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CALCIUM DEPOSITION AND OTOLITH CHECK FORMATION DURING PERIODS OF STRESS IN COHO SALMON, *ONCORHYNCHUS KISUTCH*

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Abstract—1. Periods of stress can disrupt daily growth increment formation on a fish otolith, producing a check (discontinuity). Calcium-45 was used to monitor calcium deposition on the sagittae of coho salmon, *Oncorhynchus kisutch*, during periods of check formation.

2. ^{45}Ca deposition on the otolith continued for 12 hr after transfer from ^{45}Ca water.

3. Stress applied during ^{45}Ca immersion reduced ^{45}Ca deposition.

4. Stress applied immediately after transfer from ^{45}Ca water had no effect on ^{45}Ca deposition.

5. Stress indirectly disrupted ^{45}Ca deposition on the otolith through a reduction in branchial uptake of calcium.

6. Stress did not result in resorption of otolith calcium.

INTRODUCTION

The sequence of daily growth increments in otoliths of fishes may provide a chronological record of past growth. Daily growth increments are now being used to determine the age of larval and juvenile fish (Ralston, 1976; Kendall & Gordon, 1981), back-calculate daily growth rates (Methot, 1981) and may even provide a dated record of ecological and physiological events through the lifetime of an individual fish (Pannella, 1980; Brothers & McFarland, 1981).

Checks, or discontinuities, in the daily increment sequence record periods of perturbation or stress to the fish. Transmission of light through checks is poor relative to other microstructural features, resulting in structures which are immediately distinguishable from surrounding increments. Checks also appear distinct in acetate peel replicas and scanning electron microscope (SEM) photographs. Stress due to collection, migration and starvation may all put a check on an otolith, with the intensity of the check apparently proportional to the degree of stress that the fish has undergone (Pannella, 1980; Campana, unpublished). Since daily increments provide a chronological history of past growth, the location of a check on the otolith can be related to the date of the event in question.

Daily growth increments in otoliths are bipartite structures, consisting of a relatively wide zone of calcium carbonate crystals embedded in a protein matrix, and an adjacent narrower band that is dominated by the matrix (Dunkelberger *et al.*, 1980; Mugiya *et al.*, 1981). Recent experiments indicate that the proteinaceous zone is formed near dawn as a result of a slowdown in calcium deposition (Mugiya *et al.*, 1981; Tanaka *et al.*, 1981). However, little else is known of the daily cycle of calcium deposition on otoliths (Mugiya *et al.*, 1981), and the processes responsible for check formation are not understood at all. Pannella (1980) has suggested that growth discontinuities may mark areas of otolith resorption. Otolith resorption would result in an incomplete chronological sequence of daily growth increments, and thus invalidate many applications of daily increments.

Checks are sufficiently common in wild fish to warrant understanding how and why they occur. The object of this study was to monitor calcium deposition and/or resorption on the otoliths of coho salmon (*Oncorhynchus kisutch*) before and after periods of stress. The stress used in this study was sufficient to put a moderately intense check on the sagittae of experimental fish.

MATERIALS AND METHODS

The experimental fish were collected from Tin Can Creek, Vancouver, Canada and acclimated to laboratory aquaria for a minimum of 4 days before use. Standard lengths ranged from 2.9 to 4.1 cm, with a mean of 3.5 cm. Aquaria were maintained under a 14L:10D photoperiod at 10.5 ± 0.5°C. All fish but those being stressed were fed a commercial fish food twice daily.

Experiments in which fish were immersed in ^{45}Ca water comprised the bulk of this study. They are briefly summarized in Table 1.

^{45}Ca *immersion*

It is reasonable to assume that ^{45}Ca present in the fish is not deposited instantly on bone and/or otoliths. To determine the lag time involved, retention experiments were performed in which fish were immersed for various lengths of time in ^{45}Ca water, followed by 0–24 hr in freshwater. In the first retention experiment, 19 coho were placed in a 50 l. aquarium containing 15 μCi/l of $^{45}CaCl_2$ at 10:00 hr. The radioactive solution was circulated for 12 hr prior to fish immersion. Fish were sampled after 24 and 48 hr of immersion. The remainder were removed from the aquarium after 24 hr, rinsed briefly in isothermic, uncontaminated water, and then placed in an uncontaminated aquarium for a further 24 hr before sampling. Fish were sacrificed within 5 min of sampling and their sagittae removed and prepared as described below. The retention experiment was repeated in a modified version later, when one batch of fish was sampled after 24 hr immersion and the remainder were rinsed and transferred to fresh water for 12 hr. (All

Table 1. Experiments in which coho salmon were immersed for various lengths of time in ^{45}Ca water

Experiment	Object	General Conditions
Retention t1-t2	Test for lag time of incorporation of 45-Ca into otolith	Immersed t1 hr in 45-Ca water followed by t2 hr in freshwater
IMM/IMMc	Test effect of stress while fish immersed in 45-Ca water	Immersed 36 hr in 45-Ca; IMM stressed after 12 hr
DAY/DAYc	Test effect of stress immediately after transfer from 45-Ca water	Stress period during day
NIGHT/NIGHTc	Same as DAY/DAYc	Stress period during night
RES/RESc	Test for resorption	Stress after removal from 45-Ca water and 45-Ca incorporation has finished

experimental replicates were carried out in ^{45}Ca water in which experimental fish had already been tested. Due to the lower ^{45}Ca concentration present, replicate radioactive levels are invariably lower than their precursor.)

The effect of stress upon ^{45}Ca deposition on the otoliths while fish were immersed in a ^{45}Ca solution was determined by placing 10 fish in each of 2 identical aquaria at 22:00 hr. ^{45}Ca concentrations were as above. The control fish (IMMc) were left undisturbed for 36 hr before sampling. The experimental fish (IMM) were left undisturbed for 12 hr, stressed for 12 hr and then left for a further 12 hr before sampling, all while in the radioactive calcium solution. This experiment was repeated at a later date.

Since the retention experiments indicated that ^{45}Ca within the fish remained available for deposition on the otolith for at least 12 hr, the object of the next experiment was to determine the effect of stress on ^{45}Ca deposition immediately after removal of the fish from a radioactive environment. Twenty fish were placed in a ^{45}Ca aquarium at 10:00 hr. After 24 hr, 10 control fish (DAYc) were rinsed and transferred to a fresh aquarium for 12 hr before sampling. The remaining experimental fish (DAY) were rinsed and transferred at the same time to a different aquarium, but were stressed for the subsequent 12 hr (i.e.—during the day). This experiment was repeated at a later date.

It has been suggested (Pannella, 1980; Mugiya *et al.*, 1981) that the protein matrix upon which a day's calcium is deposited, is produced near dawn. If true, ^{45}Ca deposition could vary depending on the time of stress application. Therefore, experiment DAY/DAYc (described above) was repeated with a modification. Twenty fish were immersed at 10:00 hr, but only for 12 hr, after which the control (NIGHTc) and experimental (NIGHT) fish were left undisturbed and stressed, respectively, for the following 12 hr, i.e. overnight.

To determine if resorption of previously deposited ^{45}Ca could occur under the experimental stress situation, 20 fish were placed in a ^{45}Ca aquarium at 10:00 hr. After 24 hr, 10 of the fish were rinsed and transferred to a fresh aquarium, where they were undisturbed for $2\frac{1}{2}$ days (RESc). The remaining fish were rinsed and transferred to a different aquarium, where they were left for 2 days (RES). The fish were then stressed for the final 12 hr.

^{45}Ca *injection*

To separate the effects of stress on ^{45}Ca deposition from that of ^{45}Ca uptake, 20 coho were injected IP with a ^{45}Ca solution at a titre of 0.125 μCi/g fish. Injection occurred at 14:00 hr, whereupon 1/2 of the fish were placed in an aquarium for 36 hr (Ic), and the other 1/2 were left undisturbed in a different aquarium for 24 hr, followed by 12 hr of stress (I). Sampling of all fish occurred 36 hr after injection. The experiment was repeated later.

Stress

Stress was standardized in all of the experiments. Fish remained in aquaria draped with black plastic, where they were visually isolated from laboratory activities. At the beginning of a stress period, the plastic was removed from the experimental tank, and the fish placed in 150 ml plastic containers, meshed at each end. Five fish were placed in each container and the containers were suspended directly over an airstone. Over the 12 hr stress period, the containers were lifted out of the water at 30 min intervals, drained of any remaining water, and the fish allowed to struggle in the container for 60 sec before being replaced in the water. This level of stress appeared to be significant to the coho, since experimental mortalities only occurred during the stress period. Many of the surviving, stressed fish were weak and had difficulty swimming immediately after each stress cycle, although some individuals tolerated it well.

Otolith preparation

After sacrifice, all experimental fish were treated similarly. The two sagittal otoliths were removed from each fish, brushed free of tissue, rinsed in distilled water and then dried overnight at 90°C. The sagittal pair from each fish was combined and treated as a single sample. After cooling, the otoliths were weighed to the nearest 0.01 mg and then solubilized as per Mahin & Lofberg (1966). Radioactivity was determined in a Beckman LS9000 liquid scintillation counter. The resultant DPM (disintegrations per min) values were compensated for decay, quench and chemiluminescence. Activity levels (DPM) are directly proportional to isotope concentrations and the terms are used synonymously in this paper.

Otolith check formation due to the stress period was determined by rearing 5 fish in the lab for 19 days, followed by the 12 hr stress period, and then a further 13 days of rearing. Throughout this time, photoperiod and feeding rate remained constant and the aquarium was visually isolated from laboratory activities. Water temperature was recorded daily. After removal of the sagittae, otoliths were ground and polished as described by Campana & Neilson (1982). Check position was confirmed by counting back the appropriate number of daily increments from the periphery of the otolith. The production of daily increments in coho salmon was confirmed independently.

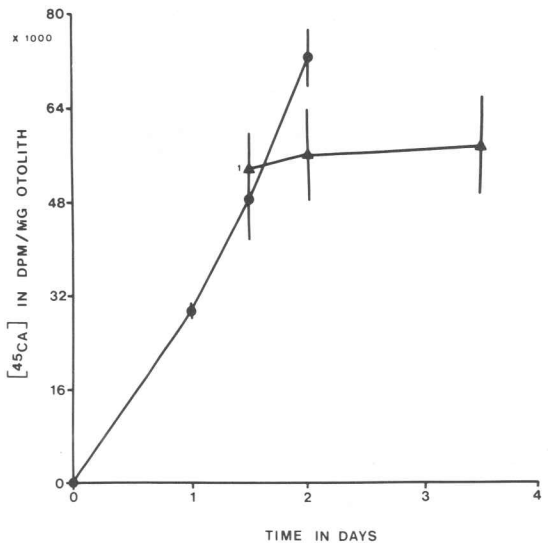

Fig. 1. Mean activity of ^{45}Ca deposited onto otoliths through time. Vertical bars represent 1 SE. Data point 1 was standardized with respect to concurrently performed Retention 24–0 experiment. ● = Kept in ^{45}Ca water. ▲ = Transferred to freshwater after 24 hr in ^{45}Ca water.

RESULTS

^{45}Ca deposition on the sagittae of immersed fish increased linearly with time for at least 48 hr. Fish that were transferred to fresh water after 24 hr immersion continued to deposit radiocalcium on the otoliths for up to an additional 12 hr. After that time, no further ^{45}Ca was deposited (Fig. 1). Upon transfer, 47% of the ^{45}Ca had yet to be deposited on the otoliths.

A moderately intense check was produced on the otolith on the day that the stress occurred (Fig. 2). Since there are only two basic components to a fish otolith, calcium carbonate and the protein otolin, the distinct appearance of the resultant check is probably due to a change in the proportion of the deposited components. Therefore, the stress used in these experiments manifested a change in the normal depositional process of the sagittae.

To determine if a change in the rate of calcium deposition was involved in check formation, stress was applied to experimental fish during ^{45}Ca immersion. When stress was applied for 12 hr during a 36 hr immersion period (Expt. IMM), ^{45}Ca deposition on the otoliths was significantly less than that of the controls (Expt. IMMc) ($P < 0.05$) (Table 2). There was a good correlation between the length of the stress period and the reduction in calcium deposition. Expt. IMM fish were left undisturbed for 67% of their immersion time. Sagittae from these fish contained 59% of the ^{45}Ca of the controls.

The reduction in calcium deposition could have occurred because of stress inhibition of the calcium deposition process, or inhibition of calcium uptake from the water. To distinguish between these alternatives, Expt. DAY fish were stressed for 12 hr immediately after transfer from the radioactive water. Since a large portion of ^{45}Ca deposition occurs after removal from ^{45}Ca water (see above), inhibited deposition should be evident during this period. Yet ^{45}Ca levels were similar in stressed and unstressed fish ($P > 0.1$) (Table 2).

If coho are easily stressed, it is conceivable that the capture, rinse and transfer of the control fish stressed them sufficiently to inhibit their calcium deposition as much as that of the stressed group. To test this hypothesis, fish were immersed in ^{45}Ca for 24 hr. One batch (Retention 24–12) was rinsed and transferred to fresh water for 12 hr and the remainder were sacrificed immediately (Retention 24–0). The former group deposited significantly more ^{45}Ca than did those fish sampled without transfer ($P < 0.05$) (Table 2), indicating that the rinse-transfer procedure did not stress fish enough to inhibit calcium deposition.

Since calcium deposition on the otolith varies with diel periodicity (Mugiya *et al.*, 1981), inhibition of calcium deposition through stress may only be possible at certain hours of the day. Fish in experiments DAYc/DAY were stressed after transfer during daylight hours. Subsequently, the experiment was repeated with the 12 hr stress period occurring throughout the night (Expts. NIGHTc/NIGHT). The results indicated that time of day was not a factor in the DAYc/DAY results, since the control (NIGHTc) and stressed fish (NIGHT) deposited essentially the same quantity of ^{45}Ca ($P > 0.1$) (Table 2).

The above results suggest that stress does not inhibit the calcium depositional process, but does inhibit calcium uptake from the water. To test this hypothesis, coho were injected with ^{45}Ca to by-pass the calcium uptake mechanism. The mean ^{45}Ca activity of the unstressed control fish was 4738 (SE = 216) DPM/mg otolith ($\bar{x}$ = 3662, SE = 201 for replicate No. 2), while that of the stressed group was 3847 (E = 481) DPM/mg otolith ($\bar{x}$ = 3307, SE = 194 for replicate No. 2). These values are not significantly different ($P > 0.05$), thereby supporting the hypothesis.

Experiment RES fish were stressed 2 days after transfer from ^{45}Ca water to determine if stress could effect resorption of previously-deposited calcium. The retention experiments indicated that all internal ^{45}Ca should have been deposited at the end of this period. Experimental (RES) and control fish (RESc) were not significantly different ($P > 0.1$) (Table 2), indicating that resorption did not occur.

DISCUSSION

Daily growth increments are formed through a circadian periodicity in the rate of calcium deposition (Mugiya *et al.*, 1981; Tanaka *et al.*, 1981). Left undisturbed, the diel cycle of calcium deposition will leave a series of very similar-appearing daily increments on the otoliths of a young fish. However, otoliths from wild fish seldom form such a series. At regular or irregular intervals, the sequence of increments is interrupted by checks or discontinuities that can mark the occurrence of a stressful period in the life of the fish. Since the checks are so visually distinctive, they are almost certainly due to a disruption in the normal cycle of calcium and/or protein deposition on the otolith.

Calcium deposition on the otolith is significantly reduced during the formation of an otolith check. This fact was made evident during the experiment in

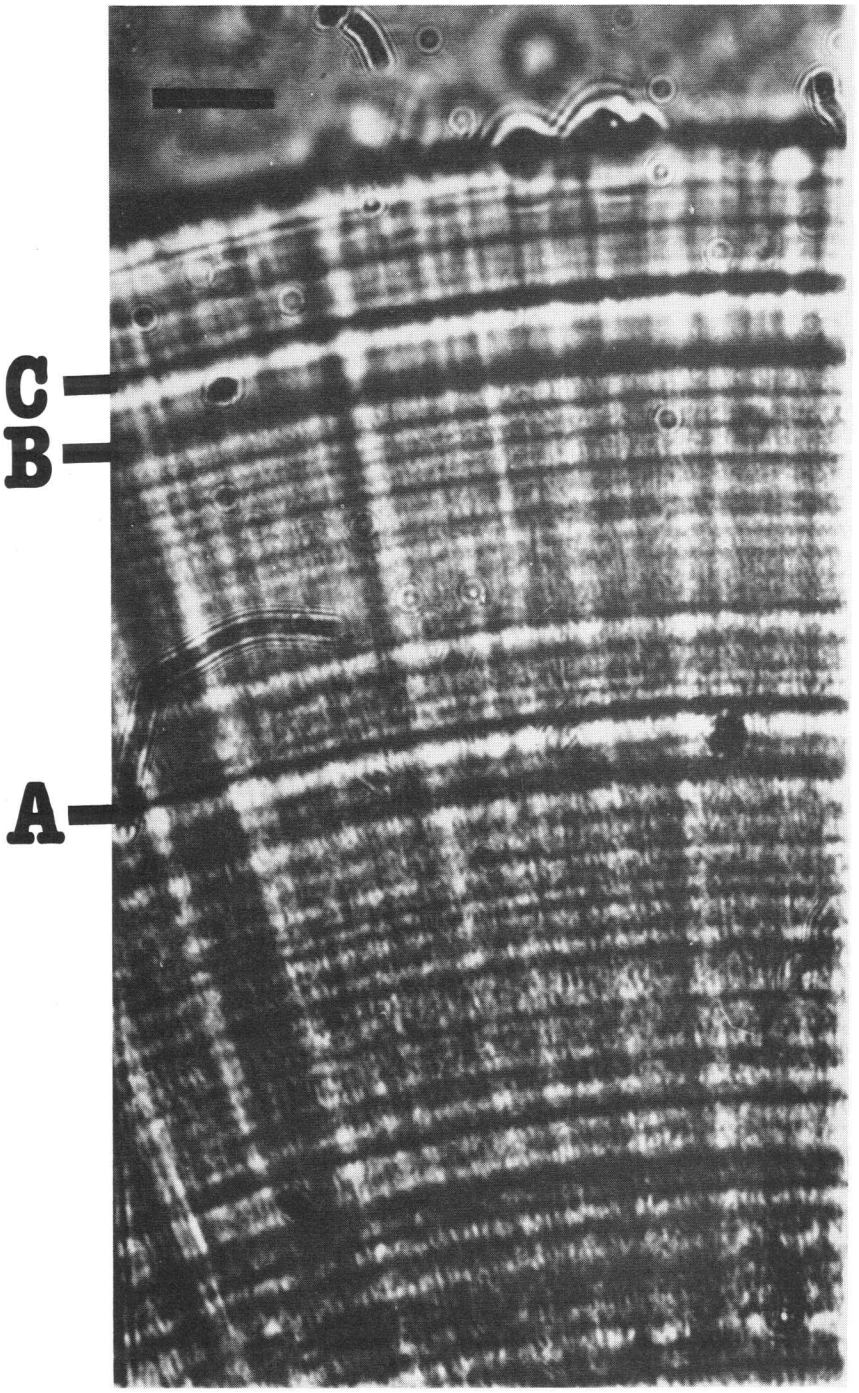

Fig. 2. Prepared sagitta of experimental coho salmon. Checks due to collection (A), experimental stress (B) and water temperature change (C) are indicated. Bar = 10 μm.

which the fish were stressed while in the ^{45}Ca water. However fish that were stressed immediately after transfer from ^{45}Ca water showed no reduction in ^{45}Ca deposition. Since the retention experiments demonstrate that almost one half of the ^{45}Ca is deposited after transfer from radioactive water, stress must interfere with calcium uptake from the water, rather than the deposition process itself. The injection experiments support this hypothesis.

Calcium pathways through the body of a teleost are poorly understood, but it is well documented that very little calcium is absorbed from food. The gills and epidermis are the main areas of calcium uptake, particularly in freshwater fish (Simkiss, 1974). And since calcium levels remain fairly constant through time, calcium influx and efflux must be under considerable control (Norris *et al.*, 1963).

Mugiya *et al.* (1981) determined that there is a

Table 2. Mean activity of ^{45}Ca deposited onto otoliths of fish kept under various experimental conditions

	Experiment	N	Total hrs. in 45-Ca //hrs. stressed	Total hrs. in FW //hrs. stressed	Mean DPM/mg otolith (X1000)	SE	Signif.
	Retention 24-0	5	24	0	29.5	1.61	
1	(Retention 24-0	6	24	0	7.96	1.27)	*
1	(Retention 24-12	9	24	12	14.4	1.78)	
	Retention 24-24	5	24	24	56.3	8.23	
	Retention 48-0	9	48	0	72.8	5.27	
	(IMMc	10	36	0	48.6	7.32)	*
	(IMM	10	36//12	0	29.2	5.16)	
1	(IMMc	10	36	0	28.4	2.73)	*
1	(IMM	9	36//12	0	16.6	2.47)	
1	(DAYc	10	24	12	34.2	2.89)	NS
1	(DAY	8	24	12//12	35.0	3.30)	
1	(DAYc	11	24	12	13.3	1.72)	NS
1	(DAY	11	24	12//12	14.3	1.56)	
1	(NIGHTc	10	12	12	6.59	0.92)	NS
1	(NIGHT	10	12	12//12	6.25	0.88)	
	(RESc	9	24	60	57.7	8.88)	NS
	(RES	10	24	60//12	51.3	3.51)	

Bracketed experiment pairs were performed concurrently in water of the same radioactivity.

1, Performed at a later date with a lower [Ca-45] in the aquarium, due to previous experiments in the same water. Therefore, experiment pairs cannot be compared among themselves without standardization.

strong correlation between a fish's plasma calcium level and calcium deposition rate on the otolith. When plasma calcium increased, calcium concentration in the ambient water decreased, implying a direct or indirect pathway from ambient water to blood plasma to otolith. Therefore, they speculated that the diel rhythm in plasma calcium and calcium deposition was due to a diel rhythm in branchial uptake of calcium from the environment. The results of this study strongly support this hypothesis. It appears that calcium uptake is the process which ultimately influences calcium deposition, and that uptake can be disrupted by factors such as stress.

If the suggestion is accurate that calcium deposition is linked to the production of a protein matrix (Dunkelberger *et al.*, 1980), one may hypothesize two basic processes by which otolith formation may occur. In neither is it likely that stress interferes with the production of the organic matrix. One possibility is that formation of the protein matrix and calcium deposition occur concurrently at the same rate, with the daily proteinaceous zone due to a cessation or reduction in calcium deposition rate. If such were the case, stress after transfer from the ^{45}Ca water should have reduced the rate of matrix formation, and consequently, the rate of calcium deposition. This did not occur. Another possibility is that the organic matrix is formed first each day, perhaps around dawn (Mugiya *et al.*, 1981), and that calcium deposition upon the matrix occurs afterwards (Pannella, 1980). If true, stress during the period of matrix formation (Expts. NIGHTc/NIGHT) should have decreased subsequent calcium deposition. This did not occur. Therefore, a reduction in calcium deposition, not matrix formation, appears to be characteristic of an otolith check.

Otolith checks are well etched by acid during preparation of an acetate replica or for viewing by SEM. Although Brothers *et al.* (1976) disagree, it appears that the zone dominated by organic matrix is most heavily etched by acid (Pannella, 1980; Mugiya *et al.*, 1981). The acid etching and the results of this study indicate that an otolith check is composed of a relatively uncalcified zone of organic matrix, making it analogous to a larger version of the organic zone formed at dawn each day (Mugiya *et al.*, 1981). This suggestion is supported by the observation that stronger checks etch widely; since protein continues to be laid down during the stress period causing the check, a long period of stress will result in a broad zone of protein, all of which is etched by acid. This could explain why the visual intensity of a check is often proportional to the magnitude and duration of the stress that caused it (Pannella, 1980; Campana, personal observation).

In this study, a 12 hr stress period did not result in any resorption of otolith calcium deposited two days previously. This does not rule out the possibility that otolith resorption may occur under different circumstances, since chronic stress situations were not tested. However, in a separate study, Campana (unpublished data) found no evidence of resorption in otoliths of starry flounders (*Platichthys stellatus*) or steelhead trout (*Salmo gairdneri*) that had been starved for periods of up to a month. Neither were checks formed in the otoliths of these fish at the time that starvation commenced.

Although no data were provided, Pannella (1980) suggests that otolith resorption occasionally occurs in wild fish, and that it is often marked by unconformities (checks that cross other daily increments). Unconformities were not produced by the stress described in this study; neither have they been observed in other coho otoliths examined by the author, although they are common in many flatfish species (Campana, unpublished data). Evidence for otolith resorption has not been obtained by other researchers (Simkiss, 1974).

Checks are ubiquitous among and within otoliths of many species of fishes. Hatching checks are often used as a temporal "benchmark" from which increment counts are made (Marshall & Parker, 1982; Neilson & Geen, in press). Checks that occur after hatching can be used to date events of interest, such as collection or rapid temperature shifts, due to the stress that these events incur. Checks formed on the otoliths of many individuals in a population on a given date can provide information on population-wide processes. However, certain checks, particularly those that delimit fortnightly or monthly patterns (Brothers *et al.*, 1976; Pannella, 1980) do not appear to have a stress-induced cause. In such cases, the reduced calcium deposition that characterizes the check may be due to inhibited calcium uptake from an endogenous source. In an analogous situation, branchial uptake of calcium virtually ceases near dawn each day during the formation of the organic zone of a daily increment. An endogenous circadian rhythm entrained to photoperiod may be indirectly responsible for the reduced calcium uptake (Mugiya *et al.*, 1981; Tanaka *et al.*, 1981). Therefore, it is possible that a biological clock based on the phases of the moon is similarly responsible for the lunar discontinuities.

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