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A comparative analysis of the fatty acid composition of sexual and asexual eggs of *Daphnia magna* and its plasticity as a function of food quality

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In cyclical parthenogenetic *Daphnia*, asexual eggs develop immediately and enable fast population growth, while sexual eggs are dormant and can survive harmful conditions. We studied whether this different function is reflected in different fatty acid profiles and explored the capacity of *D. magna* to adjust fatty acid provisioning of its eggs depending on food resources. We quantified neutral and phospholipid content of sexual and asexual eggs produced under differ ent food conditions and compared these with eggs collected from a natural pond. In eggs obtained under different la boratory food regimes, total concentration of neutral fatty acids per unit biomass was not affected by food source or egg type. Both egg types contained lower amounts of fatty acids in the neutral fraction when produced in nature than under laboratory conditions. Fatty acid concentration in the phospholipid fraction was lower in sexual than asexual eggs. Fatty acid composition of eggs largely reflected that of the food of the mothers, albeit with small modifications. Sexual eggs produced on a diet of *Scenedesmus obliquus* (no C20 PUFA), contained higher concentrations of eicosapenta enoic acid and arachidonic acid in both fractions than asexual eggs.

KEYWORDS: PUFA; neutral lipids; phospholipids; cyclic parthenogenesis; dormant eggs

INTRODUCTION

Like many aquatic organisms inhabiting inland waters, the cladoceran *Daphnia* produces eggs that develop immediately and eggs that first go through a dormant stage, the latter as a strategy to cope with temporarily harsh periods (Brendonck and De Meester, 2003). Most *Daphnia* species are cyclical parthenogens, with the dormant eggs being produced sexually while the subitaneous eggs are produced parthenogenetically. Asexual eggs are typically produced during favorable conditions and allow for rapid population growth. By the end of the growing season, when the animals are exposed to deteriorating environmental conditions, *Daphnia* start producing sexual eggs. Sexual eggs are encased in a protective envelope, the ephippium, and are deposited in the sediment where they form a dormant egg bank. These eggs can stay viable for decades and are resistant to desiccation, extreme temperatures and digestion by animals (Decaestecker *et al.*, 2009; Frisch *et al.*, 2014).

Sexual and asexual eggs have very different functions during the life cycle (Brendonck and De Meester, 2003). Dormant eggs are used to disperse in space and time, consequently the hatchlings from sexual eggs experience different growth conditions compared with hatchlings from asexual eggs, which are more likely to encounter similar conditions as the parental generation. The resulting offspring differ in life history traits (Cáceres, 1998; Arbaciauskas and Lampert, 2003) and it is conceivable that this is reflected in differences in the biochemistry of the eggs.

Dormant eggs require special adaptations to survive drought and freezing during diapause, and these adaptations may include different resource allocation strategies. In agreement with the requirements for dormancy, Pauwels *et al.* (Pauwels *et al.*, 2007) reported that sexual eggs of *Daphnia magna* contain more glycerol and heat shock proteins than asexual eggs. Both glycerol and heat shock proteins play a key role in the protection of cell metabolism during stress conditions and have also been found in anhydrobiotic cysts of other diapausing organisms, such as *Artemia fransicana* (Clegg, 1997) and certain insects (Denlinger, 2002). Early studies have reported morphological differences between parthenogenetic and dormant eggs. For instance, oocytes of sexual eggs do not contain free lipid droplets and are enclosed in three membranes, while the oocytes of asexual eggs do contain lipid bodies and only have two membranes (Zaffagnini, 1987). Based on these histological observations, sexual oocytes are expected to contain higher amounts of triglycerides, the main energy storage molecules in *Daphnia* (Peters, 1987). However, Pauwels *et al.* (Pauwels *et al.*, 2007) did not find significant differences in triglyceride concentrations between sexual and asexual eggs of *D. magna*. In another study, dormant eggs of *Daphnia pulicaria* contained much more fatty acids, especially polyunsaturated fatty acids (PUFA), than parthenogenetic eggs (Abrusan *et al.*, 2007).

The biochemical composition of parthenogenetic eggs of *Daphnia* has been shown to be highly plastic. Predation pressure, as well as both food quantity and quality in the maternal generation, have a strong influence on the biochemical composition of the eggs (Gliwicz and Guisande, 1992; Stibor, 2002; Wacker and Martin Creuzburg, 2007; Schlotz *et al.*, 2013). When exposed to low food quantity, *Daphnia* females have been reported to produce smaller clutches with larger eggs and a higher content of protein, lipid and carbon (Gliwicz and Guisande, 1992). In contrast, Tessier *et al.* (Tessier *et al.*, 1983) observed that females reproducing under low food conditions allocate fewer maternal lipids to each egg than females of the same genotype (i.e. clone) under high food conditions, and that neonates hatching from eggs with a higher triglyceride content survive longer under starvation. Besides food quantity, resource allocation into the eggs can be affected by food quality. Sterols (Martin Creuzburg *et al.*, 2005; Von Elert *et al.*, 2003) and PUFA (Brett, 1993; Muller Navarra *et al.*, 2000; Von Elert, 2002; Persson and Vrede, 2006) are both important determinants of biochemical food quality for *Daphnia*. When exposed to poor food quality conditions, total fatty acid concentrations are reduced in both somatic tissue and parthenogenetic eggs, while the concentration of cholesterol is constant in eggs but lowered in somatic tissue (Wacker and Martin Creuzburg, 2007). Not only the concentration but also the fatty acid composition of the eggs is strongly determined by the maternal dietary supply (Schlotz *et al.*, 2013). It is generally accepted that arthropods are unable or at least have limited capabilities to synthesize PUFA *de novo* from low molecular weight precursors (Leonard *et al.*, 2004). Five PUFA have been frequently discussed as being essential, of which three are *n* 3 fatty acids [i.e. α linolenic acid (ALA, 18 : 3*n* 3), eicosapentaenoic acid (EPA, 20:5*n* 3) and docosahexaenoic acid (DHA, 22:6*n* 3)] and two are *n* 6 fatty acids [i.e. linoleic (LIN, 18:2*n* 6) and arachidonic acid (ARA, 20:4*n* 6)] (Von Elert, 2002; Kainz *et al.*, 2004; Martin Creuzburg *et al.*, 2010). PUFA play important roles in cell growth and proliferation and are precursors for other important molecules. For instance, EPA and ARA are precursors for prostaglandins and other eicosanoids, which are important mediators in reproduction, the immune system and ion transport physiology (Stanley, 2006; Heckmann *et al.*, 2008). Along with sterols and proteins, PUFA containing phospholipids define the physical characteristics of cell membranes (Stillwell and Wassall, 2003; Valentine and Valentine, 2004). Both the degree of saturation and the length of the fatty acid chains within phospholipids influence membrane flexibility and permeability (Pruitt, 1990).

In this study, we explored whether the cladoceran *D. magna* is capable of adjusting fatty acid allocation to ward both asexual and sexual eggs as a function of the fatty acid profile of the food of the mother. Previous studies on the allocation of fatty acids to eggs in *Daphnia* as a func tion of food quality focused on parthenogenetic eggs only, while the one study that compared fatty acid content of sexual and asexual eggs focused on one diet only (Abrusan *et al.*, 2007). Here we compared the plasticity of fatty acid concentration and composition in asexual and sexual eggs of *D. magna*, whose mothers were reared on two algae differing strongly in their fatty acid profile and in asexual and sexual eggs produced by mothers in a natural pond. Because of the distinct functions between lipid classes, we separated the total lipid fraction into neutral lipids and phospholipids prior to fatty acid analyses, to distinguish between fatty acids that are primarily used as energy resources and fatty acids that are primarily used as cell membrane components, respectively. We expected that sexual eggs contain more neutral lipids than asexual eggs, as they are able to survive for decades. Regarding phos pholipids, we expected to find a higher plasticity in fatty acid profiles in asexual eggs than in sexual eggs as the latter may require a certain fatty acid composition to be able to withstand harsh environmental conditions.

METHOD

Cultivation and preparation of the food

In our laboratory experiments, we used the green alga *Scenedesmus obliquus* (SAG 276 3a) and the eustigmatophyte *Nannochloropsis limnetica* (SAG 18.99) to rear *D. magna*. "These two algae are characterised by highly distinct fatty acid profiles, i.e. S. *obliquus* lacks PUFA with more than 18 carbon atoms, while *N. limnetica* contains high concentra tions of C20 PUFA, especially eicosapentaenoic acid" (Von Elert, 2002; Martin Creuzburg *et al.*, 2009).

Algae were grown in batch cultures at 18°C in aerated 10 L vessels with illumination at 170 µmol quanta $m^{-2} s^{-1}$ and harvested in the late exponential growth phase. *Scenedesmus obliquus* was grown in a medium con sisting of 10 mL L^{-1} of enriched seawater nutrients (Provasoli, 1968), 5 mL L^{-1} of Walne nutrients (Walne, 1956) and the vitamins B1, B12 and H dissolved in dechlorinated tap water. *Nannochloropsis limnetica* was grown in modified Woods Hole (WC) medium with the vitamins B1, B12 and H (Guillard, 1975). Food suspen sions were prepared by concentrating the cells via centri fugation (2500 g, 5 min) followed by resuspension in tap water. Cell densities of the food suspensions were counted with an Attune® acoustic focusing cytometer (Life Technologies, Carlsbad, CA, USA).

Egg collection

To compare the biochemical composition of sexual and asexual eggs of *D. magna* produced under different condi tions, we collected sexual and asexual eggs produced by animals in a natural pond and sexual and asexual eggs derived from females fed either *S. obliquus* or *N. limnetica* in the laboratory. The laboratory animals were them selves derived from the same pond as from which the other eggs were collected.

Eggs collected from a natural population

Dormant eggs of *D. magna* were collected from "Langero devijver," a pond in Neerijse, Belgium (0°49′42.32″N, 4° 38′21.49″O). The upper 5 10 cm of the sediment, corresponding to the active egg bank (Càceres, 1998), was taken during the winter of 2011 2012, when the eggs are in diapause and before the spring hatching peak occurred. Ephippia were collected by sieving the sedi ment over first 1 mm and then 250 µm mesh sized sieves. To prevent hatching of the eggs during this process, eggs were kept in the dark and placed on ice. When the sieving was finished, all collected ephippia were stored in the dark at 4°C, awaiting further analyses. Ephippia were manually isolated from the sediment fraction and decap sulated to collect four replicate samples consisting of 100 sexual eggs for fatty acid analysis. These manipulations were performed in a room under red light (700 nm), to prevent unwanted light exposure of the dormant eggs. Samples were stored at 80°C to stop all biochemical processes in the cells.

Asexual eggs from the pond "Langerodevijver" were collected directly from the brood pouch of animals sam pled during the growing season following our winter sam pling for dormant eggs. Four samples consisting of 100 parthenogenetic eggs in the first stage of daphnid embry onic development (according to Kast Hutcheson *et al.*, 2001) were collected by dissection of the females brood chamber and stored at 80°C before freeze drying.

Laboratory cultured eggs

We conducted an experiment in the laboratory to gener ate sexual and asexual eggs from females cultured under different food conditions. Therefore, from the sediment bank of "Langerodevijver," 10 clones were hatched by ex posing the dormant eggs to hatching stimuli, i.e. a rela tively high temperature (20°C), a long day photoperiod (16L:8D) and fresh medium (tap water dechlorinated and aged for 24 h) (De Meester and De Jager, 1993). Hatched animals were reared in 0.5 L jars (density: 20 individuals per liter) filled with aged tap water (aerated for 24 h prior to use) under standard conditions (20 ± 2°C and a photoperiod of 16L:8D) for several generations. All

cultures were fed 150 000 cells mL ^{1} of *S. obliquus*, which corresponds to ~2.5 mg C L ^{1}. After this precondition ing phase, the second clutch of a new generation was sub jected to the different experimental conditions.

To obtain asexual eggs, per treatment four replicate 1 L jars per clone (10 clones), with 15 individuals in each jar, were cultured under standard conditions (20 ± 2°C and a photoperiod of 16L:8D). In the first treatment, all cultures were fed with *S. obliquus*, whereas in the second treatment all cultures were fed with *N. limnetica* (both at an algal cell density of 150 000 cells mL ^{1}). In both treatments, jars were cleaned every 2 days and food was renewed daily to keep algal concentration above the incipient limiting level. Females bearing their third clutch were dissected to collect asexual eggs in the first stage of daphnid embryonic devel opment (according to Kast Hutcheson *et al.*, 2001). From each replicate, one sample of 100 eggs (10 eggs per clone) was collected and stored at 80°C. In total, eight samples (four replicates per treatment) were put in storage.

Lastly, we induced sexual reproduction using the same clones and under the same food conditions as described above, namely the algae *S. obliquus* and *N. limnetica*. To obtain starting material, we reared 20 individuals of the same 10 clones as used for the production of the asexual eggs under standard conditions, i.e. 20 ± 2°C, a photo period of 16L:8D and an algal cell density of 150 000 cells mL ^{1}. After they had released their second clutch, four animals of every clone were mixed in a 1 L jar (replicated four times) and feeding level was raised to 250 000 cells mL ^{1}. The photoperiod was switched from long day (16L:8D) during 5 days to short day (8L:16D) photoperiod during 2 days, as this stimulates sexual re production in *Daphnia* (De Meester and De Jager, 1993). Once a week half the medium was renewed and all dormant eggs were collected. Dormant eggs were stored in Eppendorf tubes in the dark at 4°C for at least 1 month. After storage, these laboratory derived ephippia were decapsulated and four replicate samples consisting of 100 sexual eggs each were collected in each treatment. The eight samples were stored at 80°C.

In total, 24 samples (two types of eggs: sexual vs. asexual eggs, three treatments: eggs collected from a natural popu lation and *S. obliquus*- and *N. limnetica*-fed laboratory ani mals; four replicates per treatment) were freeze dried, weighed (dry mass) and transferred to Eppendorf tubes until further analysis. To reduce lipid peroxidation, egg samples were overlaid with gaseous nitrogen.

Chemical analysis

Analysis of food quality

To analyze the fatty acid composition of the laboratory reared algal food source, three replicate samples of 9.5 mg carbon (10 mL) for *S. obliquus* and 2.9 mg carbon (15 mL) for *N. limnetica* were filtered on precombusted (5 h, 550°C) GF/F filters (Whatman, 25 mm). For deter mination of the food composition of the animals in the pond, three random seston samples of 200 mL, correspond ing to 0.66 mg carbon, were taken from "Langerodevijver" and filtered on precombusted GF/F filters.

The fatty acid composition of the food sources was analyzed as described in Martin Creuzburg *et al.* (Martin Creuzburg *et al.*, 2010). Briefly, loaded filters were deposited in 7 mL of a mixture of dichloromethane and methanol (2:1) and stored at 20°C. Total lipids were extracted three times (sonication for 30 min) with dichloromethane:methanol (2:1). Pooled cell free extracts were evaporated to dryness using nitrogen. The lipid extracts were transesterified with 4 mL of 3 N methanolic HCl (60°C, 15 min) and 100 μL of internal standard (20 μg mL ^{1} 17:0 ME and 25 μg mL ^{1} 23:0 ME) was added. After cooling down to room temperature, the fatty acid methyl esters (FAMEs) were further extracted and analyzed as described for the egg samples. The absolute amount of each FAME was normalized to the carbon content of the food suspensions.

As expected, the fatty acid profiles of the food sources differed considerably (Fig. 1 and Table I). *Nannochloropsis limnetica* was rich in 20:5*n* 3 (42.7%) and ARA (4.4%). In contrast, no PUFA with more than 18 carbon atoms were detected in *S. obliquus*. The major fatty acid identified in *S. obliquus* was 18:3*n* 3 (59.5%). The lake seston con tained significantly smaller quantities of all fatty acids, but low concentrations of LIN, ALA, ARA and EPA were detected. DHA was not present in any of the samples.

Analysis of egg samples

Egg samples were analyzed using a combination of the methods described in Zhu *et al.* (Zhu *et al.*, 2006) and Martin Creuzburg *et al.* (Martin Creuzburg *et al.*, 2010). In a first step, lipid classes were separated. Total lipids were divided into several classes based on the charge of the head group. Sterols and glycerides have a less polar head group, while glycolipids, phospholipids and sphin golipids contain a more polar head group. In a second step, the composition of the liberated fatty acid chains was analyzed.

Freeze dried egg samples were homogenized in 1 mL of isopropanol. The mixture was sonicated for 30 min at 4°C. After centrifugation at 10 000 g for 10 min, the super natant was collected in a glass tube and the residue was extracted again with 1 mL of CHCl3:MeOH (2:1) during sonification for another 30 min at 4°C. After centrifugation at 10 000 g for 10 min, both supernatants were combined, and 2 mL of $CHCl_3$ and 1 mL of a 0.88% KCl solution were added. The mixture was thoroughly shaken and

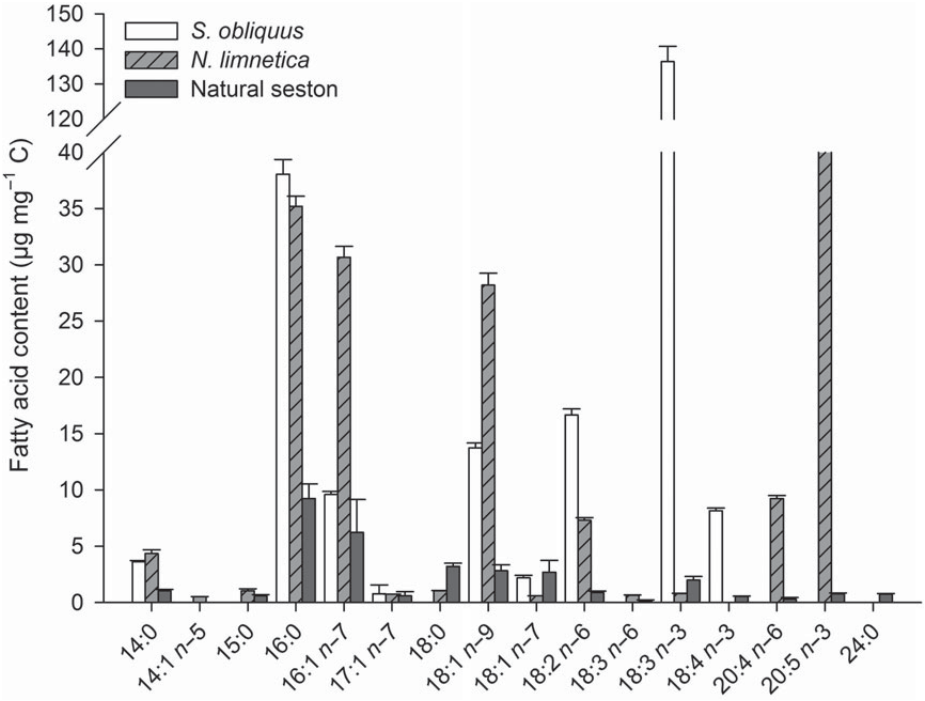

Fig. 1. Fatty acid composition of *Scenedesmus obliquus* (white bars), *Nannochloropsis limnetica* (hatched bars) and seston of lake "Langerode vijver" (dark gray bars), expressed in µg mg ^{1} carbon. Given are means of three replicates; error bars indicate one standard error.

Table I: Abundances of key fatty acids in Scenedesmus obliquus, Nannochloropsis limnetica and seston of lake "Langerode vijver"

Fatty acid	*N. limnetica*	*S. obliquus*	Lake seston
∑FA	209.97	229.13	31.8
SFA	19.82%	18.19%	46.65%
MUFA	28.67%	11.48%	38.84%
(*n* 3) PUFA	43.09%	63.06%	10.48%
(*n* 6) PUFA	8.19%	7.27%	4.03%
C18:2 *n* 6	3.48%	7.27%	2.76%
C18:3 *n* 3	0.38%	59.51%	6.27%
C20:4 *n* 6	4.40%	nd	0.09%
C20:5 *n* 3	42.71%	nd	2.45%
C22:6 *n* 3	nd	nd	nd

The total concentration (µg mg^{-1} carbon) and the percentages of saturated fatty acids (SFA), mono unsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and essential fatty acids are listed. Data are means of three replicates.

centrifuged at 4000 g for 15 min. The upper aqueous layer was aspired and 1 mL of MeOH:0.88% KCl (1:1) was added. After vortexing and centrifugation at 4000 g for 15 min, the upper phase was aspired together with the interphase. The lower phase was evaporated at 40°C under a stream of N_2. The residue (lipid extracts) was dis solved in 2 mL of $CHCl_3$:acetic acid (100:1) and applied to a silica gel column, wetted with $CHCl_3$:acetic acid (100:1), to separate the different lipid classes. First, 5.53 mL of $CHCl_3$ was applied to the column for eluting neu tral lipids, such as sterols and glycerides (Fraction 1). Second, 2.67 mL of acetone, followed by 2.67 mL of acetone:MeOH:acetic acid (100:5:1) were applied to the column for eluting glycolipids and sphingolipids (Frac tion 2). Then 4 mL of a MeOH:$CHCl_3$:H_2O mixture (100:50:40) was applied to the column for eluting the phospholipids. To this fraction, 2.25 mL of $CHCl_3$ and 3 mL of H_2O were added. The mixture was vortexed and centrifuged at 4000 g for 2 min. The upper water phase was removed and the lower $CHCl_3$ phase contain ing the phospholipids was used for further analysis (Fraction 3). All fractions were evaporated under a N_2 stream at 40°C (Zhu *et al*., 2006).

For fatty acid analysis, the dried samples were resus pended in 4 mL of 3 N methanolic HCl (Sigma Aldrich) together with 100 µL of internal standard (20 µg mL ^{1} 17:0 ME + 25 µg mL ^{1} 23:0 ME) and subsequently incubated for 20 min at 60°C in a sealed vial to transes terify fatty acids into methyl esters. After cooling, FAME were extracted three times with 1.5 2 mL isohexane. The fraction of isohexane was evaporated to dryness under a N_2 stream and the extraction procedure was repeated again with 100 µL isohexane. Afterward, the

isohexane was evaporated under a N_2 stream at 45°C and the FAME were resuspended in a volume of 10 µL isohexane. FAME were analyzed by gas chromatography (GC; Hewlett Packard 6890^{TM}) equipped with a flame ionization detector and a DB 225 (J&W Scientific, 30 m × 0.25 mm inner diameter × 0.25 µm film) capil lary column for FAME analysis. Details of GC con figurations are given elsewhere (Martin Creuzburg *et al.*, 2010). FAME were identified by comparison of retention times with those of reference compounds (Sigma Aldrich). Fatty acids were quantified by comparison with internal standards and by using multipoint standard calibration curves determined for each FAME from mixtures of known composition (Sigma Aldrich). The absolute amount of each FAME was normalized to the egg dry mass and the egg number (Martin Creuzburg *et al.*, 2010).

Data analysis

Data were analyzed using the statistical software CANOCO for windows for PCA and the packages car and phia in R (version 3.0.2) for two way analysis of variance (ANOVA), multivariate analysis of variance (MANOVA) and con trast analyses. To visualize the fatty acid distribution, or dination diagrams of principal component analysis were made with the interaction factor between food source and egg type plotted as supplementary variable. Effects of the food sources (*S. obliquus*, *N. limnetica*, lake seston) and egg types (sexual and asexual eggs) on the concen trations of the individual fatty acids of the different lipid classes were tested using a MANOVA. To test for the effect of egg type within the different food sources and for differences among the laboratory treatments and between these treatments and the natural conditions, contrast analyses were performed. Total amounts of fatty acids were compared between food sources and egg types using ANOVA; lipid classes were analyzed separately. The statistical analyses were performed on both datasets, i.e. the dry mass related dataset and the per egg related dataset.

RESULTS

Overall, the fatty acid profile of both sexual and asexual eggs reflected the fatty acid profile of the food sources en countered by the mothers (Per mg egg dry weight: Fig. 2; per egg: Supplementary data, Fig. S1). For example, C18:3*n* 3 (ALA) and C20:5*n* 3 (EPA), the principal PUFA in *S. obliquus* and *N. limnetica*, respectively (Table I), were the principal PUFA in the eggs of mothers reared on these algae (Table II).

In none of the egg samples were fatty acids observed in Fraction 2, which is supposed to contain the fatty acids derived from glycolipids and sphingolipids. For fatty acid concentrations in the other lipid classes, distinct patterns were found (Fig. 3). There is a significant interaction between food sources and egg type on fatty acid con centration for fatty acids derived from the neutral lipid fraction (Fraction 1) and from the phospholipid fraction (Fraction 3) (Table III; the MANOVA on the amount of fatty acids per egg gave similar results, see Supplementary data, Table SI). The main effect of food type is also highly significant for both the neutral lipid and the phospholipid fraction. Egg type is highly significant for phospholipids but marginally nonsignificant for neutral lipids (Table III). Given the significant interaction effects, we also explored the impact of egg type for each food class separately through independent contrasts. Contrast analysis revealed a significant effect of egg type for each food condition (Table IV; the contrast analysis on the amount of fatty

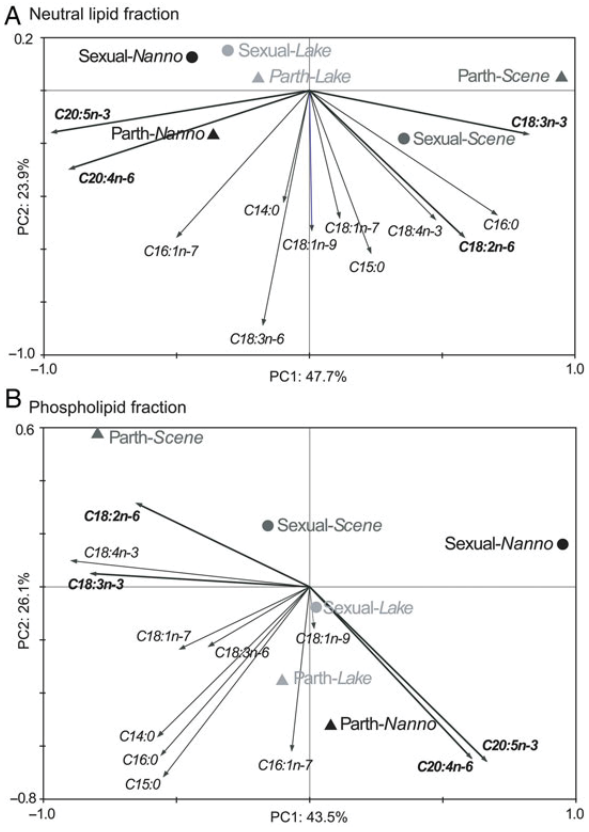

Fig. 2. Ordination diagrams of the principal component analyses (PCAs) of the fatty acid composition (**A**: fraction of neutral lipids; **B**: fraction of phospholipids) of sexual and parthenogenetic (Parth) eggs of *Daphnia magna* fed *Nannochloropsis limnetica* (Nanno) or *Scenedesmus obliquus* (Scene), and of sexual and parthenogenetic eggs directly isolated from a natural pond (Lake). Concentrations of the different fatty acids (ng mg^{-1} dry weight) were the dependent variables, while type of food (*Nannochloropsis limnetica*, *Scenedesmus obliquus*, lake seston) and egg type (resting vs. parthenogenetic) were the independent variables.

Table II: Fatty acid composition of sexual and asexual eggs of Daphnia magna produced on Nannochloropsis limnetica or Scenedesmus obliquus, and of sexual and asexual eggs directly isolated from a natural lake

	Neutral lipid fraction					
	Asexual eggs			Sexual eggs		
	N. limnetica	*S. obliquus*	Lake seston	*N. limnetica*	*S. obliquus*	Lake seston
∑FA	51.07 ± 11.65	51.7 ± 6.19	18.03 ± 2.99	37.61 ± 17.7	43.67 ± 12.3	24.97 ± 3.41
SFA	4.18%	29.10%	13.38%	2.29%	2.29%	5.26%
MUFA	35.32%	14.56%	29.01%	30.69%	30.69%	23.74%
(*n* 3) PUFA	50.94%	47.15%	43.88%	52.52%	52.52%	55.61%
(*n* 6) PUFA	9.56%	9.19%	13.73%	14.50%	14.50%	15.40%
C18:2 *n* 6	4.34%	8.79%	7.27%	6.40%	11.62%	5.39%
C18:3 *n* 3	0.95%	63.40%	17.99%	0.94%	52.22%	13.24%
C20:4 *n* 6	4.63%	0.17%	5.44%	6.97%	0.69%	8.62%
C20:5 *n* 3	48.98%	nd	22.32%	50.76%	1.45%	38.43%
C22:6 *n* 3	nd	nd	nd	nd	nd	nd
	Phospholipid fraction					
	Asexual eggs			Sexual eggs		
	N. limnetica	*S. obliquus*	Lake seston	*N. limnetica*	*S. obliquus*	Lake seston
∑FA	29.43 ± 3.60	30.02 ± 7.63	20.64 ± 1.57	14.46 ± 2.56	15.37 ± 5.98	15.33 ± 6.65
SFA	21.05%	18.78%	23.60%	16.29%	22.26%	22.68%
MUFA	30.62%	21.22%	29.49%	41.81%	29.87%	41.90%
(*n* 3) PUFA	38.22%	46.82%	34.16%	30.68%	33.47%	26.24%
(*n* 6) PUFA	10.11%	13.18%	12.75%	11.22%	14.39%	9.17%
C18:2 *n* 6	3.66%	12.56%	5.22%	5.76%	12.10%	3.88%
C18:3 *n* 3	6.12%	44.14%	8.72%	nd	27.91%	6.29%
C20:4 *n* 6	5.06%	0.21%	7.22%	5.45%	2.30%	5.30%
C20:5 *n* 3	36.49%	0.21%	23.51%	30.68%	3.48%	18.01%
C22:6 *n* 3	nd	nd	nd	nd	nd	nd

The total concentration (μg mg^{-1} dry mass) and the percentages of saturated fatty acids (SFA), mono unsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and essential fatty acids of neutral lipid and phospholipid fractions are given. Data are means of four replicates.

acids per egg gave similar results, see Supplementary data, Table SII). Likewise, the fatty acid composition of the neutral and phospholipid fraction differed significantly among the eggs from the *Nannochloropsis* and *Scenedesmus* fed laboratory cultures and among the eggs obtained in the la boratory cultures and from the natural lake (Table IV and Supplementary data, Table SII).

The total concentration of lipids in the neutral fraction expressed per mg dry weight of the eggs is only influenced by food type, not by egg type (Table V). *Post hoc* tests (Tukey's honestly significant difference) revealed no signifi cant difference between the two laboratory treatments (P 0.1), but the eggs from the laboratory cultures had significantly higher concentrations of fatty acids of neutral lipids than eggs isolated from nature ($P \leq 0.009$). When the data are expressed as concentrations of fatty acids per egg, we also observed a significant difference in the con centration of fatty acids extracted from the neutral fraction (Supplementary data, Table SIII). The concentration of fatty acids derived from phospholipids in the eggs was not affected by the food conditions the mothers were exposed to, but did differ between the two types of egg both for the amount of per unit biomass (Table V) and per egg (Supplementary data, Table SIII). There was also a signifi cant food × egg type interaction for the total amount of phospholipids per egg (Supplementary data, Table SIII). In the laboratory treatments, where high concentrations of dietary fatty acids were provided (Table I), the total con centration of phospholipid derived fatty acids was twice as high in the asexual eggs compared with the dormant eggs. The asexual eggs sampled from the lake contained 1.35 times more phospholipid derived fatty acids than the dormant eggs sampled from the sediment (Table II). Sexual and asexual eggs not only differed in their total concentration of phospholipids; we also observed higher concentrations of almost every fatty acid we measured in the asexual compared with the sexual eggs. There are only two exceptions, namely 20:4*n* 6 and 20:5*n* 3, which were detected in higher concentrations in the sexual eggs of mothers reared on a *S. obliquus* diet (Fig. 3 and Table II).

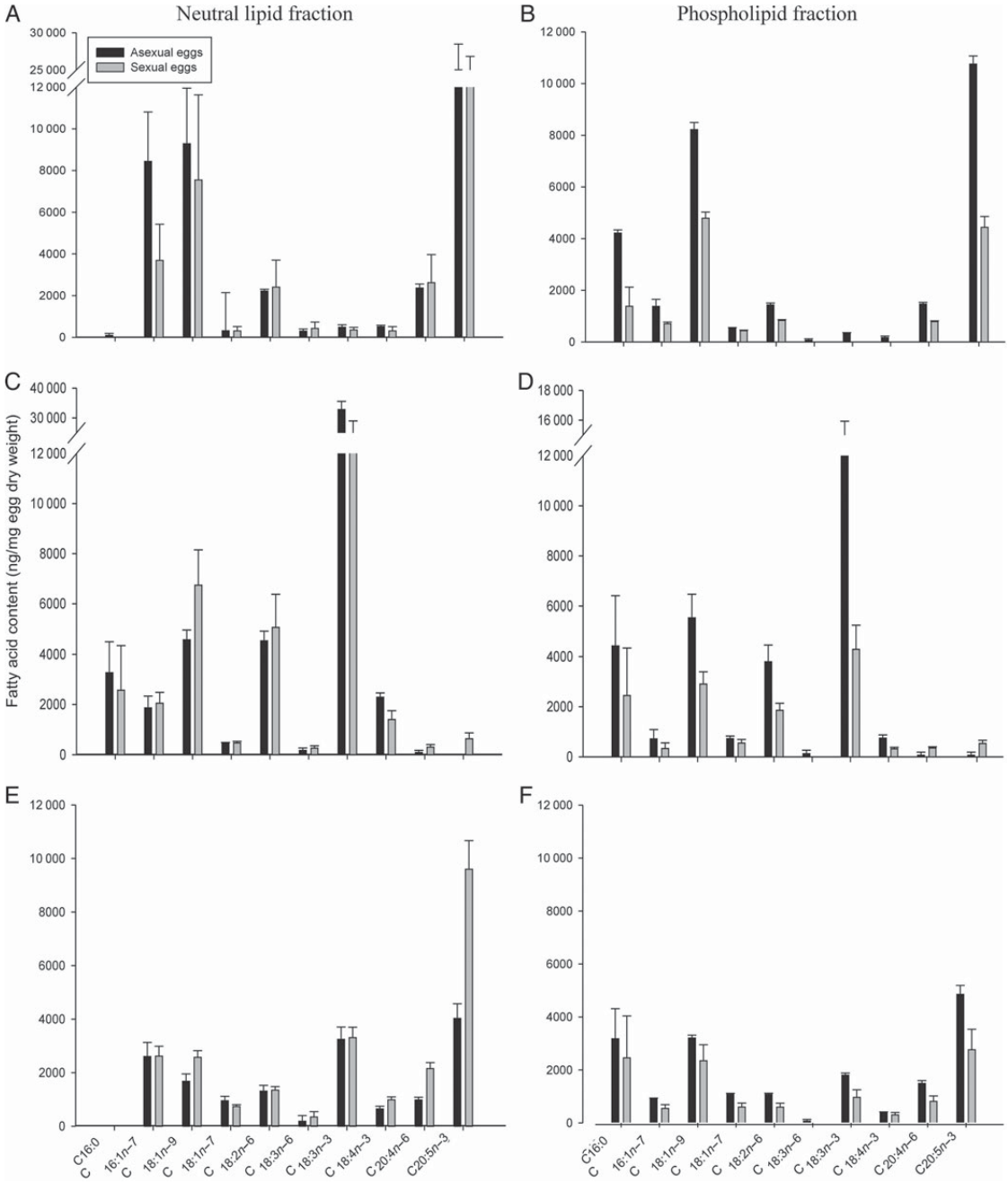

Fig. 3. Fatty acid composition of sexual (gray bars) and asexual eggs (black bars) of *Daphnia magna* (ng mg ^{1} dry mass) produced on *Nannochloropsis limnetica* (**A** and **B**) and *Scenedesmus obliquus* (**C** and **D**), and of sexual and asexual eggs directly isolated from a natural pond (LRV) (**E** and **F**). The lipids were separated into a neutral lipid fraction (A, C and E) and a phospholipid fraction (B, D and F). Data are means of four replicates; error bars indicate one standard error.

DISCUSSION

Daphnia magna females take up considerable amounts of fatty acids from their food and accumulate them as triacylglycerols or phospholipids (Goulden and Place, 1990; Becker and Boersma, 2005). Less than 2% of the accumulated fatty acids in daphnids have been reported to be synthesized *de novo* (Goulden and Place, 1990). Consequently, the fatty acid composition of somatic tissue (Von Elert, 2002; Brett *et al.*, 2006; Martin Creuzburg *et al.*, 2010) and eggs (Abrusan

Table III: Results of MANOVA comparing the fatty acid composition of sexual and asexual D. magna eggs produced on Nannochloropsis limnetica and Scenedesmus obliquus, and of sexual and asexual eggs directly isolated from a natural lake

Neutral lipid fraction				Phospholipid fraction			
Variables	df	*F* value	*P* value	Variables	df	*F* value	*P* value
Food	2	37.259	5.80E 10	Food	2	165.503	4.88E 15
Egg type	1	3.142	0.069	Egg type	1	18.864	0.0004
Food × egg type	2	6.177	0.0002	Food × egg type	2	16.271	2.95E 07

Concentrations of the different fatty acids (ng mg^{-1} dry weight) were the dependent variables and type of food (*Nannochloropsis limnetica*, *Scenedesmus obliquus*, lake seston) and type of egg (sexual vs. asexual eggs) were the independent variables. Datasets for neutral lipids and phospholipids were analyzed separately.

Table IV: Results of contrast analyses following the MANOVA (Table III) comparing the fatty acid composition of sexual and asexual D. magna eggs produced on Nannochloropsis limnetica and Scenedesmus obliquus, and of sexual and asexual eggs directly isolated from a natural lake

Neutral lipid fraction				Phospholipid fraction			
Variables	df	*F* value	*P* value	Variables	df	*F* value	*P* value
Egg type under Scenedesmus	1	11.82	0.003	Egg type under *Scenedesmus*	1	11.816	0.003
Egg type under *Nannochloropsis*	1	3.855	0.041	Egg type under *Nannochloropsis*	1	32.961	0.0004
Egg type when isolated from lake	1	13.113	0.003	Egg type when isolated from lake	1	7.284	0.007
Nanno vs. *Scene*	1	15.495	0.002	*Nanno* vs. *Scene*	1	12.226	0.002
Lake vs. Lab	1	10.151	0.003	Lake vs. Lab	1	20.971	0.0005

Upper part of the table contrasts both egg types for every food source; the lower part of the table contrasts the laboratory treatments with each other and both together against eggs isolated from the natural lake. Data on neutral lipid and phospholipid fractions were analyzed separately.

Table V: Results of ANOVA comparing the total fatty acid concentrations of sexual and asexual D. magna eggs produced on Nannochloropsis limnetica and Scenedesmus obliquus, and of sexual and asexual eggs directly isolated from a natural lake

Neutral lipid fraction				Phospholipid fraction			
Variables	df	*F* value	*P* value	Variables	df	*F* value	*P* value
Food	2	8.395	0.003	Food	2	2.156	0.145
Egg type	1	0.73	0.404	Egg type	1	33.792	1.66E 05
Food × egg type	2	1.154	0.338	Food × egg type	2	2.529	0.108

Concentrations of the total fatty acid contents (ng mg^{-1}) were the dependent variables and type of food (*Nannochloropsis limnetica, Scenedesmus obliquus*, lake seston) and type of egg (sexual vs. asexual eggs) were the independent variables. Datasets for neutral lipids and phospholipids were analyzed separately.

et al., 2007; Schlotz *et al.*, 2013) tend to strongly reflect that of their diet. This is confirmed by our data showing that in the laboratory treatments, in which the animals were cul tured under standardized conditions and provided with high food concentrations, both kind of eggs contained fatty acids in relative abundances reflecting those of the maternal food. This shows that food quality has important conse quences for both energy storage (neutral lipids) and mem brane composition (with phospholipids as their main component) in both sexual and asexual eggs.

In addition, we found that the concentrations and composition of fatty acids in both neutral lipids and phospholipids differed between the two egg types. As the main energy storage molecule in *Daphnia*, triglycerides (representing neutral lipids) are vital for proper develop ment and for survival under starvation (Tessier *et al.*, 1983; Peters, 1987). Although we observed slight differ ences in the composition of the fatty acids of these neutral lipids per mg dry weight between asexual and sexual eggs, we did not find differences in the concentra tion of the total amount of neutral lipids between egg types. On a per egg basis, the total amount of neutral lipids was found to be lower in sexual than in asexual eggs, reflecting that sexual eggs (7.7 μg) were lighter than

asexual eggs (9.4 μg). Pauwels *et al.* (Pauwels *et al.*, 2007) did not find differences in the amount of triglycerides per egg between sexual eggs collected from the sediment and asexual eggs of mothers reared on *S. obliquus* in the laboratory, but their result might have been influenced by the fact that the two egg types had a different history. Asexual eggs always contained more phospholipid bound fatty acids than sexual eggs when they were produced under the same conditions. The higher concentrations of fatty acids (neutral and phospholipid fraction) we observed in asexual compared to sexual eggs is inconsistent with the results of Abrusan *et al.* (Abrusan *et al.*, 2007), who reported that the total fatty acid content in sexual eggs of *D. pulicaria* is much higher than in asexual eggs when produced on a *S. obliquus* diet.

Asexual eggs facilitate fast population growth and are produced under favorable conditions, while sexual eggs are the vector for dispersal in time and space. As a result, in contrast to asexual eggs, sexual eggs often encounter stressful environments and must be able to survive for long periods (Hairston *et al.*, 1995; De Meester *et al.*, 2004). This is expected to be associated with a higher concentration of energy storage molecules and with a specific fatty acid composition of the membranes. The results of Pauwels *et al.* (Pauwels *et al.*, 2007) and Abrusan *et al.* (Abrusan *et al.*, 2007) reporting differences in biochemical composition between dormant and partheno genetic eggs are consistent with these expectations, as are the results of Arbaciauskas and Lampert (Arbaciauskas and Lampert, 2003), who showed that the offspring from dormant and parthenogenetic eggs differ in life history traits. In accordance with Abrusan *et al.* (Abrusan *et al.*, 2007), we found that *D. magna* maintain a certain concentration of long chain PUFA in their sexual eggs even when they are not provided by the food. In both neutral lipids and phospholipids, we found ARA (C20:4*n* 6) and EPA (C20:5*n* 3) in higher concentrations in sexual eggs than in asexual eggs when they were produced on a *S. obliquus* diet, which lacks both ARA and EPA. It is already known that ARA and EPA, presumably in their capacity to serve as eicosanoid precursors (Stanley, 2006), are important for reproduction in *Daphnia* (Becker and Boersma, 2005; Wacker and Martin Creuzburg, 2007; Martin Creuzburg *et al.*, 2010). The higher allocation of ARA and EPA together with the finding that EPA supplementation increases resting egg production (Abrusan *et al.*, 2007), suggests that C20 PUFA are very important for production of viable resting eggs. It is well established that these long chain PUFA are important in maintaining the integrity of cell membranes. Crustacea are capable of adapting the fluidity of their membranes by changing the proportions of saturated and unsaturated fatty acids (Pruitt, 1990). In addition, PUFA are crucial for acclimatization to cold temperatures (Hazel and Williams, 1990; Masclaux *et al.*, 2009) and supplementation of a C20 PUFA deficient diet (*S. obliquus*) with ARA or EPA has been shown to increase population growth rates, in particular at colder temperatures, suggesting that PUFA requirements of *D. magna* increase with decreasing temperature (Martin Creuzburg *et al.*, 2012). These functions might play a crucial role in the survival of sexual eggs during harmful conditions and, as asexual eggs do not need to cope with these stressors, this might also explain the higher allocation of EPA and ARA toward sexual eggs.

The amount and composition of fatty acids in the field differed considerably from that of laboratory grown algae, which contained high levels of key fatty acids, and these differences are reflected in the fatty acids retrieved from the eggs. For fatty acids derived from phospholipids, there were no significant differences between the concentrations in eggs collected from the pond and those produced in the laboratory. Total fatty acid concentration of the neutral fraction was lower in eggs collected from the pond than in eggs produced in the laboratory. In accordance with Tessier *et al.* (Tessier *et al.*, 1983), these results suggest that the allocation of neutral lipids, i.e. energy reserves, into the eggs increases with the dietary lipid availability. The differences may, however, in part also be due to other conditions that differed in the field compared with the laboratory. For example, temperature encountered by the mothers may have an influence on the fatty acid composition of somatic tissues (Sperfeld and Wacker, 2011; Martin Creuzburg *et al.*, 2012) and, as a consequence, potentially also on the allocation of fatty acids into the eggs. In addition, food conditions in the field may have differed during periods of dormant egg production and the active growth period. In a lake, there are typically two peaks of dormant egg production during conditions of low food quantity (clear water phases) following a population peak, while asexual eggs may be produced during more favorable conditions (Sommer *et al.*, 1986; Alekseev and Lampert, 2001). During these clear water phases, the lake seston is dominated by PUFA rich algae, mostly diatoms and cryptophytes (Ahlgren *et al.*, 1992; Muller Navarra *et al.*, 2004; Hartwich *et al.*, 2012), and consequently there might be a higher dietary PUFA availability during the production of sexual eggs than during asexual reproduction.

We conclude that both food quality and distinct allocation strategies influence the fatty acid composition of asexual and sexual eggs of *Daphnia*, with asexual eggs in general having higher concentrations of fatty acids than sexual eggs. The fatty acid composition of both asexual and sexual eggs largely reflected the fatty acid profile of the maternal food, but with an enrichment of specific

long chain PUFA, especially ARA and EPA, in the sexual eggs when the mothers were fed a diet lacking long chain PUFA. We propose that these PUFA, presumably together with other factors, such as heat shock proteins and glycerol (Pauwels *et al.*, 2007), are involved in mediating the striking resistance of *Daphnia* dormant eggs to harsh environmental conditions, including exposure to cold temperatures.

SUPPLEMENTARY DATA

Supplementary data can be found online at http://plankt.oxfordjournals.org.

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