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Neonatal line may develop after birth in the Indo-Pacific bottlenose dolphin, *Tursiops aduncus*

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Abstract: Studies using teeth to estimate age in marine mammals presume that the neonatal line (NNL) develops at birth. This study of Indo-Pacific bottlenose dolphins *Tursiops aduncus* (Ehrenberg, 1833) is the first to investigate when the NNL appears in odontocete dentine. Two to four teeth were prepared by decalcification, thin-sectioning and staining for 103 dolphins, including seven of known-age. Tooth length, prenatal and postnatal dentine and NNL widths were measured. Developmental class (foetus, young neonate, older neonate, calf <1, calf 1) was assigned using carcass external features. NNL presence/absence was categorised for individual dolphins. The NNL was absent in a near-term foetus and all except one young neonate and fully formed in 50% of older neonates, whose estimated ages were 1 week to 2 months. It was absent in a known-age dolphin aged 4–7 weeks. NNL width was greater in dolphins less than 1 year compared with those 1 year old. Factors that trigger NNL development are unknown. The present study suggests that the NNL may not be related to birth *per se* in dolphins, as has been clearly demonstrated in humans. Physiological processes, driven by diet and behavioural changes during the first few months of postnatal life, may be important drivers for NNL formation in odontocetes.

Key words: Indo-Pacific bottlenose dolphin, *Tursiops aduncus*, neonatal line, tooth dentine, incremental layers, birth, developmental class.

Introduction

Calcified structures such as bones, teeth and horns have been used to determine age and reconstruct life history events for individuals and populations of vertebrates (Klevezal 1996). Also known as ‘recording structures’, they consist of biomineralised tissues that form repeated patterns called ‘growth’ or ‘incremental’ layers. Daily rhythmic phases in mineralisation result in lines of Ebner, and when accentuated by substantial changes in collagen formation and mineralisation over a longer period of time, become contour lines of Owen (Klevezal 1996). Lines of Owen in the dentine and cementum of mammal teeth are excellent recording structures because they reflect growth and physiological changes that relate to time. In addition, teeth are not usually affected by resorption, as is sometimes the case for bone (Klevezal 1996). The processes responsible for the formation of incremental layers are not fully understood, yet they are likely to be a complex combination of endogenous and exogenous factors (Langvatn 1995). In the majority of mammal species studied (see review by Klevezal 1996), the number of incremental layers corresponds to the age of an individual.

Although discovered during the 19th century, incremental layers in marine mammal teeth were not related to age until much later (see review by Scheffer and Myrick 1980). Modern toothed whales (odontocetes) have only one set of teeth during their lifetime (monophyodont) and the dentition is often homodont, in which all teeth are simple and conical in shape (Boyd 1980; Ungar 2010). Odontocete teeth have marked incremental layers referred to as Growth Layer Groups (GLG) (Perrin and Myrick 1980), within which there is a minerally-dense and a minerally less-dense portion. Tooth-aging studies have been conducted on many odontocetes, particularly in the families Delphinidae and Phocoenidae (Perrin and Myrick

1980; Klevezal 1996). These species make good study subjects because they have many teeth and a large number of specimens is available from carcasses collected as a result of strandings and bycatch. Dentine is the primary tissue examined, sometimes in combination with cementum (Kasuya and Matsui 1984; Cockcroft and Ross 1990).

A neonatal line (NNL) is present in all mammals studied thus far. It forms in deciduous and permanent teeth (Klevezal 1996), where it is found at the dentine/cementum interface (Stoneberg and Jonkel 1966), within both enamel and dentine (Schour 1936; Iinuma et al. 2004) or in only the enamel layer (Gustafson and Gustafson 1967). In dentine and cementum the NNL is a special contour line of Owen (Bhaskar 1976). The first description of an odontocete NNL was by Nishiwaki and Yagi (1953). Since enamel forms during foetal life, the NNL is present in only dentine in odontocetes (Boyde 1980; Cooper and Maas 2018). In humans and marine mammals, the NNL consists of hypo-mineralised tissue (Gustafson and Gustafson 1967; Avery 1976; Hohn 1980a) appearing as a light-coloured band in stained thin-sections. The transparent nature of the NNL may also be a result of structural homogeneity (Boyde 1980) and/or a change in orientation of the tissue matrix at the NNL (Iinuma et al. 2004). Polarised light microscopy shows that the NNL of dolphins consists of three or more pairs of opaque and translucent layers bounded on each side by a bright translucent layer (Myrick 1980).

In humans, the NNL develops at the time of birth (Avery 1976). However, there are no investigations specifically aimed at documenting when this feature appears in marine mammals. The assumption has been that, like humans, its formation is caused by physiological disturbance associated with birth (Perrin and Myrick 1980; Locker 1995; Cooper and Maas 2018). It is important to establish when the NNL develops in marine mammals because it represents 'time zero', or birth, when estimating an individual's age (Hohn 2018).

Bottlenose dolphins (genus *Tursiops* Gervais, 1855) are particularly amenable to tooth-aging studies because they frequently strand and many have been kept in captivity. Captive dolphins have been used to confirm that each dentinal GLG represents one year of growth (Myrick et al. 1984). In addition, there are good descriptions of postnatal development of bottlenose dolphins (Herman and Tavalga 1980; Cockcroft and Ross 1990) that can be linked to tooth development. Despite the large number of bottlenose dolphins that have been aged using teeth, no study has documented the development of the NNL. One reason for this may be because the teeth of dolphins showing external signs of being very young, such as foetal folds and a folded dorsal fin, have not been prepared for aging because these animals were simply classed as ‘neonatal’, ‘perinatal’ and/or age 0.

The present study of the NNL in young Indo-Pacific bottlenose dolphins *Tursiops aduncus* (Ehrenberg, 1833) arose from a routine investigation of a large number of stranded, beach-washed and bycaught carcasses in South Australia (SA). Tooth aging studies revealed that many young dolphins lacked a NNL, thus leading to the question of whether they may have been aborted fetuses or stillborn calves. The study aimed to determine when the NNL appears by comparing internal tooth structure with developmental class defined by developmental features and body length of the carcasses. Young dolphins that died during a field study of resident *T. aduncus* helped to confirm the age at which the NNL appeared.

Materials and methods

Carcasses of 103 young *T. aduncus* (45 females, 52 males, 6 unknown sex) were collected from the coast of SA between 1981 and 2013. Species identification was based on skull features (Kemper 2004) and/or external colour pattern. Carcasses were rated for state of decomposition (category 2 = 51, 3 = 25, 4 = 14, unknown = 13; Geraci and Lounsbury 2005). Post-mortems were conducted by a team of experienced researchers, including a marine

mammal pathologist, at the South Australian Museum Adelaide (SAMA) during which body length, weight, sex and external features were recorded. Stomachs were examined and the presence/absence of a milk-like substance noted. The circumstance of death for each dolphin (as determined by post-mortem and other relevant data, see Kemper et al. 2005) included anthropogenic interactions (intentional killing, entanglement), disease, live stranding and unknown circumstance. Specimens were registered into the mammal collection of the SAMA (registration number prefixed with letter M). Skeletons, including teeth, were macerated for several months in warm water (25–35° C) and before 2000 they (but not their teeth) were degreased in boiling trichloroethylene. Cleaned teeth were air dried and stored in plastic bags or containers. The body of the single foetus studied (M25053/002) was fixed in 10% buffered formalin, followed by storage in 70% ethanol. Lung tissue from seven dolphins was prepared for histological examination (6-µm thick sections stained with haematoxylin and eosin) to determine if the alveoli were open *i.e.* the dolphin had breathed.

External developmental features were noted and photographed at post-mortem, with some confirmed later by examining photographs. These were; presence and appearance of foetal folds, presence of rostral hairs and umbilical chord, amount of closure of umbilicus, dorsal fin erectness (folded, partially folded, erect), and number and position of erupted teeth.

Seven dolphins were individually identified either by unique body markings or by association with mothers that were known to researchers carrying out long-term studies in the Port River estuary, Adelaide and Kangaroo Island, SA (Bossley et al. 2017; A. Bartram private communication, 2013). Birth date and age at death were estimated for these dolphins by knowing when their mothers were seen with or without the neonate.

Tooth preparation and age estimation

After maceration, teeth were selected from the sets available for each dolphin (*i.e.* jaw and position not known because teeth fell out during maceration). Many had major cracks due to having been stored dry. Four teeth, ranging from small to large, were prepared for 49 dolphins. For an additional 53 dolphins, two of the largest intact teeth were prepared. Four teeth were extracted from the formalin-fixed foetus: one at each of the posterior end and middle of the tooth row, in the right maxilla and mandible.

Teeth were decalcified in Hydrochloric Acid (RDO, Apex Engineering Products Corporation, Aurora, Illinois, USA) for 1 to 3.5 h, followed by rinsing in running tap water overnight. After mounting on disks using tissue freezing compound, they were thin-sectioned using a freezing microtome (Leica CM 1850) set at 25 μ m. Sections were stained with Mayer haematoxylin solution for 25–40 min, followed by bluing in a 2% solution of ammonia and rinsed in running water for 30 min. Sections were mounted on slides that were later protected by coverslips using DPX Mounting Medium (Dibutylphthalate Polystyrene Xylene, Merck, New South Wales, Australia). These methods broadly followed the recommended protocol of Myrick et al. (1983). When tooth sections split into two parts (which happened frequently), multiple examples of both parts were mounted onto slides. About 10 sections were prepared for each tooth studied.

Tooth sections were examined for presence/absence of the NNL and GLGs under a dissecting microscope at 9–75x magnification. Three evaluations were obtained by two readers who had experience in counting GLGs in bottlenose dolphins. Observations were made without knowledge of the specimen number or animal's details (*i.e.* the readings were blind). Discrepancies between readers relating to the presence/absence of the NNL were reassessed by CK. If quality of preparation was very poor for all sections from a tooth it was not included in the analyses. In some cases close inspection at the pulp cavity edge was required by CK to determine if the NNL was just forming. Age 0 was assigned to dolphins

with no visible NNL, age 0.5 to dolphins in which the NNL was visible, but the first GLG was not complete, and age 1 to dolphins for which the first, but not the second, GLG was complete.

Tooth measurements

For dolphins having four prepared teeth, tooth length and diameter were measured (to 0.01 mm), before decalcification, using digital calipers. For the remainder, tooth length was measured in mounted, stained sections using a ruler and a dissecting microscope. Mean tooth length was calculated for each tooth and dolphin.

The suitability of mounted sections for measurement of internal features was ascertained by assessing the quality of preparation (Pinedo and Hohn 2000), *i.e.* adequate staining, position of section at or close to centre of tooth, flatness of section on slide and whether the section was whole or half of the tooth. Only good quality sections were used for measurement of features. Measurements were taken at a point half way between the distal end of the pulp cavity and the intersection of the NNL and decalcified enamel (Fig. 1).

Internal tooth features were measured under a bright-field microscope (Olympus BH2) at a magnification of 400x. For teeth aged 0.5, and having a clear NNL, widths of prenatal dentine (including the decalcified enamel layer), postnatal dentine (excluding the darkly-stained layer adjacent to the pulp cavity) and the NNL were measured perpendicular to the long axis of the NNL (Fig. 1). Since at high magnification internal and external edges of the NNL were indistinct, they were estimated by eye. The halo sometimes present on the pulp cavity side of the NNL was not included in the measurement.

The number of measurements (NNL and prenatal dentine) made for each tooth and dolphin varied according to how many good-quality sections were available: dolphins aged

0.5, 2–10 per tooth, 4–40 per dolphin; dolphins aged 1, 1–10 per tooth, 8–30 per dolphin.

Mean measurements were calculated for each tooth and dolphin.

Statistical tests were performed in SPSS version 25 (IBM SPSS Statistics, Armonk, NY, USA). Means of NNL width for 0.5 and 1 year-old dolphins were compared using a two-tailed Student's *t*-test, after meeting the assumptions of equality of variances (Levene's test) and normality of distribution (Shapiro-Wilk test). For comparison of developmental classes against body length and weight, these assumptions were violated and an Independent-samples Kruskal-Wallis was performed. Linear regression was performed in SPSS. Measurement error was included as standard error of the mean.

Results

Age and developmental features

Each dolphin was assigned to a developmental class (foetus, young neonate, older neonate, calf<1 and calf 1) based on external features and tooth eruption or age (Table 1). An additional five could not be classified due to photos and/or data being unavailable or the carcass being too decomposed. Since it was possible that some of the young neonates may have been unidentified aborted foetuses, additional evidence was used when available to investigate this possibility. This was lung alveolar development ($n = 11$), stomach contents ($n = 16$), and presence/absence of an umbilical chord. No young neonates (total $n = 29$) had identifiable milk in their stomachs. Lung tissue from seven young neonates had alveoli that were either opening or fully opened, indicating that these dolphins had breathed. Of the remaining 22 young neonates, three had an intact umbilical chord and body lengths of 94–97 cm, and therefore may have been aborted near-term foetuses. However, they remained classified as young neonates for the purposes of this study. All seven known-age dolphins from field studies in the Port River estuary and Kangaroo Island were also assigned to a

developmental class (Table 2). Of the three young neonates recorded during the field studies, two were seen alive after birth.

Body size increased from young neonate to calf 1, with overlap between classes (Fig. 2, Table 3). A Kruskal-Wallis test found a significant difference ($P < 0.001$, $df = 3$) among classes for both mean body length and mean weight. Included in these analyses were known-age dolphins from field studies and for them, there was little difference in body size between young neonates and older neonates (Fig. 2).

Tooth features

Consistency of NNL presence

Young dolphins that each had four prepared teeth of assessable quality ($n = 41$) were used to test the consistency of NNL presence or absence within individuals. The length of the smallest teeth for each dolphin was 5.35–11.77 mm, the largest was 6.78–13.11 mm. There were uniform, between-teeth results for 28 dolphins (21 young neonates, 7 older neonates) where the assignment was NNL absent, six dolphins (2 older neonates, 4 calf < 1) where the assignment was NNL present and two dolphins (1 older neonate, 1 calf < 1) for which the NNL appeared to be forming at the edge of the pulp cavity. Thus consistent results were obtained in 90% of dolphins. Five other dolphins (3 young neonates, 1 older neonate, 1 unknown) had inconsistent NNL assignments amongst the four teeth. When the NNL was present in these cases, it was described as ‘just forming’ and therefore its presence may not have been detected in some teeth.

Appearance and timing of the NNL

The NNL was not visible in the teeth of 39 dolphins (Fig. 3), including the near-term foetus (99 cm body length). In this foetus, however, there was a visible change in staining

intensity about half way through the prenatal dentine (Fig. 4). An indistinct translucent line was associated with the junction in staining densities but it showed no gross similarity to a NNL. The Port River estuary older neonate, 'Mimo' (aged 4–7 weeks, body length 102 cm, weight 16 kg) had a faint, lightly-stained zone near the pulp cavity edge that is likely to have been the early stages of NNL development. The first clear indication of a NNL was a broad band of lightly-stained dentine near the edge of the pulp cavity and running its full length (Fig. 5). This was more distinct at the proximal end of the tooth, probably as a result of the cut section being more at right angles compared with the distal (cusp) end. After a thin band of postnatal dentine appeared, the NNL was clearly distinguishable as a pale line bordered by darkly-stained dentine (Fig. 1). No zonation was observed within the NNL of the animals studied. Some dolphins had a distinctly darker edge on the pulp cavity side of the NNL but again, angle of the cut section, even in teeth cut at the centre, may have influenced this observation in some individuals. In a few teeth this darker line was present for the full length of the NNL. In a 15-month-old known-age dolphin that had well-prepared teeth, the NNL was clear but the fabric of the line was disrupted by densely stained, globular dentine (Fig. 6). A junction between the NNL and decalcified enamel was observed just proximal to a slight convexity in the tooth surface (Fig. 1, insert).

The presence/absence of a NNL for each individual was compared to its developmental class (Fig. 7). None of the young neonates ($n = 28$) had a fully-formed and clear NNL. Two of the four teeth prepared for one young neonate had an indistinct pale zone along part of the edge of the pulp cavity. Although classed as 'NNL forming' (Fig. 7), this could not be confirmed. Half of the older neonates had either a forming or fully-formed NNL and all except one of the calf <1 class had a fully formed NNL. These results demonstrate that the NNL usually becomes visible in older neonates.

The age at which the NNL develops in *T. aduncus* was estimated by combining data for wild, known-age dolphins from SA (Table 2) and published information on the development of captive and wild known-age dolphins from elsewhere (Table 4). For wild SA dolphins, the NNL was absent in ‘Mimo’ (4–7 weeks) and ‘Phoebie’s calf’ M22439 (<34 days), and clearly forming at the edge of the pulp cavity of another of ‘Phoebie’s calves’ M22548 (>20 days).

Studies of captive and wild known-age bottlenose dolphins have reported variability in the timing of some external features (*e.g.* disappearance of rostral hairs, tooth eruption, Table 4). Since the discrepancy was large for tooth eruption (6 weeks vs 3 months), an approximate age of 2 months was applied in the present study. A clear pattern of when rostral hairs disappeared was not apparent in the present study or published literature and was therefore not used as a primary determinant when comparing age and developmental classes (Table 5). Older neonates were 1 week to about 2 months old and dolphins in the class calf<1 were at least 2 months. By combining all lines of evidence, the NNL is not visible at birth and appears at 1 week to 2 months of age.

NNL width and growth of postnatal dentine

Mean NNL widths for dolphins with tooth ages of 0.5 ($37.8 \pm 1.10 \mu\text{m}$) and 1 year ($43.4 \pm 1.77 \mu\text{m}$) were significantly different ($P = 0.01$, $t = -2.64$, $df = 75$). Testing if tooth size and NNL width for the 0.5 age group showed a positive linear relationship was not attempted because some assumptions were violated, as determined by examining data on residuals. Postnatal dentine width increased relative to body length (Fig. 8) which in turn was related to dolphin developmental class (Fig. 2) and age. The slope of the regression line showed that postnatal dentine grows at a rate of $6.614 \mu\text{m}/\text{cm}$ body length. In the absence of a robust data set for known-age dolphins, there was no attempt made to determine a daily postnatal dentine growth rate.

Discussion

Equating age to GLG counts made from prepared teeth is commonly used in studies of odontocetes and pinnipeds. It is somewhat surprising that, until now, there has not been an in-depth study to document the age at which the NNL appears. World-wide, there are many long-term carcass retrieval programs for small cetaceans and their teeth are often collected for aging purposes. Perhaps the emphasis has been on determining age in years and that the precise timing of NNL development has been less important.

The present study of a large sample of young *T. aduncus* concluded that the NNL was not visible at birth and that it appeared at 1 week to 2 months of age, during the older neonate developmental class. However, because the teeth of almost all the dolphins used in the study were macerated, these results may need to be treated as preliminary evidence for the NNL being formed after birth. Predentin is an organic tissue, forming the matrix for subsequent mineralisation, and it would be lost or damaged during maceration. In odontocetes it forms at the pulp cavity edge of the tooth, where the NNL forms. In the unmacerated *T. aduncus* foetus, the predentin tubules were visible (Fig. 4) but were not apparent in the illustrations of macerated teeth used in the study. If the NNL begins formation at birth (with the laying down of predentin), it would not be detected in macerated teeth. Further studies, using unmacerated teeth of neonatal *T. aduncus*, are needed to confirm that the NNL is formed after birth. Ideally, known-age captive or wild dolphins should be included, although it may take a long time to accumulate such specimens.

Another potential constraint of the present study is that it is necessary to consider the length of time taken for the NNL to develop, since it is not likely to appear instantly in the teeth. Hohn et al. (1989) estimated that the postnatal dentine growth rate during the first year was

about 2 $\mu\text{m}/\text{day}$ in common bottlenose dolphins, *Tursiops truncatus* (Montagu, 1821), a larger species than *T. aduncus* (Kemper 2004). Since mean NNL width for *T. aduncus* that are less than 1 year old is 37.8 μm , it would take a minimum of 19 days to fully form. Although this may resolve the lack of a NNL in young neonates, it does not explain why the NNL was absent in the 4–7 week-old ‘Mimo’ or just forming in Phoebe’s calf (aged >20 days) and a calf <1 (at least 2 months).

There are limited data on when the NNL develops in other odontocetes but the evidence points to a similar pattern as *T. aduncus*. The following studies used unmacerated teeth (some fixed in formalin), hence the predentin would have remained intact. The NNL was not visible in dentine of near-term fetuses of striped dolphins, *Stenella coeruleoalba* (Meyen, 1833) (Nishiwaki and Yagi 1953) or newborn short-finned pilot whales, *Globicephala macrorhynchus* Gray, 1846 (Kasuya and Matsui 1984) and spotted dolphins, *Stenella attenuata* (Gray, 1846) (Hohn and Hammond 1985). Myrick et al. (1983) stated that newborn *Stenella* spp. Grey, 1866 had part of the neonatal line but did not elaborate on how this was ascertained. Stewart and Stewart (2014) noted that there was an unknown duration of time between birth and the formation of the NNL in the beluga, *Delphinapterus leucas* (Pallas, 1776), and that the line itself was variable in extent and visibility beyond this point. Very relevant to the present study is the unpublished data for *T. truncatus* neonates referred to in Hohn and Hammond (1985, page 564). Of the 18 neonates (no umbilical chord, folded dorsal fin and flukes) some had no NNL while others had part of a NNL present.

Hohn et al. (1989) observed that the teeth of known-age *T. truncatus* from the north-west Atlantic Ocean had more dentine than expected, given the known birth month. Based on this finding, they considered the possibility that the NNL develops before birth. If correct, this would be in contrast to the results of the present study and could be explained by variability

between species and/or geographic location. The age at which the NNL forms in SA *T. aduncus* may be later than other mammals, although there are few published data for comparison. In the European bison (*Bison bonasus* (Linnaeus, 1758)) the NNL forms in dentine at about 2 weeks of age (Klevezal et al. 1991) and in sika deer *Cervus nippon* Temminck, 1838 it appears around the time birth (Iinuma et al. 2004). There are numerous studies to show that the NNL develops in human enamel at birth (e.g. Avery 1976).

In the dolphins examined during the present study, 90% had consistent results for presence/absence of the NNL between teeth taken from the same animal. This implies that the formation of incremental layers, including the NNL, is triggered by a factor or factors affecting all teeth and possibly the whole animal (Boyde 1980; Klevezal 1996). Myrick (1988) concluded that dentinal tissue alteration was related to hypocalcaemia, which is under systemic control. He and others (Nishiwaki and Yagi 1953; Hohn 1980b) noted sub-layering within the NNL, which may reflect multiple causal factors spanning a period of time. In humans, there is good evidence that birth triggers NNL formation in enamel because it is wider in children who experience protracted births (Eli et al. 1989; Sabel et al. 2008). Physiological changes from foetal to extra-uterine life have been implicated in its formation in humans (Schour 1936; Janardhanan et al. 2011). Since the NNL appears to form weeks or months after birth in SA *T. aduncus*, the process of birth *per se* is unlikely to be responsible for its development. A change in diet from intrauterine nourishment to milk is also not a plausible explanation because the newborn begins to suckle within hours of birth (McBride and Kritzler 1951; Reid et al. 1995).

Diet and physiological changes during the first few months of life may prompt NNL formation. For example, behavioural studies of bottlenose dolphins have shown that suckling is very frequent during the first week after birth and declines thereafter (Peddemors et al.

1992). During the first month they learn to swim in infant position (Mann 1997) and during that time their swim speed is only 37–52% of adult speeds (Noren et al. 2006). At 1–3 months old they spend more time away from their mother (McBride and Kritzler 1951; Cockcroft and Ross 1990; Reid et al. 1995; Mann and Smuts 1999). Mann (1997) emphasised that individuals behaved differently, which may be important in the context of the variation in timing of NNL line development in the present study. Cockcroft and Ross (1990) studied a young captive bottlenose dolphin and noted that blubber mass was greater than muscle mass (relative to total body mass) until about 4–5 weeks of age. The same dolphin also experienced a plateau in growth of body length between about 20 and 50 days, which is about the time that the NNL may have been forming. Although young bottlenose dolphins play-forage from the age of 3 weeks, the first fish are not normally caught and consumed until 4–6 months (Mann and Smuts 1999). If this timing also applies to SA dolphins, a change in diet from milk to solids would not be the primary factor responsible for the formation of the NNL.

Disruptions to incremental layers in odontocete teeth have been linked to life history and environmental events, and to toxicological and diet effects (see review by Locker 1995; Luque et al. 2009; Luque et al. 2013), including weaning (Klevezal 1996). These abnormal incremental lines can appear hypo-mineralised, much like a NNL but generally not as wide. The present study included a case of how nutritional compromise may have affected dentine development in *T. aduncus*—the near-term foetus with a disruption in the prenatal dentine (Fig. 4). A post-mortem conducted on its 19-year-old mother concluded that it was suffering from a serious, chronic mammary gland abscess and heart disease. This condition may have affected tooth development in the foetus.

There are broader implications for the conclusions reached in the present study. Firstly, because the NNL does not form until weeks or possibly months after birth, the age at the

completion of the first GLG is likely to be 13–15 months of age not 1 year. This this may have bearing on studies incorporating age-at-weaning, early postnatal growth rates and physiology of early development. Secondly, the terms prenatal and postnatal dentine are now incorrect because some of the so-called prenatal dentine is likely to be formed after birth. Thirdly, absence of a NNL in a young neonate does not mean that the dolphin is an aborted foetus, a view held by some researchers. Examining other features, such as the degree of lung alveolar development would be necessary to identify if a neonate was full-term and/or born alive.

There was preliminary evidence that the NNL was wider in older (and therefore larger) dolphins but a reason for this is unclear. It seems unlikely that the NNL would increase in width as the dolphin and teeth grow because dentine is a hard and inflexible tissue. A more plausible explanation is that larger dolphins have larger teeth and the NNL may be proportional to tooth size. To test this hypothesis, a study needs to be carried out that involves more teeth (including a range of sizes) from each dolphin and also many older neonates ranging in length from 100–130 cm.

Several other topics for further research have arisen out of the present study. Examining the teeth of neonates of other species, including *T. truncatus*, would help to identify whether there is a consistent pattern amongst odontocetes. The SAMA has a large collection of young common dolphins (*Delphinus delphis* Linnaeus, 1758) and preliminary observation of their teeth indicates that the NNL is not visible at birth. However, these have been macerated. Research could also be directed at examining the ultrastructure and composition of the NNL and surrounding dentine with a view to understanding whether diet influences its development. For example, Austin et al. (2013) used the distribution of barium in primate

teeth to establish dietary changes from milk to solid foods and Knoff et al. (2008) studied stable isotopes in bottlenose dolphin teeth to describe ontogenetic changes.

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Fig. 1. Stained thin-section of an Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) tooth from a female calf<1 (M24457). Position of measurements for prenatal dentine, NNL and postnatal dentine widths shown in insert (different dolphin). Dentinal tubules visible as fine lines running at an angle to the long axis of the tooth. Enamel refers to the organic portion of this structure after decalcification. Body length 117 cm, 18 kg.

Fig. 2. Body size of young SA Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) in four developmental classes. Labelled data points indicate known-age dolphins from the Port River estuary and Kangaroo Island (see Table 2).

Fig. 3. Stained thin-section of an Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) tooth from a female young neonate (M26198) showing no NNL. This dolphin had severe blunt trauma. Body length 93 cm, 10.5 kg.

Fig. 4. Stained thin-section of tooth from a near-term Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) male foetus (M25053/002) showing a hypo-mineralised line (arrow) that separates two regions of prenatal dentine. NNL absent. Dentinal tubules visible as fine lines running at an angle to the long axis of the tooth. Fixed soft tissue likely to be predentin. Body length 99 cm, weight not available.

Fig. 5. Stained thin-section of tooth from an Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) male calf<1 (M25892) showing NNL on edge of pulp cavity. A very thin layer of postnatal dentine is visible. Dentinal tubules visible as fine lines running at an angle to the long axis of the tooth. Body length 114 cm, 21 kg. See Fig. 1 for position of measurements.

Fig. 6. Stained thin-section of a tooth from a 15-month-old female Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) calf 1 ('Miki', M20735) showing the NNL in relation to prenatal dentine and the first completed GLG (Growth Layer Group). Inset shows detail of the NNL in the same dolphin and tooth section. Body length 170 cm, 54.5 kg. See Fig. 1 for position of measurements.

Fig. 7. Presence/absence of the NNL compared to developmental class in young Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) from SA.

Fig. 8. Relationship between postnatal dentine width and body length in Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) from SA. Data points represent animal means for older neonates, calf<1 and calf 1. The linear regression model is $y = 6.614x - 665.374$ and R^2 is 0.578.

Table 1. External features of developmental classes and number of SA Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) examined in each.

Feature	Foetus	Young neonate	Older neonate	Calf <1	Calf 1
Foetal folds	distinct, creased	distinct, deeply creased	usually faint or very faint	very faint or absent	absent
Rostral hairs	present	usually present	usually absent	absent	absent
Dorsal fin	folded	folded or partially folded	erect	erect	erect
Umbilical chord	present	sometimes present	absent	absent	absent
Umbilicus	open	open	open or partially closed	partially or fully closed	closed
Teeth	none erupted	none erupted	none erupted	some erupted	most erupted, first GLG complete
Number of dolphins	1	29 (3)	20 (2)	38	10 (2)

Note: Known-age dolphins presented in parentheses and included in total. Five dolphins not classified because of lack of information on development.

Table 2. Details for known-age Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) from field studies in the Port River estuary and Kangaroo Island, SA.

Animal no.	Name	Estimated age (field)	Age class	NNL	Seen alive	Collected
M27240	Susan	~5 days	young neonate	absent	yes	24/2/2012
M22558	Billie's calf	~7 days	young neonate	absent	yes	10/2/2004
M22439	Phoebie's calf	<34 days	young neonate	absent	no	23/2/2001
M26013	Mimo	4–7 weeks	older neonate	absent	yes	16/4/2013
M22548	Phoebie's calf	>20 days	older neonate	forming at edge	yes	9/2/2003
M26011	Indigo	~15 months	calf 1	present	yes	9/3/2013
M20735	Miki	~15 months	calf 1	present	yes	23/9/1998

Note: Un-named dolphins known by association with identified mother.

Table 3. Total body lengths for four developmental classes of SA Indo-Pacific bottlenose dolphins (*Tursiops aduncus*). Body lengths and weights are mean ± standard error and range.

	Young neonate	Older neonate	Calf<1	Calf 1
Body length (cm)	97.6 ± 1.24	110.0 ± 1.90	124.6 ± 1.86	153.1 ± 6.17
	95.1–100.2	106.0–114.0	120.8–128.3	138.8–167.3
Body weight (kg)	11.8 ± 0.57	19.6 ± 1.19	23.8 ± 0.99	38.4 ± 3.12
	10.6–13.0	17.1–22.1	21.8–25.9	31.2–45.6
<i>n</i>	23	18	37	9

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Table 4. Timing of developmental features in young bottlenose dolphins (*Tursiops* spp).

Feature	Age (relative to birth)	Source
Dorsal fin erect	Within a few hours	Tavolga and Essapian (1957)
Rostral hairs disappear	By 3–4 days	Mann and Smuts (1999)
	By 22 days	Cockcroft and Ross (1990)
	By 1 month	McBride and Kritzler (1951)
	By weaning	Herman and Tavolga (1980)
Umbilicus	Healed by 22 days	Cockcroft and Ross (1990)
	Reduced to small scar by 44 days	
Foetal folds lightly pigmented/clear	Up to several weeks	Tavolga and Essapian (1957)
	Up to 62 days	Cockcroft and Ross (1990)
	Up to 6–10 weeks	Mann and Smuts (1999)
Tooth eruption	Begins at 6 weeks	McBride and Kritzler (1951)
	By 10 or 11 weeks	Mann and Smuts (1999)
	Maxillary teeth begin by 91 days	Cockcroft and Ross (1990)
	Most teeth by 163 days	

Table 5. Comparison of age with developmental class for Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) from SA.

Developmental class	Age	Primary evidence
Young neonate	<1 week	Dorsal fin folded Foetal folds deeply creased and pale
Older neonate	1 week to ~ 2 months	Dorsal fin erect Foetal folds creased or fading No teeth erupted
Calf<1	~2 to 12 months	Some maxillary teeth erupted
Calf 1	>1 to <2 years	Most/all teeth erupted, 1 st GLG complete

Note: GLG = Growth Layer Group

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