Incremental Lines in the Dentin of Alligator Teeth before and after Hatching

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Abstract: The present study was designed to examine the differences of the incremental lines deposited in the dentin of alligator (Alligator mississippiensis) teeth before and after hatching. Before hatching, the incremental lines were uniform, and their average width was 4 \( \mu m \). After hatching, incremental lines of three types of periodicity were observed in the dentin of the decalcified tooth specimens. The average width of short-period incremental lines was 4 \( \mu m \) (1-day interval). The average width of intermediate-period incremental lines was 53 \( \mu m \) (approximately 14-day interval), but these lines were poorly defined. The average width of long-period incremental lines was 107 \( \mu m \) (approximately 28-day interval). In ground sections of tooth specimens after hatching, incremental lines of three types periodicity were observed in the dentin. The average widths of short-period, intermediate-period and long-period incremental lines were 12 \( \mu m \) (2-3 days interval), 56 \( \mu m \) (approximately 14-day interval) and 112 \( \mu m \) (approximately 28-day interval), respectively. Darker, wider and less calcified incremental lines were observed in a lunar cycle of approximately 28-day intervals. The lunar incremental lines (28 days) are due to the rhythmic deposition of minerals and organic matrix. Although the semi-lunar incremental lines (14 days) are caused by the rhythmic deposition of minerals and organic matrix, they strongly depend on the mineralization. The infradian incremental lines (2-3 days) are due to the rhythmic deposition of minerals. The circadian incremental lines (daily) are based on the rhythmic deposition of organic matrix. The intervals of incremental lines may reflect the rhythm of the odontoblast function and the fundamental biorhythm of the animals.

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孵化前後におけるワニの歯の象牙質に観察される成長線

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要旨：哺乳類の象牙質において，周期性の異なる成長線が報告され，その形成機構については古くから議論がある。しかし，爬行類以下の象牙質の成長線に関する報告は少数にとどまっている。とくに，孵化前の爬行類の象牙質の成長線に関する記載はない。そこで本研究は，ワニ類を用いて，孵化前と孵化後という環境変化に於ける成長線の周期性に違いが生じるかどうか検討し，さらに成長線の周期性の形成機構について考察を加えた。

用いた材料は，アメリカアリゲーター (Alligator mississippiensis) の孵化前の胎児及び孵化後の個体である。試料は中性ホルマリンにて固定後，脱灰標本及び研磨標本として作製した。脱灰標本はヘマトキシリン・エオジン (H-E) 染色及びポディアンのプロテイン鍍銀法を施した。脱灰及び研磨標本は光学顕微鏡にて観察し，成長線の周期は画像解析システムにて計測した。

孵化前の象牙質においては，周期間隔が平均 4 μm の細かな成長線が観察された。孵化後の象牙質においては，脱灰標本による観察では，3 種類の周期性の異なる成長線が観察された。細かい周期の成長線は，間隔が平均 4 μm であった。この細かな成長線は，一日周期の成長線と同定された。中周期の成長線は平均 53 μm の間隔であり，約 14 日周期のものであった。長周期の成長線は平均 107 μm の間隔であり，約 28 日周期であった。研磨標本による観察では，細かな周期の成長線の間隔は平均 12 μm の間隔であり，約 2-3 日周期，中周期の成長線は平均 56 μm の間隔であり，約 14 日周期，長周期の成長線は平均 112 μm の間隔であり，約 28 日周期であった。長周期の成長線は周囲より暗調であり，幅広く，より低石灰化と推定された。

これらの所見から，本論文では次の諸点にまとめられる。
1. 長周期の成長線（約 28 日周期）は基質及び石灰化の両者の形成機構リズムで形成される。
2. 中周期の成長線（約 14 日周期）は基質と石灰化による形成リズムのうち，石灰化沈着が強く影響される。
3. 短周期の成長線（約 2-3 日周期）は石灰化のリズムで形成される。
4. 一日周期の成長線は基質の沈着機構のリズムで形成される。
5. 孵化前の象牙質では一日周期の成長線だけが存在する。
6. これらの成長線の周期は象牙芽細胞の活性のリズムと動物の基本的なバイオリズムの相互作用によるものと考えられる。

Introduction

The various types of incremental lines in mammalian dentin have been debated for many years.7-11,21-25 The short-period incremental lines or von Ebner lines are considered to be daily markings indicative of circadian rhythms.1-3,14,20,25 Kleveza10 showed that the annual growth layers were the first rhythmic order in mammals, the monthly layers were the second order, and the daily or circadian layers were the third order. Ohtsuka and Shinoda17 found that the circadian increments and the ultradian increments coexisted in rat incisor dentin. However, there are few reports on the short-period incremental lines and long-period incremental lines in the dentin of the alligator teeth (Alligator mississippiensis) after hatching.18 Also, there is little information on the incremental lines in the dentin of the alligator teeth before hatching.26 Furthermore, there is no unified viewpoint about the incremental
lines before and after hatching. The present study was designed to examine the differences of the incremental lines before and after hatching and the periodicity of the short-period and long-period incremental lines.

**Materials and Methods**

Embryos of alligators (*Alligator mississippiensis*) before hatching (*N*=10) and alligators after hatching (*N*=4) were used in this study. The embryos ages were 42 (*N*=2), 48 (*N*=2), 54 (*N*=2), 60 (*N*=2) and 66 days (*N*=2) of incubation (Ferguson’s embryological stage 23–28)\(^5\). Ferguson’s embryological stage bases on the morphology of embryo. These embryos were collected from eggs gathered from nests in the marshes of the Rockefeller Wildlife Refuge, Louisiana, USA. The alligators after hatching were collected from the Atagawa Tropical and Alligator Garden, and the ages of the specimens were approximately 4 (\(N=2\)) and 10 years (\(N=2\)). Wild alligators reach sexual maturation at approximately 10 years old. The teeth of specimens were fixed in 10% neutral formalin solution. After fixation, the teeth were ground parallel to the longitudinal axis. The ground sections (80 to 100 \(\mu\)m thick) were observed by light microscopy and confocal laser scanning microscopy. Some of pre- and post-hatching specimens were decalcified with 10% EDTA (pH 7.4). The decalcified sections (4 \(\mu\)m thick) were stained with hematoxylin and eosin (H-E) staining and Bodian protein silver impregnation. Chronological analysis was made by counting the number of incremental lines and measuring the widths of adjacent incremental lines using an image processing system (Win Roof, Mitani Co., Japan) equipped with a light microscope (Olympus AX80).

![Fig.1 Micrograph of a decalcified section of *Alligator mississippiensis* at 42 days of incubation. Stage 21. Two incremental lines (arrows) are observed in the dentin. Hematoxylin and eosin staining. Bar=20 \(\mu\)m.](image1)

![Fig.2 Micrograph of a decalcified section of *Alligator mississippiensis* at 48 days of incubation. Stage 24. Narrow incremental lines (arrows) are observed in the dentin. Hematoxylin and eosin staining. Bar=20 \(\mu\)m.](image2)
Results

After 42 days of incubation (embryological stage 23), two incremental lines (arrows) were observed in the dentin of decalcified sections (Fig. 1). The width of the incremental lines was 4.47±0.74 μm. At 48 days of incubation (embryological stage 24), the incremental lines were observed in the dentin (Fig. 2). The width of the incremental lines was 4.33±0.56 μm. At 54 days of incubation (embryological stage 25), the incremental lines were also observed in the dentin (Fig. 3) at a width of 4.11±0.22 μm. After 60 days of incubation (embryological stage 25), narrower incremental lines (arrows) were observed uniformly in the dentin (Fig. 4), and the width was 4.01±0.46 μm. After 66 days of incubation (embryological stage 28), narrower incremental lines (arrows) were observed in the dentin in crown formation stage of tooth development (Fig. 5). The width of the incremental lines was 3.51±0.78 μm. In the specimen of same day, narrower incremental lines (arrows) were also observed at the root formation stage of tooth development (Fig. 6), and the width of the incremental lines was 3.72±
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Fig. 6 Micrograph of a decalcified section of *Alligator mississippiensis* at 66 days of incubation. Stage 28. Narrow incremental lines (arrows) are observed in the dentin. Root formation stage of tooth development. Hematoxylin and eosin staining. Bar=20 μm.

Fig. 7 Micrograph of a decalcified section of *Alligator mississippiensis* at 66 days of incubation. Stage 28. Narrower incremental lines (arrows) are observed in the dentin. Root formation stage of tooth development. Bodian protein silver impregnation. Bar=20 μm.

0.53 μm. The narrow incremental lines were more clearly observed using Bodian protein silver impregnation compared with H–E staining (Fig. 7). The width of incremental lines measured by Bodian protein silver impregnation was 3.50±0.39 μm.

After hatching, incremental lines of three kinds of periodicity were observed in the dentin in decalcified sections of the functional tooth from an approximately 4 year-old alligator (Fig. 8). The width of the long-period incremental lines was 103.18±4.71 μm. The intermediate-period incremental lines were weakly stained, with a width of 50.77±4.99 μm. The width of the short-period incremental lines was 4.24±0.47 μm. At the same age, incremental lines of two kinds of periodicity were observed in the dentin of tooth germ (Fig. 9). The widths of the long-period and short-period incremental lines were 111.70±24.89 μm and 4.74±0.72 μm.

At approximately 10 years old, incremental lines of three kinds of periodicites were observed in the dentin of decalcified sections of the functional tooth (Fig. 10). The width of the long
Fig. 9 Micrograph of a decalcified section of Alligator mississippiensis. After hatching, 4 years old: root formation stage of tooth germ. Incremental lines of two kinds of periodicity are observed in the dentin. 1: long-period incremental lines, 2: short-period incremental lines. Hematoxylin and eosin staining. Bar=100 μm.

Fig. 10 Micrograph of a decalcified section of Alligator mississippiensis. After hatching, 10 years old: functional tooth. Incremental lines of three kinds of periodicity are observed in the dentin. 1: long-period incremental lines, 2: intermediate-period incremental lines, 3: short-period incremental lines. Hematoxylin and eosin staining. Bar=100 μm.

Fig. 11 Micrograph of a decalcified section of Alligator mississippiensis. After hatching, 10 years old: functional tooth. Incremental lines of three kinds of periodicity are observed in the dentin. 1: long-period incremental lines, 2: intermediate-period incremental lines, 3: short-period incremental lines. Bodian protein silver impregnation. Bar=100 μm.

Fig. 12 Micrograph of ground section of Alligator mississippiensis. After hatching, 10 years old: functional tooth. Incremental lines of three kinds of periodicity are observed in the dentin. 1: long-period incremental lines, 2: intermediate-period incremental lines, 3: short-period incremental lines. 8ar=100 μm.

Narrow incremental lines were more clearly observed using Bodian protein silver impregnation than with H-E staining (Fig. 11). By Bodian protein silver impregnation, the widths of the short-period incremental lines, intermediate-period, and long-period incremental lines were 3.43±0.53 μm, 53.98±2.75 μm and 110.97±5.85 μm, respectively.

The width of the short-period incremental lines of ground section was wider than that of the decalcified section (Fig. 12). Using ground

were weakly stained. The width of the intermediate-period incremental lines was 53.70±3.88 μm. The width of the short-period incremental lines was 5.54±0.64 μm with H-E staining. Narrow incremental lines were more clearly observed using Bodian protein silver impregnation than with H-E staining (Fig. 11). By Bodian protein silver impregnation, the widths of the short-period incremental lines, intermediate-period, and long-period incremental lines were 3.43±0.53 μm, 53.98±2.75 μm and 110.97±5.85 μm, respectively.
sections, the widths of the short-period, intermediate-period, and long-period incremental lines were 12.04±3.19 \(\mu m\), 56.11±1.82 \(\mu m\) and 112.67±8.17 \(\mu m\), respectively.

Discussion

Before hatching, the incremental lines were uniform and the average width of these lines in the dentin was 4.02 \(\mu m\) in the decalcified sections. In mammals, daily incremental lines or circadian incremental lines are formed in the dentin\(^{10,17-19,23}\). This periodicity reflects the daily rhythmic deposition of organic matrix in dentin\(^{1,8,13,16,23}\). Dean et al.\(^{11}\) reported that the ameloblasts secreted an enamel matrix in a circadian manner. Ohtsuka et al.\(^{18}\) demonstrated that odontoblasts showed a circadian rhythm with regard to collagen synthesis and secretion.

After hatching, incremental lines of three kinds of periodicity were observed in the dentin of decalcified tooth sections. The average width of short-period incremental lines was 4.34 \(\mu m\). From the width, the short-period incremental lines are identified to have a circadian rhythm, similar to the incremental lines before hatching\(^{16,20}\). The average width of intermediate-period incremental lines was 52.81 \(\mu m\). Rosenberg and Simmons\(^{21}\) reported that an infradian rhythm might also exist in rabbit incisor dentin. In Eurasian lynx (Lynx lynx), the mean period of the layer formation was 14–15 days (semilunar cycle)\(^9\). From our previous study\(^{15}\) and the average width of the circadian incremental lines, the period of formation of the intermediate-period incremental lines was judged to be approximately every 14 days. The average width of long-period incremental lines was 107.24 \(\mu m\). From our previous study\(^{15}\) and the average width of circadian incremental lines, the period of formation of the long-period incremental lines was judged to be approximately every 28 days as in the lunar cycle. The incremental lines with a periodicity of approximately 28 days were intensely stained by hematoxylin.

Using ground sections, the incremental lines in the dentin of tooth after hatching were estimated to have periodicities of 2–3 days (average width: 12.04 \(\mu m\)), approximately 14 days (average width: 56.11 \(\mu m\)), and approximately 28 days (average width: 112.67 \(\mu m\)). Klev-
lunar incremental lines (approximately 14-day interval) strongly depends on mineralization. This opinion supports the hypothesis that the rates of collagen matrix production and collagen mineralization may be relatively independent. The results of the ground sections show that the infradian incremental lines (2-3 days apart) are due to the rhythmic deposition of mineralization, while the results of the decalcified sections indicate that the circadian incremental lines are caused by the rhythmic deposition of organic matrix (Fig. 13). The rhythm of matrix formation may not correspond to the rhythm of the mineralization

The intervals of incremental lines may be related to the rhythm of the odontoblast function and the fundamental biorhythm of the animals. The authors speculate that the circadian and infradian incremental lines may be independent and driven by different oscillatory mechanisms. More data concerning the implication of these findings are needed to elucidate this phenomenon.

Conclusion

1. Before hatching, the incremental lines in the dentin were uniform and had a circadian rhythm.
2. After hatching, incremental lines of four kinds of periodicity were observed in the dentin.
3. Darker, wider and less calcified incremental lines were observed following a lunar cycle of approximately 28-day intervals.
4. The lunar incremental lines (approximately 28-day interval) are due to the rhythmic deposition of both minerals and organic matrix.
5. The semi-lunar incremental lines (approximately 14-day interval) are due to the rhythmic deposition of minerals and organic matrix, but these incremental lines strongly depend on mineralization.
6. The infradian incremental lines (approximately 2 to 3-day interval) are due to the rhythmic deposition of mineralization.
7. The circadian incremental lines are due to the rhythmic deposition of organic matrix.
8. The intervals of incremental lines may be related to the rhythm of the odontoblast function and the fundamental biorhythm of the animals.

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