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## Endolymph chemistry and otolith growth in fish

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### Abstract

Otoliths are composed of 99% CaCO<sub>3</sub> in the aragonite form which is deposited daily onto an organic matrix. The mineralisation process takes place in an acellular medium, the endolymph, which is secreted by the inner-ear epithelium. The present review is mostly devoted to ionic and organic endolymph components (concentration and spatial distribution) in relation to otolith growth, with a special interest to the ionic supply from plasma to endolymph and to the biochemical relationships between endolymph and otolith matrix. *To cite this article: P. Payan et al., C. R. Palevol 3 (2004).*

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### R esum e

**Chimie de l'endolymphe et croissance de l'otolithe chez les poissons t el ost eens.** Les otolithes sont compos es de 99 % de CaCO<sub>3</sub> (aragonite), d epos e dans une matrice organique selon un rythme quotidien. Le processus de min eralisation s'effectue dans un milieu acellulaire, l'endolymphe, s ecr et e par l' epith elium de l'oreille interne. La revue rapporte les r esultats concernant les composantes ioniques et organiques (concentrations et r epartition spatiale) de l'endolymphe en relation avec la croissance de l'otolithe. Les flux ioniques   travers l' epith elium sacculaire et l'origine endolymphatique des compos es organiques pr esents dans l'otolithe sont particuli erement  voqu es. *Pour citer cet article : P. Payan et al., C. R. Palevol 3 (2004).*

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**Keywords:** Fish otoliths; Endolymph; Ions; Organic matrix; Otolith growth; Calcification process

**Mots cl es :** Otolithe ; Endolymphe ; Ions ; Matrice organique ; Croissance ; Processus de calcification

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## 1. General introduction

Otoliths (earstones) are paired calcified structures used for the maintenance of equilibrium and hearing in all teleost fishes (see Fig. 1A for the localization of the inner ears in the skull and Fig. 1B for the position of otoliths within their chambers). Otoliths contain more than 99%  $\text{CaCO}_3$  [6,12] in the aragonite form, which is deposited daily onto an organic matrix [30]. They are generally considered as biological archives and, as such, are routinely used for age and growth estima-

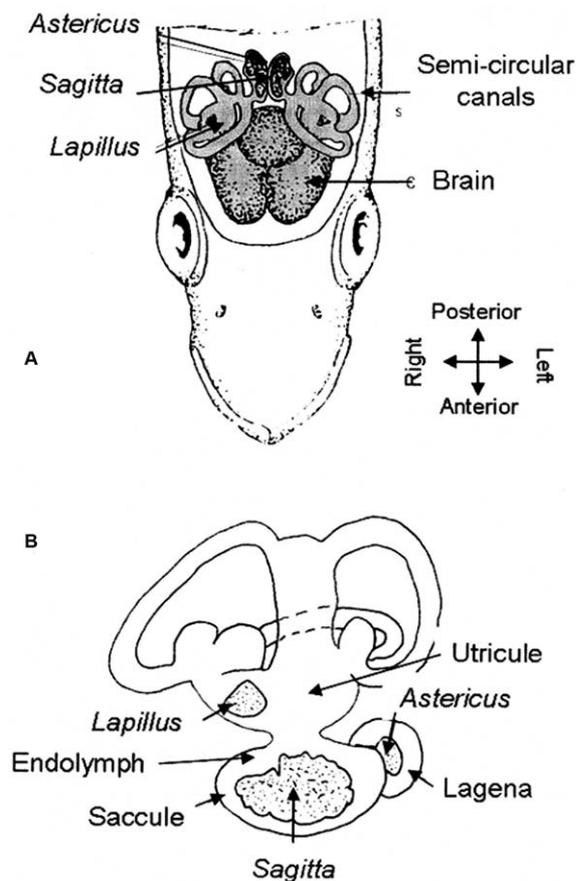


Fig. 1. Position of the otoliths within the inner ear of Teleost fish. (A) Dorsal view of the labyrinth system in a typical Teleost species. The top of the cranium is cut away (frontal section). (B) Otoliths within the labyrinth systems of a typical Teleost (modified from Panfili et al. [29]).

Fig. 1. Position des otolithes dans l'oreille interne des Téléostéens. (A) Vue dorsale du système du labyrinthe chez un Téléostéen typique, le sommet du crâne ayant été enlevé (section frontale). (B) Otolithes dans le labyrinthe d'un Téléostéen typique (modifié d'après Panfili et al. [29]).

tions, stock discrimination of exploited fish populations and characterization of events in the fish's life history [9,44]. Stock discrimination is based on the assumption that changes in physical and chemical environments will be registered by differences in otolith chemical composition; numerous studies have focused on strontium as a marker of environmental temperature and salinity [9,14]. From the environment to the otolith, the pathway is a complex multistep route involving several successive barriers/compartments (gills/intestine/skin, blood, inner ear epithelium, endolymph).

As described in Fig. 2, the calcification process consists of a  $\text{CaCO}_3$  deposition within an organic matrix framework; thus the principal substances involved in otolith growth are organic matrix (OM),  $\text{Ca}^{2+}$  and bicarbonate ions ( $\text{HCO}_3^-$ ). The formation of  $\text{CaCO}_3$  produces  $\text{H}^+$  according to the equation:  $\text{Ca}^{2+} + \text{HCO}_3^- \rightarrow \text{CaCO}_3 + \text{H}^+$ , which must be removed for calcification to proceed. Unlike most calcifying systems, e.g. vertebrate bones, enamel, mollusc shells and coral skeletons, otolith mineralisation takes place in an acellular medium, the endolymph, which is secreted by the inner-ear epithelium (saccular epithelium when considering the *sagitta*). This implies that the calcification process is strictly dependent on the endolymph chemistry and that endolymph contains all the ionic and organic precursors for otolith formation. Thus the saccular epithelial cells fulfil the critical roles of: (i) secreting the appropriate macromolecules constituting the organic matrix; (ii) providing the ionic environ-

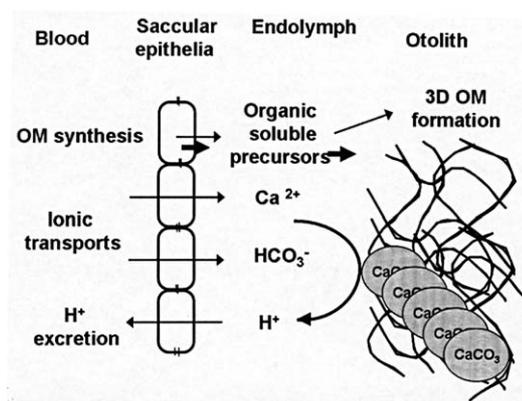


Fig. 2. Basic mechanisms acting in biomineralisation process of otolith (see text for further explanations).

Fig. 2. Mécanismes fondamentaux du processus de biominéralisation de l'otolithe (voir texte).

ment necessary for controlled mineralisation; and (iii) exerting a spatio-temporal control over these events.

Within the endolymph, two driving forces may be identified to promote the  $\text{CaCO}_3$  deposit: the ionic and organic states of the endolymph. Mineral growth of the otolith aragonite is linked to the aragonite saturation state of the endolymph [36], which can be expressed as the supersaturation ratio  $S_a$  [39] according to the equation:

$$S_a^2 = [\text{Ca}^{2+}] [\text{CO}_3^{2-}] / K_{s,a}^0$$

Concentrations are in fact the activity of the ionic species and  $K_{s,a}^0$  is the thermodynamic solubility product of aragonite.  $[\text{Ca}^{2+}]$  depends on several parameters, such as  $[\text{Ca}]_{\text{tot}}$ , pH of the fluid and nature and concentration of Ca-binding proteins.  $[\text{CO}_3^{2-}]$  depends on pH,  $p_{\text{CO}_2}$  and  $[\text{CO}_2]_{\text{tot}}$  according to the equations:

$$[\text{CO}_2]_{\text{tot}} = [\text{CO}_2]_{\text{d}} + [\text{HCO}_3^-] + [\text{CO}_3^{2-}]$$

and

$$\text{pH} = \text{p}K + \log [\text{HCO}_3^-] / [\text{CO}_2]_{\text{d}}$$

$[\text{CO}_2]_{\text{d}}$  represents the dissolved  $\text{CO}_2$  and can be calculated according to the equation:

$$[\text{CO}_2]_{\text{d}} = \alpha_{\text{CO}_2} p_{\text{CO}_2}$$

where  $p_{\text{CO}_2}$  is the partial pressure of  $\text{CO}_2$  and  $\alpha_{\text{CO}_2}$  the solubility coefficient of  $\text{CO}_2$  in the fluid. When  $S_a > 1$ , the fluid is considered as supersaturated with respect to aragonite, and  $\text{CaCO}_3$  naturally precipitates. Fish endolymph is usually considered as a highly supersaturated fluid [36,39] with a  $S_a$  around 2–3.

As in most biominerals, the otolith matrix forms only 0.1–1% of its weight [12], but it is now admitted that it has a considerable importance in the otolith crystallization processes of nucleation, growth, orientation and growth control [1,4,46]. The otolith matrix consists of proteins, carbohydrates, and lipids [22]. Most results concerning the chemical nature of matrix proteins were obtained after otolith demineralisation [2–4,6,7,12,26] and only Takagi et al. [41] characterized the presence of carbohydrates in trout otolith matrix by a lectin approach. Surprisingly, the nature of organic precursors of otolith matrix within the endolymph has received little attention [6,7].

The present review is mostly devoted to ionic and organic endolymph components in relation to otolith growth with a special interest to the ionic supply from plasma to endolymph and to the biochemical relationships between endolymph and otolith matrix.

## 2. The ionic chemistry of endolymph

In vertebrates, the labyrinth fluid is always characterized by a high  $[\text{K}^+]$  and a low  $[\text{Na}^+]$ , which is unusual for an extracellular compartment [37]. The high  $[\text{K}^+]$  value is generally related to electrophysiological event,  $\text{K}^+$  being the ion that normally carries most of the transduction current through the sensory cells in the macula. By comparison with higher vertebrates, fish endolymph shows a higher  $[\text{Na}^+]$ , a comparable  $[\text{Ca}]_{\text{tot}}$  and a higher relative alkalinity with a pH value around 8.0 (pH of plasma 7.2–7.6) and a  $[\text{CO}_2]_{\text{tot}}$  around 30 mM ( $[\text{CO}_2]_{\text{tot}}$  of plasma: 8–12 mM) [13, 15,16,18,25,31–33].

In vertebrates, the electrical potential measured on the endolymph side is always positive with respect to the plasma (from +80 mV in the cochlea to +5 mV in the utricle [37]). To our knowledge, the only published measurement in teleosts gave a saccular potential of +10 mV [15]. The calculated Nernst potentials ( $E_{\text{eq}}$ ) suggest that  $\text{Na}^+$  and  $\text{Cl}^-$  are passively distributed, whereas endolymph  $\text{K}^+$  is clearly driven by an energy-dependent mechanism ( $E_{\text{eq}}$  around –90 mV) [31].

Although many studies have been done on the composition of the fish endolymph, there is little knowledge of the mechanisms of transport across the saccular epithelium for the ionic precursors of the otolith. Series of experiments were performed using an isolated preparation of trout otolith-containing sacculus described in the Fig. 3A.

Concerning the mechanism of the epithelial  $\text{Ca}^{2+}$  supply to the endolymph, a transcellular route involving a combination of a receptor-operated  $\text{Ca}^{2+}$  channel, a  $\text{Na}^+/\text{Ca}^{2+}$  exchange and an ATP-dependent  $\text{Ca}^{2+}$  pump has been proposed (Fig. 3B, [26]). Concerning the acido-basic equilibrium of the endolymph, Payan et al., [31] measuring the in vitro excretion of titrable acidity and Tohse and Mugiya [43] using radiolabelled bicarbonate came to similar conclusions: secondary active transport processes ( $\text{Na}^+/\text{H}^+$  and  $\text{Cl}^-/\text{HCO}_3^-$  ex-

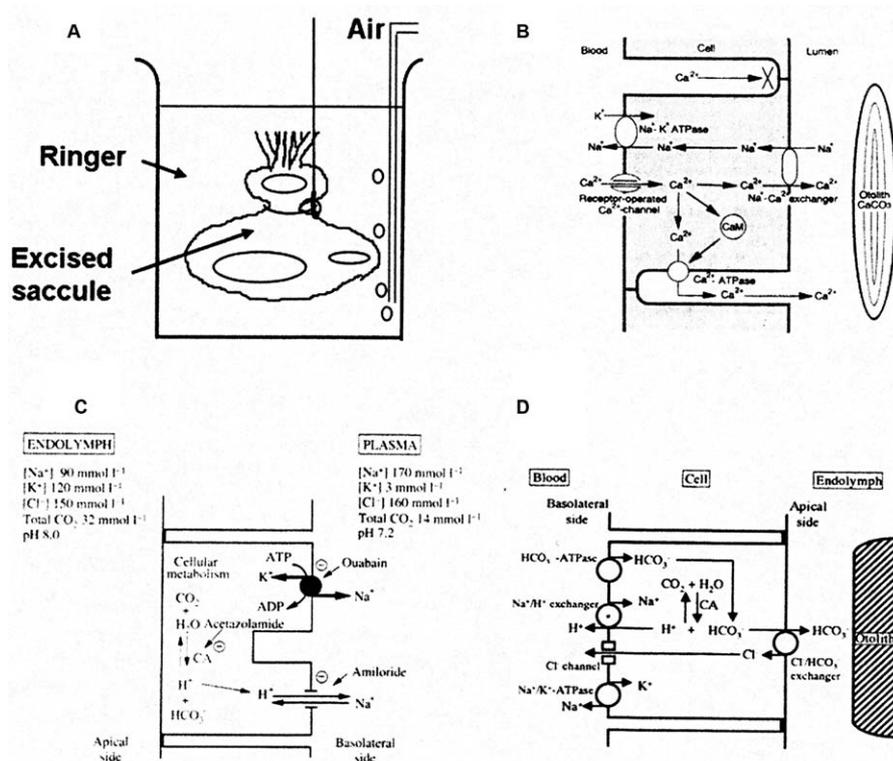


Fig. 3. The ion transporting models across the saccular epithelium. (A) Experimental set-up for studying ionic fluxes by incubating an isolated saccule. Different models of ionic fluxes: (B)  $\text{Ca}^{2+}$  transport [25]; (C)  $\text{H}^+$  excretion [31]; (D)  $\text{HCO}_3^-$  transport [42,43].

Fig. 3. Modèles de transport d'ions à travers l'épithélium sacculaire. (A) Dispositif expérimental permettant l'étude des flux ioniques par incubation du saccule isolé. Divers modèles de flux ionique : (B) transport de  $\text{Ca}^{2+}$  selon [25]; (C) excrétion de  $\text{H}^+$  selon [31]; (D) transport de  $\text{HCO}_3^-$  selon [42,43].

changes) are involved to maintain an alkaline endolymph (see Fig. 3C,D). Furthermore, carbonic anhydrase was also suggested to play a role in  $\text{H}^+$  excretion [31] and bicarbonate production for otolith calcification [43].

The active transport of ions across an epithelium is usually performed by mitochondria-rich cells also called ionocytes. In both trout and turbot, the ionocytes in the saccular epithelium were observed in two zones: the first consists of a ring of large ionocytes around the macula and the second is of smaller cubital ionocytes unevenly grouped at the opposite side of the macula (Fig. 4 and [19,35,38]).

Payan et al. [33] hypothesized that the heterogeneous distribution of ionocytes within the saccular epithelium could induce a non-uniform ionic composition of the endolymph. The microtechniques of sampling and endolymph analysis developed in trout and turbot (Fig. 5A, [31]) permitted to determine the vari-

ous chemical concentrations in single 4–5- $\mu\text{l}$  samples. Indeed, microchemical analysis of endolymph sampled at various sites around the otolith revealed proximo-distal great differences in concentrations of most parameters studied (Fig. 5B, [13,32]). The endolymph fluid may be depicted as two compartments: a proximal and a distal spaces, separated by the otolith (Fig. 5B). Sodium, calcium, phosphate, and magnesium are more concentrated in the proximal endolymph, whereas potassium, pH, and tot $\text{CO}_2$  levels are significantly higher in the distal endolymph (Fig. 5B).

The calculated Nernst potentials for the calcifying parameters through the proximal and distal saccular epithelium are presented in Table 1. The electrical potentials through the two opposite sides of the epithelia have not been measured but, by analogy with the situation in higher vertebrates, it should be positive on the endolymphatic side ([15] obtained +10 mV, prob-

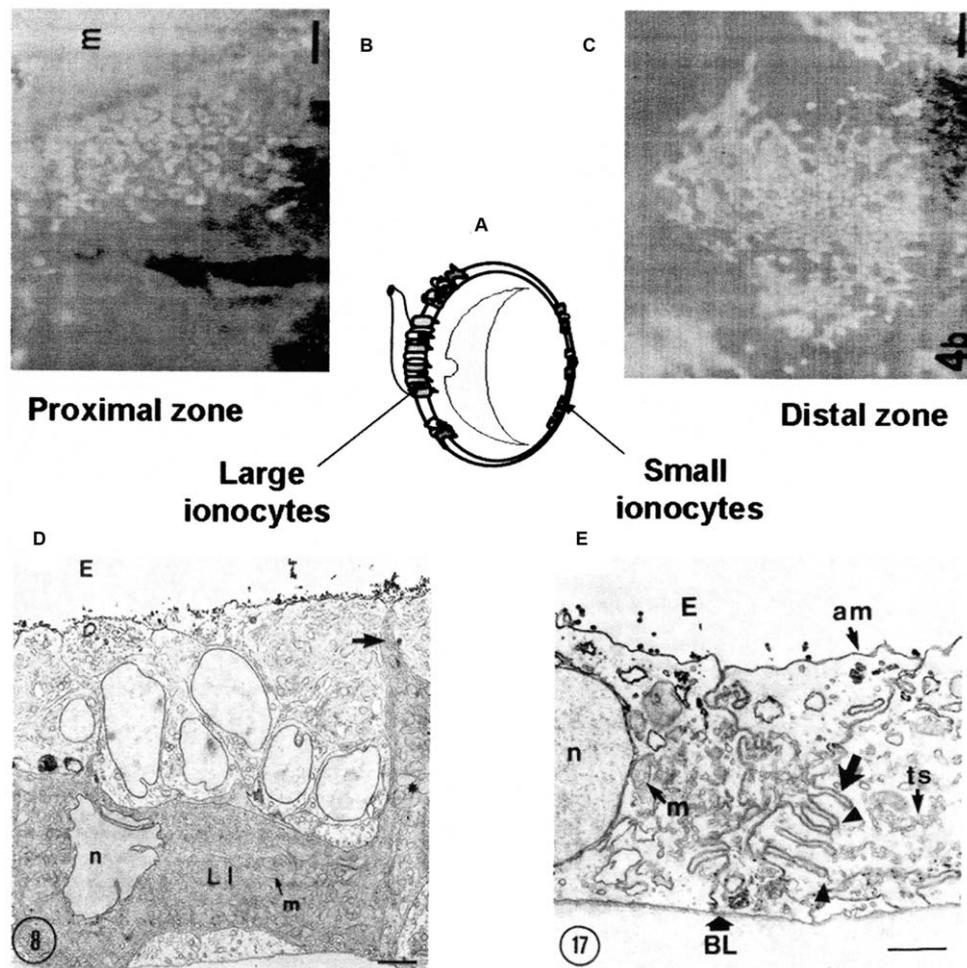


Fig. 4. Repartition of ionocytes (mitochondria-rich cells) in the saccular epithelium. (A) Schematic drawing of a left saccule (transverse section). Large ionocytes arranged in a meshwork around the macula (B) and small ionocytes grouped in patch in the distal zone (C) are visualized with DASPMI (a fluorescent probe of mitochondria). (D) and (E) are ultrastructural views of large and small ionocytes respectively (E: endolymph, LI: large ionocyte, n: nucleus, m: mitochondria, am: apical membrane, BL: basal lamina, ts: tubular system (bar: 3 μm). Adapted from [18,35].

Fig. 4. Répartition des ionocytes (cellules riches en mitochondries) dans l'épithélium sacculaire. (A) Dessin schématique d'un saccule gauche en coupe transversale. Les ionocytes volumineux sont disposés en réseau autour de la macula (B) et les petits ionocytes sont groupés en amas dans la zone distale (C). Les ionocytes sont visualisés par le DASPMI, un marqueur fluorescent des mitochondries. (D) et (E) sont respectivement des images ultra-structurales des ionocytes volumineux et des petits ionocytes (E : endolymph, LI : ionocytes volumineux, n : noyau, m : mitochondrie, am : membrane apicale, BL : lame basale, ts : système tubulaire. Échelle = 3 μm. Adapté de [18,35].

ably in distal position). These calculations suggest that energy-dependent mechanisms are involved in maintaining a high  $[K^+]$  value in both proximal and distal endolymphs and high pH and bicarbonate levels in distal endolymph (Fig. 6A).  $Ca^{2+}$  seems near its electrochemical equilibrium in both proximal and distal endolymphs; this is in agreement with the results [34]

obtained using a perfused inner ear (Fig. 6B). These authors found that (i) verapamil (a blocker of voltage-dependent  $Ca^{2+}$ -channel) or cyanide (a blocker of mitochondrial ATP production) had no effect on  $Ca^{2+}$  accumulation in the endolymph fluid and (ii) net fluxes of  $Ca^{2+}$  were linear in both proximal and distal compartments during  $Ca^{2+}$  loading and unloading experi-

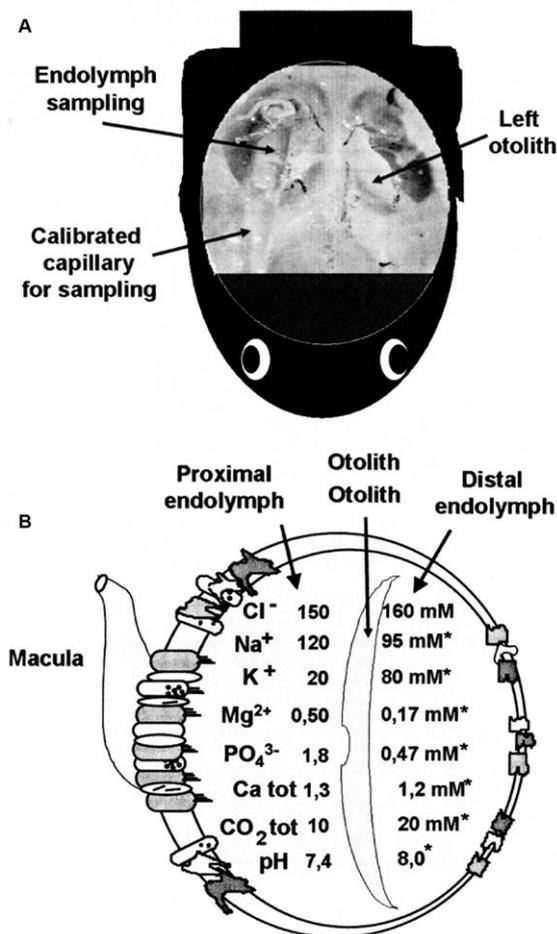


Fig. 5. Heterogeneity of the ionic composition of the endolymph. (A) Experimental sampling of distal endolymph in a right saccule of turbot (frontal section). The tip of a calibrated capillary (external diameter: 2 mm) is inserted into the distal zone of the saccule and the endolymph is slowly sucked up using a peristaltic pump (not shown). (B) Schematic drawing of a left saccule (transverse section) and heterogeneity of the ionic composition of the endolymph. The star (\*) indicates that the difference in concentration between proximal and distal endolymphs is statistically different. Adapted from [33].

Fig. 5. Hétérogénéité de la composition ionique de l'endolymphe. (A) Prélèvement de l'endolymphe distale du saccule droit chez le turbot (section frontale). L'extrémité du tube capillaire calibré (diamètre externe : 2 mm) est insérée dans la zone distale du saccule et l'endolymphe est lentement aspirée à l'aide d'une pompe péristaltique (non montrée). (B) Schéma d'un saccule gauche en coupe transversale et hétérogénéité de la composition ionique de l'endolymphe. L'astérisque (\*) indique que la différence de concentration entre endolymphe proximale et distale est statistiquement différente. Adapté de [33].

ments. Consequently, it was concluded that the Ca<sup>2+</sup> transport via the proximal epithelium was passive and could occur mainly via a paracellular way (Fig. 6A).

The most unexpected results concern the acidobasic equilibrium between the plasma and the proximal endolymph with a Nernst potential that passively favours the entry of HCO<sub>3</sub><sup>-</sup> and the exit of H<sup>+</sup> (Fig. 6A). This would mean that, in the proximal endolymph (bathing the convex shape of the otolith, which is generally characterized by a maximal growth), the supply of ionic precursors (i.e. Ca<sup>2+</sup> and HCO<sub>3</sub><sup>-</sup>) and the removal of crystallization reaction products (H<sup>+</sup>), necessary for otolith growth, involve passive transfers across the proximal epithelium. This is probably in relation with the high turnover rate that characterizes these calcifying parameters within the endolymph (see next section). Furthermore, it appeared that proximal calcification process does not necessitate a very alkaline endolymph pH, as it occurred at pH 7.4. In these conditions, the *S<sub>a</sub>* value estimated for the proximal endolymph is not always supersaturated, but is around 1 [8]. Thus small increases of the concentrations of ionic parameters could allow the saturation state of the aragonite to be reached and induce CaCO<sub>3</sub> crystallization. This has been recently proposed to explain how the night–day cycle variation in the trout endolymph could determine the alternation of CaCO<sub>3</sub> deposit in otolith [8].

### 3. The organic chemistry of the endolymph: its relationship with otolith matrix

Similarly to a lack of uniformity of most of ionic components within the endolymph, it has been shown that the organic compounds were also heterogeneously distributed [6,7,13,34]. Non-collageneous proteins, collagens, amino-acids were 4, 10 and 3 times more concentrated in the proximal endolymph, whereas proteoglycans were only detectable in the distal side (Fig. 7). The presence of an anticalcifying factor has been shown in the fluid surrounding some biominerals (statocyst fluid of cephalopods [20], chicken uterine fluid [17], endolymph of teleost fish [6]). As shown in Fig. 7 the anticalcifying activity is 2.5 times more concentrated in the proximal endolymph than in the distal. Thus, there was about 4 times more organic material in the proximal region than in the distal one.

Table 1

Ionic concentrations in plasma and endolymph and Nernst potentials across the saccular epithelium in the trout. Concentrations are expressed as mM. The Nernst potential is given with plasma as reference. Values of pH and  $[\text{HCO}_3^-]$  are from Borelli et al. [8].  $[\text{Ca}]_{\text{tot}}$  is taken as 2.3 mM in plasma, 1.2 mM in proximal endolymph and 1.0 mM in distal endolymph [8]. Concerning the  $[\text{Ca}^{2+}]$ , 2 values are given according to the percentage of  $\text{Ca}^{2+}$  estimated in the literature (47% and 72% for Mugiya [21] and Takagi [39] respectively). The  $[\text{K}^+]$  are from Payan et al. [33].

Concentrations ioniques dans le plasma et l'endolymph et potentiels de Nernst à travers de l'épithélium sacculaire chez la truite. Les concentrations sont exprimées en mM. Le potentiel de Nernst est donné avec le plasma comme référence. Les valeurs du pH et de  $[\text{HCO}_3^-]$  sont données par Borelli et al. [8].  $[\text{Ca}]_{\text{tot}}$  est égal à 2.3 mM dans le plasma, 1.2 mM dans l'endolymph proximale et 1.0 mM dans l'endolymph distale [8]. Concernant la  $[\text{Ca}^{2+}]$ , deux valeurs sont proposées en tenant compte des pourcentages de  $\text{Ca}^{2+}$  estimés dans la littérature, soit respectivement 47% pour Mugiya [21] et 72% pour Takagi [39]. Les valeurs de  $[\text{K}^+]$  sont celles de Payan et al. [33].

Species	Plasma concentration	Endolymph			
		Proximal		Distal	
		Concentration	$E_{\text{eq}}$ (mV)	Concentration	$E_{\text{eq}}$ (mV)
$\text{K}^+$	2.8	19.7	-49.1	78.6	-84.0
$\text{H}^+$ (pH)	7.56	7.38	-10.5	7.84	+16.2
$\text{HCO}_3^-$	13.3	10.3	-6.4	29.9	+20.4
$\text{Ca}^{2+}$	1.15	0.86/0.56	+3.7/+9.1	0.72-0.47	+6.0/+11.2

To our knowledge, only few studies have presented the results of endolymph electrophoresis [6,7,27,40]. Proteic patterns of endolymphs are complex and revealed major and minor bands in a wide scale of molecular weights (Fig. 8). According to Borelli et al. [6] and to Fig. 8A, SDS PAGE analysis of the endolymph shows eight major stripes (macromolecules beyond the range, three bands around 66, 52, 36, 24 and 14 kDa) and minor stripes. The comparison of proximal and distal samples of endolymph showed similar patterns (Fig. 8A) suggesting that the spatial heterogeneity of proteins is quantitative and not qualitative.

Concerning the study of the otolith matrix, four experimental approaches were used after extraction (EDTA or acetic acid): quantitative analysis using colorimetric kits, SDS-PAGE electrophoresis, molecular characterization, and antibodies dressed against the OM.

- Irrespective of the method of demineralisation (0.5-M EDTA or acetic acid), trout and turbot otoliths were found to be largely composed of proteins, collagens and proteoglycans in different proportion than those found in the endolymph (Fig. 9A,B, [6]). The presence of protein and glycoprotein complexes has been recently confirmed by Dauphin and Dufour [10] in cod otolith.
- Electrophoretic protein patterns of otolith matrix are very different (Fig. 8B–D), suggesting that the results depend mainly on the experimental procedure. Sometimes, the electrophoresis of otolith matrix shows smears (Fig. 8D) that could result from the presence of sugars and proteoglycans in the

matrix [10]. According to Borelli et al. [6], five bands were visible in SDS PAGE analysis (macromolecules(s), 56, 33, 30 and 14 kDa, Fig. 8B) and three (macromolecules, 56 and 14 kDa) had similar apparent molecular weight in the endolymph (see the arrows in Fig. 8B). It should be noted that although the same amounts of proteins were introduced into the wells (10  $\mu\text{g}$  according to Coomassie blue), the staining of the gel by Coomassie brilliant blue gave a paler coloration of the matrix in comparison with the endolymph (Fig. 8B). This reveals that otolith matrix proteins and endolymph proteins react differently versus the same colorant.

- Only 2 otolith matrix proteins were characterized using biomolecular techniques: OMP-1 (55 kDa), a major component of EDTA-soluble matrix proteins which has 40% homology to the C-terminal half of the human melanotransferrin [27] and a collagen-like protein (100 kDa) called otolin-1 and identified as a major component of EDTA-insoluble fraction obtained from the chum salmon otolith [28]. The sequence of the otolin-1 revealed a high homology with parts of a saccular collagen-type described by Davis et al., [11]. This structural protein could serve as a template for calcification.
- In order to target the precursors of the otolith matrix within the saccular epithelium and the endolymph, polyclonal antibodies were dressed against the matrix of EDTA-soluble fraction [40] or acetic acid-soluble fraction [7]. By immunohistochemistry, Takagi and Takahashi [40] identified the saccular cells responsible for the synthesis and secretion of

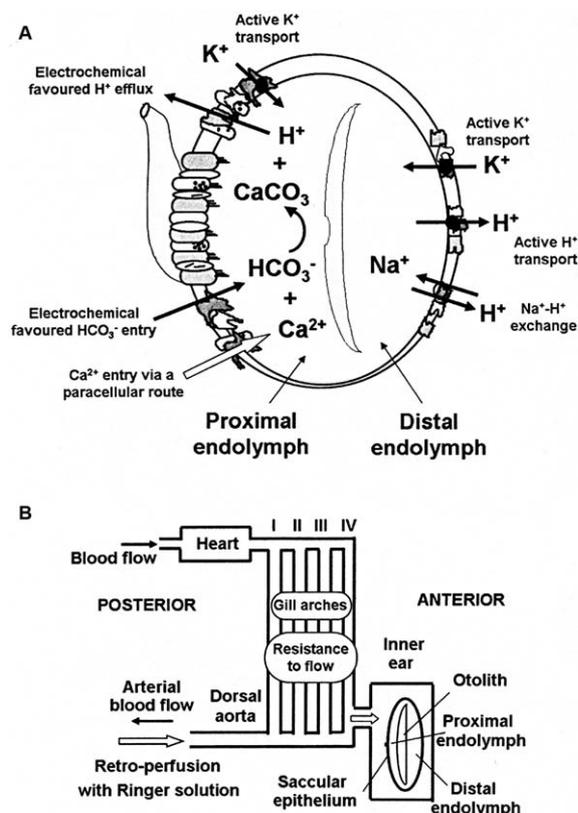


Fig. 6. The ion-transporting model across the proximal and distal saccular epithelium. (A) Hypothetic ionic fluxes across the saccular epithelium (the model combines the results of Payan et al. [31–34] and Borelli et al. [8]). See further explanations in the text. (B) Schema showing the experimental set-up to study ionic fluxes in perfused inner ears [34].

Fig. 6. Modèle de transport d'ions à travers les zones proximale et distale de l'épithélium sacculaire. (A) Flux ioniques hypothétiques à travers l'épithélium sacculaire (le modèle intègre les résultats de Payan et al. [31–34] et de Borelli et al. [8]). Voir les explications dans le texte. (B) Schéma du montage expérimental pour l'étude des flux ioniques à l'aide de la technique de perfusion de l'oreille interne [34].

the EDTA-soluble fraction of the otolith matrix. Using Western blotting, only one band (94 kDa) was detected in the endolymph with the antibodies raised against the EDTA-soluble fraction [40] and two bands (65 and 75 kDa) were observed with the antibodies raised against the acetic acid-soluble fraction [7]. The small number of proteins recognized by the antibodies within the endolymph is surprising and probably results from a weak immunoreactivity and/or unsatisfactory separation of proteins in the otolith extract.

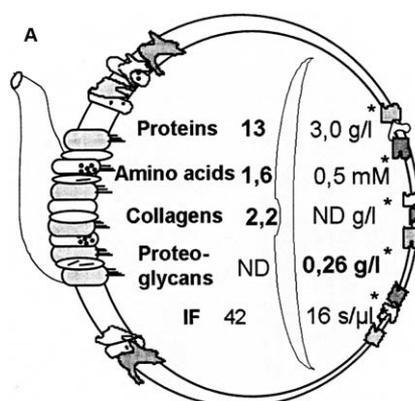


Fig. 7. Organic compound composition in saccular endolymph (adapted from Borelli et al., [6]). (A) Concentration of various organic compounds in proximal and distal endolymphs – Coomassie blue sensitive proteins (proteins), amino acids, total collagens, total proteoglycans and inhibiting factor (IF). The star (\*) indicates that the difference in concentration between proximal and distal zones is statistically different; ND means non-detectable.

Fig. 7. Composés organiques de l'endolymph sacculaire (adapté de Borelli et al. [6]). (A) Concentration de divers composés organiques dans l'endolymph proximale et distale – protéines sensibles au bleu de Coomassie, acides aminés, collagènes totaux, protéoglycans totaux et facteur inhibiteur (IF). L'astérisque (\*) indique que la différence de concentration entre endolymph proximale et distale est statistiquement différente ; ND = non détectable.

#### 4. Turnover rate of otolith precursors within the endolymph

Organic components present in the endolymph are synthesized *de novo* by specialized cells of saccular epithelium, whereas the ionic composition of the endolymph results from fluxes through this epithelium. Two kinds of ions may be considered: those that are not directly involved in the calcification process (Na<sup>+</sup>, K<sup>+</sup>, Mg<sup>2+</sup>, PO<sub>4</sub><sup>3-</sup>...) and are in equilibrium (influx = out-flux), and those that are precursors of the CaCO<sub>3</sub> formation and consumed (Ca<sup>2+</sup> and HCO<sub>3</sub><sup>-</sup>) or produced (H<sup>+</sup>) during the calcification process.

Considering the precursors of the calcification, the ratio between their endolymph pools and their daily incorporation into the otolith allows us to calculate the turnover rates and offers a dynamic vision of the overall calcification process. As summarized in Fig. 10, only a small fraction (between 0.02 and 1%) of the organics precursors present in the endolymph is used

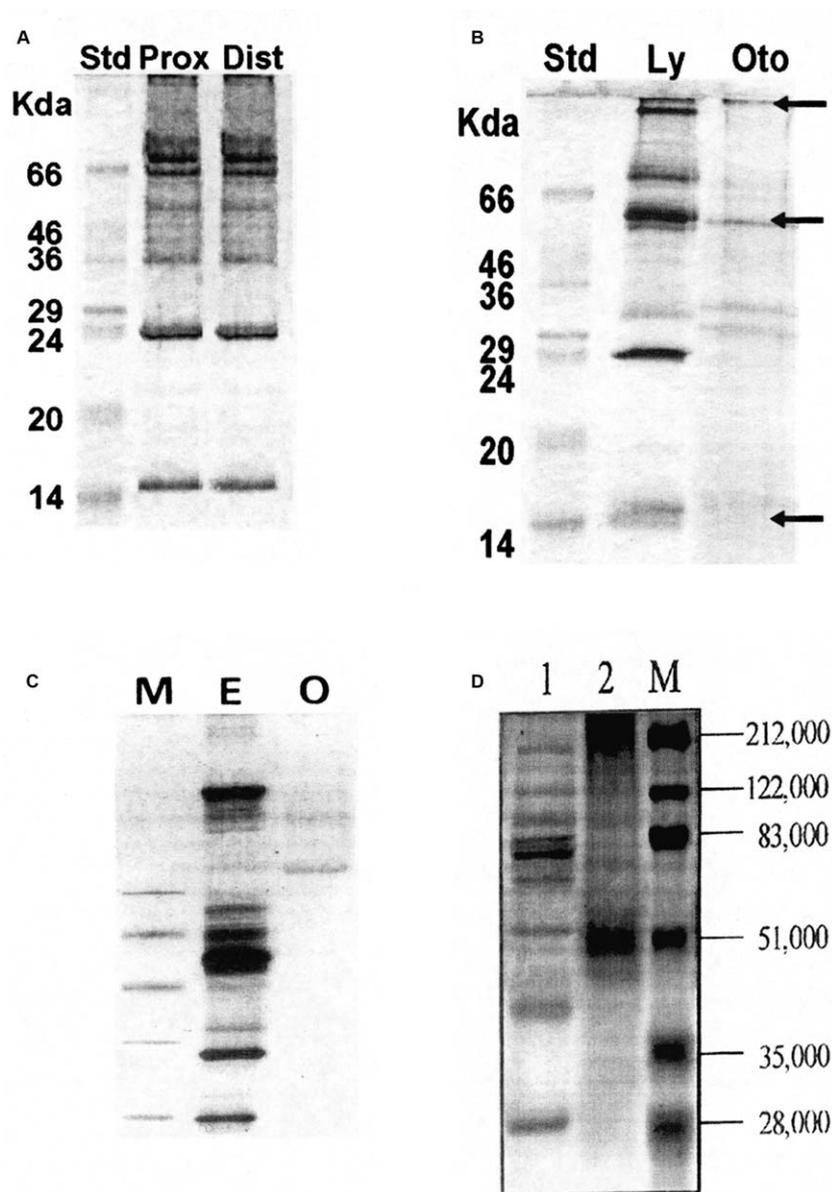


Fig. 8. SDS-PAGE of endolymph and otolith matrix in trout stained with Coomassie brilliant blue. (A) Proximal (Prox) and distal (Dist) endolymph electrophoresis under reduced conditions on 12% SDS-PAGE [6]. (B)–(D) Comparison between endolymph and the otolith matrix electrophoresis by different authors. (B) Under unreduced conditions on 12% SDS PAGE. (Std: markers, Ly: endolymph, Oto: otolith matrix, 10 µg proteins per well, Borelli et al. [6]). (C) Under unreduced conditions on 5–20% gradient SDS PAGE (markers MW 94, 67, 43, 30, 20 and 14 kDa from the top, Takagi and Takahashi [40]). (D) Under reduced conditions on 12% SDS-PAGE (lane 1: endolymph, lane 2: otolith matrix, M: markers, [27]).

Fig. 8. Électrophorèse SDS-PAGE de l'endolymphe et de la matrice de l'otolithe chez la truite (coloration au bleu de Coomassie brillant). (A) Endolymphe proximale (prox) et distale (dist) sous condition réduite sur 12% SDS-PAGE [6]. (B)–(D) Comparaison entre électrophorèses de l'endolymphe et de la matrice de l'otolithe par différents auteurs. (B) Sous conditions non réduites sur 12% SDS-PAGE (Std : marqueur, Ly : endolymphe, Oto : matrice organique de l'otolithique, 10 µg de protéines par puits, Borelli et al. [6]). (C) Sous conditions réduites, gradient de 5–20% SDS-PAGE (marqueurs MW 94, 67, 43, 30, 20 et 14 kDa depuis le haut, Takagi et Takahashi [40]). (D) Sous conditions réduites sur 12% SDS-PAGE (piste 1 : endolymphe, piste 2 : matrice organique de l'otolithique, M : marqueurs, [27]).

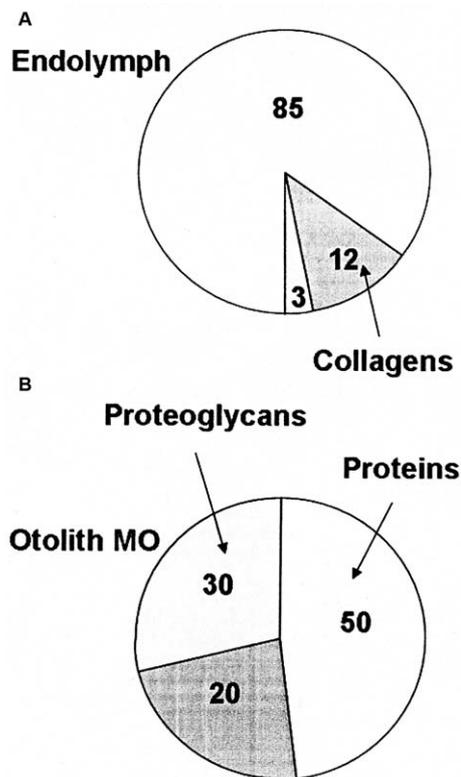


Fig. 9. Comparison of the organic compounds between endolymph and otolith matrix of trout. (A)–(B) Schematic representation of percentage (indicated as number) in proteins, collagens and proteoglycans in endolymph (A) and otolith matrix (B). Adapted from Borelli et al. [6].

Fig. 9. Comparaison des composés organiques entre l'endolymph et la matrice organique de l'otolithique chez la truite. (A)–(B) Représentation schématique des pourcentages en protéines, collagènes et protéoglycans dans l'endolymph (A) et la matrice organique de l'otolithe (B). Adapté de Borelli et al. [6].

in matrix formation per day [6]. The endolymph can therefore be considered as a reserve of organic matrix precursors in considerable excess of daily requirements. Inversely, with regard to the daily deposition of  $\text{CaCO}_3$ , the amounts of calcium and bicarbonate consumed correspond to 7 and 1 time(s) the content of the endolymph pools respectively, a much higher percentage utilization than those of the organic compounds. As previously mentioned, these needs should be related to the fact that the proximal epithelium was found freely permeable to ionic species involved in otolith growth.

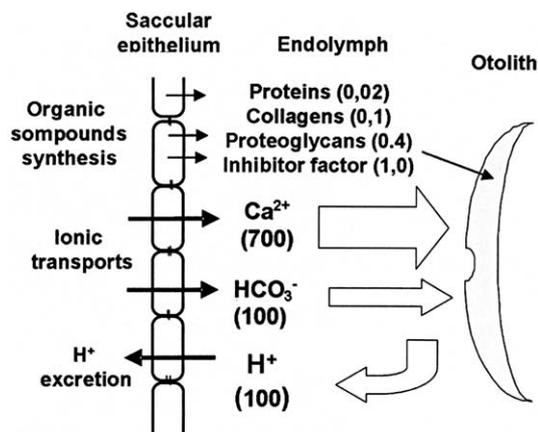


Fig. 10. Turnover rate of otolith precursors within the endolymph. The turnover rate corresponds to the ratio between the daily amounts needed for the otolith deposition and the endolymph pools of precursors. Turnover rate is expressed as  $\% \text{ j}^{-1}$  and given in brackets. Adapted from Borelli et al. [6].

Fig. 10. Taux de renouvellement des précurseurs de l'otolithe dans l'endolymph. Le taux de renouvellement correspond au rapport entre la quantité journalière nécessaire au dépôt de l'otolithe et le pool de précurseurs dans l'endolymph. Le taux de renouvellement est exprimé en  $\% \text{ j}^{-1}$  et donné entre parenthèses. Adapté de Borelli et al. [6].

### 5. Are there relationships between endolymph heterogeneity and otolith growth? (Fig. 11)

The difference in protein levels (collagenic and non-collagenic) between the proximal and distal endolymphs clearly matches the growth axes of the otolith. Actually, the proximal zone facing the macula corresponds to the convex shape of the otolith where the growth rate is generally greater than on the concave (distal) side. Thus the endolymph fraction with the highest proteins contents bathes the side of the otolith characterized by the highest growth.

The intra-endolymph repartition of proteins has a further signification in relation with the fact that in all biological fluids proteins are strong chelators of  $\text{Ca}^{2+}$ . Thus, in spite of the small decreasing proximo-distal gradient of  $[\text{Ca}]_{\text{tot}}$  (about 10%, Fig. 5B), an increasing proximo-distal gradient of  $\text{Ca}^{2+}$  should be created (Fig. 11). Finally, increasing proximo-distal gradients of  $[\text{CO}_2]_{\text{tot}}$ ,  $[\text{HCO}_3^-]$  and pH have been recorded [8,13,33]. As  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  combine to form  $\text{CaCO}_3$ , the presence of these ionic gradients would favour the formation of  $\text{CaCO}_3$  along the proximo-distal axis. These results do not agree with the otolith

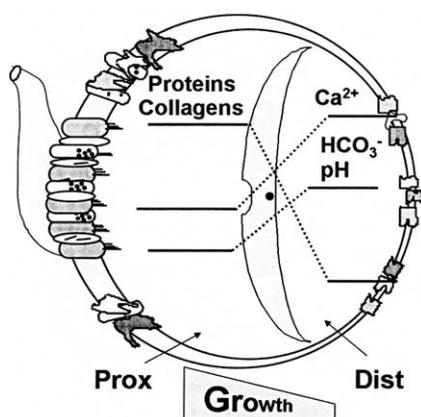


Fig. 11. Schematic representation of chemical heterogeneity within the endolymph in relation with otolith growth.

Fig. 11. Représentation schématique de l'hétérogénéité chimique de l'endolymphe en rapport avec la croissance de l'otolithe.

growth gradients (Fig. 11). Payan et al. [33] proposed that these ionic gradients would correspond to driving forces favouring (i) the buffering of the  $H^+$  produced during  $CaCO_3$  formation and (ii) the availability of ionic precursors necessary to the front of calcification.

## 6. Diurnal dynamic of otolith growth

We will mainly discuss results concerning the proximal endolymph that bathes the convex shape of the otolith characterized by the maximal growth rate [30]. Recently, Borelli et al. [8] confirmed in the trout the results observed in turbot [13] concerning the daily variation of the endolymphatic precursors of calcification. Thus the proteins (non-collagenous and collagenous) peak during the day and vary in antiphase with  $Ca^{2+}$  and  $HCO_3^-$ , which increase during the night (Fig. 12).

These findings confirm a daily variation in otolith calcification raised in previous reports [23–25,42,45,47]. It may be noted that Bettencourt and Guerra [5] observed that protein and calcium levels showed discrete variations during the day in cephalopod endolymph, which have been associated to a daily deposition of  $CaCO_3$  on cephalopod statoliths.

The daily variations of endolymph proteins cannot be explained by the formation of otolith matrix as less than 1% of the proteins present in the endolymph is incorporated during otolith increment [6,13]. Thus, the

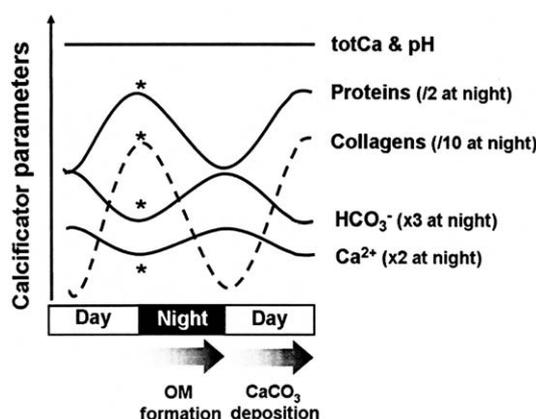


Fig. 12. Daily variations of calcification factors in the proximal endolymph. Schematic representation of overall variations in these factors observed during the day and night periods. OM: organic matrix. Adapted from Borelli et al. [8].

Fig. 12. Variations journalières des facteurs de calcification dans l'endolymphe proximale. Représentation schématique des variations totales de ces facteurs observées pendant les périodes diurnes et nocturnes. OM = matrice organique. Adapté de Borelli et al. [8].

functional significance of such huge variations of protein levels remains unresolved. Concerning the ionic precursors, their endolymph variations could result from their utilisation to build the otolith, as the daily  $CaCO_3$  deposit needs seven and one endolymph pools of  $Ca^{2+}$  and  $HCO_3^-$ , respectively. Furthermore, Borelli et al. [8] suggested that the supersaturation state of aragonite ( $S_a$ ) should fluctuate around the unity during the day–night cycle and  $CaCO_3$  precipitation should occur when saturation is reached, at the end of the night (Fig. 12). The fact that  $CaCO_3$  can precipitate at pH 7.4 at the proximal side of the otolith is a new non-classical view, as it was repeatedly proposed that very alkaline pH was necessary for such a mechanism. This reinforces the primordial importance of the organic matrix in the overall calcification process.

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