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Phospholipid-bound eicosapentaenoic acid (EPA) supports higher fecundity than free EPA in *Daphnia magna*

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Nutrition bioassays in which polyunsaturated fatty acids (PUFA)-deficient diets were supplemented with free long-chain PUFA (≥C20) consistently revealed positive effects on somatic growth and fecundity of *Daphnia*. However, free PUFA are hardly available in natural diets. In general, PUFA are bound to other lipids, especially to phospholipids and triglycerides. Here, we evaluate the potential of free and phospholipid-bound dietary eicosapentaenoic acid (EPA) to support somatic growth and fecundity of *Daphnia magna*. In a growth experiment, supplementation of a C20 PUFA-deficient diet with free or phospholipid-bound EPA improved somatic growth rates of *D. magna* equally. However, the increase in fecundity was significantly more pronounced when phospholipid-bound EPA was provided. Free and phospholipid-bound EPA were provided in the same concentrations in our experiment, suggesting that the allocation to reproduction-related processes is affected differently by phospholipid-bound PUFA and free PUFA. Our finding stresses the need to consider the distribution of dietary PUFA in different lipid classes to gain a better understanding of how PUFA influence life history traits of *Daphnids* in the field.

KEYWORDS: *Daphnia magna*; food quality; phospholipids; polyunsaturated fatty acids; reproduction; somatic growth; trophic interactions

INTRODUCTION

Animals are mostly incapable of synthesizing long-chain (≥C18) polyunsaturated fatty acids (PUFA) *de novo* (Arts *et al.*, 2001) and thus rely on an adequate dietary supply with PUFA to cover their physiological demands. PUFA are indispensable structural components of cell membranes and are involved in the regulation of various membrane properties, such as fluidity and permeability

(Valentine and Valentine, 2004). In addition, some PUFA serve as precursors of eicosanoids, bioactive molecules known to be important mediators in many physiological processes, mostly related to reproduction and immunity (Stanley-Samuelson, 2006). It has long been recognized that a dietary supply with PUFA can crucially influence the performance of aquatic consumers (Ahlgren *et al.*, 1990; Müller-Navarra *et al.*, 2000; Von Elert, 2002). Especially, the dietary supply with eicosapentaenoic acid (EPA), a representative of the n-3 family of PUFA, has been shown to enhance somatic growth and reproduction of *Daphnia* (Von Elert, 2002; Ravet *et al.*, 2003; Martin-Creuzburg *et al.*, 2010). Laboratory experiments with PUFA-manipulated diets have greatly improved our understanding of dietary PUFA requirements of *Daphnia*. In all these experiments, however, PUFA-deficient diets were supplemented with free PUFA, neglecting the fact that PUFA are hardly available in their free form in natural diets. In algal cells, PUFA are generally bound to other lipids, such as phospholipids, triglycerides and glycolipids (Dunstan *et al.*, 1993; Gushina and Harwood, 2006; Wacker *et al.*, 2016). Seston analyses suggest that EPA is more abundant in the phospholipid fraction (Bourdier, 1985). The way dietary PUFA are provided, i.e. experimentally as free PUFA or naturally bound to other lipids, may influence the biological availability and thus the potential of dietary PUFA to improve growth and reproduction of consumers. Studies on vertebrate nutrition suggest that phospholipid-bound n-3 PUFA are more efficiently absorbed than PUFA bound to other lipid classes (Gisbert *et al.*, 2005; Burri *et al.*, 2012; Kullenberg *et al.*, 2012).

To compare the effects of free and phospholipid-bound EPA (PL-EPA) on somatic growth and fecundity of *Daphnia magna*, a growth experiment was conducted in which a C20 PUFA-deficient diet was supplemented with liposomes enriched with free EPA and liposomes prepared with EPA-containing phospholipids.

METHOD

Origin and maintenance of *Daphnia*

We used a clone of *D. magna* originally collected from a floodplain pond of the River Allier, France. Several generations of females were maintained in glass containers (1 L) in Volvic® mineral water/ADaM medium (Klüttgen *et al.*, 1994) (2:1, v/v) on a 16:8 h light:dark cycle at 20°C. They were fed once a day with *Chlamydomonas reinhardtii* (SAG 7781) (carbon concentration: 2 mg C L^{-1}).

Phytoplankton culture

Chlamydomonas reinhardtii was grown in modified autoclaved WC medium (Guillard, 1975) at 20°C and at a permanent artificial light flux of 120 µmol quanta m^{-2} s^{-1}. It was cultured semi-continuously at a dilution rate of 0.2 d^{-1} in aerated (compressed sterile-filtrated air) 5 L vessels. Food suspensions of *C. reinhardtii* were prepared by centrifugation and resuspension of the cultured cells in Volvic® mineral water. The carbon concentrations of the *C. reinhardtii* food suspensions were estimated from photometric light extinction (800 nm) and from carbon-extinction regressions determined prior to the experiment. Particulate organic carbon concentrations were determined using an elemental analyzer (TOC-VCPN Shimadzu). *Chlamydomonas reinhardtii* was used as food because it does not contain long-chain (i.e. >C18) PUFA (data not shown).

Daphnia growth experiment

The growth experiment was conducted at 20°C and a 16:8 h light:dark cycle. Three generations of females (20 individuals per liter) were maintained in glass containers (1 L) in Volvic® mineral water/ADaM medium (2:1, v/v). They were fed once a day with *C. reinhardtii* at a concentration of 2 mg C L^{-1} which is well above the incipient limiting level. Daphnids were individually transferred into new medium every day. When the females produced offspring, the neonates (first generation) were separated and the mothers removed. This step was repeated to keep the third generation. After they had released their first clutch, females from the third generation were kept and the neonates removed. The third-brood offspring from the third generation were finally used for the experiments in order to limit inter-individual variability (Lampert, 1993). Third-clutch neonates (born within 8 h) were randomly assigned to glass beakers containing 200 mL of Volvic® mineral water/ADaM medium (2:1, v/v) and 2 mg C L^{-1} of *C. reinhardtii*. The experiment consisted of four different food treatments: *C. reinhardtii* (without supplementation), *C. reinhardtii* + 80 µL of a liposome suspension prepared in the absence of EPA (C+liposomes), *C. reinhardtii* + 80 µL of a liposome suspension prepared with free EPA (C+ free EPA), and *C. reinhardtii* + 80 µL of a liposome suspension prepared with phospholipid-bound EPA (C + PL-EPA). Each treatment consisted of three replicates with eight animals each. To measure the average initial dry weight (W_0), randomly selected neonates were previously transferred into pre-weighed aluminum containers (3 samples of 30 neonates), dried overnight at 60°C, and weighed on an electronic balance (Sartorius ME 36 S ± 1 µg). During the experiment, individuals were transferred

daily to new glass beakers containing freshly prepared medium and food suspensions. The experiment was stopped when the females reached maturity (Day 6). Individuals were collected and observed under a stereo microscope to determine the number of eggs in the brood chamber (fecundity). Individuals were then dried overnight at 60°C and weighed to obtain the average individual weight per replicate (W_t). Somatic growth rates (g) were calculated as: $g = (\ln W_t - \ln W_0)/t$.

Preparation of liposome suspensions

Liposome suspensions were prepared basically according to Martin-Creuzburg *et al.* (Martin-Creuzburg *et al.*, 2009). Stock suspensions were prepared from 3 mg 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) and 7 mg 1-palmitoyl-2-oleoyl-phosphatidylcholin (POPC; Sigma-Aldrich) dissolved in an aliquot of ethanol. Liposomes containing free EPA were prepared by adding 3.33 mg EPA (Sigma-Aldrich) from stock solutions in ethanol. Liposomes containing PL-EPA were prepared by adding 1, 2-di-eicosapentaenoyl-sn-glycerophosphocholine (Sigma-Aldrich) from stock solutions in ethanol instead of free EPA. The resulting suspensions were dried using a rotary evaporator and dissolved in 10 mL buffer (20 mmol L^{-1} NaP_i, 150 mmol L^{-1} NaCl, pH 7.0). Subsequently, the liposome suspensions were sonicated in an ultrasonic bath. Excess free EPA was removed by washing the liposomes in fresh buffer using an ultraspeed centrifuge (150 000 g, 90 min, 4°C). To ensure that the liposome suspensions prepared with free EPA and PL-EPA provided similar amounts of EPA to *Daphnia*, several liposome suspensions were prepared prior to the experiment by slightly varying the amounts of free or PL-EPA added during the preparation. Subsequently, aliquots of the resulting suspensions were subjected to fatty acid analysis and the ones with the most similar EPA concentrations were used for the experiment (Table I). Prior to the addition of liposomes to the experimental beakers, the liposome stock suspensions were sonicated again (2 min).

Fatty acid analyses

Fatty acid concentrations of the different liposome suspensions were analyzed 2 days before the experiments, on Days 3 and 6. Fatty acids were extracted twice using chloroform/methanol, following the method of Folch *et al.* (Folch *et al.*, 1957). Fatty acids were then converted into fatty acid methyl esters (FAME), after the addition of non-methylated 13:0 (internal standard), by acid-catalyzed transesterification (4% H_2SO_4 in methanol at 75°C for 2 h). FAME were analyzed on a gas chromatograph (Agilent technologies™ 6850) equipped with a DB-Wax column (J&W Scientific) and a flame ionization detector. The GC was operated under the following configuration: detector 250°C; split injection; carrier gas: helium; oven temperature ramp 150–240°C at 3°C min^{-1}.

Table I: Fatty acid contents (μg per mg C) of the different liposomes solutions

	16:0	18:1(n-9)	20:5(n-3)
liposomes	26.6 ± 2.4	34.4 ± 2.9	
Liposomes + Free EPA	24.1 ± 1.3	29.1 ± 0.9	18.5 ± 0.6
Liposomes + PL-EPA	22.1 ± 1.1	27.4 ± 0.7	18.6 ± 0.4

Data analyses

Effects of food sources on somatic growth rates and egg production of *D. magna* were analyzed using one-way ANOVA ($\alpha < 0.05$). Pairwise comparisons were performed using *post hoc* tests (Tukey's HSD).

RESULTS AND DISCUSSION

The aim of our study was to compare the potential of free and PL-EPA to support somatic growth and fecundity of *D. magna*. We conducted a supplementation experiment using liposomes to provide *D. magna* with either free and PL-EPA. Liposomes have been successfully used already in numerous experiments to provide *Daphnia* with well-defined dietary PUFA concentrations (Ravet *et al.*, 2003; Martin-Creuzburg *et al.*, 2009; Sperfeld and Wacker, 2011). Liposomes prepared in the presence of dissolved free PUFA encompass the provided PUFA loosely, i.e. the PUFA are not covalently bound to the phospholipids used to prepare the liposomes. To provide *D. magna* with PL-EPA we used EPA-containing phospholipids, i.e. phospholipids with EPA esterified to the glycerol backbone, to prepare the liposomes in the absence of free EPA. The liposome suspensions we used did not differ in their EPA concentrations or in the concentrations of the two other fatty acids present in the phospholipids, i.e. palmitic acid (16:0) and oleic acid (18:1(n-9)) (Table I).

Our data confirms that juvenile somatic growth rates and fecundity of *D. magna* on a C20 PUFA-deficient diet can be improved by supplementing both free and PL-EPA (Fig. 1). While the growth-promoting effects observed with free and PL-EPA did not differ (Tukey's HSD, $P = 0.99$, following ANOVA, $F_{3,8} = 12.06$, $P < 0.01$; Fig. 1), fecundity was significantly more enhanced upon supplementation with PL-EPA than upon supplementation with free EPA (Tukey's HSD, $P < 0.001$,

following ANOVA, $F_{3,8} = 114.1$, $P < 0.0001$; Fig. 2). Supplementation with PUFA-free liposomes did not influence somatic growth or fecundity of *D. magna*, suggesting that the liposome-forming phospholipids *per se* did not affect the performance of *D. magna*.

We show here that dietary PL-EPA supports a higher fecundity than free EPA in *Daphnia*. Enhancing effects of dietary EPA especially on zooplankton fecundity have been reported previously (Bec *et al.*, 2006; Wacker and Martin-Creuzburg, 2007; Martin-Creuzburg *et al.*, 2010). The emerging question here is why the supplementation with PL-EPA supported higher fecundity than supplementation with free EPA. Free oxidized PUFA are known to be toxic to *Daphnia* when provided in high concentrations (EPA: 9 $\mu g\,mL^{-1}$) (Reinikainen *et al.*, 2001; Desbois and Smith, 2010). In our study, however, free EPA concentrations did not exceed 0.037 $\mu g\,mL^{-1}$. Moreover, once incorporated into liposomes, free PUFA are presumably less affected by oxidation as proposed by Ravet and Brett (Ravet and Brett 2006). Finally, supplementation with free EPA incorporated into liposomes has been repeatedly shown to improve somatic growth and fecundity of *Daphnia*. Thus, the observed differences in the strength of free versus PL-EPA supplementation on *Daphnia* fecundity are most likely attributable to a positive effect of PL-EPA rather than a negative effect of free EPA.

Although free fatty acids are readily absorbed by the enterocytes (Iqbal and Hussain, 2009; Niot *et al.*, 2009), a higher absorption and/or utilization efficiency of PL-EPA for reproduction could potentially explain our observation. Lipid digestion and absorption in

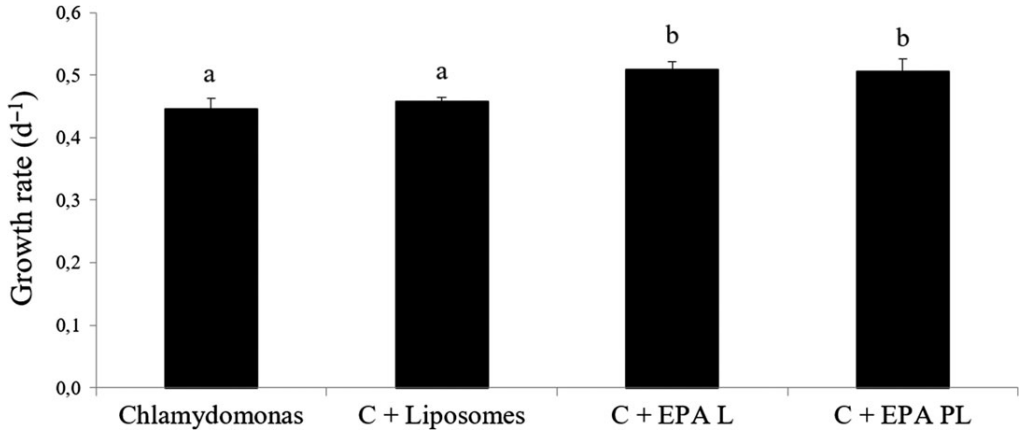

Fig. 1. Somatic growth rates (d^{-1}) of *D. magna* on *C. reinhardtii* supplemented with PUFA-free liposomes (+ Liposomes), liposomes containing free EPA (+ free EPA) and liposomes composed of EPA-containing phospholipids (+ PL-EPA). Data show means ± standard deviation ($n = 3$); bars labeled with the same letters are not significantly different (Tukey's HSD, $P < 0.05$).

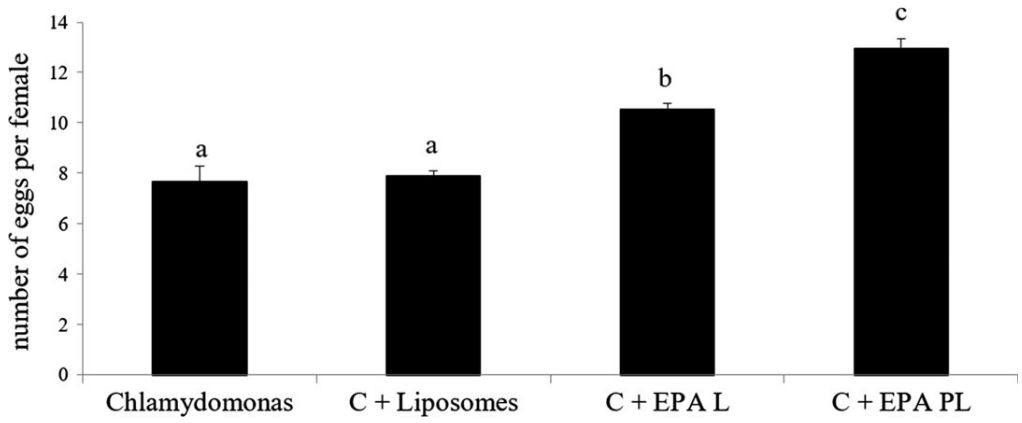

Fig. 2. Fecundity (number of eggs in first clutch) of *D. magna* on *C. reinhardtii* supplemented with PUFA-free liposomes (+ Liposomes), liposomes containing free EPA (+ free EPA) and liposomes composed of EPA-containing phospholipids (+ PL-EPA). Data show means ± standard deviation ($n = 3$); bars labeled with the same letters are not significantly different (Tukey's HSD, $P < 0.05$).

invertebrates are poorly understood and remains virtually unstudied in *Daphnia* (Hassler, 1935; Koussoroplis *et al.*, 2017). In the intestine of vertebrates, however, most of PL are hydrolyzed and taken up as free fatty acids and lysophospholipids. The hydrolysis products are then re-esterified to PL and incorporated into chylomicrons and lipoproteins before they enter the blood stream (Kullenberg *et al.*, 2012). Yet, a significant fraction of the intestinal PL may be absorbed passively without hydrolysation (Zierenberg and Grundy, 1982). Additionally, the emulsifying properties of PL are important for intestinal lipid absorption because they enable the formation of vesicles and micelles thus facilitating the uptake of lipids by enterocytes (Cohn *et al.*, 2010). To the best of our knowledge, no other study has compared the utilization efficiencies of free and PL-fatty acids in vertebrate or invertebrate taxa. Yet, there is growing evidence that, at least in vertebrates, PL-PUFA are more efficiently taken up in tissues (Brossard *et al.*, 1997; Lemaitre-Delaunay *et al.*, 1999; Graf *et al.*, 2010) and utilized than TAG-PUFA (Gisbert *et al.*, 2005; Rossmeisl *et al.*, 2012). Whether this is the case in *Daphnia*, remains to be determined.

There is emerging evidence from animal and human studies indicating that, following digestion and absorption, PL- and TG-PUFA are differently allocated to specific tissues (Burri *et al.*, 2012; Kullenberg *et al.*, 2012) or metabolic pathways. Ghasemifard *et al.* (Ghasemifard *et al.*, 2015) showed that the greater deposition of PL-PUFA in rat tissues was not due to higher absorption efficiencies for PL-PUFA than for TG-PUFA but rather due to differences in metabolic fate of PL- and TG-PUFA; they observed that TG-PUFA are more likely subjected to β-oxidization than PL-PUFA. In analogy, one could speculate that PL-PUFA might be more likely than free EPA to be deposited in the gonads or to be routed towards reproduction-related metabolic pathways such as eicosanoid metabolism (Medeiros *et al.*, 2004; Stanley-Samuelson, 2006; Schlotz *et al.*, 2012, 2016). An alteration of eicosanoid metabolism could either affect reproductive allocation strategy (e.g. more but smaller eggs) or the partition between somatic growth and reproduction (lower somatic mass but more eggs at reproduction). Such changes would be in agreement with the fact that mass-specific growth rates (soma and eggs) in our experiment did not differ between treatments of free and phospholipid-bound EPA supplementation. Unfortunately, the standard experimental design for this type of experiment, that is, pooling several individuals (with eggs) to obtain an average of individual weight does not allow us to explicitly test for these hypotheses. Provided sufficient instrumental precision, future studies should weigh daphnids individually. Finally, in the event of such allocation strategy changes, it would also be important to determine whether they positively or negatively affect population fitness.

CONCLUSION

We show here that the beneficial effects of EPA on *Daphnia* fecundity are more pronounced when this compound is covalently bound to PL. This finding may have far-reaching consequences for PUFA-related food quality research as it implies that, considering the distribution of PUFA in the different lipid fractions, is important for assessing potential PUFA limitation in the field. Future studies also need to consider that the lipid class composition of phytoplankton may be affected by the prevailing environmental conditions, such as light, temperature or stoichiometric constraints (Van Mooy *et al.*, 2009). Our results also suggest that the available thresholds for PUFA limitation obtained by supplementing free PUFA need to be verified in supplementation experiments with free and lipid-bound PUFA. Further studies are required to improve our understanding of the physiological mechanisms underlying the effects described here.

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