Development of a brown internal coloration in the scallop shell *(Pecten maximus)*: Study of microstructural characteristics and analyses of crystal organic matrices

by

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ABSTRACT

Calcification abnormalities linked to a brown coloration of the internal layer of the valves have been observed on scallops – *Pecten maximus* – in different rearing sites of Brittany.

Scanning electronic microscopic (SEM) observations demonstrate that the brown pigmentation is associated with various modifications of the foliated microstructure of the internal layer of the shell.

Very brown shells have been compared to healthy white shells with different methods. According to the amino acid analyses, the production of the soluble organic matrices appears to be preserved in the diseased shells. A small but significant decrease in the aspartic acid content has been observed in the insoluble organic matrix of the brown shells. The Ca content is the same in the two types of shells, but modifications of the Na, Mg, P, S and Zn contents have been noticed. Moreover, the preparative process for the organic matrices study demonstrates that the red brown pigmentation is exclusively located in the insoluble matrices. Finally, even after HCl hydrolysis for amino acid study, a deep brown residual can still be noticed,

demonstrating the high chemical resistance of the brown red pigment. More than a disturbance in the calcification process itself, the crystal disturbance within the internal brown layer of shells appears to be due to the production of a brown compound clearly associated with the crystalline envelopes.

INTRODUCTION

During the last decade, an important development of brown-red areas in the internal layer of scallop shells – *Pecten maximus* – has been observed in Brittany (France) by the IFREMER staff of the scallops seeding survey (DAO, in press). This usual old shell phenotype is widespread today in younger scallops (from 2 to 3 years old) especially in shallow waters and in ecologically instable places, and is often associated with important winter mortalities.

The appearance of such a brown coloration in the internal side of bivalve shells has already been reported in many species. This phenomenon consists in two main types of shell modifications. The deposit of brown organic membranes has been observed in association with parasite infestation (BARTOLI, 1976; BLAKE and EVANS, 1973) or bacterial diseases (GOULLETQUER *et al.*, 1989; GETCHELL, 1991; PAILLARD, 1992; PAILLARD and MAES, 1994), or in stressful rearing conditions (MORI, 1975; MARIN and DAUPHIN, 1992; PALMER, 1980). Other authors (JOHANNESSEN, 1973; PALMER, 1980; PASS *et al.*, 1987) have recorded the presence of a brown calcified material among more or less ecologically disturbed bivalves. According to the literature, the production of the brown material seems then to be linked to poor ecological conditions or to pathological disturbances wich affect the formation of the shell by the mantle edge.

On the other hand, calcification abnormalities have been related to poor rearing conditions and to water pollution (GOULD and FOWLER, 1991). For instance, a deformation of the shell of the scallop *Argopecten irradians* cultured in a recirculating seawater system is reported by EPIFANIO (1976) and seems to be due to an inadequate diet; chamber formation in *Crassostrea gigas* is generated by TBT antifouling paints (ALZIEU *et al.*, 1981); a modification of the shell structure in *Anodonta cygnea* is associated with diflubenzuron pollution (MACHADO *et al.*, 1990). Therefore, the study of the brown coloration of *Pecten maximus* shells was investigated, in order to check if this phenomenon was the sign of a calcification disturbance and to search for an health indicator for scallop spawners. The other objective of the study was to try to detect the parameters which can induce such calcification abnormalities in *Pecten maximus* shells. This phenomenon might be later considered as a biological index of ecologically unbalanced systems. This paper presents the results concerning the calcification disturbances associated with the brown coloration of the internal side of the shells.

MATERIAL AND METHODS

Microstructural analyses

10 white valves from the Bay of the Seine river, 10 very brown valves and 14 valves showing just light brown spots from the Bay of Brest were selected for microstructural study (for the description, see Figure 1). All the shells were from 4 to 5 year old scallops dredged in the autumn.

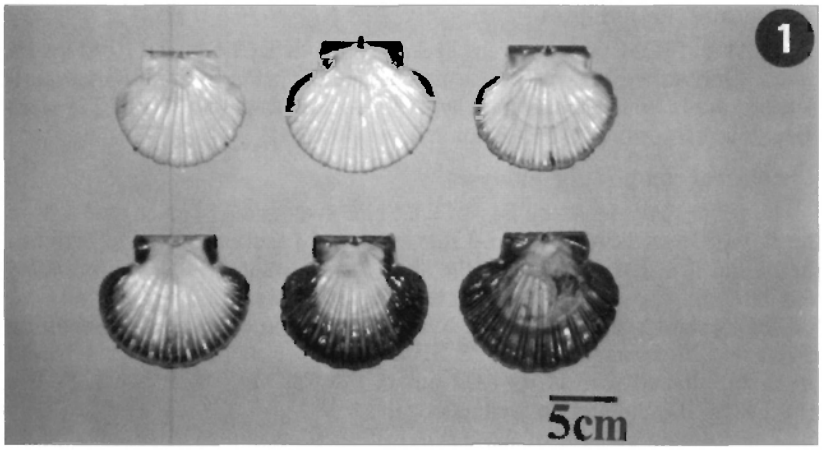

Figure 1 - Right valves of *Pecten maximus* showing increasing brown color development.

Pieces of shells were sampled between the pallial line and the edge of the valve for observation of the natural white or brown internal layer. Transversal sections sampled in the same part of the shell were polished and etched with a solution of 12% glutaraldehyde, 1% acetic acid, 0.05% alcyan blue (in the proportions 98, 1, 1) for 6 or 7 minutes. After freshwater and ultrasonic rinses, dry fragments or sections were coated with gold-palladium for microstructural observations with a scanning electronic microscope (PHILIPS SEM 505).

Colorimetric study

For a first trial, reflectance properties of five pieces of shells showing different steps of brown color intensity were analysed with a spectral analyser (Zeiss PMQ II) from 450 to 1000 nm.

Organic matrices analyses

Analysed shells provided from 5 year old scallops dredged in the autumn. 10 shells were selected in the Bay of the Seine river sample and 10 very brown shells in the Bay of Brest sample. These shells were prepared according to the method cited in the publication of DEVAUCHELLE *et al.* (in press).

Soluble and Insoluble Organic Matrices (SOM and IOM) isolated from 2500 mg of each powdered shells were lyophilisated for storage at room temperature.

Chromatographic analyses

HPLC analyses were performed on a TSK PW 3000 column at a low pH mobile phase (Citrate 0.025 M, NaCl 0.3 M, pH 3.1). The column was calibrated with BSA, Ovalbumine and Chymotrypsinogen under the same acidic phase.

5 samples of SOM isolated from brown shells and 5 samples of SOM isolated from white shells were dissolved in 500 μl of the mobile phase. 30 μl of each of these solutions were injected in the column.

Amino acids analyses

SOM and IOM samples were hydrolysed with HCl 6 N at 110°C for 24 hours. Derivatization was performed with PITC (Phenylisothiocyanate). Amino acids were separated with a reversed phase HPLC system (Beckman) according to an Acetonitrile gradient.

Mineral composition analyses

11 white shells from the Bay of the Seine river and 11 brown shells from the Bay of Brest were selected. Little transversal sections sampled between the pallial line and the edge of the shell were mounted on stubs, polished and carefully rinsed with demineralizated water, then dried and carbon coated for mineral analyses with an X-ray microprobe coupled to a Scanning Electronic Microscope (PHILIPS SEM 505). Twelve points were selected on each polished section for analyses of the Na, Mg, Al, P, S, Cl, K, Ca, Mn, Fe, Sr, Ba, Pb, and Zn contents.

The calcium content of the 2 types of shells was also checked with an atomic absorption analysis.

RESULTS

Microstructural observations

The internal layer of healthy white shells or the white areas in brown shells show parallel, flat elongated blades regularly fan-arranged in large groups usually oriented to the growing edge of the shell (Figure 2A).

The modifications of this regular structure in brown calcified areas are more or less severe. Distorted crystals have irregular margins and growing surfaces, usually with pitted or split extremities (Figures 2B and 2C). The crystallisation process seems to be reduced to the formation of isolated granules (Figures 2C and 2D): giving an aspect of anarchic growth and the microstructural units cannot be recognized. This form of crystallisation could result in the formation of a very disorganised fibrous growing system (Figure 2E). However, the disappearance of crystal lateral limits and merging of adjacent units can give a compacted aspect to the internal surface (Figure 2F). SEM observations of the internal shell layer demonstrate that the abnormal pigmentation is associated with various modifications of the foliated microstructure. Etched sections of white and brown shells have the same structure. The limits between white and brown parts cannot be detected structurally in the brown shells.

Very different stages of microstructural modifications can be seen on deeply brown shells or on light brown spots in white shells. Then, there seems to be no relationship between the intensity of the microstructural disturbances and the intensity of the brown coloration.

Colorimetric study

Reflectance decreases with increasing brown color intensity (Figure 3). The decrease is particularly high between the healthy white shell and the light brown one from 450 to 600 nm.

Organic matrices analyses

* SOLUBLE ORGANIC MATRIX

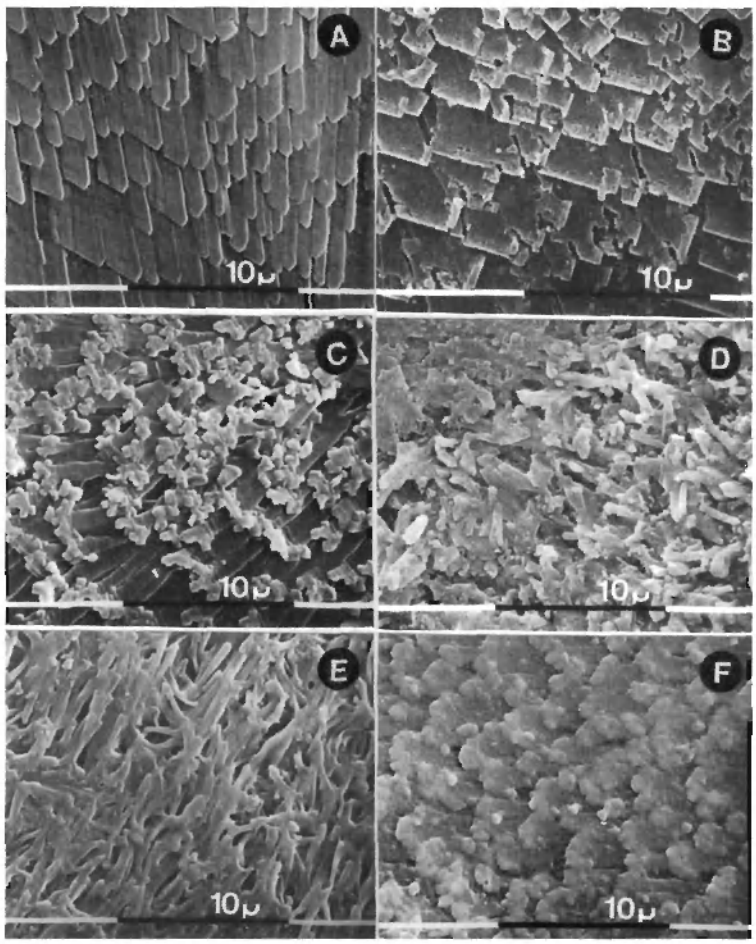

Figure 2 – The calcitic foliated microstructure of the internal layer of *Pecten maximus* shells.
A: Healthy white shell.
B to F: The different modifications observed in the brown shells.
B: The distorted crystals with pitted extremities.
C: The distorted crystals with irregular marging and splitted extremities.
D: The crystal anarchic growth.
E: The very disorganized fibrous growing systems.
F: The disappearance of crystal lateral limits and merging of adjacent units.

– *Chromatographic analysis*

Individual elution profiles are too variable to establish any global results about the molecular composition of the SOM and all the more to establish any difference between the brown and white shells. It can only be observed that the SOM of brown and white shells is mainly constituted of 2 major molecules: one about 100 to 150 kDa and the other about 20 to 25 kDa, according to our calibration. The use of the acidic mobile phase is more convenient to separate the different types of molecules of the SOM, compa-

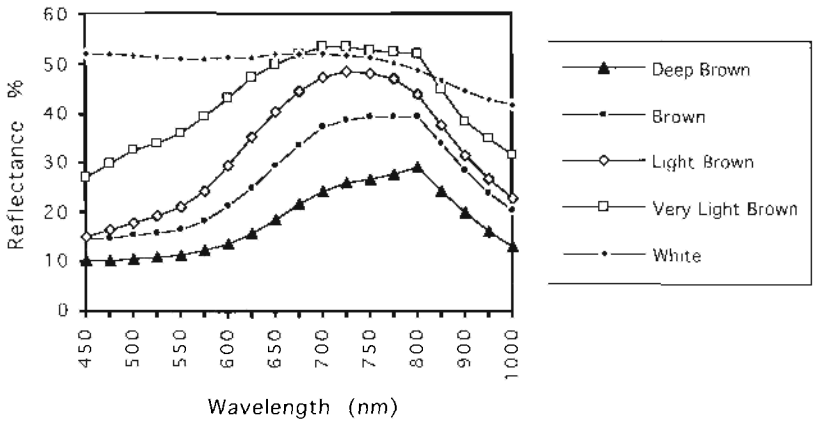

Figure 3 – Spectral analysis of shells showing increasing brown color intensity.

red to the HEPES neutral mobile phase, but it generates some problems with column calibration. Some of the standards – such as Aldolase, Catalase or Ribonuclease – exhibit elution profiles which do not correspond at all to their molecular weights.

As a consequence, HPLC analyses of this special acidic material (pI 3.2, calculated according an extension of the method of SILLERO and RIBEIRO (1989) using the amino acid composition of the SOM) requires a more precise methodological research to give any valid results. Other chromatographic study trials (chromatofocusing and ion exchange chromatography) which have not been presented herein, suggest the same conclusions.

– Amino acids analyses

SOM of brown and white shells are composed of 4 major amino acids: aspartic acid, serine, glycine and glutamic acid which represent more than 80% of the total amino acid content (Figure 4), but the results dispersion is very high (Figure 5.1 and 5.2). No significant difference has been pointed out between the amino acid content of brown and white shells SOM except in the serine content (P = 0.05).

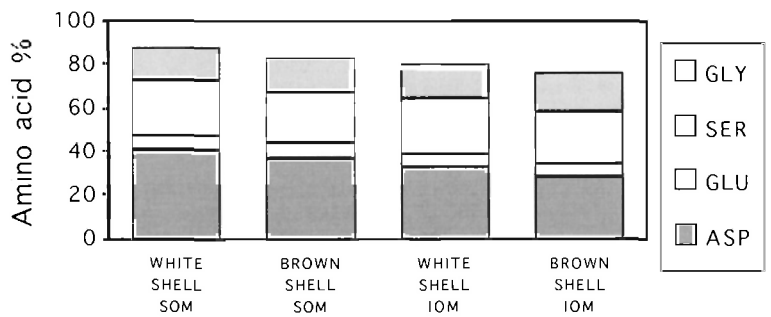

Figure 4 – Cumulative histograms of the average values of the main amino acids of the Soluble and Insoluble Organic Matrices of brown and white shells.

* INSOLUBLE ORGANIC MATRIX

The preparative process for the organic matrices study shows that the brown pigmentation is exclusively located in the insoluble matrix of the shell. An insoluble deep brown residual can still be observed after HCl hydrolysis for amino acid study.

On the other hand, the amino acid analysis of the IOM does not show much difference between the white and brown shells. A small but significant (P = 0.05) decrease in aspartic acid is observed in brown shells (Figure 5.3 and 5.4). The scatter of the results is as high as for the SOM, and the principal amino acids are the same ones.

* SOLUBLE AND INSOLUBLE ORGANIC MATRICES COMPARISON

SOM and IOM of *Pecten maximus* shells have already the same amino acid composition (Figure 5.1 to 5.4). Significant (P = 0.05) differences can only be seen in the aspartic acid, arginine and lysine contents: a decrease in the aspartic acid content and an increase in arginine and lysine content can be noted both in the IOM of white and brown shells. A slight tendancy to aspartic acid -or to principal amino acids- decrease can be noticed from white shell SOM to brown shell IOM (Figure 4).

Mineral composition analyses (Table I)

Significant differences (P = 0.001) have been pointed out between white and brown shells: an increase in the P content and decreases in the Na, Mg, S, and Zn contents have been observed in brown shells. The decrease in the Ca content has not been confirmed by atomic absorption analyses.

TABLE I

Average values of the mineral composition of brown and white shells and statistical results concerning the differences observed.

	Na	Mg	Al	Si	P	S	Cl	K	Ca	Mn	Fe	Sr	Ba	Pb	Zn
Brown Shells															
X (n=130)	**.35**	**.11**	.10	.23	**.12**	**.17**	.04	.00	**39.03**	.02	.04	.12	.04	.02	**.10**
σ	**.14**	**.06**	.05	.10	**.07**	**.08**	.03	.01	**1.56**	.03	.04	.05	.04	.02	**.09**
White Shells															
X (n=130)	**.52**	**.16**	.11	.23	**.08**	**.21**	.04	.00	**39.42**	.01	.04	.12	.03	.02	**.15**
σ	**.18**	**.13**	.06	.15	**.04**	**.08**	.03	.01	**1.24**	.03	.04	.05	.04	.02	**.14**
Statistical results	***	***	NS	NS	***	***	NS	NS	*	NS	NS	NS	NS	NS	***

DISCUSSION

Disturbances in shell microstructure have already been pointed out in bivalves cultured in poor ecological conditions. Microstructural alterations of the nacreous layer have been observed in diseased black lip pearl oyster *Pinctada margaritifera* (MARIN and DAUPHIN, 1992). PREZANT and CHALERMWAT (1983) reported that trophic and temperature conditions may act synergistically to alter the normal internal shell microstructure of *Corbicula fluminea*. Such observations have also been made in fasting scallops, in our laboratory (DEVAUCHELLE *et al*, in press). In our case, the microstructural modifications observed on brown shells could then be a response to ecological disturbances.

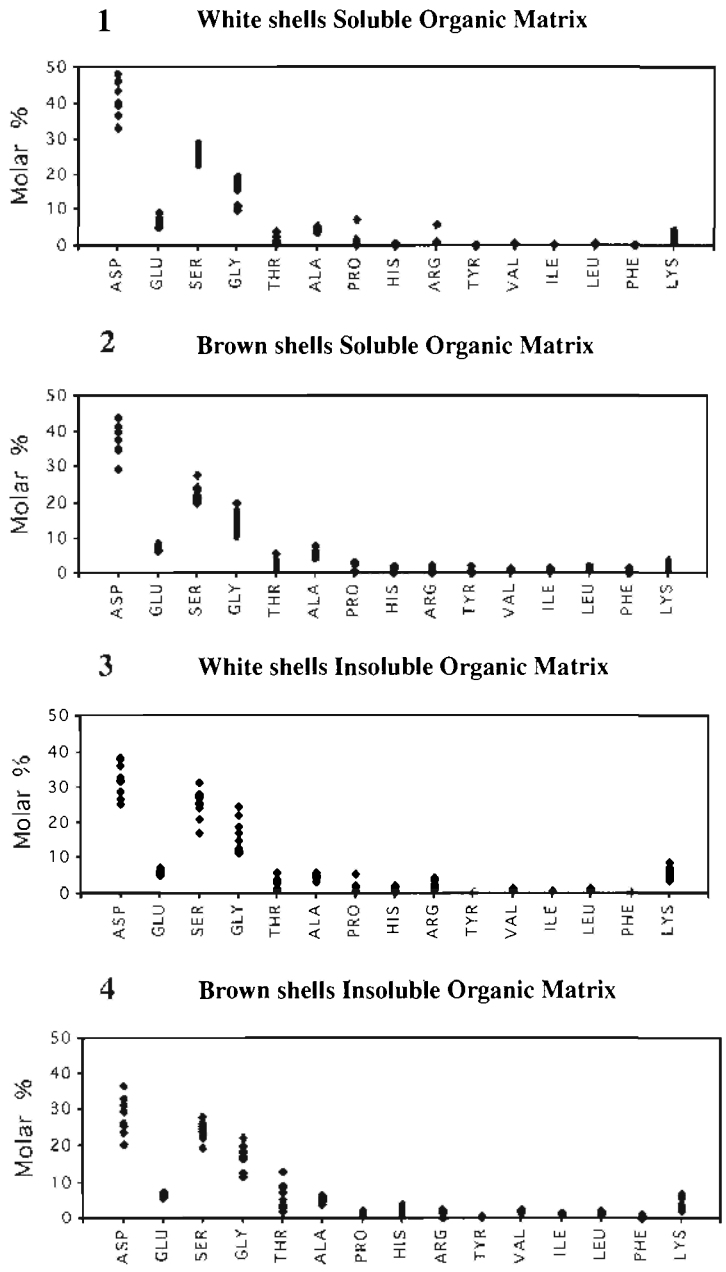

Figure 5 – Amino acids composition of the Insoluble and Soluble Organic Matrices of brown and white shells. 1: Soluble organic matrix of white shells. 2: Soluble organic matrix of brown shells. 3: Insoluble organic matrix of white shells. 4: Insoluble organic matrix of brown shells.

The study of the reflectance characteristics of the brown pigment associated with the insoluble matrix of the shell gave interesting results, which must be improved. It seems to be a practical method for detecting the presence of the brown compound, even when it cannot be detected with the naked eye. The spectroscopy of the intact shell had already been suggested by COMFORT (1951) as the only technique to study the visible spectrum of the non-extractable pigments of molluscan shells.

The production of a brown material such as brown membranes or brown calcified parts in the internal layer of the shells has been usually related to parasite infestations (JOHANNESSEN, 1973; BARTOLI, 1976; BLAKE and EVANS, 1973, GETCHELL, 1991), to bacterial disease (PAILLARD, 1992; PAILLARD and MAES, 1994) or to bad living conditions (MORI, 1975; PALMER, 1980; PASS *et al.*, 1987; MARIN and DAUPHIN, 1992). This could also be the case in our study because the rearing site where the very brown shells have been sampled is well known for its ecological instability. Some analyses run on these materials have demonstrated that the appearance of the brown color and the associated shell deformations were related to modifications of the amino acid composition of the organic matrices of the shell. For instance, GOULLETQUER *et al.* (1989) have compared the normal shell and the brown deposit in *Ruditapes philippinarum*, MARIN and DAUPHIN (1992) have analysed the brown membranes and the brown stained nacreous layer of *Pinctada margaritifera*. In an other study, KRAMPITZ *et al.* (1983) showed that the composition of the organic matter of deformed oysters *Crassostrea gigas* is different, compared to healthy normal shells. Different conclusions emerged from our work. The organic fractions of normal and brown calcified shells have already the same amino acids composition. Aspartic acid which is considered as the main Ca ligand, was present in high levels in all the analysed organic fractions and, according to the high dispersion of the values, its small decrease in the insoluble matrix of the brown shells could not probably be interpreted as the efficient cause of the disturbances generated in the foliated microstructure.

The stability of the calcium content in all the analysed shells led to the same conclusions: the biomineralization processes – such as crystal matrix production and calcium fixation – did not seem to be affected in the diseased shells.

The differences observed in the mineral composition only concerned minor elements compared to the calcium, but these should be taken into account. Even trace elements could induce biological disturbances. Then, different hypotheses could be put forward. For instance, the phosphates were considered as crystal poisons of calcification by SIMKISS (1964). According to this observation, the increase in the phosphorus content could be related to the microstructural modifications observed in the internal layer of the brown shells. A decrease in the Mg content of *Cerastoderma edule* and *Monodonta articulata* shells, has been related to a decrease in the water salinity (NERI *et al.*, 1979). In our case, decreases in the Mg and Na contents were observed. According to the preceding knowledge, this could be linked to a decrease in the water salinity, which might disturb the well-being of the scallops and the calcification processes. The influences of this environmental modifications should be later tested in our laboratory.

Nevertheless, the most evident perturbation affecting these diseased

scallops was the modification of the crystal envelopes by the production of a brown-red pigment closely associated with the insoluble organic matrix of the shells. Considering that the organic matrix controls the crystal formation (WHEELER, 1988; KRAMPITZ and GRASER, 1988; MANN *et al.*, 1991), we thought that there was probably a relationship between the production of the brown compound and the appearance of the microstructural disturbances. The characterization of this pigment will be soon be investigated in order to better understand its effects on shell microstructure.

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