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Impact of starvation and of feeding algal and artificial diets on the lipid content and composition of juvenile oysters (*Crassostrea gigas*) and clams (*Tapes philippinarum*)

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Abstract The present study evaluated the effect of starvation and of feeding various algal and lipid-supplemented diets on the lipid content and lipid class distribution in the polar and neutral lipids of early juvenile oysters (*Crassostrea gigas*) and clams (*Tapes philippinarum* L.). *T. philippinarum* was starved, fed a mixed algal diet [*Tetraselmis suecica* and *Isochrysis galbana* (clone T-Iso)] or solely *T. suecica* at three different feeding rations, either supplemented or not supplemented with lipid emulsions. *C. gigas* was fed *T. suecica* with and without the supplementation of lipid emulsions or liposomes. When *T. philippinarum* and *C. gigas* were fed solely *T. suecica*, no qualitative and only minor quantitative differences were observed between the lipid class profile of both species. The major neutral lipids were triglycerides (TAG) and free sterols plus diglycerides (FS + DAG), whereas the polar lipids were dominated by phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol plus ceramideaminoethanol phosphonate (PI + CAEP). An increase of the algal feeding ration resulted in an increase of the lipid content of spat which was associated with a significant augmentation of the TAG level (as percentage of the total lipids and as percentage of the neutral lipids). Lipid supplementation evoked a similar though more pronounced effect. Starvation resulted in a significant decline of the lipid content and a complete depletion of the TAG reserve. Contrary to the neutral lipids (NL), the relative proportion (percentage of total polar lipids, PL) of the individual PL classes was hardly affected by the diet. The importance of lipid and TAG reserves in early juveniles is discussed.

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Introduction

It is well known that seasonal variations of the lipid content and fatty acid composition of adult bivalves are closely linked to the reproductive cycle and affected by the availability and composition of the natural diet (Beninger 1984; Beninger and Lucas 1984; Beninger and Stephan 1985; Thompson and MacDonald 1990; Robert et al. 1993; Román et al. 1996; Galap et al. 1997). Recently, some population studies included detailed descriptions of the oscillations of the total polar lipid fraction and the major neutral lipid classes, but they do not focus on the different individual polar lipid classes (Beninger 1984; Abad et al. 1995; Pazos et al. 1996). Field studies indicated that lipids, besides carbohydrates and proteins, are catabolised to meet the metabolic demands during the food-depreciated winter period (Beninger 1984; Thompson and MacDonald 1990; Ruíz et al. 1992). For early juveniles, information on the role of biochemical reserves is very scarce although it is self-evident that the ability of juvenile bivalves to cope with periods of low food availability can affect their growth, survival and recruitment in natural and commercially exploited stocks (Laing and Millican 1986; Laing et al. 1987; Laing 1993; Laing and Utting 1994; Laing and Child 1996; Child and Laing 1998). Since bivalves are cultured in a subtidal area or close to the shore, their environment is influenced by the inflow of fresh water which can also include the input of pollutants. It has been shown that pesticides and types of polychlorinated biphenyl (PCB), which are rapidly accumulated in bivalves, can evoke a consumption of lipids or an alteration of lipid metabolism in contaminated bivalves (Sujatha et al. 1995; Fereirra and Vale 1998).

The complexity of the natural environment makes it hard to distinguish between the impact of the diet and other environmental (e.g. temperature) and physiological (e.g. sexual cycle) conditions on the content and composition of lipids in bivalves. Moreover, it is difficult to determine the in situ quantity and composition of the

diet consumed by natural bivalve populations (Robert et al. 1993). Controlled experimental conditions do not take into account all of the complex interactions affecting lipid anabolism and catabolism of bivalves in the field, but they can greatly reduce the interference of non-dietary parameters and thus facilitate the interpretation of the results. Compared to the numerous field studies, rather few studies describe the effect of nutritive stress on spat and adults held under laboratory conditions (Ansell and Sivadas 1973; Gabbott and Bayne 1973; Swift et al. 1980; Pollero and Brenner 1981; Lane 1986; Whyte et al. 1990; Laing 1993; Laing and Child 1996; Child and Laing 1998). There appear to be no data available from laboratory studies that investigated the effect of the diet on the polar lipid class composition of the whole tissue of larval, juvenile or adult bivalves; only two studies (Fernández-Reiriz et al. 1998; Parrish et al. 1998) report on the impact of the diet on the lipid class distribution in the neutral lipid fraction of juvenile bivalves.

The objective of the present study was to evaluate the effect of diet composition on the lipid content and lipid class distribution in the polar and neutral lipids of early juvenile oysters (*Crassostrea gigas*) and clams (*Tapes philippinarum*). Additionally, the impact of starvation on the lipid content and composition of *T. philippinarum* was studied. In lipid nutrition studies, bivalves are usually fed different algal diets selected on the basis of their particular fatty acid composition (Jonsson et al. 1999). However, apart from lipids or fatty acids, several other species-specific algal characteristics such as digestibility, palatibily and biochemical composition may interfere. This problem can partly be avoided by altering the composition of a single algal diet through manipulation of its culture conditions (Thompson et al. 1993; Parrish et al. 1998). However, recent work with various filter-feeding organisms such as *Daphnia* (DeMott and Müller-Navarra 1997), *Artemia* (Coutteau and Mourente 1996; Coutteau and Sorgeloos 1997) and bivalves (Coutteau et al. 1994, 1996; Caers et al. 1998, 1999a, 2000) have successfully used controlled supplementation studies with artificial diets to understand the effect of lipid nutrition on the metabolism and physiology of growth and reproduction. Therefore *T. philippinarum* was starved or fed *Tetraselmis suecica* at three different feeding rations, either supplemented or not supplemented with an emulsion. *C. gigas* was fed *T. suecica* with and without the supplementation of lipid emulsions and liposomes. The impact of the diet on the growth and fatty acid composition of the spat has been presented in separate publications (Caers et al. 1999b, 2000).

Materials and methods

Culture conditions and experimental design

During a period of 4 weeks, spat were fed twice daily, the water was renewed three times per week. The system for rearing spat consisted of 5-litre aquaria placed in a water bath of 21 °C. Each 5-litre aquarium contained one down-welling silo and one point aeration to keep the food in suspension. The spat was stocked at an initial concentration of 0.75 g wet weight (WW) per silo. Every week, the animals were harvested, rinsed with tap water, blotted dry with paper towel, weighed and restocked at 0.75 g per silo (WW_i). The algal diets were fed at a weight-specific feeding ration (FR). The FR is expressed as the total dry weight (DW) of the algal diet per WW of the spat per day (% DW WW^{-1} d^{-1}). Daily, the amount of food was adjusted to keep FR constant, as described in Caers et al. (1998, 1999b, 2000). The algal diet was fed alone or supplemented with an artificial diet at a concentration of 50% lipid (expressed as percentage of total DW of the algal diet). All treatments were run in triplicate aquaria and are summarised in Table 1.

Tapes philippinarum

Juvenile Manila clams (initial live weight 1.0 ± 0.1 mg) were obtained from Tinamenor S.A., Spain in spring 1995. A mixed diet of *Isochrysis galbana* (clone T-Iso) and *Tetraselmis suecica* (1:1 on a DW basis) was used as a reference diet (Iso + Tet) and fed at a weight-specific FR of 1% (Caers et al. 1999b). Starvation was used as a negative control (Starv). *T. suecica* was fed at a FR of 0.5, 1.0 and 1.5% (0.5Tet, 1.0Tet and 1.5Tet, respectively) and supple-

Table 1 Overview of the experimental design and diets used in the lipid supplementation tests with juvenile clams (*Tapes philippinarum*) and oysters (*Crassostrea gigas*). Supplementation level is expressed as % lipid per total dry weight of the algal diet; weight-specific algal feeding ration is expressed as % algal dry weight per wet weight of spat per day. Values for the initial samples of *C. gigas* and *T. philippinarum* are given in Tables 2 and 3

Treatment	Diet	Supplementation (%)	Feeding ration (% DW WW^{-1} d^{-1})
T. philippinarum			
Iso + Tet	*Isochrysis galbana* (T-Iso) + *T. suecica*[a]	0	1
Starv	Starvation	0	0
0.5Tet	*T. suecica*	0	0.5
0.5Tet + E	*T. suecica* + Emulsion Em50D	50	0.5
1.0Tet	*T. suecica*	0	1.0
1.0Tet + E	*T. suecica* + Emulsion Em50D	50	1.0
1.5Tet	*T. suecica*	0	1.5
1.5Tet + E	*T. suecica* + Emulsion Em50D	50	1.5
C. gigas			
Tet	*Tetraselmis suecica*	0	1
Tet + Lipo	*T. suecica* + liposomes	50	1
Tet + Em1	*T. suecica* + Emulsion 1	50	1
Tet + Em2	*T. suecica* + Emulsion 2	50	1

[a] 1:1 on a dry weight basis

mented with 50% lipid by means of the lipid emulsion Em50D (0.5Tet + E, 1.0Tet + E and 1.5Tet + E, respectively).

Crassostrea gigas

Juvenile Pacific oysters (initial dry weight 4.3 ± 0.2 mg) were obtained from Guernsey Sea Farms S.A., UK in spring 1996. *Tetraselmis suecica* was fed at a FR of 1%. The algal diet was fed alone (Tet) or supplemented with liposomes (Tet + Lipo), Emulsion 1 (Tet + Em1) or Emulsion 2 (Tet + Em2) at a concentration of 50% (Caers et al. 2000).

Diets

The experimental ICES emulsions (ICES, International Council for the Exploration of the Sea), prepared by INVE Technologies, Baasrode, Belgium, contained 50% lipid on a WW basis, water, emulsifiers, antioxidants, preservatives and liposoluble vitamins (Caers et al. 1999b). Emulsion Em50D, used in the *Tapes philippinarum* experiment, was based on ethyl esters. The major fatty acids were 22:6*n*-3 (45%), 18:1*n*-9 (17%) and 16:0 (16%). For the *Crassostrea gigas* experiment, emulsions Em1 and Em2 were based on soybean triglycerides (TAG) enriched with 22:6*n*-3 ethyl esters, liposomes were prepared as described in Caers et al. (2000) using Pro-liposomes (Lucas Meyer, France) and 22:6*n*-3 ethyl esters. Except for 22:6*n*-3, which was present as ethyl esters, fatty acids of the liposomes were supplied as phosphatidylcholine. Em2 was prepared to contain similar amounts of 18:2*n*-6 and 22:6*n*-3 (34% of the total fatty acids or 318 mg g^{-1} lipid), whereas the 22:6*n*-3 content of Em1 was similar to the 22:6*n*-3 content of the liposomes (158 and 153 mg g^{-1} lipid, respectively). The fatty acid profile of the liposomes was dominated by 22:6*n*-3 and especially 18:2*n*-6 which compromised 20 and 50%, respectively, of the total fatty acids. Compared to the emulsions, the percentage of 18:1*n*-9 was approximately three times lower.

Algae, grown in 4-litre batch cultures in filtered seawater enriched with Walne medium, were harvested twice daily in the exponential phase and counted with a haemocytometer (Caers et al. 1998, 1999b, 2000).

Biochemical analyses

Before sampling, spat were starved during 24 h to empty their guts. All analyses were done on freeze-dried samples, homogenised in a mortar. For each aquarium, DW (24 h at 60 °C) and ash (24 h at 450 °C) determinations were performed in triplicate. The ash-free dry weight (AFDW) defines the organic matter content of the spat, and was calculated as the difference between DW and ash. Lipid analysis was done in triplicate (one sample from each aquarium). Total lipids were extracted with chloroform:methanol (2:1, v/v) as a solvent mixture and determined gravimetrically (Caers et al. 1999b). Lipid classes were analysed using high-performance thin-layer liquid chromatography (HPTLC) on HPTLC plates (10 × 10 cm, Silicagel 60, Merck, Germany). Lipids were separated in a one-dimensional double development using methyl acetate:isopropanol:chloroform:methanol:0.25% aqueous KCl (25:25:25:10:9, v/v) and hexane:diethyl ether:acetic acid (80:20:2, v/v) as a developing solvent mixture for polar and neutral lipid classes, respectively (Henderson and Tocher 1992). For quantification of the lipid classes, plates were sprayed with 3% (w/v) cupric sulphate in 8% (v/v) phosphoric acid, charred at 160 °C for 20 min and quantified with a Sharp JX-325 scanner supported with ImageMaster™ software. Identification was done by using specific stain reagentia, lipid standards and two-dimensional development systems (Henderson and Tocher 1992).

Since plasmalogen and phosphonolipids are not separated from their corresponding diacylphospholipid analogues, they were quantified as one group. For clarity, only the names of the diacylphospholipids are used in the presentation of the results and the discussion. Similarly, the fraction named TAG includes triglycerides as well as alkyl and alkenyl forms, since they were not separated by the applied neutral solvent system.

Statistical analysis

Statistical analysis included Student's *t*-test, one- and two-way analysis of variance (ANOVA) and Tukey's honest significant difference test (Tukey HSD-test) using the software-program STATISTICA (Statsoft). The homogeneity of the variances of means was checked by Bartlett's χ^2-test. Arcsine $\sqrt{}$ transformation was used prior to statistical analyses of percentage data (Sokal and Rohlf 1995).

Results

Lipid class composition and content of *Tapes philippinarum* and *Crassostrea gigas* spat

Irrespective of the diet, the neutral lipids of oysters and clams were dominated by TAG and FS + DAG while PC, PE and PI + CAEP were the major polar lipid classes (abbreviations, see Table 2 legend). A comparison between *T. philippinarum* and *C. gigas* fed solely *Tetraselmis suecica* at a ration of 1% DW WW^{-1} d^{-1} reveals a slightly higher TL and TFA content (mg g^{-1} AFDW) in clams than in oysters. Furthermore, the % NL/TL is lower but the % SE/NL is higher in *C. gigas* than in *T. philippinarum* (Tables 2, 3).

Effect of the *Tetraselmis suecica* feeding ration in *Tapes philippinarum*

Due to the lower AFDW (as % of DW) content of spat fed 0.5Tet than spat fed 1.0Tet, the TL content (mg g^{-1} AFDW) increased by only 11% when the feeding ration doubled from 0.5 to 1.0% (Table 2). A further increase of the feeding ration from 1.0 to 1.5% did not affect the AFDW content of the spat and caused an increase of the TL content (mg g^{-1} AFDW) of 34%. This was coupled with an increase of the NL content from 42.9 to 58.8% of the TL. A higher TL content was associated with a significant increase of % TAG/TL (2.9, 11.5 and 26.7% at a feeding ration of 0.5, 1.0 and 1.5% DW WW^{-1} d^{-1}, respectively) and fatty acid content. In the NL, the drastic increase of the % TAG/NL (from 7.1 to 26.9 and 45.4% at a feeding ration of 0.5, 1.0 and 1.5% DW WW^{-1} d^{-1}, respectively) was compensated by a reduction of the other neutral lipid classes, except for the SE which remained constant. The impact of the diet on the PL class composition was of no significance compared to the modifications detected in the NL. In treatments where any limited effects at all were noticed, they can generally be summarised as a slight increase of the % PC/PL which was accompanied by small declines in the % PE/PL and/or % PS/PL.

Table 2 *Tapes philippinarum*. Impact of the diet on the total lipid content (*TL*), total fatty acid content (*TFA*), total neutral (*NL*) and polar (*PL*) lipid content (% of TL), the NL/PL ratio, and the lipid class composition of the neutral (as % of NL) and polar (as % of PL) lipids of spat fed various algal and lipid-supplemented diets. Data represent the mean of three replicate aquaria. Values with the same letter within one row are not significantly different (ANOVA, Tukey HSD-test, $p < 0.05$); significantly different levels between the initial sample and starved clams are indicated with * (*t*-test, $p < 0.05$); data not followed by a letter (except for the initial sample) were excluded from statistical analysis since they caused violations against the assumption of homogeneity of variances (*SE* sterol esters; *TAG* triglycerides; *FFA* free fatty acids; *FS* free sterols; *DAG* diglycerides; *MAG* monoglycerides; *PIG* pigments; *GLY* glycophospholipid; *PE* phosphatidylethanolamine; *UN* unknown phospholipid; *CAEP* ceramideaminoethyl phosphonate; *PI* phosphatidylinositol; *PS* phosphatidylserine; *PC* phosphatidylcholine; *LPC* lyso-phosphatidylcholine)

	Initial	Iso + Tet	Starv	0.5Tet	0.5Tet + E	1.0Tet	1.0Tet + E	1.5Tet	1.5Tet + E
TL/DW (mg g^{-1})	8.0 ± 0.2*	8.4 ± 0.4e	5.9 ± 0.6f	6.8 ± 0.4f	10.9 ± 0.1d	10.0 ± 0.4d	15.0 ± 0.6c	12.6 ± 0.3b	17.6 ± 0.4a
TL/AFDW (mg g^{-1})	81.20 ± 2.0	81.8 ± 4.5e	73.9 ± 5.1e	76.6 ± 5.5e	97.8 ± 6.5d	85.3 ± 1.1de	136.8 ± 4.8c	114.7 ± 2.5b	160.0 ± 3.2a
TFA/DW (mg g^{-1})	4.2 ± 0.2*	3.8 ± 0.1d	1.9 ± 0.1e	1.9 ± 0.1e	6.0 ± 0.1c	4.4 ± 0.2d	8.2 ± 0.2b	6.3 ± 0.2c	10.9 ± 0.5a
TFA/AFDW (mg g^{-1})	42.28 ± 1.6*	37.7 ± 1.6d	24.0 ± 2.0e	21.9 ± 0.7e	53.5 ± 3.5c	38.1 ± 1.3d	74.6 ± 2.1b	57.7 ± 1.4c	99.4 ± 4.4a
TFA/TL (%)	52.1 ± 2.0*	46.2 ± 2.9c	32.7 ± 4.7d	28.7 ± 2.5d	54.7 ± 0.2b	44.7 ± 1.3c	54.5 ± 1.7b	50.3 ± 0.8bc	62.1 ± 2.2a
NL/PL	0.8 ± 0.0*	0.8 ± 0.0d	0.4 ± 0.0e	0.7 ± 0.0d	1.0 ± 0.0c	0.8 ± 0.1d	1.4 ± 0.0b	1.7 ± 0.1b	2.0 ± 0.1a
NL (% of TL)	43.7 ± 1.2*	43.2 ± 1.4d	30.2 ± 0.5e	41.0 ± 0.7d	49.9 ± 0.9c	42.9 ± 1.6d	58.5 ± 0.8b	58.8 ± 1.2b	67.0 ± 0.7a
SE	9.7 ± 0.7*	10.7 ± 0.3b	16.1 ± 0.4a	10.6 ± 0.3b	8.8 ± 0.4ce	10.5 ± 0.7bd	7.4 ± 0.3e	10.0 ± 1.0bc	9.0 ± 0.7 cd
TAG	27.8 ± 1.2*	23.9 ± 1.6e	0.0 ± 0.0 g	7.1 ± 0.7e	46.4 ± 0.6c	26.9 ± 0.9d	51.0 ± 0.9b	45.4 ± 0.8c	55.9 ± 0.8a
FFA	9.8 ± 1.2	10.2 ± 1.1b	13.5 ± 1.0a	10.2 ± 0.6b	7.6 ± 0.6bc	8.5 ± 1.5bc	7.2 ± 0.5c	7.1 ± 0.9c	6.5 ± 0.9c
FS + DAG	39.2 ± 1.3*	40.8 ± 1.8c	56.6 ± 1.3a	52.0 ± 0.7b	27.9 ± 0.3de	40.3 ± 1.2c	25.8 ± 0.9e	28.6 ± 0.9d	21.8 ± 0.5f
MAG + Pig	13.4 ± 0.6	14.3 ± 0.4b	13.8 ± 0.5b	20.1 ± 0.9a	9.4 ± 0.3c	13.8 ± 0.7b	8.6 ± 0.4c	8.9 ± 0.2c	6.7 ± 0.4d
PL (% of TL)	56.3 ± 1.2*	56.8 ± 1.4d	69.8 ± 0.5e	59.0 ± 0.7d	50.1 ± 0.9c	57.1 ± 1.6d	41.5 ± 0.8b	41.2 ± 1.2b	33.0 ± 0.7a
GLY	5.1 ± 0.2	5.3 ± 0.9a	4.8 ± 0.1a	4.9 ± 0.3a	4.8 ± 0.5a	3.9 ± 0.5a	4.7 ± 0.2a	4.6 ± 0.9a	4.7 ± 0.2a
PE	26.9 ± 0.3	27.6 ± 0.7b	27.4 ± 0.9b	32.6 ± 0.7a	26.0 ± 0.9b	27.9 ± 0.6b	26.2 ± 1.2b	27.9 ± 3.0b	28.4 ± 1.7b
UN	6.3 ± 0.4	6.3 ± 0.4b	6.6 ± 0.4ab	7.3 ± 0.4a	7.1 ± 0.1ab	7.6 ± 0.3a	7.2 ± 0.4ab	7.5 ± 0.1a	7.4 ± 0.5a
PI + CAEP	20.0 ± 0.8	20.1 ± 0.9a	19.9 ± 0.6a	20.1 ± 0.3a	19.3 ± 0.4a	19.3 ± 0.6a	18.6 ± 0.8a	18.8 ± 1.0a	18.3 ± 0.6a
PS	7.7 ± 0.2	7.9 ± 0.3ab	8.6 ± 0.6a	7.1 ± 0.4bd	8.1 ± 0.5abe	8.6 ± 0.5a	5.8 ± 0.6d	6.9 ± 0.7de	4.3 ± 0.3f
PC	31.6 ± 0.2	30.5 ± 0.5	30.7 ± 0.3	25.3 ± 0.3	30.4 ± 2.3	29.2 ± 0.1	33.1 ± 1.4	32.9 ± 1.1	35.2 ± 1.7
LPC	2.3 ± 0.2	2.4 ± 0.2cd	2.0 ± 0.5ce	2.7 ± 0.2bc	4.2 ± 0.3a	3.6 ± 0.5ab	4.4 ± 0.4a	1.4 ± 0.2e	1.7 ± 0.0de

A 50% replacement of *T. suecica* (1.0Tet) by *Isochrysis galbana* (Iso + Tet) in the algal diet of *T. philippinarum* hardly affected the lipid composition and content of the spat.

Effect of lipid supplementation in *Tapes philippinarum*

In general, lipid supplementation had a similar, though clearly more pronounced effect, on TL and TFA content and lipid class composition of *T. philippinarum*, as discussed above for increasing algal feeding rations (Table 2). Spat fed lipid-supplemented diets showed a higher TL content which was mainly reflected in a higher % NL/TL, % TAG/TL and % TAG/NL and consequently a higher TFA content than spat fed non-supplemented diets (at the same *Tetraselmis suecica* ration). The more pronounced accumulation of TAG by *T. philippinarum* fed lipid microspheres at a lower algal feeding ration demonstrates the significant interaction (*df* effect = 2, *df* error = 12, $F = 595$, *p*-level = 0.0000) between the fixed factors feeding ration (0.5, 1.0 and 1.5% DW WW^{-1} d^{-1}) and lipid supplementation (0 and 50%).

Effect of starvation in *Tapes philippinarum*

During a starvation period of 4 weeks, clams lost 26% of their initial TL content (mg g^{-1} DW) which resulted in a complete depletion of their TAG reserves (Table 2). Due to the lower AFDW of starved spat, the loss of TL compared to the initial sample was limited to 9% (when expressed as mg g^{-1} AFDW). The TL and TFA content of starved clams was not significantly different from that of spat fed solely *Tetraselmis suecica* at a ration of 0.5% DW WW^{-1} d^{-1}.

Effect of lipid supplementation in *Crassostrea gigas*

The total lipid and fatty acid content of oysters fed lipid microspheres was significantly higher than in oysters fed *Tetraselmis suecica*, whether supplemented with liposomes or not (Table 3). The supplementation of liposomes hardly affected the lipid class composition of *C. gigas*, the only significant effect being a reduction of the % SE/NL and a small increase of the % FFA/NL. Like in emulsion-fed *Tapes philippinarum*, the increase of the TL and TFA content in emulsion-fed compared to *Tapes suecica*-fed *C. gigas* was associated with a significant increase of the % NL/TL and was greatly attributed to an increase of the % TAG/TL [from 9.9 (Tet) to 27.2 (Tet + Em1) and 25.5% (Tet + Em2)]. Like in *T. philippinarum*, the elevation of the % TAG/NL [from 26.6 (Tet) to 54.2 (Tet + Em1) and 50.8% (Tet + Em2)] was mainly compensated by a reduction of the % FS + DAG/NL and MAG/NL level. Additionally, a severe reduction was also observed in the % SE/NL [from 17.1% (Tet) to 9.8 (Tet + Em1) and 9.3% (Tet + Em2)]. The impact of the emulsion on the polar lipid classes was limited to a 50% reduction of the level of the smallest lipid class, namely GLY, which reduced from 4.8 (Tet) to 2.4 (Tet + Em1) and 2.6% (Tet + Em2) of the PL.

Discussion

As seen in many marine invertebrates including bivalves, PC and PE were the major polar lipids, whereas the neutral lipids were dominated by TAG and FS (see reviews of Joseph 1989; Vaskovsky 1989). The presence of CAEP, a sphngophosphonolipid which was first detected in the sea anemone (*Anthropleura elegantissima*),

Table 3 *Crassostrea gigas*. Impact of the diet on the total lipid content (*TL*), total fatty acid content (*TFA*), total neutral (*NL*) and polar (*PL*) lipid content (% of TL), the NL/PL ratio, and the lipid class composition of the neutral (as % of NL) and polar (as % of PL) lipids of spat fed *Tetraselmis suecica* with and without lipid supplements during 4 weeks. Data represent the mean of three replicate aquaria. Values with the same letter within one row are not significantly different (ANOVA, Tukey HSD-test, $p < 0.05$) (*nd* not determined; other abbreviations, see Tables 1 and 2)

	Initial	Tet	Tet + Lipo	Tet + Em1	Tet + Em2
TL/DW (mg g^{-1})	1.92 ± 0.05	3.86 ± 0.02d	4.69 ± 0.10c	7.42 ± 0.25a	6.61 ± 0.19b
TL/AFDW (mg g^{-1})	35.7 ± 0.8	70.5 ± 1.5b	77.4 ± 1.9b	103.7 ± 6.0a	94.2 ± 2.9a
TL/oyster (µg g^{-1})	8.3 ± 0.2	46.0 ± 3.4b	59.4 ± 4.9b	135.4 ± 14.9a	114.0 ± 9.80a
TFA/DW (mg g^{-1})	nd	1.6 ± 0.0a	2.1 ± 0.1b	3.9 ± 0.1d	3.4 ± 0.1c
TFA/AFDW (mg g^{-1})	nd	29.2 ± 1.8a	35.0 ± 1.6b	54.9 ± 2.8d	49.1 ± 0.8c
TFA/oyster (µg g^{-1})	nd	18.9 ± 1.7c	27.0 ± 1.1c	71.3 ± 6.3a	59.1 ± 4.6b
TFA/TL (%)	nd	41.1 ± 1.0b	45.5 ± 2.5b	52.7 ± 1.8a	51.8 ± 1.4a
NL/PL	0.5 ± 0.0	0.6 ± 0.0b	0.6 ± 0.0b	1.0 ± 0.1a	1.0 ± 0.0a
NL (% of TL)	35.2 ± 0.4	37.3 ± 0.6b	36.2 ± 1.5b	50.2 ± 2.1a	50.2 ± 0.4a
SE	10.4 ± 0.3	17.1 ± 1.0a	11.2 ± 1.1b	9.8 ± 2.2b	9.3 ± 1.3b
TAG	17.6 ± 0.6	26.6 ± 2.7b	27.3 ± 2.1b	54.2 ± 1.3a	50.8 ± 1.6a
FFA	10.1 ± 1.0	7.5 ± 0.1b	10.9 ± 2.2a	9.2 ± 0.4ab	11.5 ± 0.4a
FS + DAG	40.3 ± 0.7	34.5 ± 2.0a	36.5 ± 1.1a	18.9 ± 0.4b	19.8 ± 0.8b
MAG + Pig	21.6 ± 1.0	14.3 ± 0.8a	14.1 ± 0.5a	7.8 ± 0.7b	8.6 ± 0.8b
PL (% of TL)	64.8 ± 0.4	62.7 ± 0.6a	63.8 ± 1.5a	49.8 ± 2.1b	49.8 ± 0.4b
GLY	3.4 ± 0.2	4.8 ± 0.5a	4.4 ± 1.0a	2.4 ± 0.3b	2.6 ± 0.2b
PE	23.6 ± 0.6	21.6 ± 0.5a	19.8 ± 1.3a	22.3 ± 1.2a	20.8 ± 0.7a
UN	7.2 ± 0.5	5.9 ± 0.4a	6.3 ± 0.5a	6.5 ± 0.4a	6.9 ± 0.5a
PI + CAEP	22.8 ± 0.5	23.0 ± 0.2a	22.7 ± 0.1a	22.6 ± 0.4a	22.4 ± 0.4a
PS	10.1 ± 0.4	9.3 ± 0.6a	9.4 ± 0.2a	8.9 ± 0.2a	8.5 ± 0.7a
PC	29.8 ± 0.8	32.5 ± 1.1a	34.6 ± 0.6a	34.4 ± 1.8a	35.4 ± 0.7a
LPC	3.2 ± 0.2	2.9 ± 0.1a	2.8 ± 0.2a	2.9 ± 0.1a	3.5 ± 0.5a

confirms the findings of Vaskovsky (1989) that CAEP is widely distributed among mollusc species. The latter author suggested that many molluscan studies do not report the presence of CAEP due to the use of inadequate analytical techniques. Research by Sampugna et al. (1973) demonstrated the presence of phosphonolipids in diacyl, plasmalogen and glyceryl ether esters of *Crassostrea virginica*, but, in general, phosphonolipids are mainly present as components of sphingolipids (Sampugna et al. 1973; Joseph 1989; Vaskovsky 1989). Swift (1977) did not specify the individual phosphonolipids but noticed a preferential retention of the total phosphonates in starved *C. virginica* and suggested that their selective conservation indicates a unique role in membrane structure and function. In *Tapes philippinarum*, fasting did not alter the CAEP + PI level.

The relative lipid class distribution of the polar lipids was hardly influenced by the diet. Even the extra supply of PC in liposome-fed oysters did not enhance the PC levels of *Crassostrea gigas*, although there was some indication that the high supply of ethyl esters did slightly enhance the PC level in *Tapes philippinarum*. Information concerning seasonal variations in the relative amounts of the individual polar lipid classes is restricted to the work of Pollero and colleagues (Pollero et al. 1979, 1981). The latter authors observed only minor seasonal fluctuations in the composition of the polar lipids of the adult scallop *Chlamys tehuelcha* and the fresh water mollusc *Diplodom patagonicus*. Although it is not known to which extent these results can be extrapolated to young spat, our data confirm the general stability of the relative amounts of the different polar lipid classes. Since FS and PL are primarily associated with membranes, it is not surprising that the impact of the diet was limited as compared to the energy-linked TAG. Although the exact physiological function of SE remains uncertain, it has been suggested that they could serve as a reservoir for FS (Napolitano et al. 1988; Pazos et al. 1996).

Free fatty acids are generally associated with hydrolysis or breakdown of acyl lipid classes. However, Napolitano et al. (1988) suggested that under proper conditions of lipid extraction and storage, they are better characterised as ''non-esterified fatty acids'' and considered as an actual lipid class, likely a protein-bound one. Since the low FFA level detected in the present study suggests acceptable analytical and storage conditions, Napolitano's hypothesis formulated for *Ostrea edulis* larvae may also be valid for early juveniles studied in the present work.

Like in the present study, lipid supplementation of algal diets increased the lipid content of juvenile *Crassostrea gigas* (Langdon and Waldock 1981), *Mercenaria mercenaria* (Hurd et al. 1989), *Placopecten magellanicus* (Coutteau et al. 1996) and adult *C. virginica* (Trider and Castell 1980). This could be important when spat are transferred to the sea, since Laing and Millican (1986) found a positive relation between the lipid content of *Ostrea edulis* spat at the moment of planting and their subsequent growth and survival in the field. Holland and Hannant (1974) illustrated that in *O. edulis*, the changeover from neutral lipid to glycogen as the main energy source occurred 3 to 5 months after settlement. From the observation that carbohydrates contributed less to the weight loss in smaller (0.3 g DW) than in larger oysters (0.8 g DW), Rodhouse and Gaffney (1984) concluded that more lipids and proteins are catabolised in the small than in the larger individuals.

Enhanced lipid levels in *Tapes philippinarum* and *Crassostrea gigas* spat were coupled with high TAG levels which were, in turn, associated with high TFA levels since transesterification of TAG yields a higher amount of fatty acid methyl esters than polar lipids or sterol esters. Jarzebski et al. (1986) found a positive correlation between food availability and lipid content of the clam *Macoma balthica* sampled at different locations in the bay of Gdansk, and, as in the present study, high lipid contents (mg g^{-1} DW) were coupled with high TAG levels (% of TL) and a high NL/PL ratio. Similarly, the higher lipid content of clam seed (*Ruditapes decussatus*) cultured in waste water from an intensive turbot farm compared to spat cultured in seawater was attributed to increased NL contents (Jara-Jara et al. 1997). In the same way, Parrish et al. (1998) found the highest TAG level (as % of TL) in juvenile *Placopecten magellanicus* fed the diet which resulted in the highest TL content per scallop [a mixture of *Isochrysis galbana* (clone T-Iso) cultured under N-limited conditions and *Chaetoceros muelleri*]. It is well established that seasonal changes in the lipid content closely reflect variations of the NL or TAG content in adult bivalves which is linked with the food supply and the reproductive cycle (Pollero et al. 1979; Swift et al. 1980; Beninger 1984; Beninger and Lucas 1984; Abad et al. 1995; Pazos et al. 1996). It has been postulated that in bivalves, TAG do not only form a readily accessible source of energy but also form a temporary reservoir from which polyunsaturated essential fatty acids can be transferred to the polar lipids or directed to specific metabolic pathways (Napolitano et al. 1988; Coutteau et al. 1996; Caers et al. 1999b).

In *Tapes philippinarum* fed *Tetraselmis suecica* at a low algal feeding ration (0.5% DW WW^{-1} d^{-1}), a two-fold increase of the feeding ration is primarily reflected in a higher growth rate accompanied by a rather limited increase of the lipid content (% of AFDW), whereas, at a higher feeding ration (1% DW WW^{-1} d^{-1}), an elevation of the feeding ration to 1.5% DW WW^{-1} d^{-1} provokes a strong augmentation of the lipid content (Caers et al. 1999b; present study). Similar effects can be seen in the total lipid content of *Ruditapes decussatus* spat fed a daily ration of 0.25, 0.5 and 2% *Isochrysis galbana* (clone T-Iso) (Pérez-Camacho et al. 1998), whereas a slight though not significant elevation of the total lipid content of *R. decussatus* spat fed 0.5, 1 and 2% *I. galbana* (clone T-Iso) was reported by Albentosa et al. (1999). The dietary components are mainly allocated to growth when food supply is limited, whereas, at feeding rations close to optimum, they are partly used to build-

up lipid reserves. However, this process is not endless, since, at algal feeding rations above optimum, growth rates decrease (Coutteau et al. 1994). Whether increased lipid supplements (>50%) would further enhance the lipid reserves of spat and/or affect their growth remains to be investigated. At least in juvenile *Placopecten magellanicus*, the accumulation of TAG (as % of TL) was independent of scallop growth (Parrish et al. 1998). Although the lipid content of *T. suecica* is much lower than in *I. galbana* (clone T-Iso), a 50% replacement of *T. suecica* by *I. galbana* (clone T-Iso) hardly affected the lipid or TAG content of *T. philippinarum* spat. Several studies showed no correlation between the lipid content of different algal diets and the lipid content of spat fed these diets (Laing and Millican 1986; Laing 1993; Knauer and Southgate 1996; Parrish et al. 1998). This may be due to species-specific characteristics (e.g. palatability, digestibility and biochemical components other than lipids) affecting the assimilation efficiency of algal lipids. Decreasing dietary lipid levels, obtained by partial or full replacement of a lipid-rich algal diet (*I. galbana*, clone T-Iso) by wheatgerm flours, were associated with decreased lipid levels in *R. decussatus* spat fed these diets but were also coupled with increased TAG levels (expressed as % of TL) (Fernández-Reiriz et al. 1998). However, care should be taken with the interpretation of these results, as, apart from the lipid content of the diet, several other factors that vary between the algal and wheatgerm diets may have interfered. This problem can be reduced by modifying the composition of an algal diet via manipulation of the algal culture conditions (Thompson et al. 1993; Parrish et al. 1998). In this way, Parrish et al. (1998) clearly illustrated that juvenile *P. magellanicus* fed *I. galbana* (clone T-Iso) cells with the highest TAG content stored the highest amount of TAG. On the other hand, the addition of *Chaetoceros muelleri* to *I. galbana* (clone T-Iso) did not significantly alter the TL or TAG content of *P. magellanicus* as compared to juveniles fed solely *I. galbana* (clone T-Iso).

Stress-induced energy demands can force bivalves to use their endogenous reserves. If such situations occur at higher temperatures, lipid reserves will be even more quickly mobilised, as shown by Laing et al. (1987) and Laing and Child (1996) for clam (*Tapes semidecussata*, *T. decussatus* and *T. philippinarum*) and oyster (*Ostrea edulis*) spat under nutritive stress at 3, 6 and 9 °C. Recently, Sujatha et al. (1995) and Ferreira and Vale (1998) illustrated the importance of lipid-derived energy during toxicant-induced stress in clams and oysters. Even though oysters (two size classes, <50 mm and >60 mm, of *Crassostrea angulata*) were fed a high quality algal diet [mixture of *Tetraselmis suecica* and *Isochrysis galbana* (clone T-Iso)], Ferreira and Vale (1998) observed a sharp decline in the TAG levels in the first 30 d of PCB exposure after which the TAG remained at a very low level till the end of the experiment in both size classes (60 d). The lipid profile of larger clams (*Ruditapes decussatus* >35 mm) was not affected, but, after 60 d of PCB-exposure, the TAG level of the smaller clams (<25 mm) was significantly lower than in the non-exposed clams (Ferreira and Vale 1998). Sujatha et al. (1995) demonstrated a direct relation between the reduction of the lipid content in the clam *Villorita cyprenoides* var. *cochinensis* and the concentration (three sublethal concentrations) and duration (24 to 96 h) of the exposure to three pesticides (endosulfan, malathion and methyl parathion). The effect was most severe for endosulfan, which caused an excessive lipid utilisation of nearly 50% (compared to control clams) during 78 to 96 h of exposure.

The relative contribution made by lipids compared to carbohydrates and proteins towards the utilisation of energy reserves during starvation was not assessed in the present study. However, the results of a series of experiments done by Laing and co-workers (Laing 1993; Laing and Utting 1994; Laing and Child 1996; Child and Laing 1998) illustrated that lipids and carbohydrates, as well as proteins, can be the dominant energy reserve, suggesting a species-oriented preferential utilisation of the three substrates in early juveniles. Nevertheless, the relative abundance of each constituent in the endogenous reserves of larvae, juveniles or adults also seems to affect their preferential mobilisation (Gabbott and Bayne 1973; Haws et al. 1993; Laing 1993; Pérez-Camacho et al. 1998). Therefore, it is likely that spat enriched in TAG, like the ones fed lipid-supplemented diets, would primarily use their readily accessible TAG reserves before severe consumption of proteins and carbohydrates occurs. This hypothesis is supported by the findings of Beninger (1984) and Beninger and Lucas (1984), who observed an increase of the relative PL level (as % of TL) accompanied by a decrease of the relative TAG level during the period of sexual inactivity (winter) in *Tapes philippinarum* and *T. decussatus*. This indicated a faster catabolism of TAG compared to PL in this period. However, since the authors found that the net decrease (mg clam^{-1}) of PL was higher than the net decrease of TAG, they concluded that both TAG and PL contributed to the maintenance energy when food is scarce, but that PL may contribute a greater proportion under severe conditions, after the more labile reserves have been mobilised. Beninger (1984) speculated that although the metabolised phospholipid may have been derived from actual somatic reserves, the simultaneous severe reduction of proteins during the food-limited winter season indicates that probably most of the mobilised phospholipid originated from membrane autolysis.

In clam spat (present study), fasting caused a significant reduction of the lipid content, which was mainly attributed to a severe decline in the TAG reserves and resulted in a halving of the neutral to polar lipid ratio (NL/PL). Similarly, prolonged starvation brought about a deprivation of TAG in adult *Crassostrea virginica* and *Diplodom patagonicus* (Swift et al. 1980; Pollero and Brenner 1981). A severe decline of the NL was also observed in starved *Ostrea edulis* spat (Holland and

Spencer 1973). Our data agree with other studies which reported decreased lipid levels (% AFDW) in starved juvenile *C. gigas*, *O. edulis*, *Tapes philippinarum* and *T. decussatus* (Laing et al. 1987; Laing 1993; Laing and Child 1996; Child and Laing 1998), but the latter studies do not report on the importance of NL or TAG to the depletion of the lipid reserves.

This study demonstrated that artificial lipid supplements provide the tools to manipulate the level and composition of NL reserves in bivalves. Besides their application in controlled laboratory trials to investigate effects of stress conditions and food quality on the dynamics of lipid reserves, it may be interesting to study the effect of lipid reserves generated under laboratory conditions on physiology and performance in the field.

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