Helgol Mar Res (2000) 54:1–6

ORIGINAL ARTICLE

D. Medaković

Carbonic anhydrase activity and biomineralization process in embryos, larvae and adult blue mussels *Mytilus edulis* L.

Received: 28 November 1998 / Received in revised form: 30 August 1999 / Accepted: 31 August 1999

Abstract To decide whether a physiological role can be attributed to enzymatic activity with respect to crystal formation and biomineralization of the first larval shell, carbonic anhydrase (CA) activity was measured in embryos and larvae of the blue mussels *Mytilus edulis* L. Also, CA activity was determined in the mantle edge and gonads of adult mussels with different shell length and condition index. The intention was to find a possible correlation between CA activity and adult shell calcification, i.e. gonadal maturation. The comparison of CA activity in different developmental stages of mussels and the results of an X-ray diffraction study of biomineralization processes in embryonic and larval shells indicate that CA activity is maximal at the end of several developmental stages. Consequently, the increase in CA activity precedes some physiological changes, i.e. the somatoblast 2d formation and the occurrence of the first calcite and quartz crystals in embryos, shell field formation in the gastrula stage, shell gland and periostracum production in trochophores, and rapid aragonite deposition in larval prodissoconch I and prodissoconch II shells. Furthermore, it was found that in adult mussels CA activity was quite variable and that in the mantle edge it was frequently inversely related to the activity in the gonad.

Key words *Mytilus edulis* L. · Carbonic anhydrase (CA) activity · Biomineralization process · Shell development · Biomineralization curves

Introduction

The enzyme carbonic anhydrase (CA) catalyses the reversible hydration of CO_2 to HCO_3^- and H^+. This zinc metaloenzyme accelerates bicarbonate formation and plays an important role in several physiological functions: respiration, ion transport, acid-base regulation, and biomineralization. The activity of CA and its influence on physiological functions in Mollusca have been examined by many authors (Wilbur and Anderson 1950; Kawai 1955; Freeman 1960; Nielsen and Frieden 1972; Wilbur and Saleuddin 1983; Erlichman et al. 1994; Miyamoto et al. 1996). However, the effect of CA activity on biomineralization processes in adult bivalve shells as well as in the early larval stages have been insufficiently studied so far (Medaković and Lucu 1994; Medaković 1995). In the present study, CA activity was measured at different developmental stages, as well as in the gonads and the mantle edge of adult specimens of *Mytilus edulis*, in order to clarify the possible physiological role of CA activity in crystal formation and biomineralization processes.

Communicated by H.-D. Franke

D. Medaković (✉)
Ruđer Bošković Institute, Center for Marine Research Rovinj,
Giordano Paliaga 5, HR-52210 Rovinj, Croatia
e-mail: medakovic@cim.irb.hr
Tel. +385-52-811-544, Fax: +385-52-813-496

Materials and methods

Adult blue mussels, *M. edulis*, with different shell lengths (48–67 mm) were collected from the rocky shore of the island of Helgoland, North Sea, Germany. The mussels were conditioned to obtain mature gonads and to induce spawning. The conditioning stimulus was a daily temperature increase of 1°C, from 14°C up to 18°C, followed by a return to the initial conditioning temperature. Such a thermal conditioning treatment was repeated regularly for 3 weeks. Animals were induced to spawn using a combined thermal-mechanical stimulation (Loosanoff and Davies 1963; Hrs-Brenko and Calabrese 1969). Mature eggs were fertilized by adding a drop of sperm to the egg suspension. Fertilized eggs were reared in a 3-l beaker, filled with 0.45 µm filtered seawater of 30.0 ppt salinity and at 18°C. The starting densities of each culture ranged from 1000 to 5000 eggs l^{-1} of seawater. The water was changed every 2 days, using a 40-µm sieve, irrespective of embryo or larval size. Different developmental stages were separated by means of sieves of 40-, 80- or 120-µm mesh size, at the beginning every 30 min and later every few hours. After filtration the contents of each sieve were resuspended in a small volume, then classified by means of optical microscopy. Average length of embryos and larvae, respectively, was calculated after measuring 50 or more specimens in each sample. Each sample was divided into two subsamples, one for measuring enzymatic activity and the other for examination of mineral components.

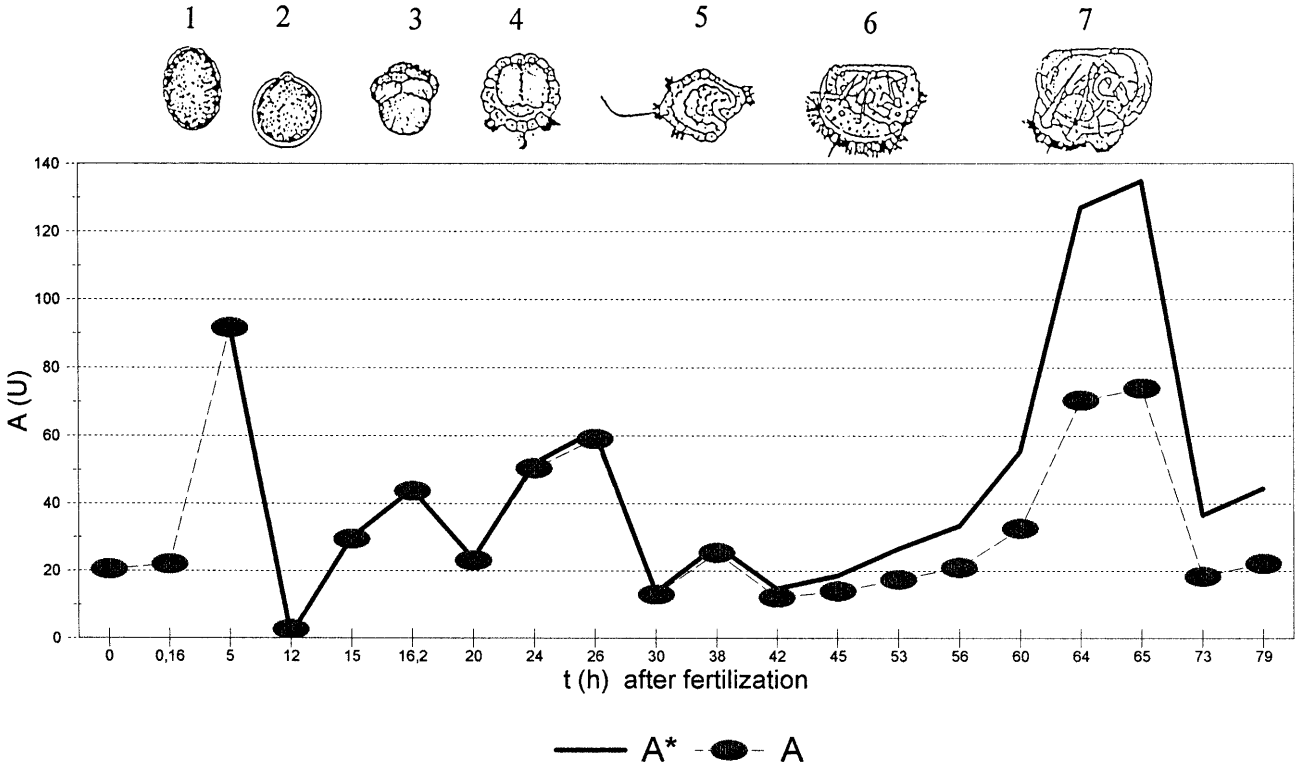

Fig. 1 *1–7* Development of *Mytilus. edulis* according to Arakawa (1980), Strathmann (1987) and Kulakowski and Fliatchinskaya (1993): *1* unfertilized egg; *2* fertilization; *3* secondary 2d somatoblast formation; *4* transition stage from late gastrula to early trochophore; *5* fully developed trochophore; *6* D-shaped or straight hinge veliger with prodissoconch I shell; *7* prodissoconch II. *Curves* show carbonic anhydrase (CA) activity *A* at different developmental stages of *M. edulis*, expressed as CA units (U) per g of wet weight of sample. *A* CA activity in whole samples. According to biomineralization curves (Fig. 2) correction of results for weight percent of shell in samples was carried out and corrected CA activities are represented by bold curve *A**

The shell length of another group of adult mussels was measured, and soft tissues and shells were weighed separately. After determination of the condition index (Baird 1985; Davenport and Chen 1987), the parts of the edge of the mantle and gonadal tissue were dissected and briefly air dried on filter paper. The dissected tissues were stored in vials at –80°C before weighing. The shells were carefully cleaned from the remaining soft tissues, washed with distilled water, air dried and stored before analysis.

Measurement of CA activity

The samples of mussel embryos and/or larvae were placed in plastic vials and centrifuged for 3 min at 600 *g*. The superfluous suspension was carefully decanted, and the samples were weighed and stored at –80°C.

Before measurement of the enzymatic activity, samples of embryos, larvae and adult mussel soft tissues were thawed and solubilized with 6.125 ml Triton X-100 per 1 g wet weight (ww), homogenized and stored at 0°C. The activity of CA was measured by a micromethod developed by Maren (1960) and modified by Bruns et al. (1986). The activity (*A*) of CA was expressed by the uncatalysed reaction time (t_0) and the catalysed reaction time (t_{enz}), using the following equation: $A=(t_0/t_{enz})-1$. One unit (U) of CA activity was defined as the enzyme activity required in the final assay volume to halve the uncatalysed reaction time ($t_0=2t_{enz}$). The CA assays of each sample were performed in triplicate and averaged. Before and after each series of samples, the uncatalysed time was measured and, for the interpretation of results, a daily average was used (e.g. $t_0=60.1\pm2.2$; $n=71$). The results are expressed as CA units (U) per milligram of wet weight of the sample.

Preparation of samples for X-ray diffraction

Preparation of embryos and larval mussels for X-ray diffraction depended on the sample quantity (Medaković et al. 1989, 1997; Medaković and Lucu 1994; Medaković 1995). After collection, each sample was washed with distilled water and dried at room temperature. When the amounts of some embryonic and larval samples were too small to fill the sample holder, they were transferred by a pipette onto the centre of the sample holder, carefully powdered, spread uniformly, and glued to prevent a dispersion of powder during the X-ray diffraction experiment.

Samples of adult mussel shells were powdered in an agate mortar and placed on the sample holder. Diffraction patterns were taken by means of an X-ray counter diffractometer (Philips, MPD 1880), equipped with a vertical goniometer and proportional counter, using monochromatized CuKα radiation. Calcite and aragonite were identified according to the JCPDS, Powder Diffraction File, cards no. 5-453 for aragonite and 5-586 for calcite. The fractions of each mineral phase were determined by application of quantitative X-ray diffraction phase analysis (Popović and Gržeta 1979; Popović et al. 1983).

Results

Figure 1 shows the CA activity in embryos and larvae of *M. edulis*. The enzymatic activity ranged from $A=0.2\times10^{-3}$ U mg^{-1} ww in the embryos to $A=136.0\times10^{-3}$ U mg^{-1} ww in the larvae 65 h after fertilization. The biomineralization curves (Fig. 2) of embryonal and larval *M. edulis*,

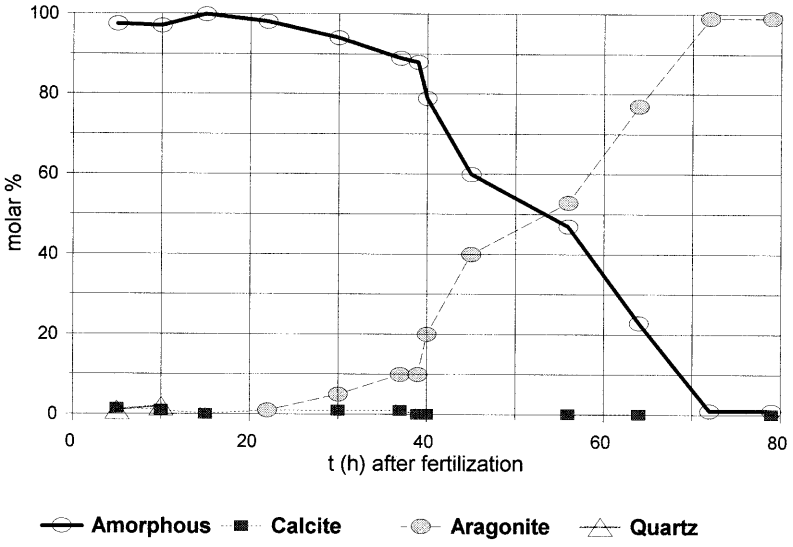

Fig. 2 *Mytilus edulis*. Biomineralization curves. Variation of fractions of amorphous tissue, calcite, aragonite and quartz during development. Immediately after fertilization only soft tissue, the amorphous phase, is observed. Five hours later, embryos which have developed a secondary 2d somatoblast contain 1.5% calcite and 1% quartz. Mussel larvae 72 h after fertilization have mostly aragonitic shells, mineral composition is constant, and prodissoconch II shell has been formed

constructed according to diffractograms obtained by X-ray diffraction, show that some later embryonic and all larval samples contained inorganic components, i.e. minerals of the first shells. These samples were not decalcified in order to prevent a possible chemical influence on CA activity. To express real activities in soft tissues, a correction of results for the weight percent of shells was required and presented by a bold curve in Fig. 1.

The enzymatic activity of the mantle edge of adult mussels ranged from A=58.3×10^{-3} U mg^{-1} ww (shell length 60 mm) to A=327.0×10^{-3} U mg^{-1} ww (shell length 56 mm). The CA in mussel gonads had lower minimal A=7.0×10^{-3} U mg^{-1} ww and higher maximal activities A=415.0×10^{-3} U mg^{-1} ww than those measured in the mantle edges. However, the average activity in gonads, (A=208.5×10^{-3} U mg^{-1} ww) was higher than in the mantle epithelium (A=167.5×10^{-3} U mg^{-1} ww). Figure 3a shows the CA activity in both the mantle edge and the gonads as a function of shell length. A comparison of CA activity and the condition index in gonads and the mantle edge is presented in Fig. 3b.

Discussion

In the recent literature, except for preliminary results (Medaković and Lucu 1994; Medaković 1995), no data are available on CA activity in embryos, larvae and adult *M. edulis*. Consequently, to interpret the results appropriately, a thorough discussion of literature data related to other molluscs, especially bivalve species, and certain hypotheses on biomineralization processes and possible CA activity influence on biomineralization of bivalve shells is indispensable.

The present results indicate that average CA activity in mussel embryos and larvae was approximatively fourfold lower than in the adult mantle edge and fivefold lower than in the gonadal tissue. In contrast, Wilbur and Anderson (1950), reported that in snail larvae of *Busycon carica* the CA activity increased 47-fold at the beginning of shell formation in comparison to adults. Nielsen and Frieden (1972) stated that the great difference in CA activity between oysters *Ostrea equestris* and *Crassostrea virginica* may be caused by particular physiological or environmental differences between the two species. Physiological and ecological differences between the gastropod *Busycon carica* and the mussel *M. edulis* may also explain the relatively high differences in enzymatic activity between these two species. Also, although CA activities in embryos and larvae of *M. edulis* are lower than in adult mussel tissues, a significant increase in enzymatic activity in some developmental stages (Fig. 1) confirmed the findings of Wilbur and Anderson (1950), indicating a connection between CA activity and shell formation. The same authors assumed that CA activity is not constant but may be altered by physiological changes in the organism. The results of the present study are strongly in accordance with this assumption. The comparison of CA activity in different developmental stages (Fig. 1) and the biomineralization curves of embryos and larvae (Fig. 2) indicate that CA activity reached a maximum at the end of each developmental stage connected with biomineralization. Unfertilized eggs have low CA activity, but immediately after fertilization CA activity slowly increases. During later cell division, the first CA activity maximum is reached 5 h after fertilization. Strathmann (1987) and Lutz and Kennish (1992) reported that 5 h post-fertilization mussel embryos have 16–32 cells. Kulakowski and Fliatchinskaya (1993) noted that *M. edulis* embryos 5 h after fertilization have only 8 cells. The present study shows that immediately after the first CA maximum embryos had 12 cells and the first somatoblast 2d was formed. According to Verdonk and Cather (1983), the 2d somatoblast and in some rare cases the 2c somatoblast are the only cells in the embryo forming the initial shell field, the shell gland and at least the first shell. Also, the biomineralization curves (Fig. 2) show that in such em-

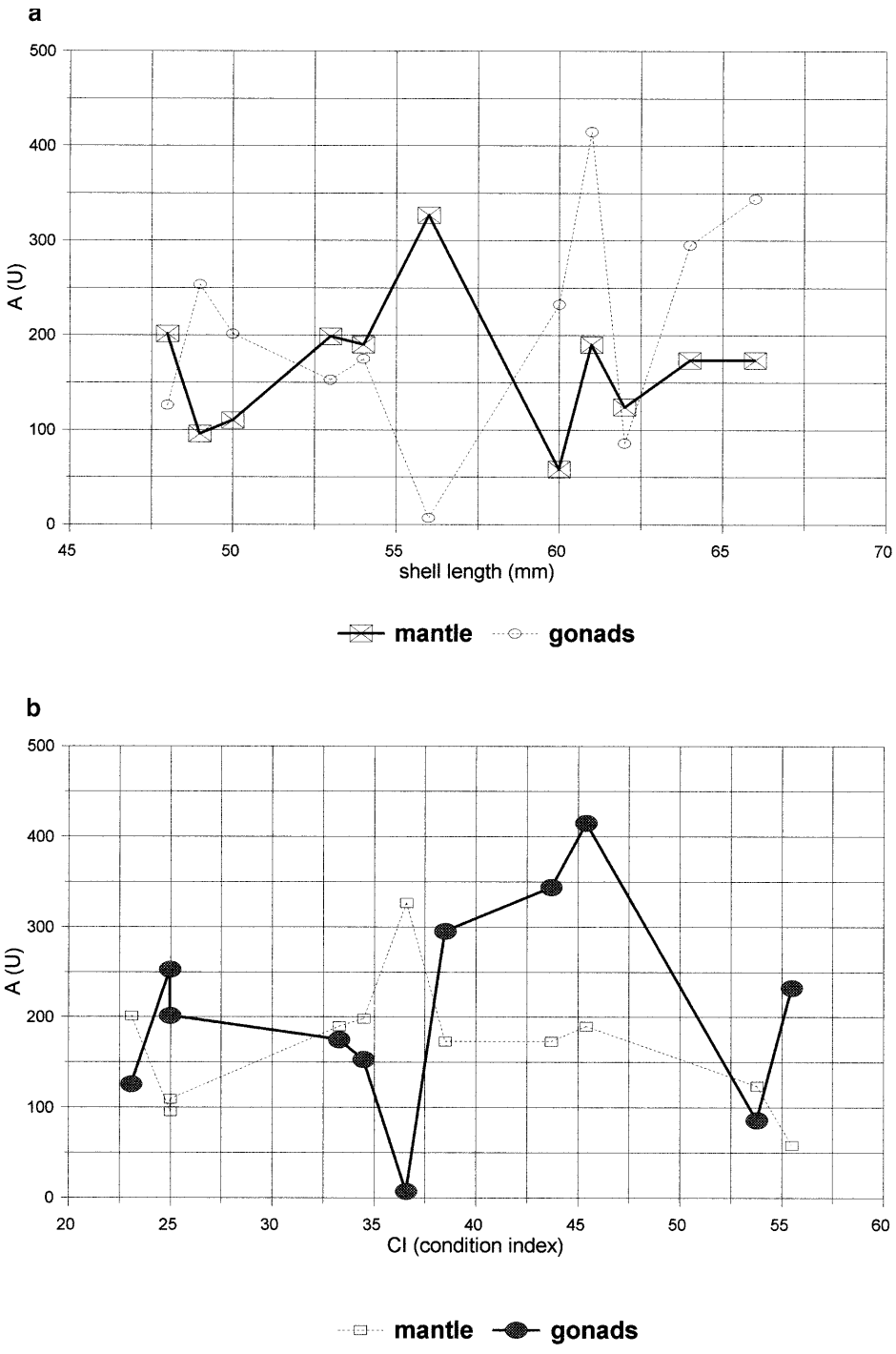

Fig. 3 *Mytilus edulis*. Carbonic anhydrase activity (*A*), expressed as CA units (*U*) per g of wet weight of sample in both mantle edge and gonad of adult mussels as a function of shell length (**a**) and condition index (**b**). Activity in the mantle edge is frequently inversely related to activity in the gonad

bryos the first mineral components are present, i.e. calcite in amounts of 1.5 molar %, and quartz 1%, respectively. The occurrence of small calcite amounts in mussel embryos is expected since a similar quantity of calcite was found in embryos of the Mediterranean mussel *Mytilus galloprovincialis* and oyster *Ostrea edulis* (Medaković et al. 1989, 1997; Medaković 1995). Calcite crystals play a role in the further development of larval shells, acting as aragonite crystallization centres. Small quantities of mineral quartz were also found in early embryos of *M. galloprovincialis* (Medaković 1995). It is known that quartz and some other minerals can be incorporated into or "captured" in adult molluscan shell layers during rapid shell growth or as the result of some disturbances in the biomineralization processes (Carriker et al. 1982; Medaković 1995). Nevertheless, the presence of this mineral in embryos of both *Mytilus* species remains to be explained.

After formation of the first somatoblast 2d, CA activity rapidly decreases and reaches its lowest value in the blastula, 7 h after fertilization (Fig. 1). During gastrulation, CA activity again increases and reaches a further

maximum 16 h after fertilization, indicating shell gland formation in the late gastrula stage. In this period the biomineralization curves show a dominance of the organic-amorphous component; calcite is present in small amounts and quartz is not recorded (Fig. 2). In the subsequent developmental period, during transition from gastrula to early trochophore, an increasing CA activity immediately precedes the formation of the shell field, which is situated on the posterior side of the trochophore (Kniprath 1979, 1981). Later during the invagination process, the shell field forms the prechonchiolin gland of the outer noninvaginated cells. This gland secretes the first organic periostracum, i.e. the beginning of the prodissoconch I shell (Kniprath 1979; Waller 1981; Kasyanov 1984). The periostracum can be detected by optical microscopy in a trochophore 26 h after fertilization (Fig. 1), although biomineralization curves show that mineralization of the first periostracum, characterized by the first appearance of aragonite crystals, has already begun in the trochophore 22 h after fertilization (Fig. 2). In the oyster *O. edulis*, appearances of the first aragonite crystals indicate a transitional phase between late trochophore and early veliger (Medaković et al. 1997). These results show that in *M. edulis*, although calcification of the periostracum begins earlier, the first veliger larvae are formed 38 h after fertilization. This is in accordance with the findings of Kulakowski and Fliatchinskaya (1993), who reported that the first veligers of *M. edulis* are formed 30–34 h after fertilization. The later CA activity increase (Fig. 1) followed by a rapid aragonite increase and proportional soft tissue weight decrease (Fig. 2) shows that prodissoconch I shell formation is completed in veliger larvae 55 h after fertilization, while the prodissoconch II shell is formed 72 h after fertilization (Figs. 1, 2). Waller (1981) noted that the formation of prodissoconch II shells of the oyster *O. edulis* begins immediately after the formation of the prodissoconch I, the shell of which encloses the whole larval body. This statement is confirmed by an intensive CA activity increase in the period of prodissoconch I to prodissoconch II formation (Fig. 1), and also by a rapid aragonite increase (Fig. 2).

High CA activity precedes physiological changes and biomineralization processes during early development stages of *M. edulis*. These findings confirmed earlier hypotheses that CA activity is linked with shell formation (Wilbur and Jodrey 1955; Freeman 1960; Medaković and Lucu 1994; Medaković 1995).

The determination of the CA activity in different soft tissues of adult *M. edulis* partially confirmed several previous ideas about the role of this enzyme in adult shell biomineralization. Kawai (1955) noted that CA activity in particular tissues of the pearl oyster *Pinctada martensii* decreased sharply during the first year of life and continued to decrease, though less markedly, during the second and third years of the organism. The decrease in this enzyme activity with age was related to the decreased rate of shell formation. Results of the present study show that CA activity of the mantle edge, which is responsible for shell growth, is not connected with shell length (Fig. 3a), at least in *M. edulis*.

Also, the CA activity of gonads does not depend on the condition index (Fig. 3b), suggesting that it is not influenced by the quantity and maturity of gametes. All mussels were collected at the same time, so the stage of reproduction should have been the same. There is a connection between CA activity in the mantle edge and the gonad. A high mantle activity is accompanied by low activity in gonads, and vice versa. The low divergences of CA mantle epithelium activities in different size classes of mussels from an average indicate a relatively stable and constant biomineralization process. The results support the hypothesis that CA activity is essential for a rapid shell development but may be insignificant at slow growth (Freeman, 1960).

Acknowledgements The hospitality and facilities at the Biologische Anstalt Helgoland (BAH) were very much appreciated. I am grateful to Professor F. Buchholz, Director of the BAH, for his kind assistance, Professor Č. Lucu for his introduction to carbonic anhydrase determination and valuable intellectual support, and Professor Susan Ford for her helpful advice, critical reading of the manuscript and for improving the English.

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