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Analysis of the soluble matrix of vaterite otoliths of juvenile herring (*Clupea harengus*): do crystalline otoliths have less protein?

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Abstract

Otoliths are calcium carbonate concretions laid down in the inner ear of fish and used in fish age estimation. Otoliths precipitate in the form of aragonite but aberrant precipitation may result in vaterite formation instead of aragonite. Vaterite otoliths are more translucent than aragonite. The quantity of HCl-soluble proteins (SP) was measured in the vaterite otoliths and their aragonite pairs of one year old reared herring *Clupea harengus* to assess the changes induced by the precipitation of vaterite in the amount of soluble proteins in the otolith. Results showed that vaterite otoliths had as much soluble proteins as their aragonite pairs ($p>0.05$). Due to the lower density of the vaterite, vaterite otoliths were lighter than their aragonite pairs ($p<0.05$) which explained that protein concentrations were significantly higher ($p<0.05$) than in aragonite otoliths. These results indicate that the precipitation of vaterite in otoliths did not affect the inclusion of soluble proteins. Furthermore, they suggest that soluble proteins do not affect the translucent or opaque appearance of otoliths. Differences in translucency may instead be caused by the amounts of insoluble proteins or by differences in the physical properties of proteins. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the otolith proteins revealed two bands at 50 and 62 kDa in both aragonite and vaterite otoliths suggesting that the precipitation of vaterite in the otolith is not controlled by either of these two proteins present in the otolith.

Keywords: Protein; Opaque; Translucent; Calcium carbonate polymorph; SDS-PAGE

1. Introduction

Otoliths are $CaCO_3$ concretions laid down in the inner ear of fishes, formed by the precipitation of mineral crystals on an organic matrix (Wright et al., 2002). The daily, lunar and seasonal cycles in the growth rhythms of fish are reflected in the otoliths as growth marks in the form of opaque and translucent bands which are widely used in age estimation of fish (Wright et al., 2002). Opaque bands have been shown to have higher concentrations of protein compared to translucent bands (Mugiya, 1965; Ans et al., 1982) which has resulted in the assumption that the opacity in parts of the otolith is caused by the higher amounts of protein, i.e. that it is the proteins that stop the light penetrating the surface of the otolith.

Recent studies have shown that otolith proteins can be separated into two major categories in relation to their solubility in aqueous solutions: soluble (SP) and insoluble proteins (IP). SP are probably involved in the regulation of otolith growth while IP have a structural role (de Pontual and Geffen, 2002). SPs regulate crystal growth, and are therefore more closely linked to the crystals (Sasagawa and Mugiya, 1996).

In this study, otoliths with vaterite (a morph of $CaCO_3$ which is more translucent than the predominant otolith $CaCO_3$ morph, aragonite) were analysed to assess the effect

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of vaterite precipitation on soluble protein content of the otolith. The different crystal structure makes otoliths with vaterite easily identifiable. These are glassy in appearance, and are often referred to as "crystalline" because they are more translucent than aragonite otoliths. Since the precipitation of vaterite could affect the inclusion of protein and ultimately explain differences in translucency we compared the protein content of vaterite otoliths with their aragonite pairs sampled from fish used in a parent study (Tomás and Geffen, 2003).

The differences in protein were also studied by identifying the proteins extracted from vaterite otoliths using Sodium Dodecyl Sulfate Polyacrilamide Gel Electrophoresis (SDS-PAGE) and comparing them with the proteins extracted from their fully aragonite pairs. In vitro studies have shown that one or few proteins are sufficient to change the $CaCO_3$ polymorph (calcite, aragonite or vaterite) being precipitated (Falini et al., 1996) but to date no studies have been carried out in aberrant otoliths of fish.

2. Materials and methods

2.1. Otoliths and fish

Otoliths with vaterite (hereafter referred to as vaterite otoliths) were obtained from one-year-old juvenile herring (*Clupea harengus*, Linnaeus., 1758) sampled from two laboratory reared populations (Celtic Sea and Clyde). Details of the rearing have been reported elsewhere (Tomás and Geffen, 2003). Fish were sampled in the tanks at the time of weaning between February and March 1999 when they were around 1 year old. Every fish was measured (total length), weighed and the otoliths removed. Once extracted, otoliths were double rinsed in distilled water, air dried and stored in acid washed polypropylene vials. Aberrant otoliths were clearly distinguishable from normal, fully aragonite otoliths because of their glassy appearance (Fig. 1). Aragonite and aberrant otoliths were screened for polymorph determination using X-ray diffraction spectrometry and Raman spectrometry as reported by Tomás and Geffen (2003). Both techniques showed that the polymorph present in the aberrant otoliths was vaterite. The Raman shift spectrum for the vaterite of one otolith is presented in Fig. 2. The incidence of vaterite otoliths in herring is small (Tomás and Geffen, 2003) and only 16 pairs of otoliths were available for the comparison. Dry left and right otoliths of each fish were weighed to the nearest 0.001mg using a Cahn G-2 electrobalance. The otolith dimensions (area, perimeter, length and width) including the area occupied by the aragonite in each vaterite otolith were measured on calibrated digital images of the otoliths using an image analysis software (Optimas version 5.1) (Table 1). The extent of vaterite replacement in vaterite otoliths varied between 20% and 90% of the total area of the otolith (Fig. 1, Table 1).

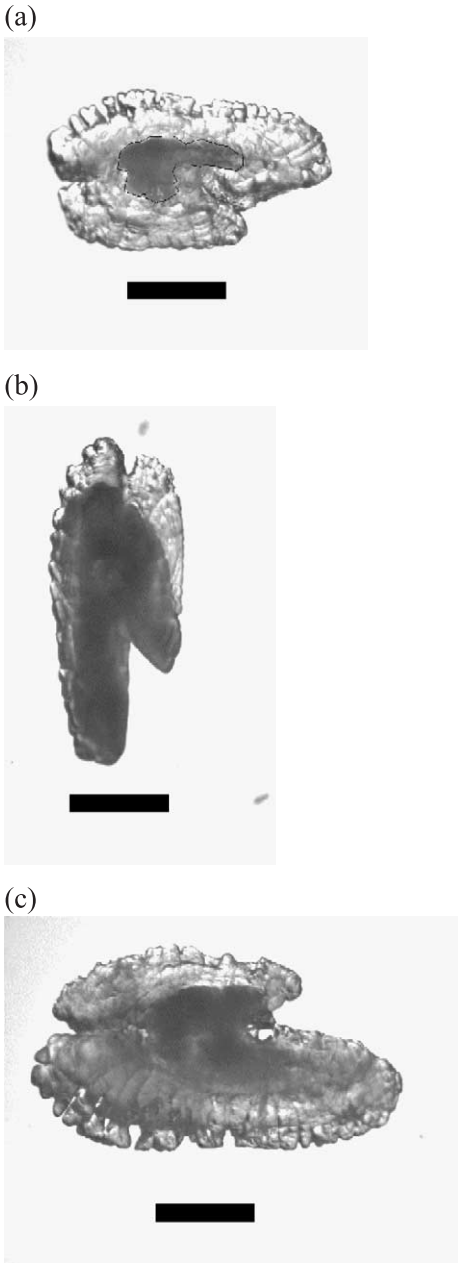

Fig. 1. Vaterite otoliths of three juvenile herring (*Clupea harengus*) exhibiting the typical inner aragonite part with a vaterite ring around, more translucent (scale bar=1 mm). The inner aragonite region is outlined in (a).

2.2. Protein determination

Isolating the vaterite from the aragonite in the vaterite herring otoliths is a delicate and difficult process. Given the small number of samples we did not attempt it but instead, protein was measured in whole otoliths. Aragonite otoliths were used as a control and in vaterite otoliths the area occupied by the vaterite was measured by subtracting the area of the inner aragonite zone from the total area of the otolith, as an estimate of the extent of the vaterite replacement.

Protein determination was carried out using Lowry's method (Lowry et al., 1951) that is widely used to estimate

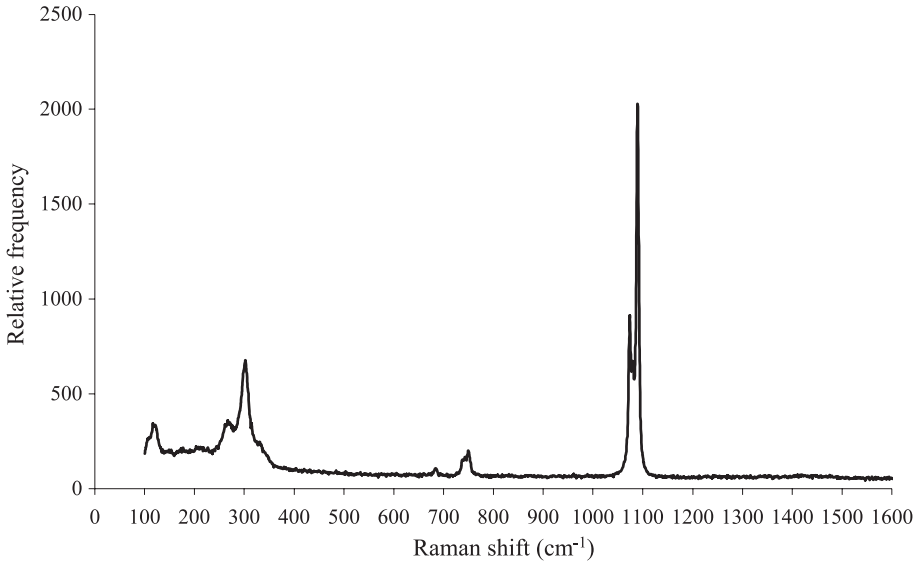

Fig. 2. Raman spectrum from one vaterite otolith of herring analysed with the Raman spectrometer displaying the characteristic peaks of the vaterite at 106, 266 and 301 cm^{-1}.

protein in biological samples (Smith, 1982) including otoliths (Wright, 1991; Asano and Mugiya, 1993). Otoliths were dissolved in 0.1 mL 1 M HCl for 48 h at 1.5 °C then diluted to 0.5 mL with distilled water and subsequently treated with 0.15 mL 2% sodium deoxycholate (Sigma, St. Louis, MO, USA) and 4M NaOH (0.15 mL). Three replicated standard solutions of standard albumin solution were prepared with a range of 20–40 μg of total protein (Albumin Bovine Fraction V, BDH). Replicated blanks were prepared using 0.1 mL of 1M HCl and diluted to 0.5 mL with distilled water. Samples, standards and blank were left to stand for 10 min. The reagent was prepared fresh every day by mixing 0.5 mL of $CuSO_4$ (2% solution) with 0.5 mL of NaK-tartrate (4% solution) and 49 mL of sodium carbonate (2% solution). The reagent (3 mL) was added to the samples, standards and blank and left to stand for 10 min. The Folin-Ciocalteau's phenol reagent (0.3 mL, BDH, UK) was subsequently added, and the solution shaken with a vortex mixer and allowed to stand for 30 min at room temperature. Samples, standards and blank were centrifuged for 10 min at 1000×g. Absorbance of the supernatant was measured in a spectrophotometer at 750 nm, subtracting the blank.

2.3. Gel electrophoresis of protein from aragonite and vaterite otoliths (SDS-PAGE)

An additional four pairs of vaterite-aragonite otoliths were used for gel electrophoresis. The molecular weights of proteins extracted from vaterite otoliths were compared to those extracted from their fully aragonite pairs to assess whether different proteins than those present in the aragonite otoliths could be detected in the vaterite otoliths. All four fish came from the Celtic Sea stock, three were juveniles hatched and reared in the laboratory like the ones used for soluble protein quantification while the fourth was an adult herring caught in the wild. The aberrant otolith of the adult fish (C94) had 81% of its area filled with vaterite whereas the three juveniles had less vaterite: 39% for sample C41, 56% for C41 and 62% for sample C108. In order to avoid interference from

Table 1
Summary of the otolith dimensions of fully aragonite otoliths and their vaterite pairs (including the dimensions of the inner aragonite part of vaterite otoliths) of juvenile herring from the Celtic and Clyde Sea populations

	Otolith area (μm^2)	Otolith perimeter (μm)	Otolith length (μm)	Otolith width (μm)	Otolith mass (mg)	Percent of otolith area replaced by vaterite
Fully aragonite otoliths						
Mean	2348541.3	7460.6	2544.7	1329.7	1.2	0
St dev	430398.6	905.2	306.2	104.9	0.3	0
Otoliths with vaterite						
Mean	2513058.8	9027.4	2618.7	1343.6	1.0	66.5
St dev	540970.5	1646.3	347.8	110.0	0.2	19.1
Inner aragonite sector within vateritic otoliths						
Mean	747474.3	4228.4	1429.3	797.2	–	–
St dev	351813.8	1421.5	446.9	222.7	–	–

other compounds in the otoliths, proteins were partially purified by homogenising in 10% trichloroacetic acid, dissolved in acetone (300 μL per otolith) using an acid-washed plastic pestle and then sonicated with a microprobe sonicator (Branson) at 25 W intensity for three cycles of 10 s each. Samples were then left overnight in a freezer to precipitate all proteins. The precipitated protein was spun down for 30 min at 14,000×*g* in a refrigerated micro-centrifuge and the supernatant (containing potentially interfering compounds) was discarded. The pellet was re-dissolved in 0.2 M sodium carbonate buffer containing 4% sodium dodecyl sulphate (SDS) and 10 μL were sampled for protein. SDS was necessary in order to completely re-solubilise the protein, but it also interfered with the Lowry method (Smith et al., 1985). Therefore, protein was quantified using the bicinchoninic acid (BCA) method, with bovine serum albumin as a standard (Smith et al., 1985, Sigma Kit BCA-1), using Sigma Kit BCA-1. Protein extracts were then prepared for gel electrophoresis by adding loading buffer containing dithiothreitol, glycerol and bromothymol blue, loaded on an equal protein basis (based on BCA assays), and separated by SDS-PAGE on precast 5–40% gradient gels (BioRad) (Laemmli, 1970; Berges and Falkowski, 1998). The gel was run between 5 and 200 kDa. Proteins were stained with the fluorochrome SYPRO Orange (Molecular Probes, Leiden, The Netherlands) (Steinberg et al., 1996a,b), photographed under UV illumination using a Kodak DC40 digital camera, and analysed using Kodak 1D Image analysis software (Version 2.01).

3. Results

3.1. Aragonite and vaterite otoliths

Vaterite otoliths had areas and lengths not significantly different from those of aragonite pairs (Area: ANOVA, $F_{30,1}$=0.90, p>0.05; Otolith length: ANOVA, $F_{30,1}$=0.41,

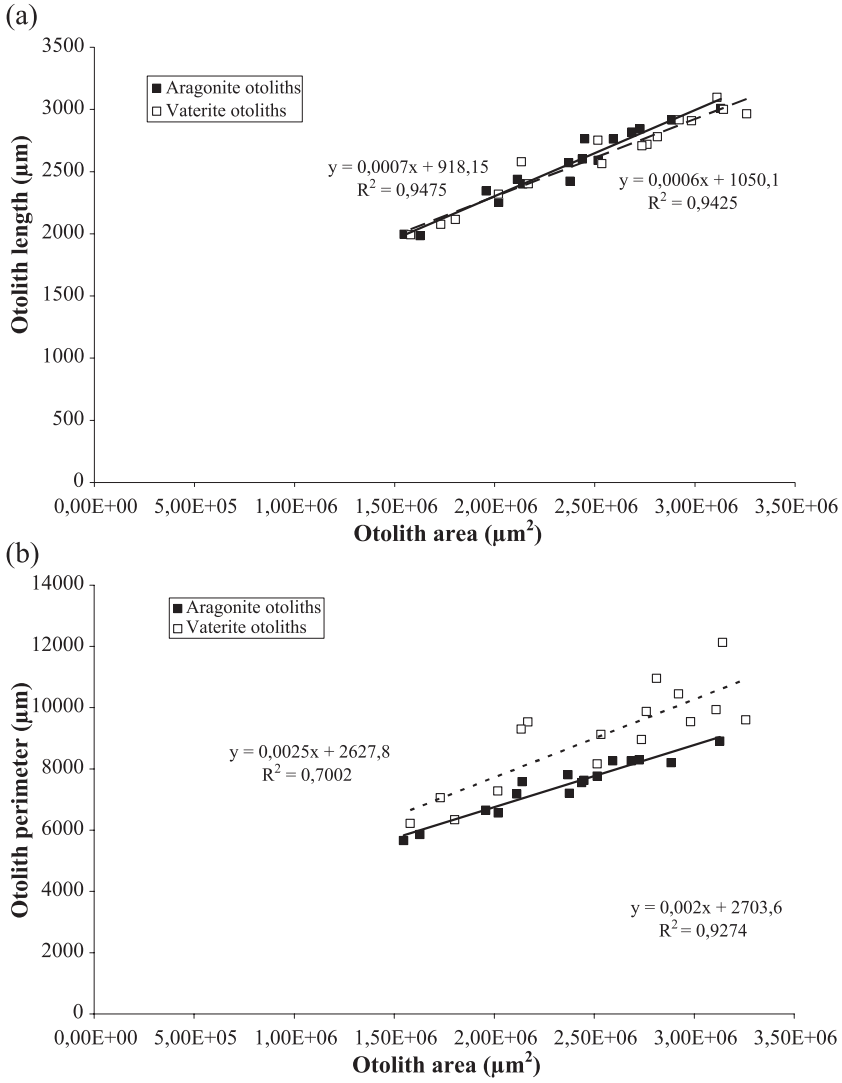

Fig. 3. Relationship between otolith dimensions in the aragonite and vaterite otoliths. (a) Relationship between otolith length and otolith area. (b) Relationship between otolith perimeter and otolith area.

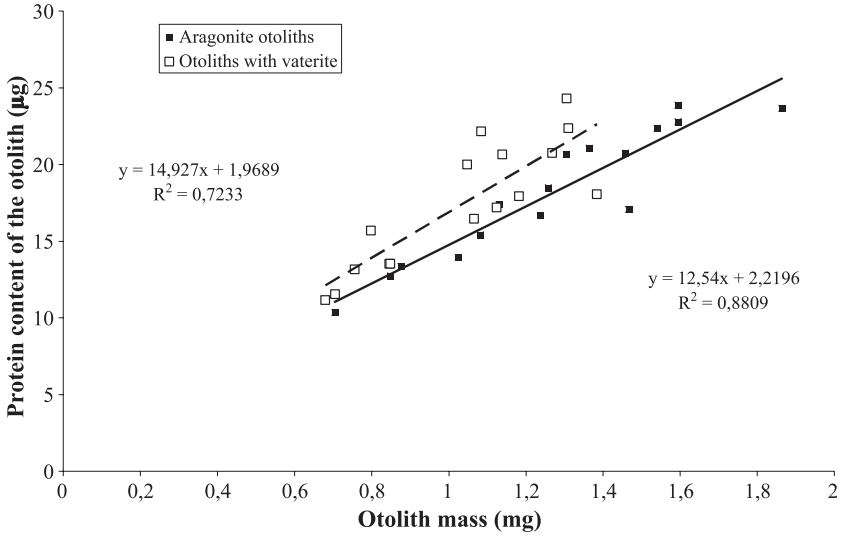

Fig. 4. Relationship between SP content in the otolith and the otolith mass in aragonite and vaterite otoliths. Left equation: vaterite otoliths; right equation: aragonite otoliths.

$p>0.05$), but had greater perimeters (ANOVA, $F_{30,1}=9.9$, $p<0.05$) than the aragonite otoliths. The relationship between otolith area and otolith length in vaterite otoliths was not significantly different (ANCOVA, $F_{29,1}=1.3$, $p>0.05$) from that of their aragonite pairs (Fig. 3a). Nonetheless, the relationship between otolith perimeter and otolith area was significantly different between vaterite and aragonite otoliths (ANCOVA, $F_{29,1}=23.19$, $p<0.001$) (Fig. 3b). Vaterite otoliths were significantly lighter than the aragonite pairs (ANOVA, $F_{30,1}=5.89$, $p<0.05$) as a result of the lower density of vaterite compared to aragonite (Tomás and Geffen, 2003). Overall, these results showed that the otolith dimensions of vaterite otoliths were similar to their aragonite pairs with the exception of the perimeter (due to their more crenulated outline) and the otolith mass.

Protein was positively correlated with otolith mass in both aragonite and vaterite otoliths (Fig. 4) and significant differences existed in the protein: mass relationship between vaterite and aragonite otoliths (ANCOVA, $F_{29,1}=10.92$, $p<0.01$). SP concentration (SP content ratioed to otolith mass) was significantly higher in vaterite otoliths (t-test for paired samples, $n=18$, td=−7.96, $p<0.001$). Nonetheless, no significant differences in the SP content of the otolith were detected between aragonite otoliths and their vaterite pairs (t-test for paired samples, $n=16$, td=1.81, $p>0.05$) and so irrespective of the area occupied by the vaterite (Fig. 5). Higher concentrations in vaterite otoliths were expected since vaterite otoliths were lighter than their aragonite pairs and this resulted in higher values relative to the otolith mass. The amounts of soluble protein present in vaterite otoliths were the same than their aragonite pairs. It is unlikely that

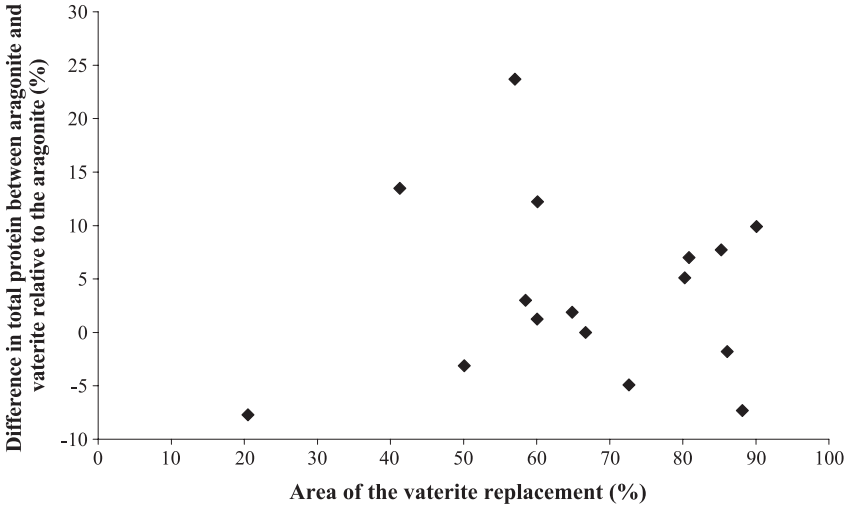

Fig. 5. Relationship between the difference in total protein between the aragonite otolith and its vaterite pair (relative to the weight of the aragonite otolith) and the extent of the vaterite area in the vaterite otolith.

all the protein extracted from the vaterite otoliths was located in the inner aragonite area for there was no relation between the extent of the vaterite replacement and the differences in protein between the vaterite otolith and its fully aragonite pair (Fig. 5). Consequently, the vaterite areas include soluble proteins. Furthermore, vaterite deposition does not seem to alter the amount of soluble proteins included in the otolith.

The lanes with protein extracted from aragonite and vaterite otoliths and analysed with SDS-PAGE contained only two dominant bands in the gel at 62 and 52 kDa (Fig. 6). Other bands were much fainter and could not be consistently measured. Vaterite samples seemed to have in general less protein in the bands but this was more evident in the otolith of the adult fish which had higher amounts of protein and a relatively larger vaterite area (lane 1, Fig. 6a). Since each pair of lanes was loaded on an equal protein basis, results suggest that some protein in the vaterite samples detected by the assay did not resolve into detectable bands. Vaterite samples thus may have proteins different from that of the aragonite samples, but not abundant enough to form a visible band and allow identification, at least between 5 and 200 kDa.

4. Discussion

4.1. Protein in vaterite otoliths

Vaterite deposition did not alter the amount of soluble proteins incorporated to the otolith in the herring otoliths analysed in this study. Despite its different crystal structure compared to aragonite, the amount of soluble protein included in the mineral matrix was not altered by the precipitation of vaterite instead of aragonite. The switch from aragonite to vaterite might be caused by different soluble proteins as shown in mollusc shells with aragonite and calcite (Belcher et al., 1996; Feng et al., 2000) but does

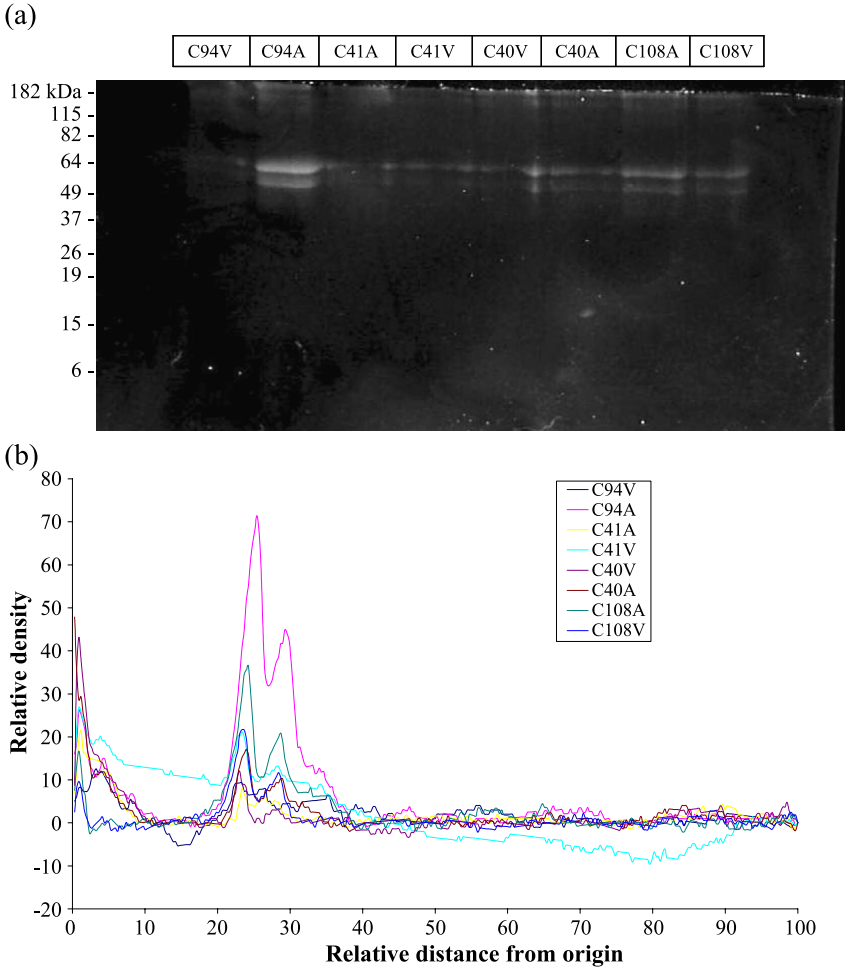

Fig. 6. Result of the SDS-PAGE (4–20% gradient) of the proteins extracted from the otoliths of juvenile herring (*Clupea harengus*), stained with SYPRO Orange and observed under UV illumination. (a) The two bands correspond to 50 and 62 kDa. Eight lanes can be observed, each one corresponding to one otolith. Each fish is thus represented by two contiguous lanes. The row at the top indicates the sample number (V=vaterite and A=aragonite). Starting from the left hand side, the first and second lanes correspond to the proteins extracted from the otolith of the adult herring from the wild, hence its greater intensity. (b) Densitometric profile of the gel presented in (a).

not seem to affect the quantity of soluble protein incorporated to the otolith.

These results may have some implications to the interpretation of the relationship between protein inclusion and translucency in fish otoliths. The opacity of parts of the otolith has been explained so far by the ratio of organic matrix to mineral. However, our measurement of SP: vaterite ratios indicate that the accepted model of protein inclusions in the mineral stopping light penetration is too simple, for it does not stand for the vaterite. Mugiya (1965) showed that translucent bands in the otoliths of several fish species concentrated less protein than opaque bands. Moreover, the glassy otoliths of the trout had less protein than the more opaque or milky otoliths of two cod species (*Gadus macrocephalus* and *Theragra chalcogramma*). Morales-Nin (1985) quantified the protein of calcite otoliths of *Genypterus capensis* and found a higher organic content than in aragonite otoliths. Our results suggest that the differences in translucency in otoliths, if caused by protein inclusion between crystals, are not related to the amounts of soluble proteins. At the micro structural level, and under transmitted light, the dark zones (D-zones) of the incremental unit are filled with organic material, most probably protein (Watabe et al., 1982). These authors suggested that organic deposition onto the growing surface of the otolith would continue even in the absence of mineral deposition resulting in a D-zone (Watabe et al., 1982). If opaque bands result from an aggregation of proteins resulting from the reduction of mineral deposition then these proteins are probably not soluble proteins. Could insoluble proteins be responsible instead for opacity in opaque zones? IP play a structural role in the accretion of the otolith (Campana, 1999) and build up the framework onto which crystals precipitate. Insoluble proteins, although accounting for as much as SP in quantitative terms, at least in the otoliths of *Oreochromis niloticus* (Asano and Mugiya, 1993) may be more unevenly distributed in the otolith than SP (which are closely bound to the crystal and thus found all over the otolith) and be responsible for the opacity of parts of the otolith. Nonetheless, these are speculations that will require further study. IP seem to be involved in the definition of the otolith overall shape which is precisely one of the few otolith characteristics that is not altered in vaterite or calcite otoliths (Morales-Nin, 1985; Gauldie, 1986; Strong et al., 1986).

Other factors may increase or decrease the light transmittance properties of the otoliths. Among these, the physical properties of the mineral (such as crystal density, crystal orientation or crystal habit) or the presence of other organic compounds as inclusions between crystals such as proteoglycans (Borelli et al., 2001), lipids (Mugiya, 1968) and sugars (Dauphin and Dufour, 2003). Both SP and IP play a role in the build up of the crystalline system that may ultimately determine the density and other properties of the mineral. For instance, differences in crystal nucleation and in the degrees of orientation of the crystals are a function of the nucleating sheet, made of insoluble proteins, as shown in in vitro experiments (Belcher et al., 1996; Belcher et al., 1998). Insoluble proteins influence the density of nucleation, the sizes and the quantities of crystals (Feng et al., 2000). Thus, added to the quantitative measurement of IP in the opaque and translucent zones of the otolith it would be advisable to analyse their nucleating capacities and abilities to bind to SP.

The inclusion of other compounds between crystals will lower the density of crystals in those otolith zones, an aspect that may ultimately be controlled by the nature of the nucleating sheet. In that sense, the analysis of mineral density may ultimately explain differences between opaque and translucent zones.

4.2. Differences between polymorphs

The proteins detected in the vaterite samples were not different from the aragonite samples. Nonetheless, the results suggest that some of the protein migrating in the lanes with vaterite remained undetected probably because of their low concentration. These proteins could be those responsible for polymorph switch if vaterite precipitation in herring otoliths is determined by proteins. Polymorph biomineralisation in the shells of molluscs has confirmed that the alternating growth of aragonite and calcite is governed by the interaction and cooperation of a number of polyanionic soluble proteins (Belcher et al., 1996; Belcher et al., 1998). These studies showed that the precipitation of aragonite was related, without the deposition of a new nucleating sheet, to the secretion of a group of polyanionic soluble proteins different from that extracted from the calcite layer. This implies that soluble proteins alone are capable of polymorph selection and orientation (Belcher et al., 1996). Nonetheless, proteins are not the unique cause of polymorph switch. In the particular case of vaterite, it has been shown that this polymorph could precipitate in the presence of glycoproteins extracted from both the calcite and the aragonite layers of molluscan shells under certain conditions of solubility of the solution from which they precipitate (Falini et al., 1996). These authors reported that vaterite precipitated in the presence of aragonite-determining proteins, without an organic substrate for nucleation. Although the precipitation of aragonite and calcite seemed to be controlled by glycoproteins, the shift from aragonite to vaterite depended on the solubility of the solution (Falini et al., 1996). The control of ion flux at the matrix interface is crucial in biomineralisation and may explain the transition from aragonite to vaterite (Bianconi et al., 1991). Vaterite formation occurs (in order of decreasing solubility) after calcium carbonate monohydrate and before aragonite and calcite (Falini et al., 1996; Dalas et al., 2000). Therefore, the shift from aragonite to vaterite in the otoliths of juvenile herring could also be caused by changes in the solubility of the endolymph. Our results do not allow to conclude if vaterite precipitated in the herring otoliths because of some proteins, most probably SP, but unidenti-

fied for the moment, or by changes in the solubility of the endolymph at the time of $CaCO_3$ precipitation. Larger sample sizes of vaterite from otoliths will be required to identify any of these proteins, especially if there are species differences as described by Dauphin and Dufour (2003).

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