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Daily variations of endolymph composition: relationship with the otolith calcification process in trout

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Summary

Ionic and organic parameters of the otolith calcification process in the trout *Oncorhynchus mykiss* were analysed in plasma and endolymph over the day:night cycle. Plasma pH remained constant and total CO_2 concentration was significantly lower (by 21%) during the day than at night. Calcifying parameters (total CO_2, total calcium concentration) were measured in the proximal and distal endolymphs and were unchanged in the latter during the day:night cycle, but fluctuated in the former. Non-collagenous protein and collagen concentrations in endolymph were higher (1.5- and 10-fold, respectively) during the day than at night. As there was no change in total calcium concentration, we propose that Ca^{2+} increases during the dark period and was maximal by the end of the night when the total CO_2 concentration has also increased (by 14%). Measurements of endolymph pH *in situ* revealed significant differences between samples from proximal and distal endolymph (7.38 and 7.87, respectively), but no variation between values obtained during the day and at night. Thus, the saturation state of aragonite (Sa) in the proximal endolymph should fluctuate around unity during the day:night cycle, and $CaCO_3$ precipitation should occur when supersaturation is reached. The electrophoretic pattern of proximal endolymph showed variations in both major and minor components. Immunoblotting of endolymph, using a rabbit antiserum raised against the otolith soluble organic matrix revealed an increase in the expression of two proteins (65 kDa and 75 kDa) during the day period. We propose that organic matrix and calcium carbonate deposition on the otolith vary antiphasically: organic matrix deposition begins by the end of the day period, when the concentration of organic precursors is maximal in the endolymph, whereas $CaCO_3$ precipitation starts once the solubility of $CaCO_3$ is exceeded.

Key words: otolith, calcification, day–night cycle, endolymph.

Introduction

A general characteristic of calcified biominerals is a periodic growth increment producing concentric density bands. These structures are useful for age determination on a daily or annual scale, and their microanalysis constitutes the basis for reconstruction of paleoclimatic records or studies of population biology using organisms as diverse as coral skeletons (Knutson et al., 1972), bivalve shells (Carell et al., 1987), squid statoliths (Arkhipkin, 1997) or fish otoliths (Campana, 2001). Otoliths in teleost fish are calcified structures located in the inner ear and involved in hearing and maintenance of equilibrium (Fay, 1984; Popper et al., 1993). Otolith consists of a predominant (>90%) mineral phase of $CaCO_3$ in aragonite form (Carlstrom, 1963) enmeshed into an organic matrix (OM) that only accounts for 0.01–10% of the total mass (Degens et al., 1969). The OM consists of a complex network of macromolecules having Ca^{2+}-binding capacity and often expressing an anticalcifying activity (Morales-Nin, 1987; Wright, 1991a; Asano and Mugiya, 1993; Sasagawa and Mugiya, 1996). Recent studies on trout otolith have also shown the presence of collagens in the matrix (Borelli et al., 2001; Murayama et al., 2002). The OM plays an important role in different steps of the calcification process, such as nucleation, orientation, inhibition, crystal nature and growth regulation (Wheeler et al., 1981, 1988; Belcher et al., 1996; Falini et al., 1996). However, the literature still presents a confusing picture of the timing of density band formation that impairs optimal use of these environmental archives (Barnes and Lough, 1996; Hernaman et al., 2000). Some of these difficulties arise from our poor knowledge of the basic control mechanism of calcification at the crystal level, and more particularly the relationship between ionic and organic control.

As with other calcified biominerals, the otolith structure displays alternate optically dense layers (rich in organic materials) and translucent layers (rich in minerals), forming concentric rings (Pannella, 1980; Morales-Nin, 1987). In many fish, the deposition of these two layers produces a recognisable daily increment (Pannella, 1971). Several studies have suggested a close relationship between the cyclic process of otolith calcification and photoperiod (Taubert and Coble, 1977; Radtke and Dean, 1982). Reversal of the light:dark cycle was shown to induce an inversion of the two layers in *Tilapia nicolica* (Tanaka et al., 1981). However, in juvenile fish, the daily increment of the two layers was maintained when fish were kept under constant dark or constant light, suggesting a control by endogenous factors (Mugiya, 1987; Wright et al., 1991b, 1992). Finally, Mugiya (1984) showed that there is a seasonal reversal in the rhythm of trout otolith calcification that can be associated with a seasonal inversion in plasma calcium cycle.

Otolith growth is an acellular process, carried out far from the saccular epithelium, implying that calcification process is strictly dependent on endolymph fluid chemistry. This morphological particularity provides a unique opportunity to sample and analyse calcifying fluid in order to establish relationships between its composition and the mineralisation process. The composition of endolymph is characterised by a high K^+ concentration, an alkaline pH, and saturated Ca^{2+} and HCO_3^- concentrations (Enger, 1964; Mugiya and Takahashi, 1985; Kalish, 1991; Payan et al., 1997; Takagi, 2002). The endolymph also expressed an anticalcifying activity, probably involved in the mechanism of regulation of calcification (Borelli et al., 2001, 2002). It has been suggested that the otolith calcification process is related to the specific composition of the endolymph (Romanek and Gauldie, 1996; Borelli et al., 2001; Takagi, 2002), and recent studies have shown a lack of uniformity in the spatial distribution of ionic and organic endolymphatic components that could also be involved in the otolith formation (Payan et al., 1999; Borelli et al., 2001).

To our knowledge, there have only been two studies following plasma and endolymph parameters during the circadian cycle. Mugiya and Takahashi (1985) found simultaneous variations in trout plasma and endolymph for pH, with a nocturnal maximum (+0.4 pH unit), and total CO_2 concentration, which peaked in the daytime (+25%). More recently, simultaneous variations in total CO_2 concentration in turbot plasma and endolymph have been observed (Edeyer et al., 2000); however, the peak was nocturnal. In this last work, the concentrations of protein in endolymph samples clearly decreased during the night, and the non-uniformity described for the endolymph components in this species (Payan et al., 1999) was maintained during the circadian cycle. However some important components of endolymph that play a role in calcification, such as calcium, collagen and the anticalcifying factor (Borelli et al., 2001; Payan et al., 2002), were not measured.

The aim of the present work was to examine ionic and organic modifications of trout endolymph composition during the day:night cycle, taking into account recent work, in order to determine limiting key factor(s) in the regulation of calcification. An integrated view of our results is presented to link the observed daily variations of endolymphatic precursors with the cyclic process of otolith calcification, which takes place at the endolymph–otolith interface where the $CaCO_3$ deposition occurs.

Materials and methods

Fish handling

Trout *Oncorhynchus mykiss* Walbaum, body mass 200–350 g and 12 months old, were obtained from a local fish farm. They were maintained in running tapwater at 17°C under a constant photoperiod of 12 h:12 h light:dark, the light period starting at 08.00 h, for at least 3 weeks before experimentation. Experiments were performed in February and March and the fish were fed once a day every morning.

Collection of plasma, endolymph and otolith

The experiments reported in this paper complied with the Principles of Animal Care of the National Institute of Health (publication no. 86, revised 1985) and the French laws for experiments on animals (decree no. 87-848).

Blood was sampled from the caudal vessels and the pH measured immediately; the samples were then centrifuged and the plasma kept on ice until analysis.

Endolymphs were collected as described by Payan et al. (1997) between 13:00 h and 15:00 h (as day reference) and between 03:00 h and 05:00 h (as night reference). Briefly, the fish were decapitated and the operative field washed 3 times with 150 mmol l^{-1} NaCl. The saccular epithelium was incised to allow the collection of endolymph as two samples, one from the proximal zone (the space between the macula and the otolith) and the other from the distal zone (the space facing the opposite side of the otolith). Samples were stored at –20°C until analysis.

Otoliths (sagittas) were washed with 1 mol l^{-1} NaOH, rinsed with deionised water, wiped and ground into powder. The powder was decalcified using acetic acid (maintained at pH 4). After centrifugation (10 000 ***g***, 10 min) the soluble and insoluble fractions were separated. The supernatant, containing the soluble fraction of the organic matrix, was ultrafiltered (Amicon, Saint-Quentain, France; CO 3 kDa) at 4°C and lyophilised. This freeze-dried OM was used to generate polyclonal antibodies in a rabbit (Eurogentec, Angers, France).

Measurements

The pH was measured with a Tacussel (Villeurbane, France) pH meter connected to a Metler Toledo electrode (InLab 423; Paris, France). Endolymph pH was measured *in situ* by inserting a minielectrode (VIP, Mini Combo, Stevenage, UK; tip diameter 450 μm) into the proximal and distal zones through a small incision. The pH value was recorded within the 40 s period following the incision of the saccular wall.

These endolymph pH measurements were performed in January on a batch of trout of the same size, and measurements were done in daytime (08:00–12:00 h).

Total calcium and total CO_2 concentrations were measured by spectrophotometry using kits (Sigma, Saint-Quentain, Fallavier, France). Protein concentration was measured by colorimetry using Coomassie Blue (G 250) with bovine serum albumin as standard. Collagen concentration was determined using a Sircol kit (Biocolor) with acid-soluble type I collagen as standard. In this assay, the dye reagent binds specifically to the $[Gly\text{-}X\text{-}Y]_n$ helical structure found in all collagens and analysis of Sircol dye binding *versus* hydroxyproline (tested by the manufacturer) showed a linear and significant correlation.

SDS-PAGE and western blotting

Electrophoresis was performed on 7.5% or 12% Tris–glycine polyacrylamide gels under reduced conditions, 5 μg protein per well, following the method of Laemmli (1970). Gels were run at a constant voltage of 150 V for 1 h at 4°C. Standard proteins used were 'kaleidoscope prestained standards' (Biorad, Marne la Coquette, France): myosin (208 kDa), β galactosidase (132 kDa), bovine serum albumin (91 kDa), carbonic anhydrase (45.2 kDa), soybean trypsin inhibitor (35.1 kDa), lysozyme (18 kDa) and aprotinin (7.6 kDa). Proteins were stained with silver using the 'silver stain plus kit' (Biorad). The densitometric profiles were analysed using software developed by the Research Services branch of the National Institute of Health (NIH).

Western blotting of endolymph proteins on 7.5% gels was done using a nitro-cellulose membrane (Sigma; 0.45 μm pore size) and immunoblotting performed following the method of Towbin et al. (1979). The membrane was first incubated with 5% milk for 1 h to block non-specific binding sites. Then, the membrane was incubated (overnight at 4°C, antiserum diluted 1:2000) with a rabbit antiserum raised against the soluble OM of trout otolith. After rinsing, the membrane was incubated with peroxidase-conjugated goat anti-rabbit globulin (Biorad). Localisation was visualised by the ECL technique (Perkin Elmer, Courtaboeuf, France). The reactivity of the rabbit antiserum was tested by comparison of the labelling obtained with pre-immune and immune serum on the OM of trout by dot blots. The immune serum reacted whereas the pre-immune serum did not (results not shown). The antiserum (dilution 1:200) did not recognise trout plasma proteins on western blots (results not shown).

Calculations and statistical analysis

The partial pressure of CO_2 (P_{CO_2}) was calculated using the Henderson–Hasselbach equation $(pH=pK+\log[HCO_3^-]/d^{[CO_2]}$ with a pK value for CO_2/HCO_3^- of 5.75 and a CO_2 solubility coefficient of 0.057 mmol l^{-1} $mmHg^{-1}$ at 17°C; Albers, 1970).

Results are expressed as means ± S.E.M. (N) and analysed statistically using STATVIEW Software (Brain Power, Inc., Berkeley, CA, USA). Comparison of the means was made by a one-way analysis of variance (ANOVA). Differences were considered significant at $P<0.05$.

Results

Diurnal variation of plasma composition

Protein and total calcium concentrations were slightly but significantly increased in plasma (12% and 7%, respectively) during the night (Table 1). In plasma, values of pH remained stable while a significant increase in total CO_2 concentration was observed at night (+2.9 mmol l^{-1}, i.e. 21%). The calculated P_{CO_2} was significantly higher during the night (+1.3 mmHg, where 1 mmHg=133.3 Pa; i.e. 31%).

Endolymph composition in day samples

Calcium and CO_2 concentrations in endolymph samples are shown in Table 2. Total calcium concentration $[Ca]_{tot}$ was significantly greater (by 20%) in the proximal samples than in the distal ones, in contrast to $[CO_2]_{tot}$, which was threefold greater in the distal samples. Direct measurements of pH within the endolymph revealed significant differences between proximal and distal endolymph (7.38±0.047, N=8 and 7.87±0.078, N=8, respectively, $P<0.0001$; not shown). The protein concentration was approximately threefold greater in the proximal than the distal endolymph in day (Table 3). Collagen concentration was only measured in pooled proximal endolymph. These results demonstrate that the composition of endolymph surrounding the otolith is not uniform for the components measured.

Diurnal variation of endolymph heterogeneity

The $[Ca]_{tot}$ measured during the night was not significantly different from that measured during the day (Table 2), but the significant difference between proximal and distal samples was lost during the night. During the night, the $[CO_2]_{tot}$ was significantly increased (by 30%) in the proximal endolymph,

Table 1. *Diurnal variations in plasma composition*

	Protein (g l^{-1})	Total Ca (mmol l^{-1})	Total CO_2 (mmol l^{-1})	Measured pH	Calculated P_{CO_2} (mmHg)
Day	30.7±1.92 (20)	2.3±0.04 (20)	13.5±0.48 (10)	7.56±0.240 (20)	4.2±0.31 (8)
Night	34.9±0.97 (20)	2.5±0.04 (20)	16.4±0.74 (11)	7.52±0.280 (20)	5.5±0.50 (8)
P values	0.0400	0.0054	0.0040	0.5220	0.0426

Values are means ± S.E.M. (number of fish).
Differences between day and night were considered significant at $P\leq0.05$.

Table 2. *Diurnal variations of ionic parameters in proximal and distal endolymphs*

	Total Ca (mmol l^{-1})		*P*	Total CO_2 (mmol l^{-1})		*P*
	Proximal	Distal	(proximal/distal)	Proximal	Distal	(proximal/distal)
Day	1.2±0.09 (10)	1.0±0.04 (9)	0.0265	10.5±0.61 (8)	31.1±3.09 (10)	0.0001
Night	1.1±0.11 (10)	1.1±0.05 (9)	0.5482	13.6±0.76 (10)	32.2±3.19 (9)	0.0001
P (night/day)	0.3796	0.0681		0.0069	0.8015	

Values are means ± S.E.M. (number of fish).
Differences between day and night, and between proximal and distal endolymphs, were considered significant at $P \leq 0.05$.

Table 3. *Diurnal variations of organic parameters in proximal and distal endolymphs*

	Protein (g l^{-1})		*P*	Collagen (g l^{-1})
	Proximal	Distal	(proximal/distal)	(proximal)
Day	15.2±0.70 (38)	5.3±0.79 (39)	0.0001	2.5
Night	10.5±0.46 (40)	3.9±0.68 (40)	0.0001	0.20
P (night/day)	0.0001	0.1782		

Values are means ± S.E.M. (number of fish) except for collagen.
Collagen concentration was measured on 10 pooled endolymph samples.
Differences between day and night, and between proximal and distal endolymphs, were considered significant at $P \leq 0.05$.

but was unchanged in the distal endolymph (Table 2), and although the magnitude of the difference between the proximal and distal endolymph was reduced during the night, it was still significant.

Protein concentrations in endolymph were followed in a preliminary experiment and the protein levels increased after the dark–light transition, peaking approximately 5 h after the light period began (Fig. 1). The differences between the night and day samples in proximal endolymph was highly significant. The results of this experiment and those obtained previously on turbot (Edeyer et al., 2000) were used to select the sampling periods for the main experiment. Protein concentration decreased by 25–30% in both proximal and distal zones, but the decrease was only significant for the proximal value (Table 3). Although collagen was only measured on pooled proximal endolymph, the decrease (90%) during the night was important.

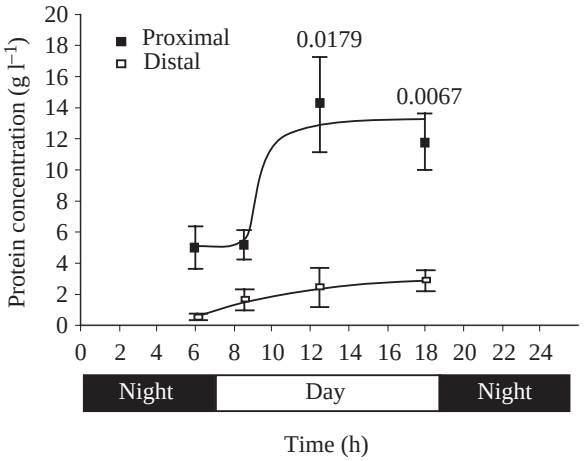

Fig. 1. Protein concentrations in trout endolymph during the day:night cycle. Solid squares, proximal and open squares, distal endolymph. Values are means ± S.E.M. (N=4). Comparisons between reference (6:00 h) and day and night values were considered significant at $P \leq 0.05$.

Qualitative diurnal variation of endolymphatic organic compounds

Protein patterns in proximal endolymph samples collected during day and night periods were compared by gel electrophoresis (Fig. 2A). Whatever the collecting period, the endolymph displayed a complex protein pattern composed mainly of major bands together with several minor bands, covering a large range of molecular mass. After densitometric analysis, comparison between day and night samples (Fig. 2B), revealed that the profiles were globally similar in number and position of most bands. While some peaks were similar in size in both samples, peak(s) of high molecular mass were denser in the day samples whereas several bands (approximately 65–80, 35 and 21 kDa) were denser in the night samples (arrows in Fig. 2B).

Presence of organic precursors of the otolith organic matrix in the endolymph

Western blotting of proximal endolymph, using the antiserum raised against the soluble OM of trout otolith, revealed only two bands of estimated molecular mass 65 kDa and 75 kDa. They were detected in samples from both day and night (Fig. 2C), but comparative densitometric analysis (Fig. 2D) clearly showed that both bands were more

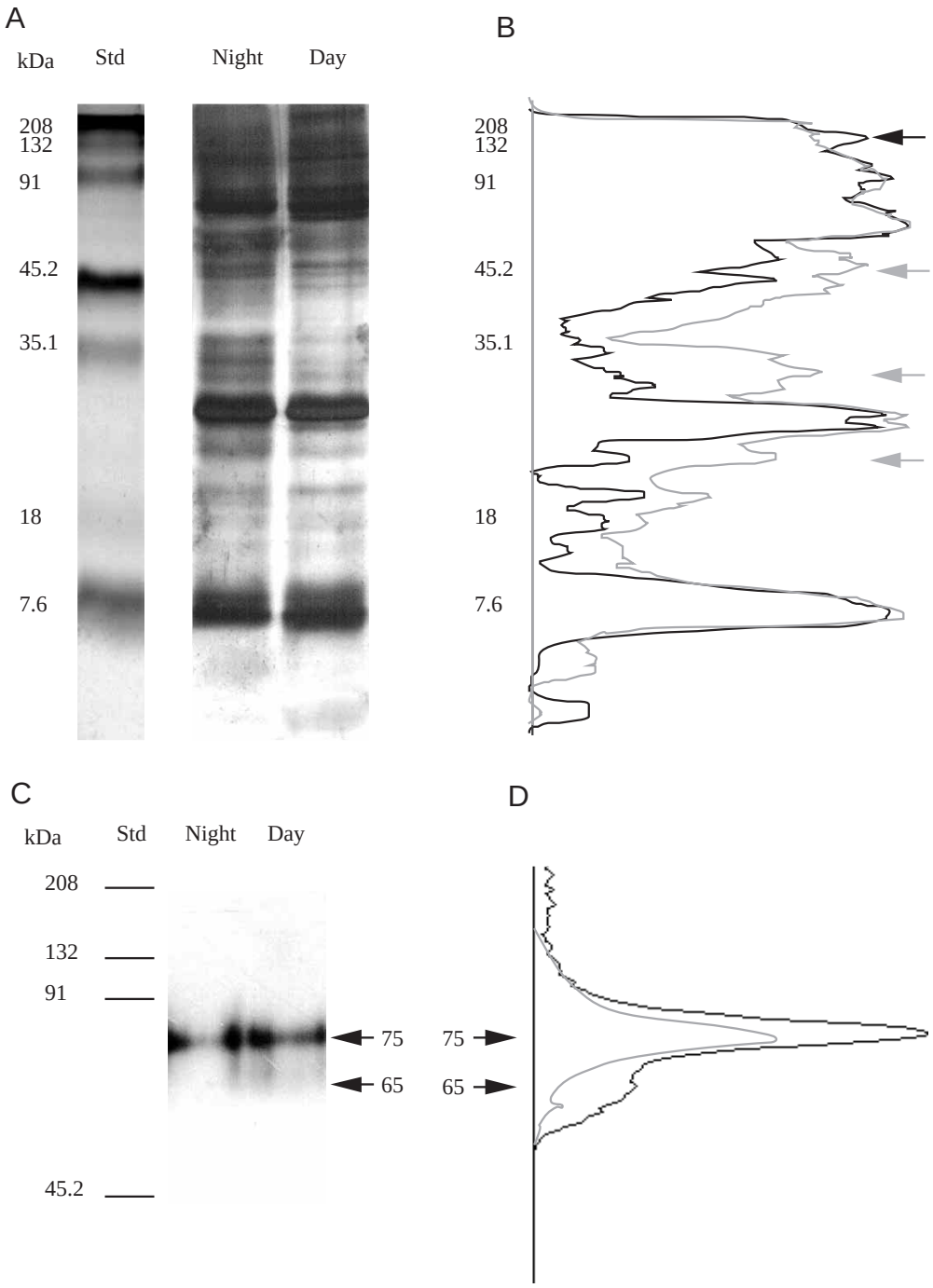

Fig. 2. Comparative analysis of endolymph samples collected during day and night periods. (A) Electrophoresis of proximal endolymph. Samples were run at 150 V for 1 h at 4°C on 12% SDS gels under reduced conditions. 5 μg protein was loaded per well. Proteins were stained with 'silver stain plus kit'. (B) Analysis of the variations in endolymph composition was performed using N.I.H. software (see Materials and methods). Dark (day) and light (night) traces represent the level of band density on gel electrophoresis. Arrows represent the main variations observed in day and night samples. (C) Western blot of endolymph, showing the presence of specific precursors of otolith organic matrix in endolymph. Western blots were performed using 7.5% gel electrophoresis under the conditions described in Materials and methods. Nitrocellulose was probed using rabbit anti-OM (1/2000 dilution), followed by horse radish peroxidase-conjugated goat anti-rabbit globulin. (D) Densitometric analysis of the immunoblot in C. Dark (day) and light (night) traces represent the level of band density on western blotting. Arrows represent the main variations observed in day and night samples.

concentrated (1.6 and 4-fold, respectively) in the day sample than in the night one.

Discussion

The present work performed on trout confirms the results of Edeyer et al. (2000) on turbot and presents new data concerning the evolution of plasma and endolymph concentrations of ionic and organic components during a circadian cycle in relation to the calcification process. Both studies showed variations in the endolymph composition, which reinforces the hypothesis of a daily variation in otolith calcification raised in previous reports (Takana et al., 1981; Mugiya, 1984, 1987; Watabe et al., 1982; Wright et al., 1992).

Importance of Ca^{2+} and CO_3^{2-} endolymphatic levels in daily otolith increments

The calcification process is strictly a function of endolymph fluid chemistry (see Introduction). Recently, Takagi (2002) calculated the aragonite saturation state (Sa) of the trout endolymph from direct quantification of electrolyte concentrations and concluded that endolymph is supersaturated

with respect to aragonite. We will mainly discuss results concerning the proximal endolymph that bathes the proximal face of the otolith, characterised by the maximal growth rate (Panella, 1980).

Many studies have suggested that otolith calcification is related to plasma calcium concentration (Edeyer et al., 2000; Mugiya, 1984; Wright et al., 1992). Consequently, changes in plasma calcium levels would be expected to have a direct effect on calcium levels in the endolymph. This hypothesis was directly confirmed by Payan et al. (2002) on trout during experiments *in vivo* (induced hypercalcemia) and *in vitro* (perfused isolated inner ear). These authors observed a tight relationship between $[Ca]_{tot}$ in perfusing fluid and endolymph. Calcium diffuses *via* a paracellular pathway located in the proximal area of the saccular epithelium with a very high turnover rate of the endolymphatic calcium pool (approximately 200% h^{-1}). These results suggest that rapid variations of $[Ca^{2+}]$ in plasma induce same variations in the endolymph. In the present study, we confirmed that $[Ca]_{tot}$ in plasma varied daily as previously noted (Mugiya, 1984, 1987; Mugiya and Oka, 1991; Edeyer et al., 2000; Wright et al., 1992). However, although [protein] and $[Ca]_{tot}$ in the plasma were both significantly increased during the night (Table 1), it is not evident that plasma $[Ca^{2+}]$ was increased.

In fact, $[Ca^{2+}]$ depends on several factors such as $[Ca]_{tot}$, pH of the fluid, and the nature and concentration of Ca^{2+}-binding proteins. Concerning $[Ca^{2+}]$ in the endolymph, Payan et al. (2002) failed to measure it *in situ* using a Ca^{2+}-sensitive mini-electrode. Mugiya (1966) measured the ultrafiltrable fraction (72%) and Takagi (2002) found that 47% of the calcium of trout endolymph was ionised, thus showing that the endolymphatic calcium was partly bound to proteins. Our results show for the first time that in the proximal endolymph of trout, protein levels are minimal at the end of the night, whereas $[Ca]_{tot}$ and pH were unchanged from daytime values. This suggests that the Ca^{2+} activity in the proximal endolymph varies, being maximum at the end of the night. Similar conclusions were proposed by Edeyer et al. (2000) on turbot from the variations detected in plasma concentration of $[Ca]_{tot}$ and proteins. It must be noted that Bettencourt and Guerra (2000) observed that protein and calcium levels showed discrete antiphasic variations during the day in cephalopod endolymph. There were significantly higher protein levels in the morning than in the evening and the opposite trend was seen for calcium. These authors associated these variations to a daily deposition of $CaCO_3$ on cephalopod statoliths.

Our results agree with previous reports of diurnal variations in $[CO_2]_{tot}$ in plasma and saccular endolymph (Mugiya and Takahashi, 1985; Edeyer et al., 2000), with a maximum at the end of the night for autumn–winter experiments (present results; Edeyer et al., 2000). $[CO_3^{2-}]$ in the endolymph can be calculated from the dissolved CO_2 and pH values. The pH of endolymph was calculated using the constants of the Henderson–Hasselbach equation for the plasma (see Results), assuming that the P_{CO_2} in endolymph was similar to that in plasma. In the proximal endolymph, we estimated the pH values to be 7.39±0.047 (N=8) during the day and 7.40±0.051 (N=8) during the night. In the distal endolymph, we estimated the pH values to be 7.84±0.078 (N=8) during the day and 7.76±0.081 (N=8) during the night. These results clearly suggest that the pH of endolymph remained unchanged during the day:night cycle and that the proximal endolymph was more acidic than the distal one. Direct measurements of endolymph pH for the day period are in accordance with these calculations (see Results). An alkaline pH value for endolymph has been repeatedly proposed (Mugiya and Takahashi, 1985; Payan et al., 1997, 1998; Gauldie and Romanek, 1998; Takagi, 2002) but the samples were probably more representative of distal endolymph as the distal space is larger than the proximal space in the sacculus. On the other hand, the pH value of 7.4 in proximal endolymph was quite unexpected and raises the following question: is the proximal endolymph supersaturated with respect to aragonite crystallisation? From the results of Takagi (2002), when the pH value switched from 8.0 to 7.4 both the $[CO_3^{2-}]$ and the Sa should decrease by a factor of about 3, so the Sa value estimated for the proximal endolymph would not be supersaturated but be around the unity.

In conclusion, our results show that, in the proximal endolymph bathing the convex shape of the otolith characterised by maximal growth, the Ca^{2+} and CO_3^{2-} concentrations were in-phase variations during the day:night cycle, whereas the pH remained unchanged. The increase in both Ca^{2+} and CO_3^{2-} levels at the end of the night period should increase the saturation state of the aragonite within the proximal endolymph, thus promoting the $CaCO_3$ deposit. Under these conditions, we propose that the $CaCO_3$ deposit would occur only when the concentration thresholds of the ionic parameters are reached. Thus, the synchronous increase of Ca^{2+} and CO_3^{2-} in the endolymph will not be sufficient to cause an immediate deposit of $CaCO_3$ as previously proposed (Mugiya, 1984, 1987; Mugiya and Takahashi, 1985; Edeyer et al., 2000). Under our conditions this would indicate that $CaCO_3$ deposition starts once the solubility product of $CaCO_3$ in the proximal endolymph is exceeded, probably at the beginning of the day. This hypothesis is in agreement with the results of Wright et al. (1992), who undoubtedly showed that light led to ^{45}Ca deposition into trout otolith. However, supersaturation of the endolymph calcium carbonate cannot alone be the cause of the aragonite precipitation, being necessary but not sufficient to precipitate aragonite. Indeed, the organic matrix plays a major role in the calcification process and could regulate the timing of $CaCO_3$ precipitation.

Importance of organic precursors levels in daily otolith increments

Our results revealed a strong variation in protein concentration in trout proximal endolymph during the circadian cycle, with maximum levels during the daytime. This confirms the results of Edeyer et al. (2000) on turbot, the only difference being the level of these variations (–4.8 g l^{-1} i.e. 30%, and –2.5 g l^{-1} i.e. 60%, in trout and turbot, respectively, for proximal endolymph at night compared with daytime).

However, as in turbot (Edeyer et al., 2000), otolith matrix formation cannot reasonably be responsible for the decrease of the endolymphatic protein concentration during the night. Indeed, fewer than 1 per 1000 proteins present in the endolymph is incorporated daily during otolith increment (Borelli et al., 2001).

One major point of interest from our study concerns the tenfold variation of collagen concentration in the proximal endolymph through the daily cycle. The daily collagen increase corresponds to 16 μg/saccule, whereas daily incorporation into the otolith matrix accounts for only 11 ng (Borelli et al., 2001). Thus, as for other proteins, matrix formation cannot explain the magnitude of the variation in collagen concentration observed in the proximal endolymph. All collagens are synthesised and secreted into the extracellular space in the form of soluble precursors, called procollagens (Hulmes, 2002). Then, proteolytic processing of N- and C-terminal propeptides by specific procollagen N- and C-proteinases leads to the production of mature collagen molecules, which spontaneously assemble into fibrils (Kadler et al., 1996). Therefore, it is probable that the molecular mechanisms determining the three-dimensional architecture of otolith involve the presence of complex enzymatic activities within the endolymph rather than a simple association of organic components previously synthesised and secreted by the saccular epithelium. Furthermore, in-phase diurnal fluctuations of collagen and non-collagenous proteins, both being present in the organic matrix of the otolith (Borelli et al., 2001), strongly support the hypothesis that they may be linked to the cyclic process of the otolith calcification.

To our knowledge, only two studies have presented the results of endolymph electrophoresis (Takagi and Takahashi, 1999; Borelli et al., 2001), and both were done on day samples. The procedures, particularly the staining, were different, which could explain why more components were detected in the present study. This is the first time that a comparison of endolymphs at day and at night has been done, but the variations in the composition of endolymph protein appear complex, as both main and minor bands are involved. Whether or not these unknown molecules are involved in the formation of the otolith matrix is a focus point for further work.

There has been only one study showing that OM immunoreactive material is present in the endolymph (Takagi and Takahashi, 1999). However, although their antiserum was also raised against the soluble fraction of the organic matrix of the otolith of trout, the reactivity on the endolymph revealed molecules of different molecular mass. The molecule recognised in the previous work clearly had a molecular mass of more than 94 kDa, whereas we observed two bands (of approximately 75 and 65 kDa). Possible explanations for their discrepancy include the procedure used to obtain the soluble OM, the immune response of the rabbit to the material injected, and/or the fact that the sample tested in the present work was a pool of proximal endolymphs. The results show for the first time that there are differences in the labelling of these molecules in endolymph samples obtained during the day and at night, suggesting that they could be precursors involved in the diurnal deposit of organic material onto the otolith. The nature of the two protein molecules is still unknown, but 75 kDa is the molecular mass of the collagen monomer. Complementary studies are necessary to determine the exact nature of these molecules.

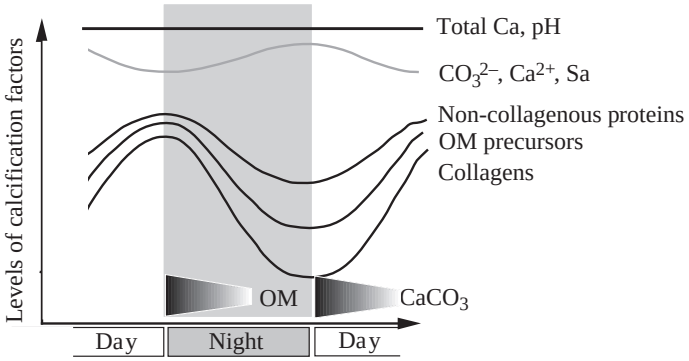

Fig. 3. Daily variations of calcification factors in the endolymph. Schematic representation of overall variations in these factors observed during the day and night periods. OM, organic matrix; Sa, saturation state of aragonite.

Our study shows that both ionic and organic precursors present within the endolymph may act not only as substrates for otolith formation but also as regulatory parameters: the levels of Ca^{2+} and CO_3^{2-} influence the aragonite saturation state, similar to the way that levels of non-collagenous and collagenous proteins may trigger the formation of the organic matrix. Thus, as shown in Fig. 3, we propose that the bilayer rings of otolith growth during the day:night cycle correspond to antiphasic mechanisms: organic matrix deposition starts at the end of the day, when the concentration of organic precursors is maximum in the endolymph, whereas $CaCO_3$ deposition starts once the solubility product of $CaCO_3$ is exceeded (i.e. at the beginning of the day).

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