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Oocyte and embryo quality in *Crassostrea gigas* (Portuguese strain) during a spawning period in Algarve, South Portugal

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Abstract — Egg quality is one of the most important factors determining larval viability. The study of oocytes released by 'wild' broodstock will contribute to the definition of quality criteria applied to hatcheries as well as being an indicator of the oyster reproduction potential and aquaculture ongrowing systems. The aim of this study was to determine the relationship between condition of *Crassostrea gigas* spp. *angulata* (Portuguese strain) from 'wild' broodstock (condition index, gonadal maturity and number of oocyte released), oocyte biochemistry and hatching rate of eggs during the spring/summer of 1995. Stages of gonadal maturity and condition index indicated that natural spawning of this species occurred between April and August with the highest spawning intensity occurring in June. Analyses demonstrated that *C. gigas* oocyte organic matter consist of 44–74 % proteins, 16–38 % lipids and 7–12 % carbohydrates. A significant relationship was observed between some biochemical parameters – organic matter and lipid content – and broodstock condition index and hatching rate. It seems that these two oocyte parameters (organic matter and total lipids) could be used to define quality of oocyte and thus larval viability. The oocyte protein content was also found to be associated with broodstock and larval quality. In summary, these data are a contribution to the knowledge of the reproductive cycle of oysters in Europe and may be useful in improving hatchery management of this species. © 1999 Ifremer/Cnrs/Inra/Ird/Cemagref/Éditions scientifiques et médicales Elsevier SAS

***Crassostrea gigas* / Portuguese strain / condition index / gonadal maturity / oocyte biochemical composition / larval viability**

Résumé — La qualité des ovocytes et des embryons de *Crassostrea gigas* (souche portugaise) pendant une période de ponte en Algarve, au Sud du Portugal. Les relations entre les caractéristiques des huîtres de souche portugaise, *Crassostrea gigas* spp. *angulata* sont étudiées en relation avec le profil biochimique de leurs ovocytes et le taux d'éclosion des œufs. Les géniteurs provenant d'un stock « sauvage » sont caractérisés par leur indice de condition, leur indice de maturité des gonades et le nombre d'ovocytes émis sous l'effet d'un choc thermique. Les teneurs des ovocytes en matière organique, en protéines, en lipides et en glucides sont estimées. Les ovocytes fécondés ont été placés dans des structures d'élevage et leur taux de développement a été calculé après 24 h. Des corrélations entre le niveau de maturité, la fécondité, les paramètres biochimiques et d'élevage ont été recherchées. Les résultats montrent que les émissions de gamètes sont maximales en juin. Les lots d'ovocytes analysés ont des teneurs en protéines qui varient de 44 à 74 %, des teneurs en lipides varient de 16 à 38 %, et les teneurs en sucres de 7 à 12 %. Les teneurs en matière organique et en lipides totaux sont corrélées de façon positive à l'indice de condition des reproducteurs et au taux d'éclosion des embryons. L'ensemble de ces données contribue à développer la connaissance du cycle de reproduction des huîtres en Europe et à préciser quels sont les indicateurs de la qualité de leurs produits sexuels. Ces indicateurs sont utilisables, d'une part pour améliorer la qualité des productions de larves en écloserie de mollusques et, d'autre part, pour évaluer le potentiel de reproduction des populations d'huîtres naturelles ou cultivées en zone littorale. © 1999 Ifremer/Cnrs/Inra/Ird/Cemagref/Éditions scientifiques et médicales Elsevier SAS

***Crassostrea gigas* / souche portugaise / indice de condition / maturité gonadique / composition biochimique des ovocytes / viabilité larvaire**

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1. INTRODUCTION

Techniques introduced by Loosanoff and Davis [18] and Walne [28] have made it possible to extend the spawning season of shellfish to all year round. However, results of experiments carried out with 'wild' and conditioned broodstock show a significant variability in larval survival. The oocyte quality and larval viability of laboratory-reared *Crassostrea gigas* spp. *angulata* (Portuguese strain) seems to depend on intrinsic broodstock characteristics, as well as on conditioning, spawning induction and larval culture [7].

Most of the published data on oocyte quality have concentrated on the lipid and fatty acid content of the gametes since it is generally accepted that vitelline reserves appear as lipids. A more complete biochemical characterisation of oocytes (total lipids, proteins and carbohydrate) may help to understand the relationship between environmental conditions and the accumulation of reserves and their transfer to the gonads. Correlation between such parameters, gametogenic cycles and larval survival may lead to a better understanding of the variability of oocyte and larval quality. Previous studies [6] on the effect of external and internal factors on hatchery success for breeding this species suggest it is important to define criteria for spawning and larvae quality and apply them to hatchery production.

In general, the biochemical content of bivalve oocytes, aimed at establishing spawning quality criteria, has only been characterised for conditioned broodstock [9, 17]. The study of oocytes released by 'wild' broodstock will contribute to the definition of quality indices. Such indices can be applied to hatcheries and aquaculture ongrowing systems and can also contribute to the knowledge of the oyster reproduction potential in Europe.

The aim of this work is to define criteria based on biochemical indicators, which can be used in the prediction of spawning quality in hatcheries.

2. MATERIALS AND METHODS

2.1. Broodstock sampling and characterisation

Samples were collected every 15 days between April and September 1995 from Ria Formosa (Algarve, South of Portugal) so as to obtain data corresponding to the spawning period [27]. Each sample comprises 50 individuals, of which ten were used to determine the condition index, ten to study gonadal development and the remainder were kept in the laboratory for spawning induction.

Condition index (CI) was calculated according to Walne [29]:

CI = dry meat weight (g) × 1 000/internal shell cavity volume (mL)

Gonadal development was evaluated by macroscopic observation of ten oysters using the following gametogenic scale (adapted from Vilela [27]): stage 1) immature gonad, stage 2) gonad almost mature, stage 3) mature gonad, stage 4) partial spawning, stage 5) spawning completed. The sex ratio was determined by microscopic observation of gametes.

2.2. Spawning, oocytes and larval sampling

Oysters were induced to spawn by thermal stimulation, with temperature increasing from 20 to 30 ± 1 °C. To avoid uncontrolled fertilisation, females once identified were stored in individual containers for spawning.

From each female, three samples of 50 000 oocytes were taken, rinsed with iso-osmotic ammonium formate (3 % w/v) to remove salt, frozen and stored in liquid nitrogen for future lyophilisation and biochemical analysis.

The remaining oocytes were fertilised by addition of a mixture of sperm from various males, to provide a ratio of ten spermatozoa per oocyte. One hour later, the fertilisation rate was determined based on two 1-mL aliquots.

The embryos from each female were incubated in triplicate 5-L tanks, with filtered and UV-irradiated seawater, maintained at 22 ± 2 °C, at a density of 100 eggs per mL. After 24 h, the normal (D-larvae) and abnormal (misshapen or exhibiting defective swimming) veliger rates relative to the initial number of eggs (hatching rate) were calculated based on two 1-mL aliquots per tank.

At the beginning of September, only a limited number of oocytes was spawned; therefore, no studies on hatching rate were possible. In late September no spawning occurred.

2.3. Biochemical composition of oocytes

Lyophilised oocyte samples were fractionated according to Holland and Gabbott [13].

Protein concentration was determined using the Lowry et al. [19] method as modified by Bensadoun and Weinstein [2] and Hess et al. [11] using bovine serum albumin as a standard. A volume of 450 μL of reagent I (25 μL 0.8 N NaOH + 18 mL reagent A + 2 mL reagent B; reagent A: 0.2 g $C_4H_4O_6Na_2$ + 10 g Na_2CO_3 + 69 mL 0.8 N NaOH made up to 100 mL with distilled water; reagent B: 2 g $C_4H_4O_6Na_2$ + 1 g $CuSO_4$ + 12.5 mL 0.8 N NaOH made up to 100 mL with distilled water) and 300 μL of reagent C (Folin-Colciteau reagent [19]/distilled water (1:2)) were added to 50 μL of protein sample. The samples were shaken and a further 300 μL of reagent C were added. After shaking, samples were incubated in darkness for 45 min. Absorbance was determined at 750 nm.

Total lipids were analysed according to Marsh and Weinstein [20] using tripalmitine as a standard. Samples (50 μL) of chloroform containing lipid extracted from the oocytes were dried for 20 min at 37 °C. Then, 500 μL of H_2SO_4 were added, the samples shaken and heated for 15 min at 200 °C and

1.5 mL of water were added before the absorbance was determined at 375 nm.

Carbohydrate concentration of oocytes was determined according to Holland and Gabbott [13] using glucose as a standard. Samples (200 µL) of the trichloro-acetic acid supernatant from the fractionation were heated at 95 °C for 10 min with 500 µL of 0.625 % NaOH, 100 µL of 0.2 % potassium ferricyanide and 200 µL of sodium carbonate/cyanide reagent. The samples were cooled and 500 µL of water added. Samples were then centrifuged for 10 min at 800 *g*. The absorbance of the supernatant was determined at 420 nm. The organic matter was the sum of proteins, total lipids and carbohydrates. From each of the triplicate of 50 000 oocytes two samples were used for analysis of biochemical composition with the exception of total lipids where a single sample was measured. All data are expressed as mean values per sampling.

2.4. Statistical analysis

A statistical analysis ANOVA (one-way) was performed ($P < 0.05$) to assess whether there were significant differences in normal veliger hatching rate among triplicate samples for each female (total of 28 females).

The experimental results for normal and abnormal veliger hatching rates and gross biochemical composition of oocytes were analysed by the ANOVA procedure using the SAS statistical package [24]. Prior to analysis, data expressed as percentages were converted to arcsine values [25].

The relationships with a significance level higher than 95 % were represented by linear regression.

3. RESULTS

3.1. Broodstock characterization

Figure 1a shows the seawater temperature during the experimental period, and the stages of gonadal maturity during the experimental period were shown in *figure 1b*. Water temperature and the percentage of oysters with stage 4 gonads appear to be correlated. A temperature decrease observed during early July separated two distinct spawning periods: a first intense spawning followed by a second less important one.

Microscopic observations showed that gonadal development in males and females was synchronous. Spawning occurred from April to August with the highest intensity in June.

The sex ratio (*figure 1c*) was approximately 1:1 until August, except in late May when 83 % of the animals were females. During September, the number of females decreased and the number of undetermined oysters increased.

Variation in the condition index of oysters is shown in *figure 1d*. Generally, mean values decreased from April to October. The highest (192 ± 61) and the lowest (52.5 ± 18.6) values were recorded at the end of May and September, respectively.

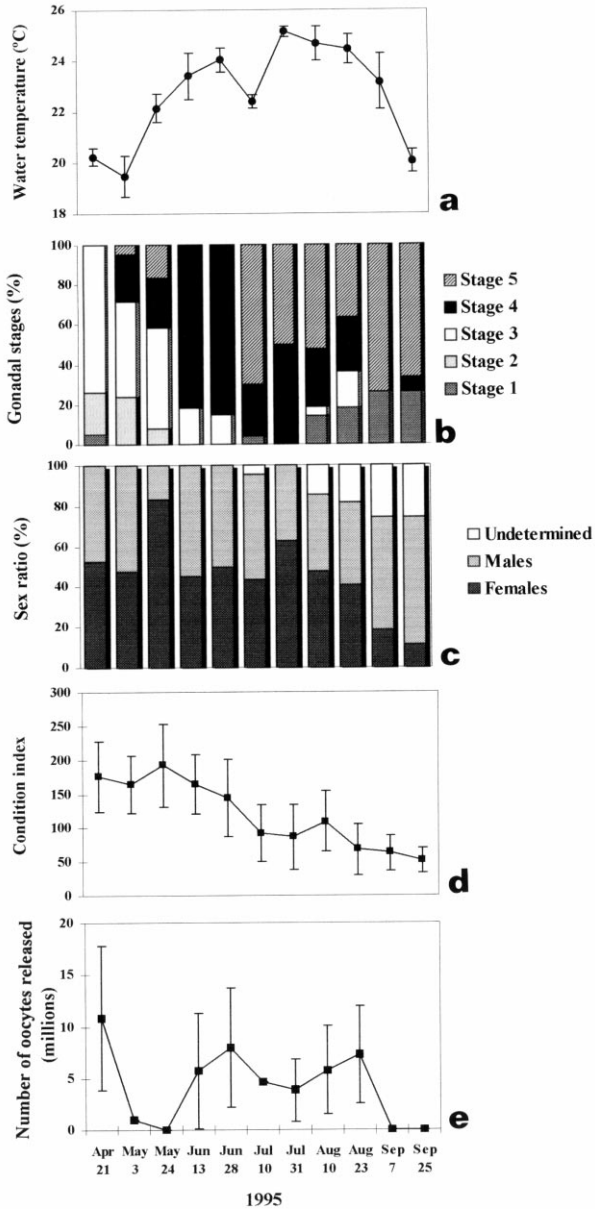

Figure 1. *Crassostrea gigas* (Portuguese strain) broodstock characterisation during the experimental period: a) water temperature, b) stages of gonadal maturity, c) sex ratio, d) condition index, and e) mean number of oocytes released. Error bars represent standard deviations.

Peaks in the maximum number of oocytes released (*figure 1e*) were observed in April (11 ± 7 million), late June (8 ± 6 million) and late August (7 ± 5 million). The large standard deviations are due to the partial spawning behaviour of this species. Data from the late May sampling are related to sex ratio (females were dominant (83 %)).

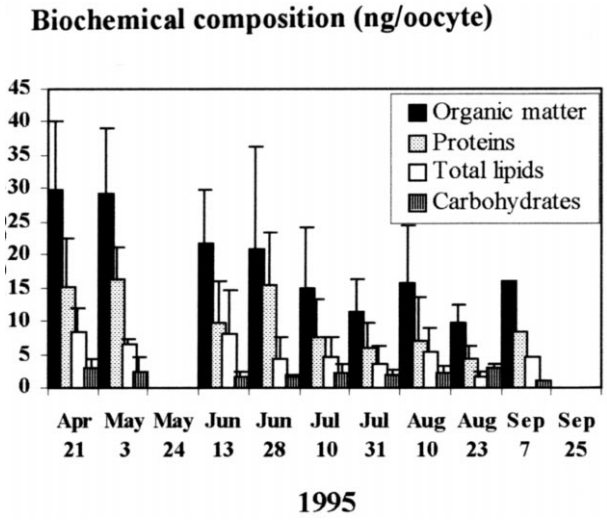

Figure 2. Mean variation in oocyte biochemical composition of *Crassostrea gigas* (Portuguese strain) oocytes (organic matter, proteins, total lipids and carbohydrates) during the experimental period. Error bars represent standard deviations.

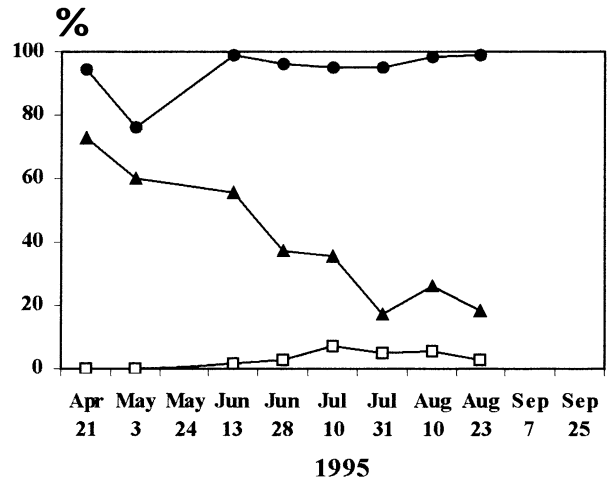

Figure 3. Variation of *Crassostrea gigas* (Portuguese strain) fertilisation, normal and abnormal veliger rates during the experimental period. —▲—, normal veliger rate; —□—, abnormal veliger rate; —●—, fertilisation rate.

3.2. Biochemical composition of oocytes

Changes in the biochemical composition of oocytes (organic matter, proteins, total lipids and carbohydrates) during the experimental period are shown in *figure 2*. Generally, the organic matter decreased during the sampling period, except in early August and September. The highest values (29.9 ± 9.4 and 29.3 ng·oocyte^{-1}) were recorded in April and May and the lowest (9.7 ± 2.6 ng·oocyte^{-1}) were recorded in late August. Protein was the predominant dry oocyte constituent (44–74 %) followed by total lipids (16–38 %) and carbohydrates (7–12 %).

The statistical analysis (ANOVA) showed significant differences ($P < 0.05$) among sampling dates for all biochemical compounds during the experimental period. However, among females from the same sampling significant differences were not found, except for proteins.

3.3. Larval viability

During the experimental period, fertilisation rate did not vary. However, the hatch rate of normal veliger decreased (*figure 3*). The percentage of abnormal veligers was generally very low and was recorded only from June to the end of the experimental period.

In seven out of a total of 28 females, significant differences were found between the triplicate samples set up to measure hatching rate of normal veligers from each female ($P < 0.05$). Using ANOVA significant differences were found ($P < 0.05$) between temporal samples and between females from the same sampling.

3.4. Relations broodstock, oocytes and larvae

Condition of broodstock is related to oocyte biochemical composition, which in turn affects larval viability ($P < 0.05$). Condition index also shows an important relationship to the normal veliger hatching rate. The two most important relationships were found between oocyte organic matter/normal veliger rate and oocyte total lipids/normal veliger rate (*table I*). The oocyte carbohydrates constituted the single factor not related to any of the studied parameters.

4. DISCUSSION

Inherent physiological and biochemical variability among bivalve larvae can always be expected, and understanding the source of this variability is crucial to reducing it in culture situations. Broodstock condition and consequently oocyte quality are important factors determining larval viability.

Stages of gonadal maturity, condition index and number of oocytes released have allowed characterisation of the spawning period of this species in Ria Formosa and the fluctuations that occurred during spring/summer 1995, from April to August with the greatest spawning activity in June. The large seasonal variation in spawning found in the present work may be a consequence of variations in environmental conditions as discussed by Fernandez Castro and Vido De Mattio [10]. During the present study, the temperature variations appeared to be associated with two distinct phases of spawning, the first more intensive and the other, despite the high water temperatures, was less intense. These seemed to be associated with maturation of different oocytes cohorts.

Table I. Relationship between condition of broodstock, oocyte biochemistry and hatching rate of eggs to the experimental period, represented by equations obtained by linear regression ($P < 0.05$)

X	Y	Equation	R	n
Condition index	organic matter*	y = 0.115x + 5.939	0.656	9
Condition index	proteins*	y = 0.064x + 2.927	0.584	9
Condition index	total lipids*	y = 0.04x + 0.845	0.741	9
Condition index	normal veliger rate	y = 0.367x – 2.46	0.718	8
Proteins*	normal veliger rate	y = 3.372x + 5.922	0.79	8
Total lipids*	normal veliger rate	y = 7.757x – 0.916	0.9	8
Organic matter*	normal veliger rate	y = 2.537x – 8.344	0.951	8
Organic matter*	abnormal veliger rate	y = - 0.268x + 8.294	0.795	8

* Of oocytes.

Condition index decreased during the experimental period and the range of values recorded (from 192 ± 61.2 to 52.5 ± 18.6) is in agreement with that reported for *Crassostrea gigas* [3], in British Columbia (Canada). Comparison of the results from the present study with those from different latitudes [8, 26] confirms the fact that timing and duration of gametogenesis of *Crassostrea* vary according to location. This variation in reproductive strategy can be considered as an adaptation to environmental conditions.

The unusually high percentage of females (83 %) found in late May may have been caused by the phenomenon of sex change. However, further histological analysis will be required to confirm this hypothesis. The small proportion of oysters that spawned in late May may be associated with sex ratio. According to Paniagua-Chávez and Acosta-Ruiz [21] spawning depends on sex ratio with an optimum of 1:1.

Condition index, the biochemical composition of the oocytes (proteins, total lipids and carbohydrates) and the normal veliger rate decreased during the experimental period. This showed that the partial spawning behaviour of *C. gigas* (Portuguese strain) may be a source of variability in oocyte quality and consequently in normal veliger rate. Lee and Heffernan [16] and Widowati et al. [31] have also identified a similar phenomenon in other species of oysters.

The relative amount of protein (44–74 %), total lipids (16–38 %) and carbohydrate (7–12 %) measured in *C. gigas* (Portuguese strain) oocytes were similar to those of other bivalve oocytes [12, 15, 16].

The relationship between condition index and lipid content of oocytes suggests that condition index is related to de novo synthesis of lipid during gametogenesis as stressed by Costa Muniz et al. [4] and Fernandez Castro and Vido De Mattio [10]. Several authors have shown that broodstock lipids increase before spawning and decrease immediately after spawning, probably as a result of losses of lipid in spawned oocytes, leading to a decrease in the condition index of the adults [22, 23, 30].

The interactions found in this study between condition index, total lipids and normal veliger rate suggest that larval survival is highly dependent on the initial quantity of endogenous total lipid supplied to the oocytes by the parents during vitellogenesis. This has been demonstrated in *Pecten maximus* [9, 17]. Also Bayne et al. [1] show that lipids of unfertilised mussel eggs were correlated directly with early larval development. On the other hand, the positive relationship found between organic matter and the normal veliger rate and the inverse one with abnormal veliger rate indicates that in the present study oocyte energy was sufficient to allow normal development.

Studies showing the importance of oocyte protein on larval viability are lacking. The relationship between oocyte protein and normal veliger rate, found in the present study, suggest the importance of protein synthesis on normal veliger development. However, further studies are necessary to determine the real importance of oocyte protein on early larval viability.

The absence of a relationship between oocyte carbohydrate and normal veliger rate may be related to its reduced importance in embryo development compared to that for juveniles. According to the *Ostrea edulis* study [14], there is no evidence of carbohydrate (particularly polysaccharide material) being used as an energy source at the larval development stage.

The problem of understanding larval development, growth and survival is essential for the expansion of this area of aquaculture [5]. This study of oocytes released by 'wild' broodstock is a contribution to the definition of quality criteria applied to hatcheries as well as being an indicator of the 'wild' oyster reproduction potential in Europe and aquaculture ongrowing systems.

This study provides a valid control for the time of year corresponding to the spawning period. From the biochemical parameters studied, the organic matter and the lipid content of spawned oocytes must be taken as the most important ones for indicating potential viability of D-larvae.

From a practical point of view, the organic matter can provide a valid and easy indication of oocyte quality. As a more precise indicator of physiological condition, lipids must be considered, since it has been shown that this parameter is highly indicative of larval viability.

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