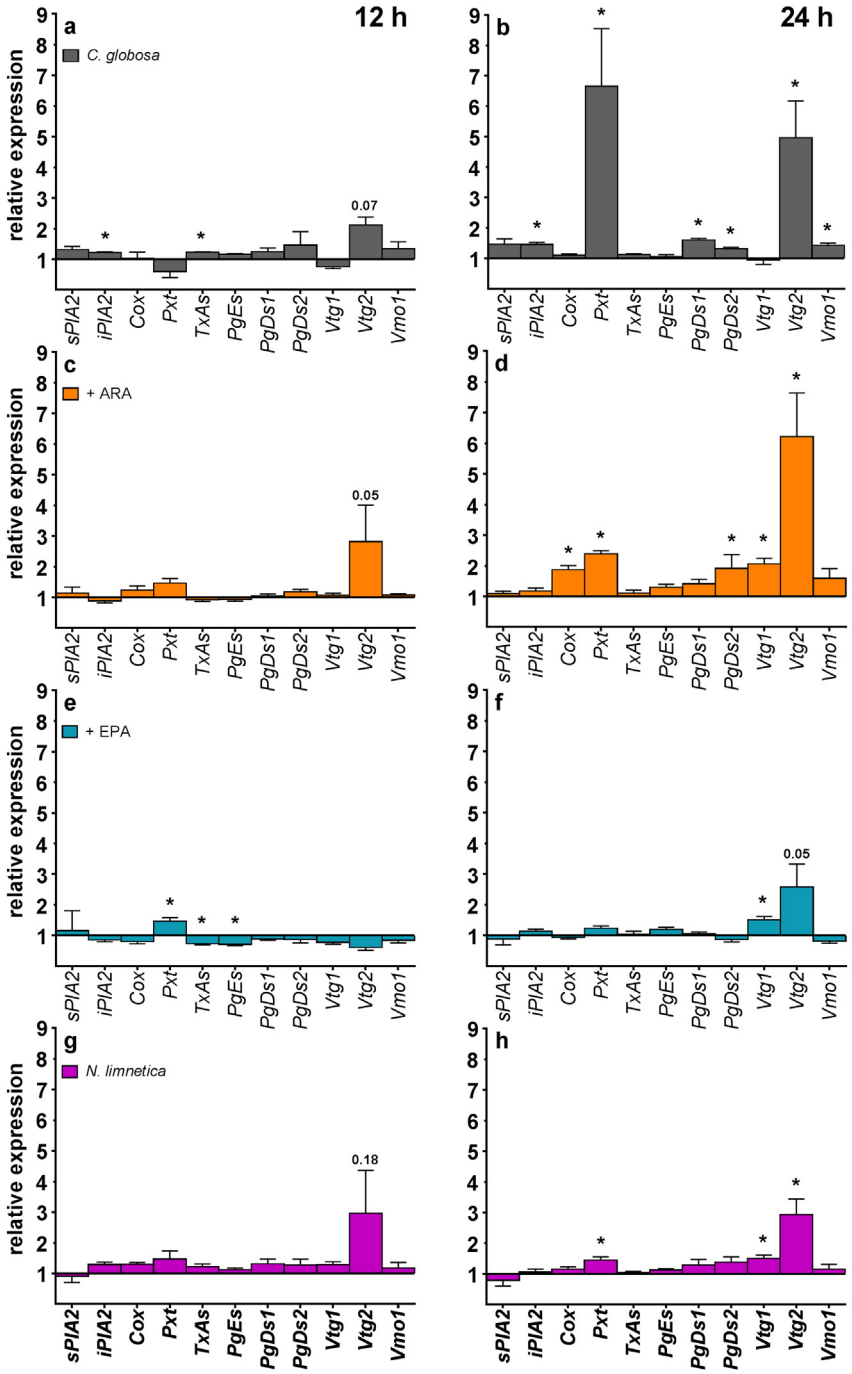

Fig. 3. Parasite-induced changes in relative target gene expression. Expression levels recorded 12 h (left panels) and 24 h (right panels) after parasite exposure. Colors indicate the different food treatments: black (a, b) = *C. globosa*; orange (c, d) = *C. globosa* + ARA; blue (e, f) = *C. globosa* + EPA; magenta (g, h) = *N. limnetica*. For illustration, the expression of each gene in the parasite-exposed animals was normalized to its expression in the mock-infected (control) animals in the respective food treatment. Data are means of 4 replicates ± standard error of the mean (SEM). Asterisks indicate the statistically significant ($p \leq 0.05$) up- or down-regulation of a particular gene in response to parasite exposure.

challenge. However, supplementation of a C20 PUFA-deficient diet with EPA seems not to translate into immune-suppression as no increase in susceptibility to the parasite was observed in a previous experiment (Schlotz et al., 2013).

Vitellogenins are often used as markers for reproductive success and vitellogenin gene expression is known to be strongly correlated with egg production (Kime et al., 1999; Okumura and Aida, 2000; Quinitio et al., 1994). *Daphnia* feeding on ARA- or EPA-containing diets have been shown to reach maturity earlier and to produce more eggs than animals feeding on a diet deficient in these PUFAs (Martin-Creuzburg et al., 2010; Schlotz et al., 2013). However, the three oogenesis-related genes used in our study were less responsive to the dietary PUFA supply than expected. Oogenesis-related gene expression is following a cyclical regime of gene activation and repression, paralleling oogenesis, egg deposition, and offspring release (Hannas et al., 2011; Kim et al., 2011). Thus, a "snapshot" of gene expression at a certain time might give a wrong impression of the actual reproductive capacity. In our data set, oogenesis-related gene expression tended to be equal or lower in animals reared on C20 PUFA-containing diets than in animals reared on *C. globosa*, which is inconsistent with previous findings (Schlotz et al., 2012). We propose that this discrepancy results from an asynchronous progression of the reproduction cycle in animals feeding on the C20

Table 2
PUFA composition of *C. globosa*, *N. limnetica* and PUFA-containing liposomes (+ARA/+EPA). Data are means of three replicates ± SD expressed in μg mg C^{-1} (*n.d.* = not detectable).

	C. globosa	+ARA	+EPA	*N. limnetica*
18:2n-6 (LIN)	5.6 ± 0.3	*n.d.*	*n.d.*	11.1 ± 0.2
18:3n-6 (GLA)	*n.d.*	*n.d.*	*n.d.*	*n.d.*
18:3n-3 (ALA)	152.5 ± 9.9	*n.d.*	*n.d.*	5.1 ± 1.8
18:4n-3 (STA)	8.2 ± 0.1	*n.d.*	*n.d.*	*n.d.*
20:3n-6 (DGLA)	*n.d.*	*n.d.*	*n.d.*	*n.d.*
20:4n-6 (ARA)	*n.d.*	31.3 ± 0.4	*n.d.*	19.8 ± 1.1
20:5n-3 (EPA)	*n.d.*	*n.d.*	31.4 ± 0.4	185.2 ± 10.6

PUFA-containing diet as compared to those feeding on PUFA-deficient *C. globosa*.

The treatment-spanning induction of vitellogenin gene expression upon parasite exposure suggests that vitellogenins, besides their classical function as egg yolk protein precursors, are involved in pathogen recognition, as has been suggested for crayfish, fish, nematodes, mosquitoes, and honeybees (Amdam et al., 2004; Fischer et al., 2013; Hall et al., 1999; Li et al., 2008; Liu et al., 2009; Raikhel et al., 2002). However, this parasite-induced increase in vitellogenin gene expression may also result from an early manifestation of a *P. ramosa*-mediated dysregulation of reproductive parameters with the aim to castrate the host or a parasite-induced terminal reproductive investment of the host prior to castration.

Table 3
Results of the two-way ANOVA used to analyze food and time effects on gene expression. Indicated are sum of squares (SS), degrees of freedom (d. f.) as well as *F*- and *p*-values. Asterisks indicate significance levels (* = $p \le 0.05$, ** = $p \le 0.01$, *** = $p \le 0.001$, **** = $p \le 0.0001$).

		Control				Exposed			
		SS	d. f.	*F*	*p*	SS	d. f.	*F*	*p*
sPlA2	Food	0.989	3	7.0	**	2.571	3	19.6	****
	Time	2.572	1	54.3	****	2.525	1	57.9	****
	Food × time	1.143	3	8.0	***	1.128	3	8.6	***
	residuals	0.995	21			1.004	23		
Cox	Food	2.765	3	42.7	****	3.566	3	168.4	****
	Time	1.096	1	50.8	****	0.957	1	135.5	****
	Food × time	0.050	3	0.8	0.53	0.083	3	3.9	*
	Residuals	0.454	21			0.169	24		
TxAs	Food	0.073	3	4.7	*	0.105	3	13.5	****
	Time	0.391	1	75.5	****	0.321	1	123.9	****
	Food × time	0.074	3	4.7	*	0.070	3	9.0	***
	Residuals	0.109	21			0.062	24		
PgDs1	Food	0.593	3	38.4	****	0.801	3	46.7	****
	Time	0.864	1	167.6	****	0.582	1	101.8	****
	Food × time	0.107	3	6.9	**	0.101	3	5.9	**
	Residuals	0.108	21			0.137	24		
Vtg1	Food	0.240	3	8.6	***	0.220	3	12.6	****
	Time	1.557	1	166.5	****	0.832	1	143.0	****
	Food × time	0.193	3	6.9	**	0.149	3	8.6	***
	Residuals	0.168	18			0.140	24		
Vmo1	Food	0.541	3	18.9	****	1.361	3	38.8	****
	Time	0.737	1	77.3	****	0.622	1	53.2	****
	Food × time	0.071	3	2.5	0.09	0.147	3	4.2	*
	Residuals	0.200	21			0.281	24		
iPlA2	Food	0.089	3	9.0	***	0.114	3	11.8	****
	Time	0.163	1	49.2	****	0.077	1	23.9	****
	Food × time	0.048	3	4.8	*	0.084	3	8.7	***
	Residuals	0.066	20			0.078	24		
Pxt	Food	4.627	3	30.7	****	5.334	3	35.4	****
	Time	2.407	1	47.9	****	0.445	1	8.9	**
	Food × Time	0.287	3	1.9	0.16	1.514	3	10.0	***
	Residuals	1.006	20			1.156	23		
PgEs	Food	0.051	3	3.1	*	0.094	3	13.4	****
	Time	0.247	1	45.0	****	0.083	1	35.6	****
	Food × time	0.145	3	8.8	***	0.114	3	16.3	****
	Residuals	0.115	21			0.056	24		
PgDs2	Food	1.525	3	41.1	****	2.606	3	50.7	****
	Time	1.566	1	126.6	****	1.363	1	79.6	****
	Food × time	0.095	3	2.5	0.08	0.197	3	3.8	*
	Residuals	0.260	21			0.411	24		
Vtg2	Food	1.127	3	5.4	**	2.237	3	15.4	****
	Time	1.362	1	19.7	***	0.177	1	3.7	0.07
	Food × time	0.272	3	1.3	0.30	0.164	3	1.1	0.36
	Residuals	1.312	19			1.015	21		

5. Conclusions

Of the 11 target genes examined here, the *Cox*-like *Pxt* was most responsive to both the dietary ARA and EPA supply (i.e. substrate availability) and parasite exposure (i.e. immune challenge), supporting the idea of prostanoid eicosanoids acting in both reproduction and immunity of *D. magna*. If both reproduction and immunity rely on the same C20 PUFA-responsive enzymes, this may favor a potential trade-off between both traits, especially when dietary PUFA are in short supply. Future research should aim at elucidating the physiological role of *Pxt* in *Daphnia* and other invertebrates and at elaborating the final link from dietary PUFA supply to reproduction and immunity. Investigating the role of vitellogenins during parasite challenge could further our understanding of infection processes in this and other model systems and thus add to our picture of invertebrate defense mechanisms.

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