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Characterization of short-period and long-period incremental markings in porcine enamel and dentine—Results of a fluorochrome labelling study in wild boar and domestic pigs

Simon Emken[1] | Carsten Witzel[1] | Uwe Kierdorf[1] | Kai Frölich[1,2] | Horst Kierdorf[1]

[1]Department of Biology, University of Hildesheim, Hildesheim, Germany

[2]Tierpark Arche Warder e.V, Warder, Germany

Correspondence
Horst Kierdorf, Department of Biology, University of Hildesheim, Universitätsplatz 1, 31141 Hildesheim, Germany
Email: kierdorf@uni-hildesheim.de

Abstract

Mammalian dental hard tissues exhibit incremental markings that reflect the periodic variation of appositional growth rates. In order to use these markings to characterize dental growth processes and to infer life-history traits, an unequivocal identification of their periodicities is required. We performed a fluorochrome labelling study on forming enamel and dentine in molar teeth of wild boar and domestic pigs to establish the periodicity and temporal correspondence of incremental markings in enamel and dentine. The dominant incremental markings in enamel (laminations) and dentine (von Ebner lines) recorded in the pig teeth are of a daily nature. In addition, long-period incremental markings with a periodicity of 2 days were recorded in enamel (striae of Retzius) and dentine (Andresen lines). The 2-day growth rhythm was also expressed at the lateral crown surface, as evidenced by the pattern of perikymata. In enamel, also markings with a sub-daily periodicity, representing an ultradian growth rhythm, were observed. Our study provides experimental evidence for the periodicity of incremental markings in porcine enamel and dentine. The findings correct previous misconceptions on incremental markings in dental hard tissues of pigs and other ungulates that had led to erroneous conclusions regarding crown formation parameters.

KEYWORDS
Andresen lines, laminations, perikymata, striae of Retzius, von Ebner lines

1 | INTRODUCTION

Mammalian dental hard tissues show periodic variation in their appositional growth rates (Boyde, 1989; Dean, 2000; Hillson, 2014; Maas & Dumont, 1999; Ohtsuka & Shinoda, 1995; Okada, 1943; Zander & Hürzeler, 1958). As a result, enamel, dentine and cementum exhibit characteristic incremental markings that constitute a permanent record of the growth process for the respective tissue, analogous to the growth rings of trees (Dean, 1987, 2000; Hillson, 2005, 2014; Hogg, 2018; Klevezal, 1996; Risnes, 1998; Waugh et al., 2018).

The cyclic modulation of growth rates and the periodic variation of various other (physiological and behavioural) processes are controlled by biological rhythms. These are understood as biological adaptations to environmental cycles of different lengths, including recurring events with a tidal, daily, lunar or annual periodicity (Baker, 1938; Floessner & Hut, 2017; Hogg, 2018). Biological rhythms are governed by genetically controlled endogenous mechanisms

referred to as 'biological clocks'. The signals from the internal biological clocks occur with periodicities (e.g. circadian or circannual) that are relatively close to those of the environmental cycles. The latter act as external timing signals (Zeitgeber) that entrain the internal rhythms to those of the environment (Mohawk et al., 2012; Zhang et al., 2013). The most prominent external timing signals are the day–night cycle caused by the Earth's rotation and the seasonal variation in day length caused by its revolution around the sun in combination with the tilt of the Earth's rotational axis (Honma, 2018). Biological clocks operate at different levels from cellular to organismal. Organismal clocks are linked to and operate via the endocrine system (Neumann et al., 2019). In mammals, the primary circadian clock (master pacemaker) is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Reppert & Weaver, 2002; Yamazaki et al., 2000). In addition, many cell types have been demonstrated to harbour molecular clocks and to show rhythmic transcription of clock genes (Heyde & Oster, 2019).

The regular incremental markings in mammalian dental hard tissues exhibit periodicities that range from sub-daily, reflecting ultradian internal rhythms, to yearly, reflecting circannual rhythms (Boyde, 1989; Bromage, 1991; Dean, 2000; FitzGerald, 1998; Hogg, 2018; Kierdorf et al., 2013; Klevezal & Mina, 1990; Lieberman, 1993; Papakyrikos et al., 2020; Smith, 2006; Waugh et al., 2018). Two main types have been defined as either short-period (reflecting circadian internal rhythms and partially also subsuming ultradian rhythms) or long-period (reflecting infradian internal rhythms) incremental markings (Boyde, 1989; Dean et al., 1993; Hillson, 2014; Risnes, 1998; Smith et al., 2003). They can be studied with different microscopic (Dean, 2006; Kierdorf et al., 2013, 2019; Orlandi-Oliveras et al., 2019; Schwartz et al., 2006; Smith, 2006) and tomographic (Newham et al., 2020; Tafforeau et al., 2007) techniques, and their analysis allows the reconstruction of dental growth processes and inferences of life-history traits. A crucial prerequisite for this approach is the correct identification of the incremental markings and their periodicities (Dean, 2000).

An established method to study the periodicity of incremental markings in mineralized tissues is *in vivo* labelling with substances that are incorporated at the growth (mineralization) front of the respective tissue and there produce a signal that can be identified microscopically (Bromage, 1991; FitzGerald, 1998; Kierdorf et al., 2013; Lieberman, 1993; Okada & Mimura, 1938, 1940; Papakyrikos et al., 2020; Schour & Poncher, 1937; Smith, 2006; Yilmaz et al., 1977). This signal can be the substance itself, for example lead (Okada & Mimura, 1938, 1940; Papakyrikos et al., 2020), a specific fluorescence emitted from fluorochrome labels (Bromage, 1991; van Gaalen et al., 2010; Iinuma et al., 2004; Kierdorf et al., 2013; Smith, 2006), or a structural alteration caused by a temporary disruption of the growth process, as in the case of higher dosages of fluoride or tetracycline (Kawasaki & Fearnhead, 1975; Kierdorf et al., 2013; Schour & Hoffman, 1939; Schour & Poncher, 1937; Smith, 2006).

Mammalian dental enamel exhibits different types of short-period incremental markings. In humans and other primates, the most prominent short-period markings in enamel are prism cross-striations (Boyde, 1989; Hillson, 2014). On microscopic inspection of thin ground sections, they appear as alternating transverse dark and bright bands along the enamel prisms, which are bundles of apatite crystallites formed in relation to the distal portion of the Tomes' process of the ameloblasts (Boyde, 1989; FitzGerald & Rose, 2008; Hillson, 2014; Nanci, 2013). Prism cross-striations are generally considered daily incremental markings, with a dark and a bright band together reflecting 1 day of enamel growth (Antoine et al., 2009; Asper, 1916; Boyde, 1989; Li & Risnes, 2004; Okada, 1943). A second type of short-period incremental markings in enamel is referred to as laminations. These are present as parallel lines that mark successive positions of the forming front during enamel growth (Kodaka et al., 1995; Ripa et al., 1966). In primate enamel, laminations are primarily found in cuspal enamel or close to the enamel-dentine junction (EDJ) (Smith, 2006; Smith et al., 2004), but they can also be observed in prismless surface enamel (Kodaka et al., 1989, 1991, 1995). In prismatic enamel, laminations can form at prism and interprism growth sites, and the visibility of these markings in different regions of the enamel layer depends on the variation in volume occupancy by prismatic and interprismatic enamel portions (Kierdorf et al., 2013, 2014). Laminations are the most prominent incremental markings in the enamel of perissodactyls and cetartiodactyls (Iinuma et al., 2004; Jordana & Köhler, 2011; Kierdorf et al., 2012, 2013, 2014, 2019; Nacarino-Meneses et al., 2017; Okada & Mimura, 1940; Tafforeau et al., 2007).

Long-period (supra-daily) incremental markings in primate enamel, which reflect an infradian activity rhythm of the ameloblasts, are known as striae of Retzius or Retzius lines (Boyde, 1989; Dean, 2000; Hillson, 2014; Risnes, 1998). Their orientation corresponds to that of the laminations, and they likewise mark successive positions of the enamel forming front (Boyde, 1989; Kierdorf et al., 2019; Risnes, 1990). The time interval in days between two consecutively formed striae of Retzius, known as Retzius periodicity, Retzius interval or repeat interval (FitzGerald, 1998; Hogg, 2018; Kierdorf et al., 2013; McFarlane et al., 2014, 2021; Smith et al., 2004), varies between species and among different individuals of the same species (FitzGerald & Rose, 2008; Hillson, 2005, 2014). The long-period increment between two successive striae of Retzius is also referred to as Retzius band (Tafforeau et al., 2007) or Retzius increment (Kierdorf et al., 2015). Retzius periodicity in enamel is established by counting daily prism cross-striations between consecutive striae of Retzius (Hillson, 2014; Mahoney et al., 2018; O'Meara et al., 2018; Reid & Dean, 2006; Reid & Ferrell, 2006; Smith et al., 2004). The number of prism cross-striations (counting either the dark or the bright bands) plus one equals the number of days between the formation of two consecutive striae of Retzius. For primate enamel, Retzius periodicity has been reported to vary between 1 and 12 days (Bromage et al., 2012; Hillson, 2014; Reid & Dean, 2006; Reid & Ferrell, 2006). For other mammals, Fukuhara (1959) reported the presence of between 2 and 11 prism cross-striations between consecutive striae of Retzius.

Thus far, it has generally been assumed that Retzius periodicity is identical in all teeth of an individual (FitzGerald, 1998; Hillson, 2014).

However, this assumption was recently questioned by McFarlane et al., (2021) who reported cases of intra-individual variation of Retzius periodicity among human permanent teeth.

Along the crown flanks of teeth, the cyclic modulation of enamel growth is reflected by the presence of perikymata at the outer enamel surface (OES) that consist of regularly alternating horizontal grooves and ridges (Boyde, 1989; Hillson, 2014; Risnes, 1984). There is a clear correspondence between internal and external (surface) enamel incremental markings in that each stria of Retzius terminates in a perikyma groove at the OES (Boyde, 1989; Hillson, 2014; Kierdorf et al., 2015; Risnes, 1984). The increment margin or increment boundary present at the cervical edge of a perikyma groove marks the resumption of matrix secretion after the formation of a stria of Retzius, that is, the start of a new long-period cycle of secretory ameloblast activity (Hillson, 2014).

The dentine, the hard tissue forming the bulk of a tooth, also exhibits incremental markings of different periodicities. The daily incremental markings in dentine are known as von Ebner lines (Dean, 1998; Ohtsuka-Isoya et al., 2001). They are the temporal equivalent of daily prism cross-striations and daily laminations, and likely reflect the same underlying biological rhythm (Dean & Scandrett, 1996). When studying incremental markings in ground sections it should, however, be noted, that while the daily incremental markings in enamel mark the position of the forming front along which deposition and initial mineralization of the proteinaceous matrix occur (almost) simultaneously, the markings in the dentine reflect the position of the mineralizing front that follows the matrix (predentine) formation front at some distance (Dean, 1998). On sections viewed under transmitted light, the von Ebner lines appear as alternating dark and bright bands (Dean, 2000; Yilmaz et al., 1977). In addition to the von Ebner lines, long-period dentinal incremental markings have been reported in the teeth of primates and various other mammalian species (Dean & Scandrett, 1996; Hillson, 2005, 2014). These markings are referred to as Andresen lines and constitute the temporal equivalent of the striae of Retzius in enamel (Dean, 2000; Hillson, 2014). Corresponding to the situation in enamel, several studies have reported the additional presence of sub-daily incremental markings in mammalian dentine (Kawasaki et al., 1980; Newman & Poole, 1974; Ohtsuka & Shinoda, 1995; Ohtsuka-Isoya et al., 2001; Papakyrikos et al., 2020; Smith et al., 2004).

Most studies on the periodicities of incremental markings in enamel and dentine have addressed primate teeth (e.g. Antoine et al., 2009; Boyde, 1989; Bromage, 1991; Bromage et al., 2012; Dean, 2000; Hillson, 2014; Smith, 2006). These investigations demonstrated that the dominant incremental markings in primate enamel are daily prism cross-striations and supra-daily striae of Retzius.

Different mechanisms involving the autonomic nervous system have been proposed to control long-period growth rhythms and to cause the formation of features like striae of Retzius (Appenzeller et al., 2005; Bromage et al., 20092009). Other authors suggested that the formation of striae of Retzius reflects an interaction of two or more long-period rhythms whose nadirs overlap at regular time intervals (Newman & Poole, 1974; Smith, 2006). It has further been argued that striae of Retzius show structural similarities to accentuated daily prism cross-striation (Boyde, 1989; Kierdorf & Kierdorf, 1997; Kierdorf et al., 2004; Li & Risnes, 2004; McFarlane et al., 2021; Risnes, 1998). However, more research is needed to reveal the mechanisms underlying the formation of striae of Retzius.

The primate pattern of incremental markings in enamel detailed above is not typical of mammals in general. Thus, several studies showed that the dominant incremental features in ungulate enamel are laminations. Contrary to primate enamel, striae of Retzius are less prominent in ground sections of enamel from these taxa (Iinuma et al., 2004; Jordana & Köhler, 2011; Kierdorf et al., 2013, 2014, 2019; Nacarino-Meneses et al., 2017; Okada & Mimura, 1940; Tafforeau et al., 2007). Due to their parallel orientation and similarity in appearance, long-period (striae of Retzius) and short period incremental markings (laminations) can be difficult to distinguish histologically in ungulate enamel (Kierdorf et al., 2013, 2014, 2019).

The situation is further complicated by the presence of sub-daily incremental markings that have been described in the enamel of primates (Mahoney, 2012; Smith, 2006), sheep (Kierdorf et al., 2013) and pigs (Kierdorf et al., 2014, 2019). The histological appearance of these sub-daily markings resembles that of daily prism cross-striations (Kierdorf et al., 2014; Smith, 2006). In consequence, when used in a purely morphological sense, that is without reference to a specific underlying periodicity, the term 'cross-striation' can mean either a daily or a sub-daily incremental marking. It is therefore recommended to always use a qualifier and to characterize the respective markings as either daily or sub-daily cross-striations.

Incautiously applying the 'primate pattern' of interpretation of histological structures to non-primate mammalian teeth is likely to result in the misidentification of enamel incremental markings. Mistaking daily laminations for long-period striae of Retzius and sub-daily cross-striations for daily ones will lead to major errors in the assessment of crown growth parameters. For example, the crown formation time of 1035 days reported for first molars of *Gazelli granti*, a medium-sized African bovid (Macho & Williamson, 2002) was strongly questioned and discussed as a possible case of misidentification of incremental markings (Kierdorf et al., 2013, 2014). A previous study had concluded that enamel formation time for individual gazelle first molars is at most 4 months (Kohn et al., 1998). Likewise, the low daily enamel secretion rate and the related long crown formation time reported for horse teeth by Hoppe et al., (2004) have been refuted and interpreted as a case of misidentification of incremental markings (Nacarino-Meneses et al., 2017).

More recently, there has also been some controversy about the interpretation of incremental features in porcine enamel. In accordance with earlier studies (Okada & Mimura, 1940), Kierdorf et al., (2014) had demonstrated that the dominant incremental markings in the enamel of domestic pigs are daily laminations. Later, however, Bromage et al., (2016) interpreted this type of linear markings as striae of Retzius with a periodicity of 5 days. A further study on the enamel of unlabelled teeth from domestic pigs and wild boar (Kierdorf et al., (2019) then concluded that Bromage et al., (2016) had apparently mistaken daily laminations for striae of Retzius and

sub-daily prism cross-striations for daily ones, and that this misidentification was likely facilitated by a scaling error.

To provide definitive experimental evidence for the types and periodicities of incremental markings in porcine enamel and dentine, we conducted a fluorochrome labelling study in wild boar and domestic pigs. We further investigated the relationship between internal enamel incremental markings and growth marks at the crown surface of pig teeth.

2 | MATERIALS AND METHODS

The study was performed on two captive-born wild boar (*Sus scrofa*) and two domestic pigs (*Sus scrofa* f. domestica), with one male and one female in each group. All four animals were housed in the Tierpark Arche Warder e.V. (Warder, Germany). Both wild boar were born on 11 April 2016 and slaughtered on 7 March 2017. The two domestic pigs of the breed 'Swedish Linderöd' were born on 21 April 2016 and slaughtered on 30 March 2017. The experiment was conducted in accordance with all current animal care regulations in Germany and with permission (including ethical approval) of the responsible veterinary authorities of the federal state of Schleswig-Holstein (Ministerium für Landwirtschaft, Umwelt und ländliche Räume des Landes Schleswig Holstein; Az. V312-72241.123–34). The experimental animals were earmarked individually, and the two groups (wild boar and domestic pigs) were kept in separate stables with access to outdoor pens during daytime and exposure to the natural photoperiod. The animals were fed commercial feed, had permanent access to water, and were under constant veterinary control.

Starting on 6th July, at an age of 76 days in the domestic pigs and 86 days in the wild boar, the experimental animals received alternating intramuscular injections of calcein (Sigma Aldrich, product no. C0875, buffered to pH 7, a total of six injections) at a dosage of 8 mg/kg body weight and oxytetracycline (ursocycline, Serumwerk Bernburg AG, product no. 09932159, a total of 6 injections) at a dosage of 40–80 mg/kg body weight. Oxytetracycline dosage was lowered over time to reduce injection volume in the rapidly growing individuals. The faster-growing domestic pigs received an oxytetracycline dose of 80 mg/kg body weight in the first, and doses of 50 mg/kg body weight in the second and third injections. The more slowly growing wild boar received 80 mg/kg body weight, each, in the first and second oxytetracycline injections, and a dose of 50 mg/kg body weight in the third. Thereafter, oxytetracycline dosage was reduced to 40 mg/kg body weight for the remaining injections in all four experimental animals. Injections were given at 14- or 21-day intervals (Table 1) and were always performed between 9 and 12 a.m. The two male individuals were castrated prior to the first fluorochrome injection.

After termination of the experiment, the animals were killed using approved humane methods, and their heads were removed and macerated, without a final bleaching step to prevent alterations of the fluorochrome labels. The macerated and dried

TABLE 1 Labelling scheme showing animal age in postnatal days (0 = day of birth) at the dates of fluorochrome injection (period from 6th July 2016 to 11th January 2017) and age at death.

Individual	Race[a]	Sex	Date of Birth	Injected Fluorochrome[b]										Age at Death
				Ca	T	Ca	Ca	T	Ca	T	T	Ca	T	
50315	wb	male	11th April 2016	86	100	114	135	156	170	184	205	226	240	330
50337	wb	female	11th April 2016	86	100	114	135	156	170	184	205	226	240	330
50369	dp	male	21st April 2016	76	90	104	125	146	160	174	195	216	230	343
50367	dp	female	21st April 2016	76	90	104	125	146	160	174	195	216	230	343

Note: [a]wb, wild boar; dp, domestic pig.
[b]Ca, calcein; T, oxytetracycline.

skulls were photographed and X-rayed, followed by the removal of the mandibular first and second molars. The extracted molars were immersed in a proteolytic solution (Enzyrim OSA, Bauer, Switzerland) for 24 hours, followed by thorough rinsing in water and drying. Tooth surfaces were analysed in a scanning electron microscope (Zeiss EVO MA 15; Jena, Germany) operated in low-vacuum mode at 20 kV accelerating voltage, using a backscattered electron (BSE) detector.

For analysis of ground sections, the teeth were embedded in epoxy resin (Biodur E12, Biodur products; Heidelberg, Germany) and subsequently sectioned axio-buccolingually through the highest point of the anterior (mesial) lobe (Figure 1). Ground sections (thickness of about 50 µm) were produced as detailed earlier (Kierdorf et al, 2019). The sections were viewed and photographed with an Axio Imager 2 microscope (Zeiss; Jena, Germany) equipped with a digital monochrome camera (Axiocam 503 mono). Fluorescence was recorded with specific filter sets to detect calcein labels (excitation filter (ex) 470/40 nm band-pass; dichroic mirror (dm) 495 nm; emission filter (em) 535/50 nm band-pass) and oxytetracycline labels (ex 390/40 nm; dm 452 nm; em 562/40 nm). The collected fluorescence light was converted to either green (calcein) or red (oxytetracycline) false colour, using the image analysis and processing software of the microscope (ZEN 2.6 blue edition, Jena, Germany). For microscopic analyses, images of identical areas of the ground sections were captured with transmitted light (using either phase contrast or differential interference contrast) and the two fluorescence channels, and overlay images from the three recording channels were produced (Figure 2). The acquired images were stitched and analysed with the tools of the Fiji freeware image processing package (NIH; Bethesda, USA).

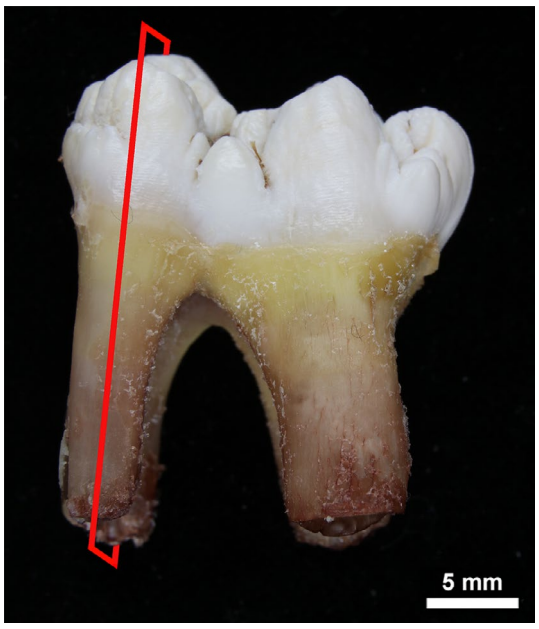


FIGURE 1 Left mandibular second molar of the male domestic pig, buccal view. The red frame indicates the sectioning plane that runs through the highest points of the anterior cusps

3 | RESULTS

The repeated fluorochrome injections had labelled successive positions of the growth (mineralization) fronts of both enamel and dentine. In dentine, conspicuous fluorescent labels were produced by both fluorochromes. In contrast, in the enamel only the calcein labels were clearly visible, whereas the oxytetracycline labels were only faintly discernible or not visible at all (Figure 2). Elimination of tetracycline-related fluorescence during enamel maturation has been attributed to the preferential binding of this fluorochrome to the organic enamel matrix (Hammarström, 1967).

In the wild boar second molars, all six calcein injections were represented by labels in the enamel (Figure 2a). In contrast, in the second molars of the domestic pigs, only the first four calcein injections had produced labels in the enamel (Figure 2b), thereby indicating that enamel growth in these teeth had already ceased prior to the penultimate calcein injection. In the wild boar second molars, the calcein label from the fourth injection was the first to reach the OES, whereas in the second molars of the domestic pigs this was the case for the calcein label from the third injection. Due to the earlier development of the first compared to the second molar, no labels were present in the enamel of the M_1 from the domestic pigs, whereas in the wild boar only the label from the first calcein injection (at day 86) was present in the (cervical) enamel of this tooth. In contrast to the enamel, the dentine of the first and second molars exhibited labels from all fluorochrome injections.

Inspection of ground sections at higher magnifications (using phase-contrast enhancement) revealed the presence of a regular pattern of prominent parallel lines, identified as laminations, in the enamel of all analysed teeth (Figure 3). The number of laminations (defined here as the bright lines seen in the phase-contrast images) between two consecutive calcein labels in the enamel corresponded to the number of days minus one between the two calcein injections producing them. Thus, 27 laminations were located between labels from injections given 28 days apart (Figure 3). The relationship between fluorescent labels, laminations (daily incremental markings), and daily increments in porcine enamel is schematically illustrated in Figure 4. The spacing between consecutive laminations varied between inner and outer enamel as well as along the vertical crown axis.

In certain crown areas, enamel incremental markings with a much closer spacing than that of the laminations were recorded and diagnosed as sub-daily markings (Figure 5). The latter were orientated in parallel to the laminations and best visible when sections were viewed at higher magnifications using differential interference contrast microscopy. Unfortunately, laminations were typically not clearly discernible in ground sections viewed with this method. The sub-daily incremental markings showed a regular pattern of alternating broader bright and narrower dark lines, and typically five pairs of these bright and dark sub-daily markings could be identified in the enamel stretch representing a daily growth increment. In places, still finer incremental markings (n = 10–12) were visible in an

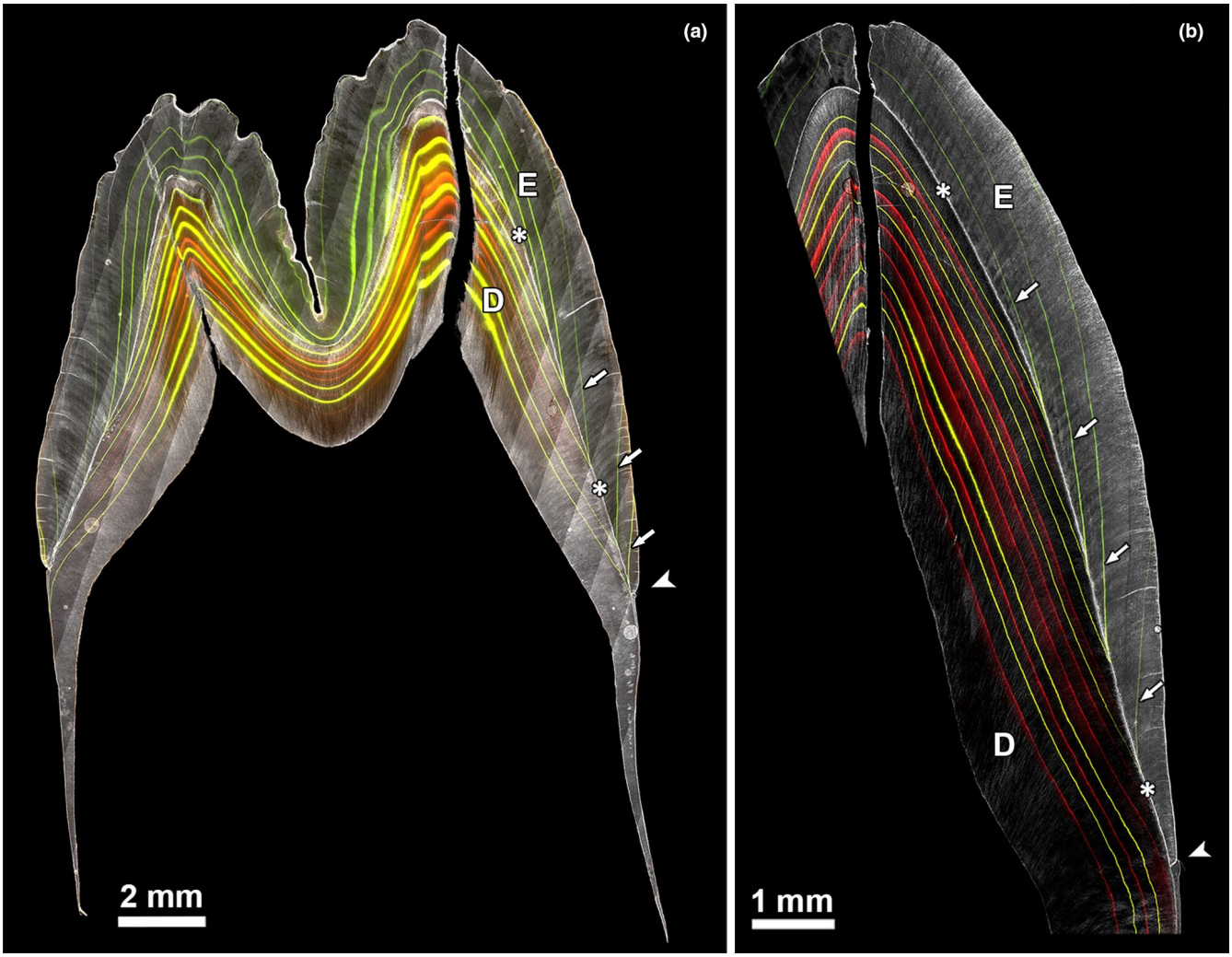


FIGURE 2 Micrographs of buccolingual ground sections through porcine mandibular second molars showing fluorochrome labels (calcein = green or yellowish/green, oxytetracycline = red). Contrary to the dentine (D), in which labels from both fluorochromes are visible, in the enamel (E) only the calcein labels are discernible. Asterisks: Enamel-dentine junction (EDJ). Arrowhead: Crown-root-border. Occlusal to top, lingual to the right. Overlays of transmitted light images (phase contrast) and the two fluorescence channels. (a) Left mandibular second molar of the female wild boar. Labels from all six calcein injections are visible in the enamel. Of these, only the labels (arrows) from the last three calcein injections given at days 170, 226 and 254 terminate at the enamel surface. (b) Lingual crown flank of the left mandibular second molar from the male domestic pig. In the enamel, only four calcein labels (arrows) caused by the injections at days 76, 104, 125 and 160 are visible. Enamel formation had, thus, already ceased prior to the calcein injection on day 216, and the labels from this and the last calcein injection at day 244 are only present in the dentine

enamel stretch representing a daily increment (Figure 5), suggesting modulation of ameloblast secretory activity with an even shorter periodicity.

The lateral crown surface of the teeth exhibited a pattern of perikymata with regularly alternating perikyma ridges and perikyma grooves (Figure 6). To determine the periodicity of these incremental markings, we compared the number of perikyma grooves present along a certain stretch of the crown surface with the number of days between consecutively formed calcein labels terminating at the enamel surface that delimited this stretch. In all four experimental animals, we counted 14 perikymata between two consecutively formed calcein labels from injections given 28 days apart, indicating that the perikyma grooves represented incremental markings with a periodicity of 2 days (Figure 7).

In the outermost enamel, more prominent incremental lines were occasionally discernible in the ground sections. Each of these prominent lines ('accentuated laminations') terminated in a perikyma groove, identifying them as striae of Retzius. Between two striae of Retzius, a non-accentuated ('normal') lamination reached the enamel surface (Figure 8). Striae of Retzius were only traceable to a depth of about 50–150 μm below the OES, whereas deeper within the enamel they were indistinguishable from normal laminations. Between two successive calcein labels caused by injections 56 days apart, 27 striae of Retzius were discernible in the enamel of the experimental animals (Figure 10). Thus, the striae of Retzius showed a 2-day periodicity in wild boar and domestic pigs. The relationships between 1) laminations and daily increments, 2) internal long-period markings (striae of Retzius) and 3) perikyma grooves are schematically illustrated in Figure 9.

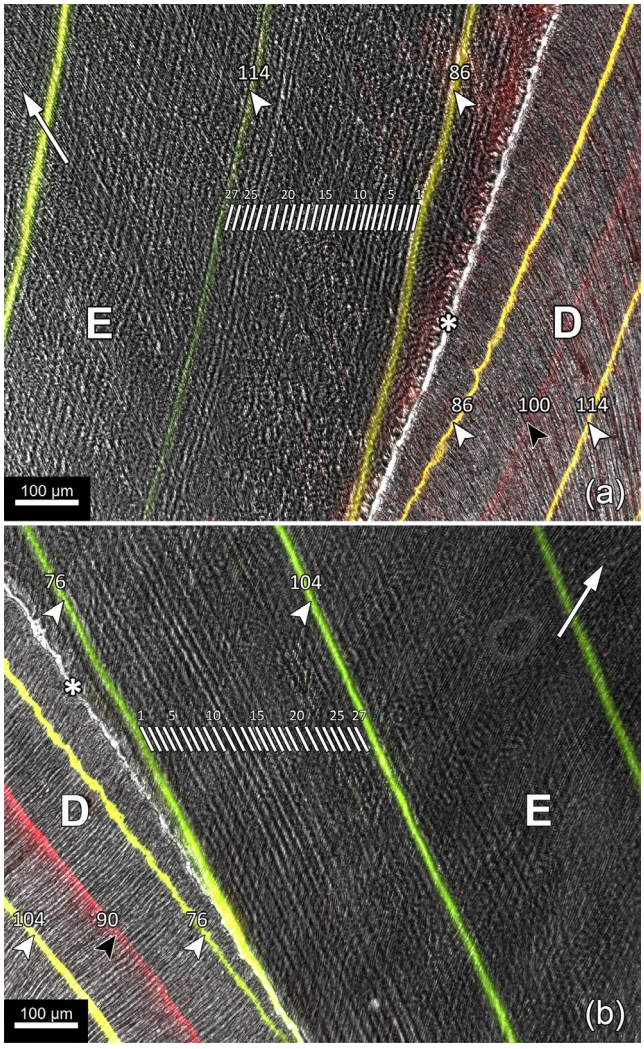


FIGURE 3 Micrographs of buccolingual ground sections of the left mandibular second molars of the male wild boar (a) and the male domestic pig (b) showing buccal enamel (E) and dentine (D). Occlusal to top. Asterisks: EDJ. Arrows: overall prism direction. Overlays of transmitted light images (phase contrast) and the two fluorescence channels. (a) Calcein labels from injections at days 86 and 114 are marked by white arrowheads in enamel and dentine. An oxytetracycline label (black arrowhead) from the injection at day 100 is only visible in the dentine. Short white lines mark 27 laminations between the two consecutive calcein labels caused by injections 28 days apart (days 86 and 114). OES to the left. (b) Calcein labels from injections at days 76 and 104 are marked by white arrowheads in enamel and dentine. An oxytetracycline label (black arrowhead) from the injection at day 90 is only visible in the dentine. Short white lines mark 27 laminations between two consecutive calcein labels caused by injections 28 days apart (days 76 and 104). OES to the right

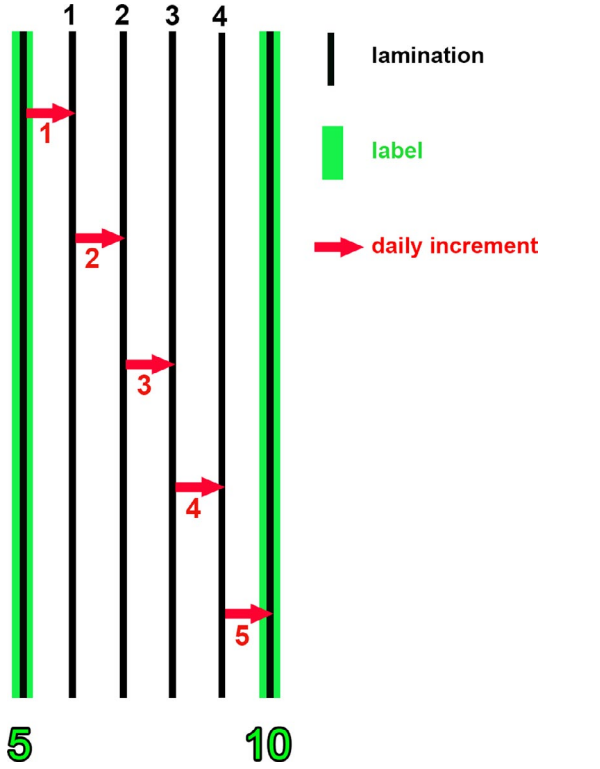


FIGURE 4 Schematic illustration of the relationship between fluorescent labels, incremental markings and daily increments in enamel. Four laminations (1–4) and five daily increments (red arrows) are present between two fluorescent labels (green lines) from (fictitious) consecutive injections administered 5 days apart (day 5 and day 10). The laminations formed at the days of the respective fluorochrome injections are also indicated (black line within green label)

In ground sections viewed in transmitted light with phase contrast, two types of incremental markings were visible in the dentine. The first type comprised regularly alternating bright and dark lines. The number of bright lines present between two consecutive fluorescent labels in the dentine equalled the number of days minus one between the fluorochrome injections that had produced these labels (Figure 10). Thus, each stretch of dentine comprising a bright and a dark line represented a daily growth increment, and the bright lines were identified as von Ebner lines. While the calcein labels in the dentine were quite distinct and typically coincided with only a single von Ebner line, the tetracycline labels appeared more blurred and extended over two von Ebner lines (Figure 10a).

A second, more faintly visible type of incremental marking was only occasionally discernible in the dentine and exhibited a longer periodicity (Figure 11). Between two consecutive fluorescent labels caused by injections 14 days apart seven growth increments, each consisting of a bright and a dark band, were visible. Thus, each pair of these bands represented a dentine formation period of 2 days. The bright bands are therefore considered Andresen lines with a periodicity of 2 days, thus matching the periodicity established for the striae of Retzius in the enamel of the experimental animals.

4 | DISCUSSION

Our study provides experimental evidence that enamel and dentine formation in pig teeth are governed by the same short- and

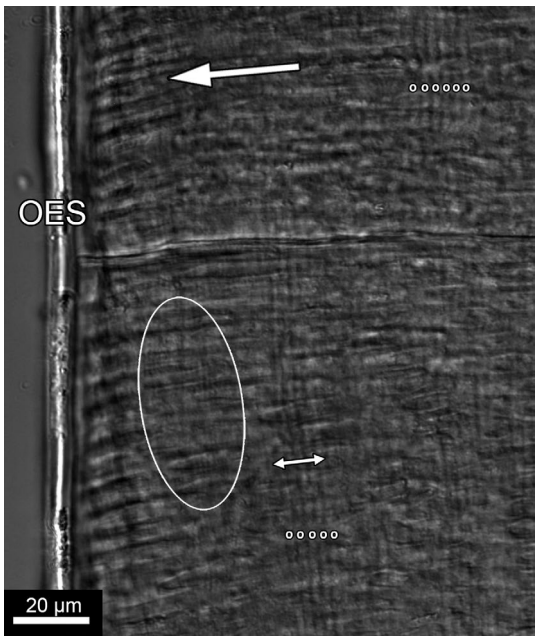


FIGURE 5 Micrograph of buccolingual ground section of the left mandibular second molar of the male domestic pig, demonstrating sub-daily incremental markings (small white circles) in buccal outer enamel. Laminations are not discernible in the image. Considering that in the depicted enamel area the average width of a daily increment is around 13 µm (indicated by the double-headed arrow), it is evident that the labelled incremental markings are sub-daily in nature. In places, even finer sub-daily incremental markings are visible (within white oval). White arrow: overall prism direction. Occlusal to top. OES: outer enamel surface. Ground section viewed in transmitted light with differential interference contrast

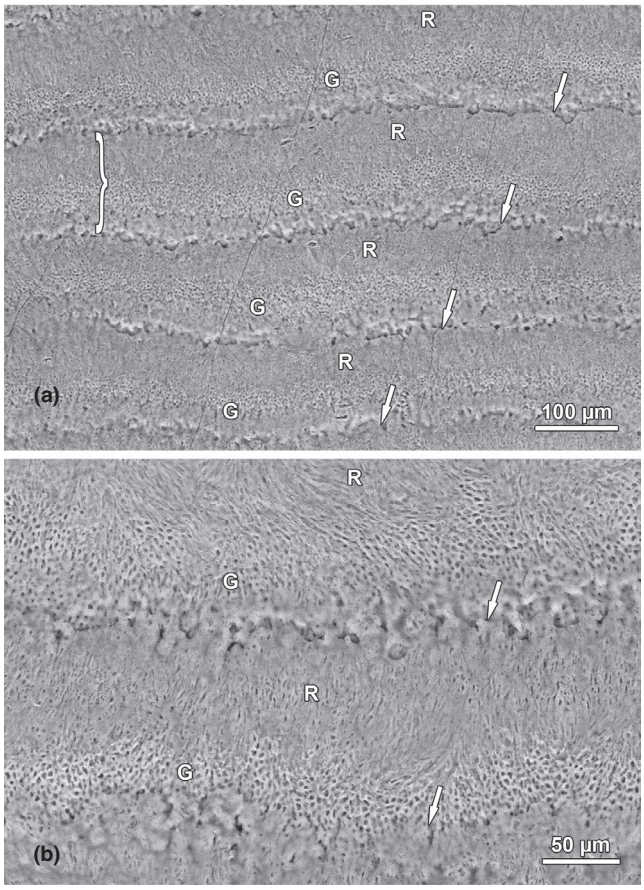


FIGURE 6 BSE-SEM micrograph of the buccal enamel surface in the mid-lateral crown region of the left mandibular second molar from the female wild boar. (a) Perikymata pattern with alternating perikyma ridges (R) and grooves (G). Numerous Tomes' process pits are present in the grooves, whereas the ridges show a smoother surface. Arrows: increment margins; bracket: long-period increment at the enamel surface. (b) Higher magnification of perikymata showing the ridge and groove pattern and the irregular course of the increment margins (arrows) Occlusal to top

long-period rhythms. We moreover conclusively demonstrate the temporal equivalence of internal and external long-period growth increments in porcine enamel.

Laminations represent the most prominent incremental markings in porcine enamel. In ground sections, these laminations could be traced throughout the whole thickness of the lateral enamel from the EDJ to the OES. This is in accordance with previous findings in other cetartiodactyl and perissodactyl species, where laminations likewise constituted the dominant incremental features of enamel (Iinuma et al., 2004; Jordana & Köhler, 2011; Jordana et al., 2014; Kierdorf et al., 2012, 2013, 2014, 2019; Nacarino-Meneses et al., 2017; Okada & Mimura, 1940; Tafforeau et al., 2007). The number of laminations present between consecutive labels equalled the number of days minus one between the two fluorochrome injections producing them, whereas the number of daily growth increments located between consecutive fluorochrome labels matched the number of days between the injections.

Previously, circumstantial evidence for the daily nature of laminations in porcine enamel was provided by the close match between lamination counts and known crown formation times (in days) of unlabelled pig teeth (Kierdorf et al., 2014, 2019). This finding was later corroborated by Skinner and Byra (2019) who recorded the number of laminations between accentuated lines (Wilson bands) in the enamel of domestic pigs caused by exactly dated stress events. The present study provides direct experimental evidence from fluorochrome labelling for the daily nature of laminations in pig enamel, thereby corroborating the findings of the early labelling studies by Okada and Mimura (1940). Using the same approach, the daily nature of laminations was also demonstrated in the enamel of dogs (Okada & Mimura, 1938), deer (Iinuma et al., 2004), sheep (Kierdorf et al., 2013) and primates (Bromage, 1991; Smith, 2006).

In addition, our study demonstrated the presence of finer, more closely spaced (sub-daily) prism cross-striations between successive laminations (Figure 5). These sub-daily incremental markings were predominantly discernible in the outer enamel where the enamel prisms are orientated in parallel and follow a straight course towards the OES (Kierdorf et al., 2014, 2019). Visibility of the different incremental features in ground sections varied with magnification and with the imaging methods used. Thus, laminations were best visible at lower magnifications using transmitted light with phase contrast, whereas sub-daily incremental markings were best seen at higher

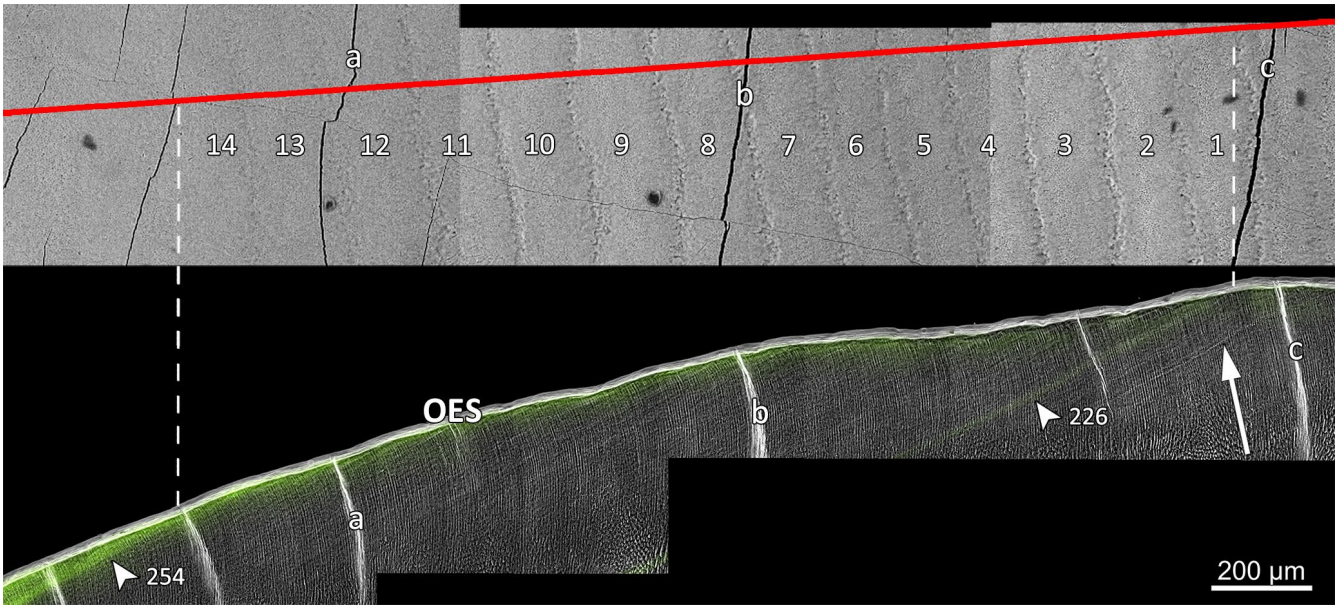


FIGURE 7 Parallelization of the appearance of enamel incremental markings on the lingual crown surface (BSE-SEM image, top) of the left mandibular second molar of the female wild boar and the associated internal structure of the outer enamel as seen in a ground section (overlay of transmitted light image (phase contrast) and the calcein fluorescence channel, bottom). Red line in the BSE-SEM image indicates approximate location of the section plane of the ground section. Corresponding larger cracks visible in both images are labelled with the same letters (a, b and c). Two calcein labels (white arrowheads) caused by calcein injections 28 days apart (days 226 and 254) are visible in the ground section. Their approximate termination at the OES is indicated by the vertical dashed lines. In the BSE-SEM image, 14 long-period increments are marked, indicating a repeat interval of 2 days. Occlusal to the right. White arrow: overall prism direction

magnifications using differential interference contrast. Therefore, it was not possible to simultaneously demonstrate both types of incremental markings in a single micrograph. However, using the typical distance between daily incremental markings in a certain enamel area as a reference, it was possible to clearly demonstrate the sub-daily nature of the finer, more closely spaced incremental markings.

The presence of sub-daily incremental markings has previously been reported in the enamel of macaques, sheep and pigs (Kierdorf et al., 2013, 2014, 2019; Smith, 2006). In ovine and porcine enamel, typically five sub-daily growth increments were recorded within a daily growth increment. The findings of this study are in principle accordance with these earlier studies in also demonstrating a sub-daily, approximately 5-hour periodicity of secretory ameloblast activity. The occasional observation in the present and a previous study (Kierdorf et al., 2019) of even finer and more closely spaced incremental markings suggests the existence of a still shorter ameloblast activity cycle.

This study furthermore provided experimental evidence for the presence of a long-period growth cycle of 2 days in the enamel of wild boar and domestic pigs. This periodicity could be demonstrated both in the internal structure of the outer enamel and at the crown surface of the studied teeth (Figure 7). Internally, long-period incremental markings (striae of Retzius) were identified that in lateral enamel terminated in perikyma grooves. However, visibility of striae of Retzius was restricted to the outermost enamel zone (Figure 8), whereas a deeper in the enamel daily and supra-daily incremental markings exhibited a uniform morphological appearance. This observation matches previous microscopic findings in the enamel of pigs (Kierdorf et al., 2014, 2019; Okada & Mimura, 1940), deer (Iinuma et al., 2004) and sheep (Kierdorf et al., 2013).

The fact that laminations rather than prism cross-striations constitute the dominant short-period enamel incremental markings in ungulate teeth has been related to their higher enamel secretion rates compared to primates (Kierdorf et al., 2019; Tafforeau et al., 2007). Thus, reported mean daily enamel secretion rates range between 11.0 and 24.0 µm in pig third molars (Kierdorf et al., 2014), 11.6 and 17.0 µm in sheep first molars (Kierdorf et al., 2013), and 8.9 and 12.1 µm in deer first molars (inner enamel only) (Iinuma et al., 2004). Much lower values with a lower limit of 2–3 µm/day and an upper limit of 6–7 µm/day (Berkovitz & Shellis, 2018; Hillson, 1996; Smith, 2006) have been recorded in primate enamel.

The morphological similarity of short-period and long-period incremental markings in the teeth of pigs, sheep and various other ungulates was likewise related to the high enamel secretion rate in these taxa (Kierdorf et al., 2013, 2014, 2019). We previously hypothesized that during most of the crown formation period, matrix secretion rate does not drop below a threshold level causing the formation of a structurally accentuated long-period incremental marking (stria of Retzius). Only near the end of their secretory lifespan, the secretory activity of ameloblasts appears to periodically drop below a threshold associated with the formation of a morphologically distinct stria of Retzius (Kierdorf et al., 2019).

A previous study reported a repeat interval of 3 days for long-period enamel incremental markings in mandibular third molars of

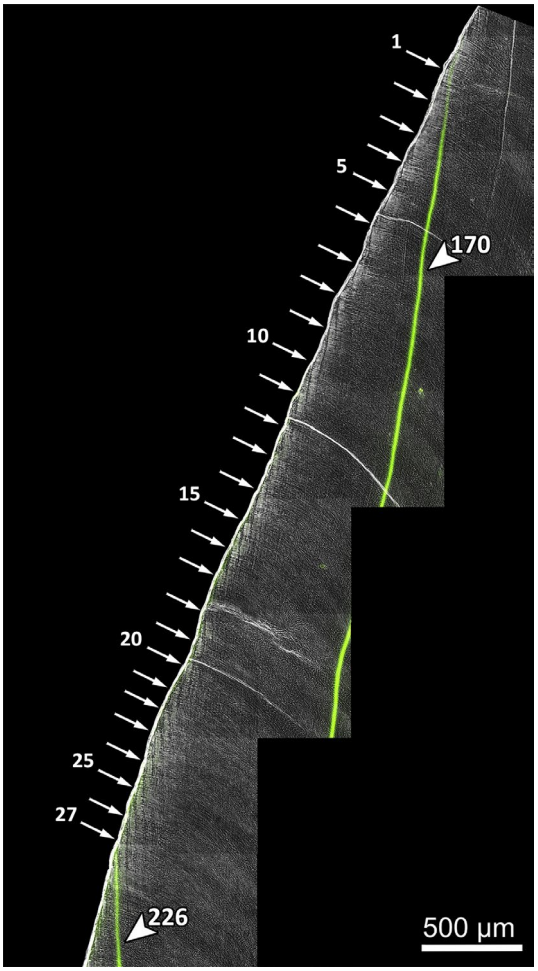


FIGURE 8 Micrograph of buccolingual ground section of the left mandibular second molar of the male wild boar showing mid-crown buccal surface enamel. Twenty-seven long-period incremental markings (white arrows) terminating at the OES between two calcein labels (arrowheads) from injections 56 days apart (days 170 and 226) are discernible in the outermost enamel. This indicates a 2-day periodicity of the long-period markings. Occlusal to top, buccal to left. Overlay of transmitted light image (phase contrast) and the calcein fluorescence channel

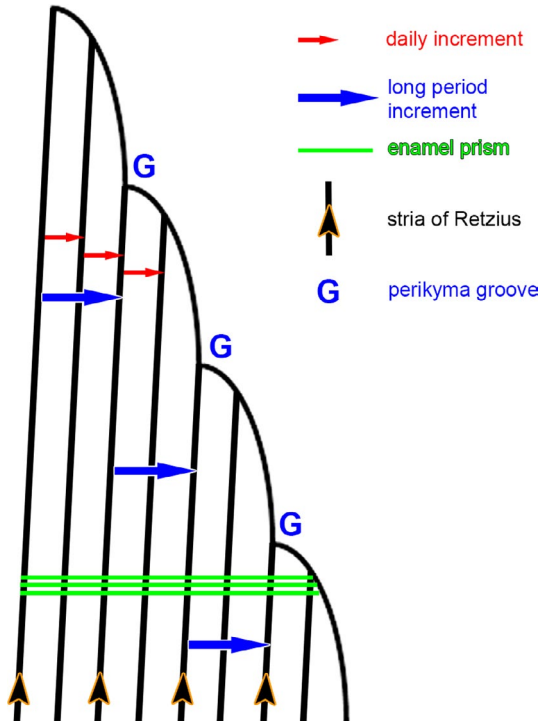


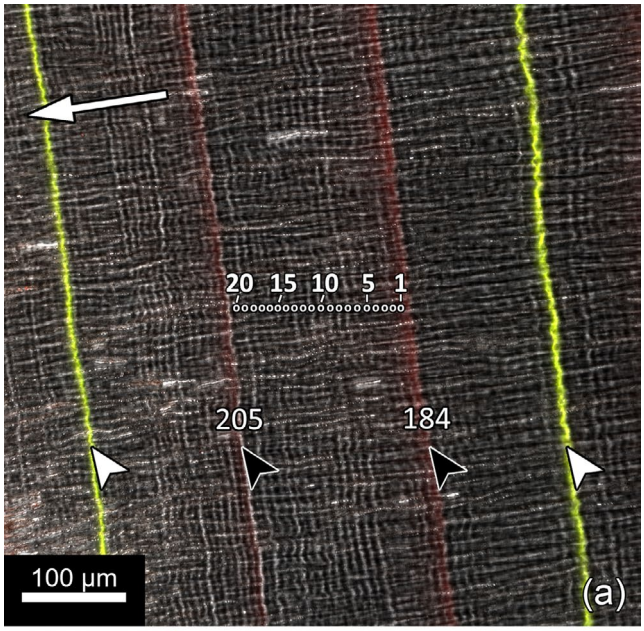
FIGURE 9 Schematic illustration of the relationship between daily and long-period (supra-daily) increments, striae of Retzius and perikyma grooves in porcine enamel. Note the presence of one lamination between two consecutive striae of Retzius

wild boar (Kierdorf et al., 2019). Other authors have reported longer repeat intervals of, respectively, 11 (Fukuhara, 1959), 6 (Bullion, 1987) and 5 days (Bromage et al., 2016) for porcine enamel. However, the latter study was shown to be flawed by a scaling error and a misidentification of incremental markings, that is, mistaking laminations for striae of Retzius. Such a misidentification has probably also occurred in the studies by Bullion (1987) and Fukuhara (1959). In the latter case, this suggestion is supported by the low daily enamel secretion rate of only 4 µm given by this author for pig enamel. It is therefore concluded that the only reliable repeat intervals for long-period incremental markings in porcine enamel available so far are in the range of 2–3 days.

With respect to incremental markings in pig dentine, our study showed that the dominant incremental markings exhibit a daily periodicity, and thus constitute von Ebner lines (Figure 10). This confirms the findings by Yilmaz et al., (1977) who first provided experimental evidence for a 1-day periodicity of these lines in pig dentine. Previously, Kawasaki and Fearnhead (1975) had demonstrated the relationship between tetracycline labels and incremental markings in the dentine of pig teeth, but these authors did not attempt to establish the periodicity of the incremental lines. The daily nature of von Ebner lines has also been demonstrated in labelling studies on other mammalian species (Dean & Scandrett, 1996; Iinuma et al., 2002; Kawasaki et al., 1980; Ohtsuka-Isoya et al., 2001; Papakyrikos et al., 2020; Rosenberg & Simmons, 1980; Waugh et al., 2018).

In places, a second, more faintly expressed type of incremental markings with a periodicity of 2 days was recorded in pig dentine (Figure 11). These long-period incremental markings (Andresen lines) exhibited the same periodicity as the long-period incremental markings (striae of Retzius) in enamel and the markings at the crown surface (perikyma grooves) of the pig teeth. Thus, our study provided evidence that, in addition to a circadian rhythm, enamel and dentine formation in porcine teeth is also controlled by an infradian (2-day) rhythm. Corresponding findings demonstrating a common long-period growth rhythm for enamel and dentine formation have previously been reported in primates (Dean, 1995; Dean et al., 1993; Dean & Scandrett, 1996; Smith & Tafforeau, 2008). For the dentine of first molars from Sika deer (*Cervus nippon*), Iinuma et al., (2004) reported a mean long-period repeat interval of 2.3 days.

The presence of dentine incremental markings reflecting an ultradian rhythm has previously been observed in stained histological



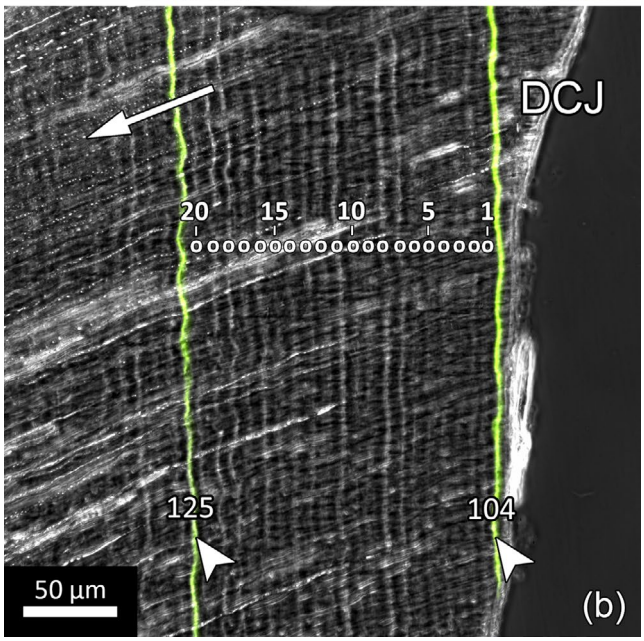


FIGURE 10 Micrographs of buccolingual ground sections of the left mandibular first molars of the female wild boar (a) and the male domestic pig (b). (a) Lingual root dentine exhibiting calcein (white arrowheads) and oxytetracycline (black arrowheads) labels. White circles mark 20 von Ebner lines between two consecutive oxytetracyline labels from injections at days 184 and 205. Occlusal to top, dentine-cementum junction (DCJ) to the right. White arrow: overall direction of dentinal tubules. Overlay of transmitted light image (phase contrast) and the two fluorescence channels. (b) Lingual root dentine. White circles mark 20 von Ebner lines between two consecutive calcein labels (white arrowheads) from injections at days 104 and 125. Occlusal to top. White arrow: overall direction of dentinal tubules. DCJ: dentine-cementum junction, the cementum originally covering the root dentine had flaked off during specimen preparation. Overlay of transmitted light image (phase contrast) and the calcein fluorescence channel

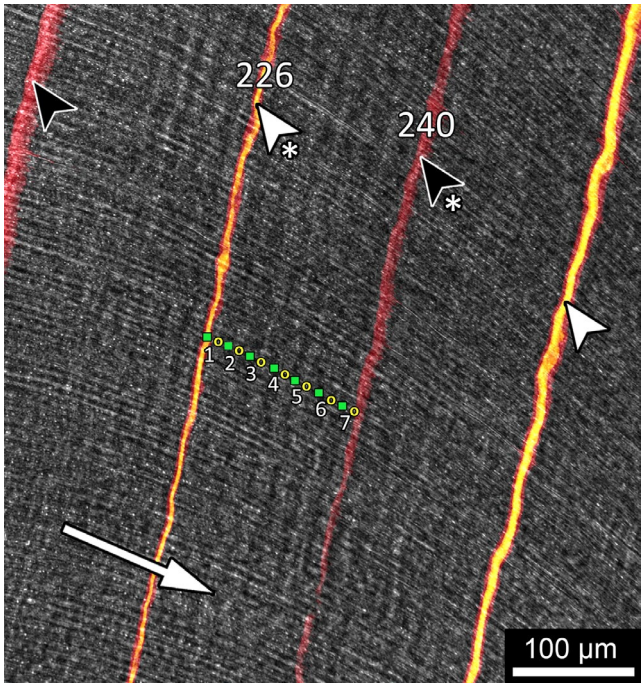


FIGURE 11 Micrograph of ground section through the left mandibular second molar of the female wild boar showing buccal crown dentine with calcein (white arrowheads) and oxytetracycline (black arrowheads) labels. Between two consecutive fluorescent labels (arrowheads with asterisks) caused by injections at days 226 (calcein) and 240 (oxytetracycline) seven long-period increments, each consisting of a dark (green square) and a bright (yellow circle) band are discernible. Occlusal to top, EDJ to the left. Large white arrow: overall direction of dentinal tubules. Overlay of transmitted light image (phase contrast) and the two fluorescence channels

sections of vitally labelled rabbit and rodent teeth (Dean, 1998; Ohtsuka & Shinoda, 1995; Papakyrikos et al., 2020; Rosenberg & Simmons, 1980). In the ground sections of the pig molars studied by us, we could not demonstrate the occurrence of such ultradian incremental markings in the dentine. However, as we were able to demonstrate their presence in the enamel of these teeth, we conclude that their non-identification in the dentine can most likely be ascribed to the method (analysis of undecalcified ground sections) applied in our study.

The ambiguous use of terms constitutes a general problem in scientific communication. The inconsistent use of the term perikymata has in detail been discussed by Risnes (1984). In our view, a similar problem also exists with respect to internal enamel structures. As is schematically illustrated in Figures 4 and 9, for establishing a correct repeat interval it is required to distinguish between the number of daily incremental markings and the number of daily enamel growth increments. To obtain a correct repeat interval (in days), it is necessary to either count the number of daily growth increments or to add 1 day to the number of incremental markings present between consecutive long-period markings. Furthermore, regarding the terms 'prism cross-striation' and 'lamination' the use of a qualifier (daily or sub-daily)

would help to avoid ambiguities. Caution is also needed when designations like dark or bright are used to describe the appearance of growth marks in a microscopic section, as their appearance depends on the preparation method (ground section vs. stained decalcified histological section) and the imaging modalities used to visualize them. An example of the varying appearance of laminations in ground sections viewed with either plain transmitted light or transmitted light with phase contrast was demonstrated by Kierdorf et al., (2014). It has further been emphasized that the visibility of incremental markings also depends on the thickness of ground sections (Tafforeau et al., 2007).

In conclusion, our study provided experimental evidence that the dominant incremental markings in porcine enamel and dentine are of a daily nature. Furthermore, we demonstrated the presence of long-period incremental markings with a periodicity of 2 days in the enamel and dentine of pig teeth. We furthermore showed that the distance between consecutive perikyma grooves on the crown surface represents the same formative period as the distance between the corresponding striae of Retzius that terminate in these grooves.

The results of this study are in accordance with the findings of the early experimental studies by Okada and Mimura (1938, 1940) and support our previous conclusion (Kierdorf et al., 2014, 2019) that long-period incremental markings (striae of Retzius) in porcine enamel are, except for a zone near the OES, morphologically indistinguishable from daily incremental markings (laminations).

The differences between the enamel incremental markings in ungulates and primates are likely to cause major problems when the interpretive scheme established for the latter is incautiously applied to the former. This caveat probably also holds for the interpretation of enamel microstructure in other taxa with high enamel secretion rates. As discussed by O'Meara et al., (2018), this can pose problems in the reconstruction of enamel growth parameters in fossil taxa, for which data on the timing of dental development are not available.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Horst Kierdorf, Uwe Kierdorf and Carsten Witzel designed the study. Kai Frölich supervised the animal experiments. Simon Emken and Carsten Witzel performed specimen preparation and histological analysis. Simon Emken, Horst Kierdorf and Uwe Kierdorf drafted the manuscript and prepared the figures. All authors critically revised the manuscript and approved the submitted version.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Uwe Kierdorf https://orcid.org/0000-0003-0531-2460
Horst Kierdorf https://orcid.org/0000-0001-6411-9631

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