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## Changes in fatty acid and sterol composition during oogenesis in the pearl oyster *Pinctada margaritifera*

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### Abstract:

The fatty acid and sterol composition of the oyster *Pinctada margaritifera* during oogenesis and in eggs was analysed. No major differences were observed during oogenesis, but the egg composition was significantly different from that of gonads. The amount of saturated fatty acids was the highest in eggs and the C16:0 predominant ( $P < 5\%$ ); by contrast, the amount of 22:6(n-3) was significantly lower ( $P < 5\%$ ) than in gonads. No major differences were observed for the polar lipid (PL) composition during oogenesis. The main free sterols in gonads and eggs were cholesterol and brassicasterol. Among free sterols, the proportion of cholesterol diminished continuously from the beginning to the end of gonad maturation, and this decrease persisted in eggs after spawning. Cholesterol represented 40% to 55% of the sterol ester encountered in gonad and eggs. This study allowed us to investigate the fatty acid and sterol composition during oogenesis of the pearl oyster *P. margaritifera*, leading to a clearer understanding of the nutritional requirements of pearl oyster during the reproduction process.

# 1. Introduction

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The rapid expansion of the cultured pearl industry in French Polynesia relies on natural spat collection from a number of atolls: however, uncontrolled practices have caused ecological and economic problems. For example, transfer of seed oysters between islands has caused the dissemination of anemones which have colonized oysters under culture. The production of seed oysters in a hatchery would limit such problems and enable a production of standard quality seed oysters. Therefore, control of reproduction is essential in any hatchery program and requires a comprehensive understanding of qualitative and quantitative nutritional requirements.

In French Polynesia, *Pinctada margaritifera* undergoes continuous gametogenesis because trophic conditions and temperature are favorable year round, with as a consequence, a relatively short maturation period of about 1 to 2 months (Pouvreau *et al.*, 2000). The relative availability of mature stock has meant that basic knowledge of the qualitative aspects of nutritional requirements of *P. margaritifera* broodstock is lacking.

Among dietary components of the food given to mollusk broodstock, lipids mainly affect eggs composition (Helm *et al.*, 1991, Utting & Doyou, 1992). Lipids deposited in eggs during maturation play a major role as an energy source during embryonic and early larval development. During oogenesis, oocytes acquire their lipid reserves directly from the food (Beninger *et al.*, 1991) and by transfer from muscle reserves and digestive gland (Barber & Blake, 1985, Napolitano & Ackman, 1993). In *Pecten maximus*, fatty acid and sterol composition of neutral lipids in gonads and oocytes are strongly influenced by diet during the reproductive process (Delaunay *et al.*, 1993, Soudant *et al.*, 1996). Marine mollusks cannot synthesize polyunsaturated fatty acids (PUFA) and sterols and must acquire them from ingested particulate matter. Most of these nutrients are precursors of membranes and play a role in metabolic regulations. PUFA with 20 and 22 carbons and more than three double bonds are essential for the survival and growth of mollusks (Trider & Castell, 1980, Langdon & Waldock, 1981, Uki *et al.*, 1986). The ability to elongate and desaturate 18:3n-3 to produce 20:5n-3 and 22:6n-3 is limited in mollusks (Waldock & Holland, 1984, De Moreno *et al.*, 1976, Delaunay *et al.*, 1993).

The objectives of this study was to describe the accumulation of fatty acids and sterols of neutral and polar fractions in the gonad and eggs during oogenesis. Since oogenesis is a continuous process between the beginning of maturation and spawning, the sampled females were classified according to the degree of development of their gonad. This study will thus determine the changes in levels of neutral and polar fractions of lipids in the gonads during maturation.

## 2. Material and methods

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### 2.1. Sample preparation

Adult *P. margaritifera* were collected in Rangiroa lagoon in November. Female were selected by biopsy of the gonad and their maturity index was determined by macroscopic observations according to Pouvreau (1999) based on the observations of Tranter (1958). The selected females were opened and the visceral mass dissected. One piece of gonad from each female was excised from the gonadal mass located at the intersection of the byssus gland and the pearl pocket (Fougerousse & Herbaut, 1994). Each part of gonad was put in 6 mL chloroform-methanol (2:1 v/v) for analysis of fatty acids and sterols and an amount of internal standard C23:0 was added.

Eggs were obtained after spawning induced by thermal shock. Female were isolated individually to collect the eggs that were washed and rinsed on 80µm and 10µm plankton mesh successively. Eggs were then filtered onto pre-ignited (450°C) GF/C filters. The filters were then placed in tubes containing the chloroform-methanol mixture and an amount of internal standard C23:0 was added. Samples were then frozen at -20°C under nitrogen.

### 2.2. Analytical procedures

Total lipids were extracted according to Folch *et al.* (1957). To separate neutral and polar lipids, 2:1 chloroform-methanol extracts were evaporated to dryness under vacuum. The extract was recovered and rinsed three times with 500 ml of 98:2 chloroform-methanol. Neutral and polar lipids were separated chromatographically on a silica gel microcolumn (30 mm x 5 mm, Kiesegel Merck, 70-230

mesh). Neutral lipids were eluted with 10 ml chloroform–methanol 98:2 and polar lipid with 10 ml methanol. The fractions were collected in tapering vials.

### Fatty acids

Fatty acids methyl esters were prepared by transesterification with 14% BF<sub>3</sub>-MeOH (Sigma), and were purified by High Performance Chromatography (Waters 600 equipped with a diode array detector PDA-996 Waters). The chromatography was performed on two columns mounted in series: Modulo cart Lichrosorb 5 µm DIOL Interchim 250 x 4.0 mm and Modulo cart Lichrospher 5µm Silice Interchim 250 x 4.0 mm. Separation of fatty acids methyl esters was made using a gradient program elution with hexane and hexane-isopropanol,(90:10, vol:vol) at 1 ml/min of flow.

Three fractions were collected. The second fraction collected from 6.5 to 13.5 min contained fatty acids esters which were analyzed using a gas chromatograph (VARIAN GC-3800, Les Ulis, France) equipped with a flame ionisation detector (FID) and a split/splitless 1079 VARIAN injector, two capillary columns mounted in series, a OV101 capillary guard column (J&W scientific) and a DB-Wax capillary column (J&W scientific). Injector and detector temperatures were 240°C and 280°C respectively. The column temperature was programmed at 49°C during 2 min, then from 49°C to 160°C at a rate of 50°C/min, 160°C to 170°C at a rate of 1°C/min, 170°C to 185°C at a rate of 2°C /min, 185°C to 240°C at a rate of 3°C/min and a final plateau at 240°C during 10 min. Ultra High Purity Helium N55 was used as the carrier gas with a constant flow of 2 ml /min. One µl of extract was injected with a splitless injection program, splitless during 1 min and split (ratio 1/50) after 1 min till the end of the run. Fatty acids esters were identified by their retention times with reference to the known standards and designated following the formula C:X (n-Y) where C is the number of carbon atoms, X the number of double bonds and Y the position of the first double bond counted from the CH<sub>3</sub> terminal. Fatty esters quantification were determined using tricosanoic acid as internal standard.

### Sterols

The esterified and free sterols were separated by high performance liquid chromatography (HPLC) in a Hewlett Packard system, equipped with a Lichrosorb 5µm Diol 250 x 4.0 mm column (Interchim, France) and a diode array UV detector. Three fractions were collected:

Esterified sterols + fatty acids methyl esters + triglycerids : 1 to 4.5 min (fraction 1)

Free sterols + free fatty acids + DG + MG : 4.5 to 9 min (fraction 2)

Diols (pavlovols) : 9 to 13 min. (fraction 3)

A known amount of cholestane was added as an internal standard to fractions 1 and 2. Fractions 2 and 3 were put together, evaporated to dryness and dissolved in 80 µl of hexane for injection into GC. The esterified sterol fraction 1 was evaporated to dryness and transesterified with 2 ml of methoxide (MeONa) for 2 h at ambient temperature. The sterols thus were extracted in hexane, evaporated to dryness, dissolved in 80 µl of toluene and injected directly into GC. The methylated and free sterols were analyzed with a Hewlett Packard gas chromatograph equipped with a Restek RTX-65, 15 m x 0.25 mm ID x 0.25 µm film thickness capillary column, a flame ionisation detector (FID) 250°C and a split injector 250°C. Helium gas vector pressure was fixed to 100 kPa at the front of the column. The column temperature was programmed at 90 °C for 2 min, then from 90°C to 170°C at a rate of 49.9°C /min, at 170°C during 1 min, 170°C to 260°C at a rate of 5 °C /min, 260°C to 280°C at a rate of 2°C /min and a final plateau at 280°C for 5 min. One µl of extract was injected with a splitless injection program, splitless during 1 min and split (ratio 1/75) after 1 min until the end of the run.

### Statistics

Percentage data were transformed to arcsine values for statistical analysis. One way analysis was used to assess significant differences at the level of p<0.05. The Student-Newman-Keuls test was used as post hoc test.

## 3. Results

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### 3.1. Fatty acids in the gonads and eggs

#### 3.1.1. Fatty acid in the neutral lipids

No major changes were observed in the composition of the neutral lipids during gametogenesis translated as an index of maturation (Table 1). However, there was a significant difference between the eggs and the gonad composition. There was a significant increase in total saturated fatty acids in eggs ( $F=8.885$ ,  $p=0.0006$ ,  $df = 4$ ) (Table 1). This increase of the total saturated fatty acids in eggs is partly due to higher contents in 16:0 of the eggs (Fig. 1). Among monounsaturated, the proportion of (n-9) was significantly higher in gonad than in eggs ( $F=3.663$ ,  $p=0.0251$ ,  $df = 4$ ) (Table 1). The total of PUFA was significantly lower in eggs than in gonad ( $F=3.083$ ,  $p=0.0443$ ,  $df = 4$ ) (Table 1). The decrease of PUFA in eggs was mainly due to the significant decrease of the (n-3) ( $F=5.653$ ,  $p=0.0044$ ,  $df = 4$ ) (Table 1) and especially of 22:6(n-3) (Fig. 1).

The (n-3)/(n-6) ratio also decreased significantly in eggs compared to the gonad ( $F=9.094$ ,  $p=0.0006$ ,  $df = 4$ ) (Table 1). Lastly, the 22:6/20:5 (n-3) ratio decreased significantly in eggs compared to the gonad ( $F=3.353$ ,  $p=0.0357$ ,  $df = 4$ ) (Table 1).

#### 3.1.2. Fatty acid in the polar lipids

No significant changes were observed in the fatty acid composition of the polar lipids during gametogenesis (Table 2). There was a significant difference between the eggs and the gonad composition. There was a significant increase in total saturated fatty acids in eggs ( $F=4.226$ ,  $p=0.0138$ ,  $df = 4$ ) (Table 2). This increase of the total saturated fatty acids in eggs is partly due to higher contents in 18:0 of the eggs (Fig. 2). The total of PUFA was significantly lower in eggs than in gonad ( $F=5.016$ ,  $p=0.0068$ ,  $df = 4$ ) (Table 2). The decrease of PUFA in eggs was mainly due to the significant decrease of the (n-3) ( $F=5.107$ ,  $p=0.0063$ ,  $df = 4$ ) (Table 2) and especially of 22:6(n-3) (Fig. 1).

The 22:6/20:5 (n-3) ratio changed significantly during maturation and in eggs ( $F=9.328$ ,  $p=0.0004$ ,  $df = 4$ ) (Table 2), the Student-Newman-Keuls *post hoc* test determined that the value of the 22:6/20:5 (n-3) ratio was significantly lower at stage 4 compared to stage 1 (Table 2) and stage 2 and the 22:6/20:5 (n-3) ratio in eggs was significantly lower compared to the gonad (Table 2). The 22:5/20:4 (n-6) ratio also decreased significantly in eggs ( $F=6.401$ ,  $p=0.0022$ ,  $df = 4$ ) (Table 2).

### 3.2. Sterols in the gonads and eggs

#### *Sterol esters*

The major sterol ester present in gonads and eggs was the cholesterol ester representing about 40 to 55 % of the sterol esters. Cholesterol ester diminished gradually significantly during the maturation process and was the lowest at stage 3 and 4, while in the eggs, cholesterol ester increased significantly ( $F=6.328$ ,  $p=0.0014$ ,  $df=4$ ) (Fig. 3).

#### *Free sterols*

Cholesterol and brassicasterol were the major free sterols present in the gonad and the eggs (Fig. 4). Among all the free sterols detected and quantified, the amount of cholesterol decreased significantly throughout the maturation process and the decrease was also seen in the eggs ( $F=7.614$ ,  $p=0.0005$ ,  $df=4$ ) (Fig.4). The level of cdihydro significantly increased in eggs ( $F=6.63$ ,  $p=0.0011$ ) (Fig. 4).

## 4. Discussion

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The main objective of this study was to determine the fatty acid and sterol composition of the female gonad and eggs of the pearl oyster *P. margaritifera*. During oogenesis of *P. margaritifera*, few changes were observed. The mass of gonad increased by the accumulation of reserves including lipids but the results showed that the proportions of the various neutral and polar fatty acids were stable during maturation; there was thus no preferential accumulation of certain molecules as in *Pecten maximus* (Duinker et al, 2004). But our results showed that the lipid compositions in gonad and eggs were

different in *P. margaritifera*. The saturated fatty acids, neutral and polar, were more abundant in eggs than in the gonad while the amount of polyunsaturated fatty acids was lower in eggs. Difference in lipid composition between gonad and eggs were previously observed in *C. gigas* (Robinson, 1992) for the saturated and monounsaturated fatty acids content but not for PUFA. Change of lipid profile from gonad to eggs in *P. margaritifera* could be due to the lipid composition of tissues composing the gonad such as haemoplasm, haemocyte and membrane of the gonadic tubules. Few data are available in this field.

The major molecules present in the gonad and the oocytes were palmitic acid 16:0 and 22:6(n-3). Fatty acids 14:0, 16:0 and most unsaturated C18s were highly used during *P. margaritifera* embryo development and as such, may be good indicators of egg quality (Acosta-Salmon, 2004). The prevalence of 16:0 and 22:6(n-3) has already been described in several bivalves including *Pecten maximus* (Soudant *et al.*, 1996), *Crassostrea gigas* (Soudant *et al.*, 1999) and *Argopecten purpuratus* (Caers *et al.*, 1999). However the content of these 2 fatty acids was highest in *P. margaritifera*. The 22:6(n-3) plays a major role in the structure and function of the cellular membranes involved in oogenesis and embryogenesis. Hatching rates could be associated with the content of 22:6(n-3) (Soudant, 1995). An important difference characterized in *P. margaritifera* was the higher level of 20:5(n-3) compared to species from temperate areas. The arachidonic acid content in *P. margaritifera* was similar with that observed in *P. maximus* (Soudant *et al.*, 1996a). The comparison of 22:6(n-3)/20:5(n-3) and 20:4(n-6)/ 20:5(n-3) ratios of the neutral and polar lipids indicated that the DHA and the EPA were selectively accumulated compared to the 20: 5(n-3). Moreover, the ratio 22:6(n-3)/20:5(n-3) would act as a nutritional indicator in *P. maximus* since the value of the ratio varies greatly according to the diet (Soudant, 1995).

Cholesterol is the major compound of sterol esters in the gonad of *P. margaritifera* as was found in *P. maximus* (Soudant, 1995). Sterols and their evolution conform with the pattern observed in other marine bivalves. Among free sterols, brassicasterol and cholesterol had similar proportions. The main change concerning the cholesterol content was a decrease during maturation. Such decrease was not thought to have resulted from food supply but probably related to maturation and egg formation. The oyster could act also by selecting or rejecting molecules specifically. Cholesterol plays a role to rigidify membrane structure and thus sterol content directly influences membrane fluidity (Irazu *et al.*, 1988). Esterified sterols are generally considered as a reserve, and used by embryo for membrane biosynthesis.

In order to provide pearl oysters with PUFA and sterols they require during conditioning for reproduction in hatchery, it is obvious that these elements be provided by food. The requirement of pearl oyster in 20:4(n-6) and 20:5(n-3) was not high, but these fatty acids are very essential. *Chaetoceros sp.* is able to provide 20:4(n-6) (Soudant *et al.*, 1996a, Renaud *et al.*, 1999, Martinez-Fernandez *et al.*, 2006), 22:6 (n-3) is more present in *T. Isochrysis* (Soudant *et al.*, 1996a, Martinez-Fernandez *et al.*, 2006). The main sterols in gonad of *P. margaritifera* were cholesterol and brassicasterol which are abundant respectively in *Chaetoceros calcitrans* and *T. Isochrysis* (Soudant *et al.*, 1998).

Our study allowed us to investigate the fatty acid and sterol composition of female *P. margaritifera* oyster gonads during their maturation and in oocytes. This knowledge could lead to a clearer understanding of the nutritional requirements of pearl oyster and could assist further adaptation of pearl oyster aquaculture to enhance the reproduction process. In particular, unicellular algae could provide the qualitative and quantitative nutritional needs in term of fatty acids and sterols during oogenesis of the oyster.

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## Tables

Table 1: Categories of fatty acids and ratios of essential fatty acids in the neutral lipids of female gonads and eggs of *Pinctada margaritifera* (% of total fatty acids). (mean±standard deviation, n=3-5 oysters)

Fatty acid categories	gonadal maturity index								eggs	
	1		2		3		4			
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
TO.SAT.	23.98 <sup>B</sup>	9.19	37.11 <sup>B</sup>	3.34	37.46 <sup>B</sup>	2.71	35.74 <sup>B</sup>	5.46	55.51 <sup>a</sup>	7.14
TO.DMA	0.59	0.49	0.11	0.10	0.44	0.17	1.43	2.46	1.19	1.72
TO.MONO	11.63	3.83	10.49	0.49	10.14	1.22	9.98	2.29	17.17	8.58
TO.(n-9)	4.32 <sup>b</sup>	0.54	4.91 <sup>b</sup>	0.12	4.76 <sup>b</sup>	0.54	5.27 <sup>b</sup>	0.41	6.51 <sup>a</sup>	1.67
TO.(n-7)	3.59	0.83	4.14	0.18	3.81	0.58	3.50	1.33	4.13	2.56
TO.POLY	53.12 <sup>a</sup>	4.76	50.78 <sup>a</sup>	2.71	50.79 <sup>a</sup>	3.74	51.78 <sup>a</sup>	3.37	34.41 <sup>b</sup>	21.72
TO.(n-4)	1.31	1.29	0.40	0.29	0.36	0.29	0.84	0.51	0.78	0.61
TO.(n-6)	6.52	1.37	4.73	1.42	5.65	0.60	7.20	1.51	7.43	2.89
TO.(n-3)	43.22 <sup>a</sup>	1.66	44.31 <sup>a</sup>	4.10	43.16 <sup>a</sup>	3.17	41.87 <sup>a</sup>	4.71	22.21 <sup>b</sup>	17.10
TO. NMI	1.98	1.62	1.18	0.24	1.47	0.38	1.60	0.98	3.89	2.61
(n-3)/(n-6)	6.79 <sup>a</sup>	1.94	7.97 <sup>a</sup>	0.76	7.69 <sup>a</sup>	0.68	6.09 <sup>a</sup>	1.69	2.75 <sup>b</sup>	1.94
22:6/20:5 (n-3)	7.60 <sup>a</sup>	0.62	7.73 <sup>a</sup>	0.43	7.57 <sup>a</sup>	0.71	7.02 <sup>a</sup>	1.53	5.32 <sup>b</sup>	0.65
22:5/20:4 (n-6)	1.08	0.42	0.71	0.09	0.66	0.09	0.62	0.22	0.38	0.11
20:4(n-6)/20:5(n-3)	0.23	0.34	0.22	0.14	0.31	0.04	0.42	0.18	0.72	0.42

Within each line, homogenous groups are marked with the same alphabetic letters.

Table 2: Categories of fatty acids and ratios of essential fatty acids in the polar lipids of female gonads and eggs of *Pinctada margaritifera* (% of total fatty acids) (mean±standard deviation, n=3-5 oysters)

Fatty acid categories	gonadal maturity index								eggs	
	1		2		3		4			
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
TO.SAT.	18.87 <sup>b</sup>	4.14	16.94 <sup>b</sup>	1.70	17.48 <sup>b</sup>	0.69	18.40 <sup>b</sup>	1.02	38.31 <sup>a</sup>	23.49
TO.DMA	5.24	5.88	7.75	5.73	5.38	4.16	10.61	2.29	2.48	1.89
TO.MONO	10.97	3.01	10.47	0.64	10.75	1.04	10.31	0.78	13.60	3.52
TO.(n-9)	4.21	2.62	4.57	2.14	4.26	1.63	5.59	1.81	5.47	2.31
TO.(n-7)	2.21	1.17	1.75	0.50	1.67	0.43	1.95	0.59	3.88	2.68
TO.POLY	63.72 <sup>a</sup>	5.84	58.85 <sup>a</sup>	2.27	58.99 <sup>a</sup>	2.09	59.05 <sup>a</sup>	1.92	39.89 <sup>b</sup>	20.55
TO.(n-4)	4.33	4.17	1.52	0.36	2.11	0.98	1.93	0.44	1.01	0.51
TO.(n-6)	10.95	2.87	10.35	0.36	10.16	0.33	10.53	0.55	8.37	3.14
TO.(n-3)	41.18 <sup>a</sup>	4.97	40.90 <sup>a</sup>	3.15	40.38 <sup>a</sup>	1.35	40.48 <sup>a</sup>	1.58	25.46 <sup>b</sup>	14.30
TO. NMI	7.13	1.97	5.90	0.82	6.18	0.34	5.95	0.34	4.87	2.85
(n-3)/(n-6)	4.01	1.26	3.96	0.40	3.98	0.14	3.85	0.25	2.84	0.84
22:6/20:5 (n-3)	6.81 <sup>a</sup>	0.48	6.71 <sup>a</sup>	0.48	6.42 <sup>ab</sup>	0.73	5.61 <sup>b</sup>	0.43	4.33 <sup>c</sup>	0.01
22:5/20:4 (n-6)	0.33 <sup>a</sup>	0.03	0.34 <sup>a</sup>	0.05	0.32 <sup>a</sup>	0.02	0.31 <sup>a</sup>	0.01	0.16 <sup>b</sup>	0.14
20:4(n-6)/20:5(n-3)	1.34	0.71	1.04	0.19	1.05	0.12	1.02	0.14	1.01	0.02

Within each line, homogenous groups are marked with the same alphabetic letters.



## Figures

Fig 1

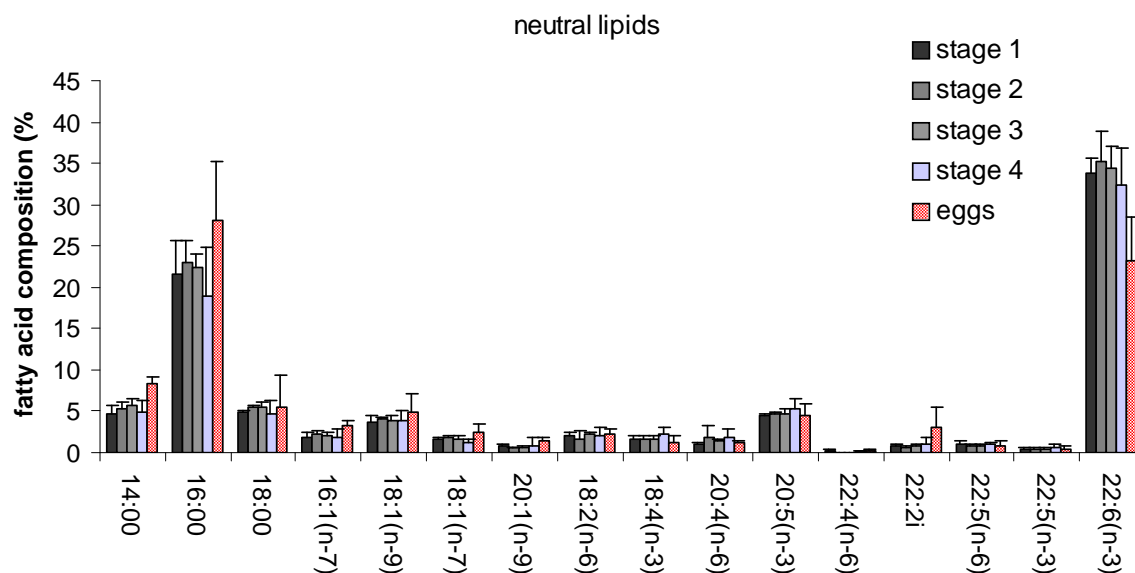


Figure 1: Simplified composition of neutral fatty acids in female gonads and eggs. The results are expressed as the percentage of total fatty acids of the fraction. (mean±standard deviation, n=3-5 oysters).

Fig 2

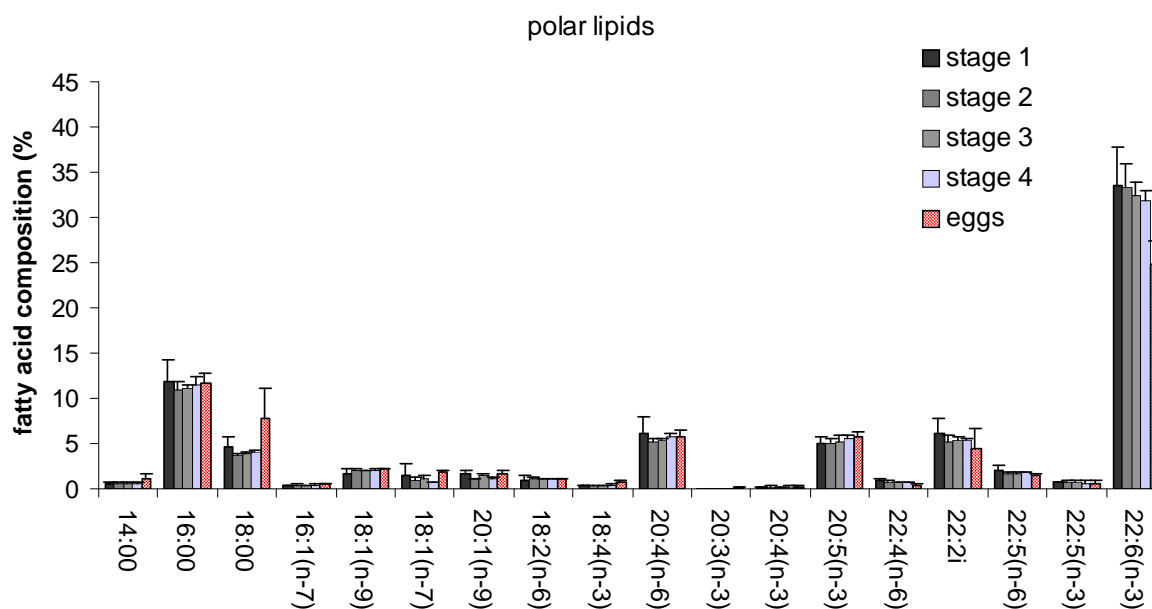


Figure 2: Simplified composition of polar fatty acids in female gonads and eggs. The results are expressed as the percentage of total fatty acids of the fraction. (mean±standard deviation, n=3-5 oysters).

Fig 3

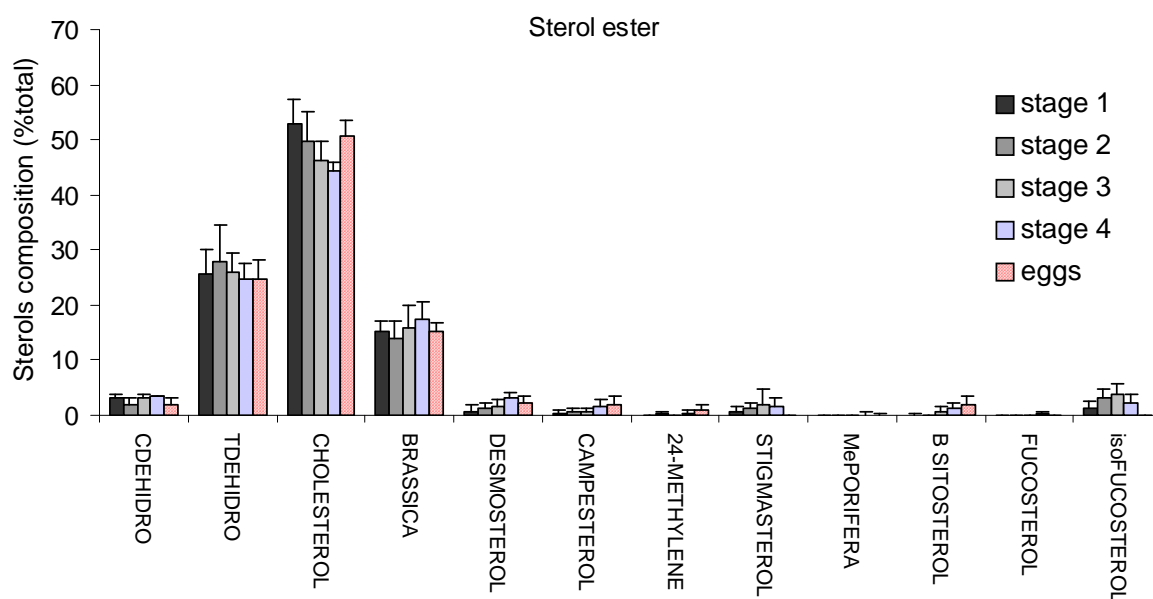


Figure 3: Composition of sterol esters in female gonads and eggs. The results are expressed as the percentage of total free sterol of the fraction. (mean±standard deviation, n=3-5 oysters).

Fig 4

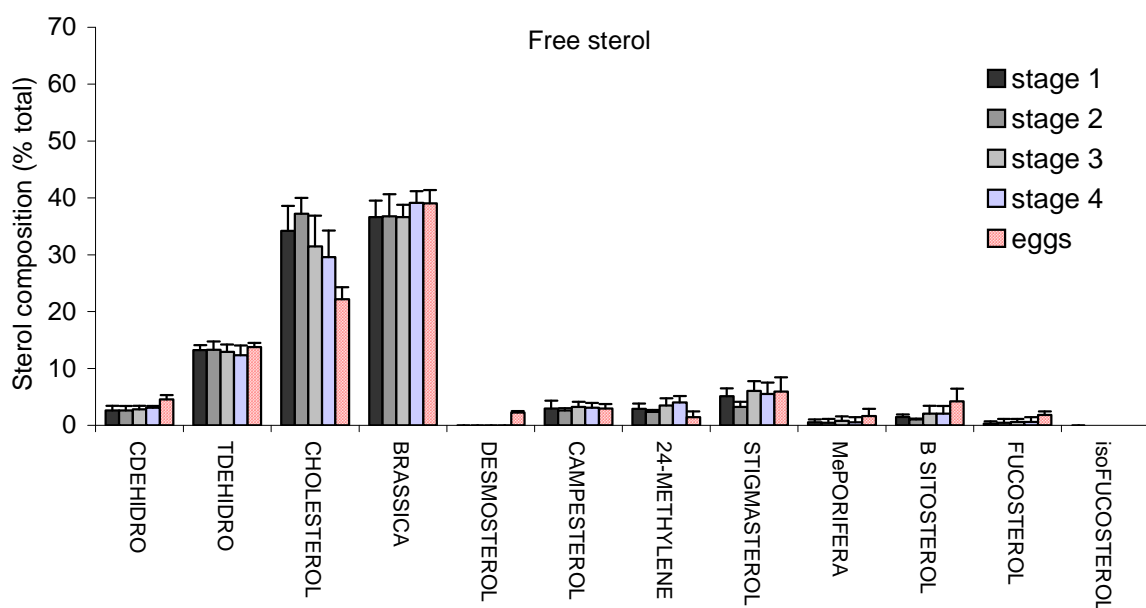


Figure 4: Composition of free sterols in female gonads and eggs. The results are expressed as the percentage of total sterol ester of the fraction. (mean±standard deviation, n=3-5 oysters).