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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.

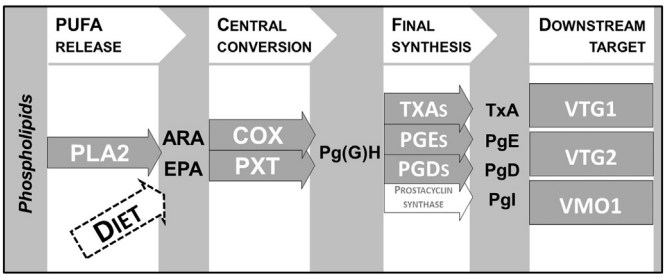

Fig. 1. Schematic overview of the prostanoid pathway based on current evidence from invertebrate models including potential reproduction-related downstream targets. Displayed are key enzymes (arrows) and major metabolites. Gray arrows/boxes indicate genes measured in this study (PLA_2 = phospholipase 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